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REVIEW

Mechanisms in photodynamic therapy: part two—cellular signaling, cell metabolism and modes of cell death

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Available online 6 May 2005

KEYWORDS

Photodynamic therapy;
Signal transduction;
Lipid metabolism;
Apoptosis;
Necrosis;
Reactive oxygen species;
Stress response;
Cell adhesion

Summary Photodynamic therapy (PDT) has been known for over a hundred years, but is only now becoming widely used. Originally developed as a tumor therapy, some of its most successful applications are for non-malignant disease. In the second of a series of three reviews, we will discuss the mechanisms that operate in PDT on a cellular level. In Part I [Castano AP, Demidova TN, Hamblin MR. Mechanism in photodynamic therapy: part one—photosensitizers, photochemistry and cellular localization. *Photodiagn Photodyn Ther* 2004;1:279–93] it was shown that one of the most important factors governing the outcome of PDT, is how the photosensitizer (PS) interacts with cells in the target tissue or tumor, and the key aspect of this interaction is the subcellular localization of the PS. PS can localize in mitochondria, lysosomes, endoplasmic reticulum, Golgi apparatus and plasma membranes. An explosion of investigation and explorations in the field of cell biology have elucidated many of the pathways that mammalian cells undergo when PS are delivered in tissue culture and subsequently illuminated. There is an acute stress response leading to changes in calcium and lipid metabolism and production of cytokines and stress proteins. Enzymes particularly, protein kinases, are activated and transcription factors are expressed. Many of the cellular responses are centered on mitochondria. These effects frequently lead to induction of apoptosis either by the mitochondrial pathway involving caspases and release of cytochrome *c*, or by pathways involving ceramide or death receptors. However, under certain circumstances cells subjected to PDT die by necrosis. Although there have been many reports of DNA damage caused by PDT, this is not thought to be an important cell-death pathway. This mechanistic research is expected to lead to optimization of PDT as a tumor treatment, and to rational selection of combination therapies that include PDT as a component.

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Introduction

Photodynamic therapy (PDT) involves the combination of non-toxic dyes known as photosensitizers (PS) and visible light of the correct wavelength to be absorbed by the PS which in the presence of oxygen leads to the generation of reactive oxygen species that can damage cellular constituents leading to cell death. The use of PDT as a cancer therapy is particularly attractive because of its intrinsic dual selectivity. The PS localizes in the malignant tissue and the light is also spatially focused on the lesion. Precisely why cells die when subjected to the PDT-generated reactive oxygen species (ROS) has been the subject of intense investigations in recent years. The discovery of programmed cell death or apoptosis [1] has revolutionized the field of cytotoxic therapies in general [2] and PDT in particular [3]. The realization that apoptosis played a major role in embryonic development [4] and in the immune system [5] led to a search for ways in which this intrinsic property of cells could be harnessed to remove unwanted tissue and in particular cancerous cells. It should be emphasized that while ionizing, radiation and chemotherapeutic drugs efficiently induce apoptosis in cancer cells, there are important differences between these modalities and PDT. Radiation and chemotherapy tend to damage DNA and lead to apoptosis via cell-cycle checkpoints, growth arrest and p53 activation [6,7]. PDT, on the other hand, tends to operate via an acute stress response involving mitochondrial damage, cytochrome c release and formation of an apoptosome involving caspase.

A second discovery that has had major impact on cell-based PDT research is the field of transcription factors. These are proteins that are often induced by acute insults and bind to certain regions on DNA and therefore lead to transcription of genes and the consequent production of a multitude of proteins that affect cell function and cell death or survival [8].

The search which more closely define the molecular targets of PDT is inherently complex. Different cell types, different PS and different incubation and illumination conditions can all significantly alter the outcome of PDT. The question of whether there are particular cellular proteins that are more susceptible to oxidation by singlet oxygen or other PDT-generated ROS is just starting to be addressed. This question is closely related to the intracellular localization of the PS that was covered in Part I [9]. Another important question to be addressed is whether cancer cells are more susceptible to PDT-induced cell death than normal cells. The various mutations in cancer cells caused by the expression of oncogenes can lead to increased or decreased susceptibility to PDT. However, it is thought that many of the mutations that lead to resistance emerging in cancer cells exposed to radiation or chemotherapy do not lead to cross-resistance to PDT [10]. If this proves to be the case, then it would encourage the use of PDT against tumors that recur after conventional therapy, and repeated PDT treatments against the same tumor could also be envisaged. Another advantage of PDT may be that it does not lead to cumulative toxicity in the patient, and there is no known maximum

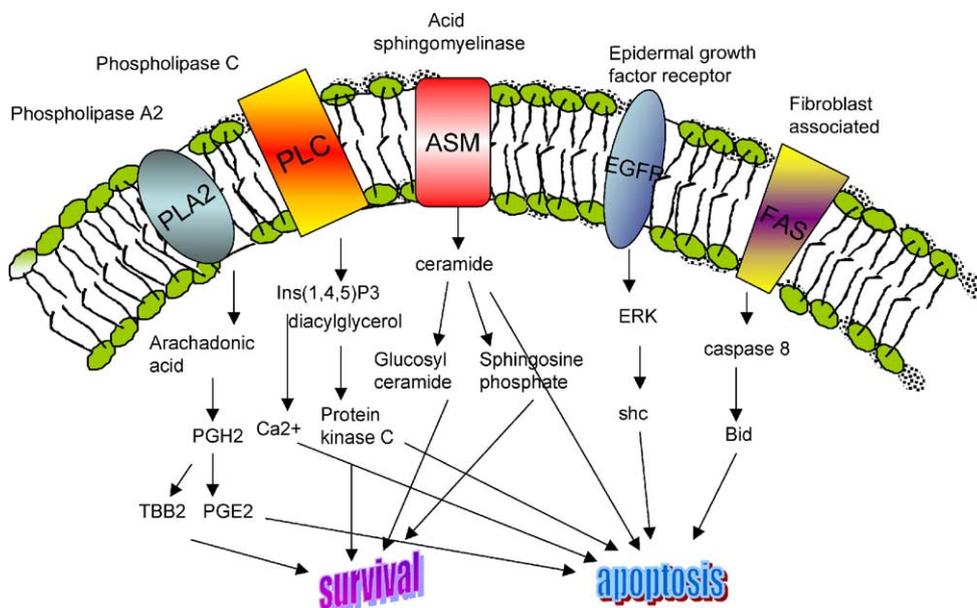


Figure 1 Signal transduction pathways activated after PDT. Events occur at receptors located at the plasma membrane and lead to alterations in cellular metabolism. These may tend towards increasing apoptosis or increasing cell survival.

cumulative dose as exists with both chemotherapy and radiation.

Signaling and changes in cellular metabolism

One of the largest areas of research in the field of mechanisms in PDT in recent years has paralleled the explosion of knowledge in cell biology about signal transduction pathways, transcription factors and regulation of cell cycle control, inflammation and cell death. Although we have attempted to divide these reports into subsections for the sake of clarity, it must be remembered that there is necessarily much overlap between these pathways in living cells, and reports that could have been assigned to several different sections have been assigned only to one. Fig. 1 gives a graphical representation of some of these signal transduction pathways activated by PDT.

Calcium

PDT of various cell types *in vitro* has been shown to raise the levels of free calcium within cells and this has been associated with cell death, and in certain occasions and conditions, with cell survival. The Ca^{2+} rise upon PDT has been proposed to occur via the influx of Ca^{2+} through ion channels [11,12], release of Ca^{2+} sequestered in internal stores in the endoplasmic reticulum (ER) and mitochondria

[13,14], and/or activation of ion exchange mechanisms [15]. Exposure of Chinese hamster ovary (CHO) cells and T24 human bladder transitional carcinoma cells treated with the PS aluminum phthalocyanine (AlPc) and respectively to red light caused an immediate increase of cytoplasmic free calcium, $[\text{Ca}^{2+}]_i$, reaching a peak within 5–15 min after exposure and then returning to basal level (approximately 200 nM) [11]. Loading the cells with the intracellular calcium chelator's quin2 or BAPTA prior to light exposure, enhanced cell killing. This indicates that increased $[\text{Ca}^{2+}]_i$, caused by extracellular Ca^{2+} influx after PDT, contributed to survivability of the treated cells by triggering a cellular rescue response. Ding et al. [16] showed that PDT with hematoporphyrin monomethyl ether (HMME)-induced cell death by apoptosis and necrosis and using sodium azide (the singlet oxygen quencher) or d-mannitol (the hydroxyl radical scavenger) they could protect HeLa cells from the death. Sodium azide or d-mannitol also inhibited HMME-PDT-mediated $[\text{Ca}^{2+}]_i$ elevation. Cytochrome c release from mitochondria into cytosol, and caspase 3 activation after HMME-PDT were inhibited by BAPTA/AM (an intracellular calcium chelator). These results demonstrated that ROS generated in HeLa cells by HMME-PDT-induced apoptosis may be through $[\text{Ca}^{2+}]_i$ elevation which mediates cytochrome c release and caspase 3 activation and initiates the apoptosis.

The effects of aminolevulinic acid (ALA)-PDT (induction with 1 mM ALA for 4 h followed by a blue light dose of 18 J/cm^2) were studied [17]

on the human promyelocytic leukemia cell line HL60 using biochemical and electron microscopy methods. It was seen that ALA-PDT activates the mitochondrial apoptotic pathway and the level of endoplasmic reticulum Ca^{2+} -binding chaperones ERp57 and ERp72 and of anti-apoptotic proteins Bcl-2 and Bcl-xL was decreased whereas that of Ca^{2+} -binding protein calmodulin and the stress protein HSP60 was elevated following ALA-PDT. Inhibition of the initiator caspase 9, execution caspase 3 and Ca^{2+} -dependent protease m-calpain, did not prevent DNA fragmentation.

PDT using the photosensitizer benzoporphyrin derivative (BPD) has been previously shown to induce rapid apoptosis via a mitochondrial-caspase activation pathway. Granville et al. [18] analyzed the impact of PDT on other cellular organelles such as the endoplasmic reticulum (ER). The effect of PDT on $[\text{Ca}^{2+}]_i$ in control and Bcl-2-overexpressing HeLa cells was assessed. A greater $[\text{Ca}^{2+}]_i$ transient was observed for Bcl-2 overexpressing cells in response to PDT. The PDT-induced Ca^{2+} release was due to the emptying of Ca^{2+} from ER and possibly mitochondrial stores and was not due to an influx of Ca^{2+} from the medium. Studying PDT effects in cell cultures of rat bladder epithelial cells for the hydrophilic tetrasulfonated aluminum phthalocyanine (ALPcS4) and using confocal microscopy, Ruck et al. [19] showed transient calcium elevation during the irradiation process, especially in the cell nuclei, followed by a more sustained increase.

The activation of the membrane-localized enzymes phospholipase C (PLC) and phospholipase A₂ (PLA₂) is a very early event in the induction of PDT-induced apoptosis because intracellular Ca^{2+} acts as a second messenger in cellular signaling in response to a wide range of stimuli and may link activation of PLA₂ to activation of PLC. PLC hydrolyzes phosphatidyl inositol-4,5-diphosphate after activation and subsequently produces inositol-3-phosphate (IP₃) and diacylglycerol (DAG). It is known that DAG activates protein kinase C (PKC) and that IP₃ promotes increases in intracellular Ca^{2+} [20,21]. Ca^{2+} binds to calmodulin in cells, which therefore acts as an intermediary protein that senses calcium levels and relays signals to various calcium-sensitive enzymes, ion channels and other proteins. This frequently happens via a complex with a second protein calcineurin. Once the complex is formed, Ca^{2+} /calmodulin/calcineurin can in turn act to dephosphorylate the transcription factor 'nuclear factor of activated T cells' (NFAT). Activated NFAT can regulate transcription through binding its own cognate DNA-binding site. One marker of keratinocyte differentiation, the p21 gene, is activated by NFAT

by a different mechanism, with NFAT activating the p21 promoter by acting as a coactivator for the transcription factors Sp1 and Sp3 [22]. In general, it is possible to think that Ca^{2+} is a link between many of the pathways activated by PDT and plays an important role in the effect PDT has on many cellular functions.

Lipid metabolism

As discussed in the previous section, PDT can lead to rapid changes in intracellular Ca^{2+} and there are many interconnected pathways between Ca^{2+} and lipid metabolism largely due to the activation of phospholipases. The rapid release of arachidonic acid metabolites seen in many cases after PDT [23–27] may result from the activation of PLA₂, an enzyme activated by Ca^{2+} . Penning et al. [22] found that HPD-PDT of human bladder cancer cells led to release of prostaglandin E₂ (PGE₂) and thromboxane B₂ (TBX₂), and this was reduced by calcium chelation with EGTA resulting in inhibition of PLA₂, or by using indomethacin to inhibit cyclooxygenase. These treatments also increased the cell death suggesting that arachidonic acid metabolism can protect cells from PDT killing. Nevertheless, the role of cyclooxygenases in PDT may depend on the cell type and/or the PDT dose, since preincubation of C6 glioma cells with indomethacin increased the number of cells surviving after PDT with HPD, whereas the survival rate for endothelial cells was decreased in the presence of the inhibitor when higher HPD concentrations were used [28]. Fingar et al. [29] showed that there was a PDT dose-dependent increase in serum thromboxane after photofrin (PF)-mediated PDT of rats bearing chondrosarcoma tumors. These authors went on to show [30] that a thromboxane synthetase inhibitor, a thromboxane receptor antagonist and an inhibitor of platelet shape change in combination with PF-PDT reduced vasoconstriction, inhibited vessel permeability and reduced tumor cure. Ferrario et al. [31] showed that both porphyrin- and chlorine-based PS were able to elicit PDT-mediated cyclooxygenase-2 (COX-2) expression after PDT in vitro and in vivo. COX-2 (but not COX-1) mRNA and protein levels were increased in radiation-induced fibrosarcoma (RIF-1), BA (mouse mammary carcinoma), LLC (Lewis lung carcinoma) cells and in RIF-1 tumors in mice together with release of PGE₂ after PF-PDT. These authors also combined PDT with the selective COX-2 inhibitor NS-398 in RIF-1 tumors and demonstrated enhanced PDT responsiveness and decreased induction of both PGE₂ and vascular endothelial growth factor in treated tumors.

Ceramide is a stress-induced second messenger that is generated from sphingophospholipids (which are part of the cell membrane) by sphingomyelinases. These enzymes cleave sphingophospholipids such as sphingomyelin to yield ceramide and phosphorylcholine, and in addition ceramide can be generated by de novo synthesis by a ceramide synthase [32,33]. The sphingolipid ceramide has proven to be a powerful second-signal effector molecule that regulates diverse cellular processes including apoptosis, cell senescence, the cell cycle, and cellular differentiation. Ceramide has been shown to activate a number of enzymes involved in stress signaling cascades including both protein kinases and protein phosphatases [34]. Dolgachev et al. [35] showed that the oxidative stress induced by phthalocyanine (Pc4)-PDT in Jurkat human T lymphoma and CHO cells was accompanied by increases in ceramide with a concomitant decrease in sphingomyelin. Sphingomyelin synthase, as well as glucosylceramide synthase, was inactivated in a dose-dependent manner and the activity of serine palmitoyltransferase (the enzyme catalyzing the initial step in the sphingolipid biosynthesis) was profoundly inhibited after treatment. Niemann-Pick disease lymphoblasts, which are deficient in acid sphingomyelinase (ASMase) activity, failed to respond to Pc4-PDT with ceramide accumulation and apoptosis, suggesting that ASMase may be a Pc4-PDT target [36]. However, this finding appears to be cell-type specific because in mouse embryonic fibroblasts isolated from ASMase knockout and wild-type mice Pc4-PDT led to increased caspase 3 activity and subsequent apoptosis in both cells [37]. Similarly, ceramide levels were elevated in both cell types post-PDT.

Tyrosine kinases

Signal transduction networks provide mechanisms for cells to receive external stimuli and respond to the signals in an appropriate manner. The mitogen-activated protein kinase (MAPK) signaling pathways play an important role in signal transduction in eukaryotic cells, where they modulate many cellular events including: mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development, cell movement and apoptosis, as well as cell and neuronal differentiation [38,39]. These evolutionarily conserved pathways are organized in "three-kinase modules" consisting of a MAP kinase, an activator of MAP kinase (MAP kinase kinase or MEK) and a MAP kinase kinase kinase (MEK kinase or MEKK). There are at least three distinct MAP kinase signal transduction pathways in mammalian cells, each named after the particular

MAPK associated with it. These include the extracellular signal-regulated kinases, ERK1/2, the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) and the p38 kinases (analogs of HOG1 yeast protein).

MAPK family activation following BPD-PDT was found in a transformed murine keratinocyte cell line, Pam212 [40]. PDT caused a strong dose- and time-dependent activation of both SAPK and p38 HOG1 but not ERK. Both L-histidine and N-acetyl-L-cysteine showed a significant inhibitory effect on PDT-induced SAPK and p38 HOG1 activation indicating the effect was partially mediated by reactive oxygen intermediates (ROI).

Western blot analysis performed on the proteins of LY-R and CHO cells at various times following lethal (90–99% cell kill) doses of Pc4-PDT found of the three MAPK types, only the p46 and p54 SAPK/JNKs were activated [41]. PDT did not affect ERK and p38/HOG activation in LY-R cells. In the case of CHO cells, however, ERK2 was slightly activated at 5 min post-PDT, then declined, and p38/HOG was strongly activated from 5 to 60 min post-PDT. This study suggests that PDT can stimulate SAPK and p38/HOG cascades and that the latter participates in both rapid and slow PDT-induced apoptosis.

Hypericin-PDT of human cancer cells led to up-regulation of the inducible COX-2 enzyme and the subsequent release of PGE2 [42]. The activation of p38 MAPK alpha and beta mediated COX-2 up-regulation at the protein and messenger levels. The p38 MAPK inhibitor, PD169316, abrogated COX-2 expression in PDT-treated cells, whereas overexpression of the drug-resistant PD169316-insensitive p38 MAPK alpha and beta isoforms restored COX-2 levels in the presence of the kinase inhibitor. The half-life of the COX-2 messenger was drastically shortened by p38 MAPK inhibition in transcriptionally arrested cells, suggesting that p38 MAPK mainly acts by stabilizing the COX-2 transcript. Hence, the combination of PDT with pyridinyl imidazole inhibitors of p38 MAPK may improve the therapeutic efficacy of PDT by blocking COX-2 up-regulation, which contributes to tumor growth by the release of growth- and pro-angiogenic factors, as well as by sensitizing cancer cells to apoptosis.

The role of ERKs in the cell survival after PDT was studied by Tong et al. [43]. They examined the response of ERK1/2 in PF-PDT-resistant (LFS087) and PDT-sensitive (GM38A) cells. ERK1/2 activity was induced rapidly in both cell types after PDT but was transient in GM38A cells and by 3 h had returned to a level significant lower than basal levels, whereas the induction of ERK1/2 was sustained in LFS087 cells and lasted for at least 11 h. Blocking of the

sustained ERK activity with PD98059, an inhibitor of MAPK/ERK significantly decreased cell survival of LFS087 after PDT. PDT also induced the expression of MAPK phosphatase (MKP-1).

ALA-PDT of HaCaT cells led to a six-fold elevation of cellular JNK activity; phosphorylation of p38 MAPK was enhanced to a similar extent. p38 was also phosphorylated by ALA-PDT in the human melanoma cell lines Bro and SkMel-23, applying doses that lead to 80–95% cell death after 24 h. The effects of ALA-PDT on MAPKs are similar to stresses such as UV irradiation or exposure to hydrogen peroxide with respect to activation of JNK and p38 MAPKs [44].

The epidermal growth factor receptor (EGFR) is a tyrosine kinase involved in the initiation and progression of various cancers especially their proliferative, angiogenic, invasive, and metastatic aspects [45]. Wong et al. [46] used ALA- and PF-PDT on human cancer cell lines: hypopharyngeal carcinoma FaDu; cervical adenocarcinoma HeLa; and hepatocellular carcinoma HepG2, and studied the cells response to cytokines, IL-6 and EGF, after PDT. PDT-induced the complete loss of EGFR on the cells membrane. Another study reported [47] the involvement of the EGFR-pathway during antiproliferative responses of Pc4-PDT in A431 cells and during ablation of murine skin papillomas. Pc4-PDT of A431 cells was found to result in a time-dependent down-modulation of the protein expression and phosphorylation of EGFR and Shc (an immediate downstream molecule in EGFR-pathway). In chemically as well as ultraviolet B radiation-induced squamous papillomas in SENCAR and SKH-1 hairless mice, Pc4-PDT resulted in a time-dependent: inhibition of protein expressions of EGFR; and tyrosine phosphorylation of EGFR and Shc; and induction of apoptosis, during the regression of these tumors.

JNK are group of MAPK comprising three protein kinases: JNK1, JNK2, and JNK3, whose genes are alternatively spliced to create 10 isoforms. JNK binds and phosphorylates the N-terminal activation domain of transcription factor c-Jun, resulting in increased transcription activity of c-Jun. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is restricted to brain, heart, and testis. JNK can be activated by treatment of cells with cytokines tumor necrosis factor- α (TNF- α) and interleukin 1 (IL-1) or by exposure of cells to a wide variety of environmental stresses [48,49]. A study [50] reported that PDT with hypericin induced a strong and persistent activation of the JNK and p38 MAPK signaling pathways while inhibiting ERK2 activity. There was a protective role for the JNK/p38 MAPK pathways during PDT-induced apoptosis.

Protein kinase B (Akt/PKB) itself is a serine/threonine protein kinase and phosphorylates a variety of substrates involved in regulation of key cellular functions such as growth control and survival. Akt/PKB can phosphorylate ATM, and ATM can phosphorylate BRCA1 and p53, and has also been suggested to interact with DNA mismatch repair proteins. Akt/PKB is thought to mediate various insulin-dependent biological processes [51,52]. The distinct functions of individual Akt/PKB isoforms still remain to be fully elucidated. Photosensitization of the murine fibroblast cell line NIH 3T3 with Rose Bengal (RB) increased the phosphorylation of Akt, which is taken as a sign of kinase activation [53]. This effect was mediated by activation of PI3-K, but was independent of activation of growth factor receptors and of focal adhesion kinase (FAK). Indeed, photosensitization with RB decreased FAK phosphorylation activity, which may explain the reduction in cell adhesion.

The effects of singlet oxygen on ERK and Akt/PKB pathways were analyzed in human dermal fibroblasts [54]. While basal ERK phosphorylation was lowered in cells exposed to either UVA or RB-PDT, Akt was moderately activated by PDT in a phosphoinositide 3-kinase-dependent fashion. Likewise, both singlet oxygen and UVA-induced ceramide generation in human skin fibroblasts. Epidermal growth factor (EGF)-induced tyrosine phosphorylation of the EGF receptor was strongly attenuated by PDT but unimpaired by C2-ceramide.

Transcription factors

Transcription factors are proteins that bind to the enhancer or promoter regions of genes and interact such that transcription occurs from only a small group of promoters in any cell. Most transcription factors can bind to specific DNA sequences, and these trans-regulatory proteins can be grouped together in families based on similarities in structure. Within such a family, proteins share a common framework structure in their respective DNA-binding sites, and slight differences in the amino acids at the binding site can alter the sequence of the DNA to which it binds. Transcription factors act as intracellular “third messengers” that couple receptor-generated signals to activation-associated changes in gene expression, often forming large transcriptional complexes with a variety of other transcription factors and accessory proteins at response elements within the promoters of the genes the transcription of which they modulate.

Activator protein-1 (AP-1) is a homo- or heterodimeric protein complex composed of the products from the proto-oncogenes c-jun and c-fos or

related family members. The association between c-jun and c-fos is required for binding to DNA and involves a structural motif known as a leucine zipper. Hydrophobic interactions between leucines located every 7th amino acid in an alpha-helix region of each sub-unit, hold the two sub-units together. AP1 binds to DNA sequences (transcription response elements) in the promoter region of many genes, which are involved in regulating cell proliferation. AP1 transcription factors are activated by a variety of physical and chemical stresses and have been related to both induction and prevention of apoptosis, depending on the tissue and on its developmental stage [55].

Gomer et al. [56] studied the PDT-mediated induction of the early response genes, c-fos, c-jun, c-myc, and egr-1, in murine RIF-1 cells. Incubation of exponentially growing cells with porphyrin-based PSs in the dark also induced an increase in mRNA levels of early response genes. Nevertheless, the xanthine PS RB, produced increased c-fos mRNA levels only following illumination. PDT with PF also increased transiently c-jun, c-myc and egr-1 mRNA in human adenocarcinoma HeLa cells [57]. Furthermore, mRNA stability experiments showed an increased half-life of c-fos and c-jun transcripts in HeLa cells sensitized with PF, and a concomitant increase in AP-1-DNA-binding activity was also observed. The AP-1 pathway was found to be responsible for the rapid increase of IL-6 expression observed after PDT [58].

Using the spontaneous transformed murine keratinocyte cell line, Pam212, it was shown that PDT with BPD could induce a time- and dose-dependent activation of stress-activated protein kinase (SAPK) and p38 high osmolarity glycerol protein kinase (p38 HOG1) [40]. SAPK and p38 HOG1 pathways are implicated in the transduction of stress signals and stimulation by inflammatory cytokines and are responsive to stimuli such as TNF- α , IL-1, UV irradiation, heat, change in osmolarity, and increase in intracellular reactive oxygen intermediates (ROIs). Stress kinases can induce activation of AP-1, and possibly a related transcription factor AP-2, thus enabling ROS-mediated gene expression. Depending on the pattern of gene expression induced, cellular responses may range from inflammation, degradation and immunosuppression to triggering of apoptosis.

The transcription factor, nuclear factor kappa B, (NF- κ B) was initially discovered as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin. NF- κ B is present in the cytoplasm as homo- or heterodimers, formed by association of sub-units belonging to the Rel protein family. These complexes are se-

questered in the cytoplasm by proteins belonging to the inhibitor of NF- κ B (I κ B) family. Stimuli leading to NF- κ B activation typically initiate a specific signal transduction cascade leading to phosphorylation of I κ Bs. Once phosphorylated, I κ B is selectively ubiquitinated and degraded by the 26S proteasome, releasing NF- κ B that is then translocated to the nucleus where it participates in transcriptional activation. NF- κ B has been linked to the regulation of many cellular genes including those encoding a number of cytokines and growth factors such as IL-1, IL-2, IL-6, IL-8, granulocyte macrophage colony stimulating factor (GM-CSF), and TNF- α . Other genes include adhesion molecules such as intracellular adhesion molecule, E-selectin, and many other proteins involved in various processes, including immune responses, acute phase reaction and inflammation such as inducible nitric oxidase synthase.

Activation of NF- κ B upon photosensitization was first shown in studies using mouse leukemia L1210 cells and PF as a sensitizer [59]. PDT of the lymphocytic ACH-2 cell line with methylene blue led to the degradation of I κ B α and increased NF- κ B DNA-binding activity [60] and similar results were found using proflavine (a PS that intercalates into DNA) that did not cause AP-1 activation [61]. The activation of NF- κ B and its role in cell death upon PDT was investigated using the PS pyropheophorbide methyl ester (PPME) in HCT-116 human colon carcinoma cells which resulted in NF- κ B activation by triggering the signaling pathway mediated by the IL-1 receptor. This involved degradation of the cytoplasmic I κ B α pool that contributed to the increase in NF- κ B DNA binding [62]. NF- κ B activation also occurred in human ECV 304 and HMEC-1 endothelial cells after PDT with PPME and this was prevented by antioxidants. The activation of NF- κ B was proposed to occur by a mechanism independent of the activation of I κ B kinases and required the activity of a tyrosine kinase [63]. NF- κ B has been shown to either promote or inhibit apoptosis, depending on the cell type and the type of inducer [64,65]. Apoptosis and NF- κ B activation was observed with verteporfin PDT in HL60 cells as shown by transient transfection with a NF- κ B-luciferase reporter construct [66]. However, less intensive PDT regimens can also affect NF- κ B, increasing the antiapoptotic mechanisms of survival.

E2F is a transcription factor that controls the transition from G1 to S phase in the cell cycle. The induced genes encode DNA replication activities such as DNA polymerase, proliferating cell nuclear antigen, nucleotide biosynthetic activities including thymidine kinase, thymidylate synthase and ribonucleotide reductase, and cell cycle regulatory

activities [67]. E2F also directs the synthesis of both cyclin E and cyclin-dependent kinase 2 (cdk2), creating the kinase activity responsible for activation of replication [68]. The retinoblastoma (Rb) gene was initially identified as a genetic locus associated with the development of an inherited eye tumor and the realization that it was a loss of function of Rb that was associated with disease established the tumor suppressor paradigm. Subsequent work identified the E2F transcription factor activity as a key target for the growth suppressing action of the Rb protein. Additional work demonstrated that Rb function, including the ability to interact with E2F, was regulated by phosphorylation and that the primary kinase responsible was the D-type cyclin-dependent kinases.

Studies have shown [69] that Pc4-PDT results in an induction of the cyclin kinase inhibitor WAF1/CIP1/p21 which, by inhibiting cyclins (E and D1), cdk2 and cdk6, results in a G0/G1-phase arrest followed by apoptosis in human epidermoid carcinoma cells A431. This group went on to show a decrease in the hyper-phosphorylated form of pRb at 3, 6 and 12 h post-PDT with a relative increase in hypo-phosphorylated pRb, which provided evidence for the involvement of pRb-E2F/DP machinery in PDT-mediated cell cycle arrest leading to apoptosis [70]. Upregulation in WAF1/CIP1/p21 protein levels was also observed upon PDT of human ovarian carcinoma (OVCAR-3)-bearing athymic nude mice with Pc4 [71] but not in SW480 colon cancer xenografts subjected to PDT with the same PS suggesting that this mechanism is tumor-type dependent [72].

Cell adhesion

Mammalian cells adhere to the extracellular matrix and to each other through specific membrane protein receptors. These are classified into the following groups: integrins, immunoglobulin G superfamily, selectins and cadherins. Integrins are ubiquitous trans-membrane adhesion molecules that mediate the interaction of cells with the extracellular matrix and also control cell–cell interactions. Recent research indicates that integrins also function as signal transduction receptors triggering a number of intracellular signaling pathways that regulate cell behavior and development [73].

The selectins mediate transient interactions between leukocytes and endothelial cells or blood platelets. There are three members of the selectin family: I-selectin, which is expressed on leukocytes; E-selectin, which is expressed on endothelial cells; and P-selectin, which is expressed on

platelets. The selectins recognize cell surface carbohydrates. One of their critical roles is to initiate the interactions between leukocytes and endothelial cells during the migration of leukocytes from the circulation to sites of tissue inflammation. The selectins mediate the initial adhesion of leukocytes to endothelial cells [74].

This is followed by the formation of more stable adhesions, in which integrins on the surface of leukocytes bind to ICAMs, which are members of the Ig superfamily expressed on the surface of endothelial cells. The firmly attached leukocytes are then able to penetrate the walls of capillaries and enter the underlying tissue by migrating between endothelial cells. Other members of the Ig superfamily mediate homophilic interactions that lead to selective adhesion between cells of the same type. There are more than 100 members of the Ig superfamily, which mediate a variety of cell–cell interactions.

The fourth group of cell adhesion molecules, the cadherins, also displays homophilic-binding specificities. They are not only involved in selective adhesion between embryonic cells but are also primarily responsible for the formation of stable junctions between cells in tissues. For example, E-cadherin is expressed on epithelial cells and homophilic interactions between E-cadherins lead to the selective adhesion of epithelial cells to one another; during cancer progression, however, E-cadherin mediated adhesion is frequently lost [75]. Other members of the cadherin family mediate selective adhesion of other cell types [76].

The alterations in the attachment of cancer cells to the substratum and each other are amongst important consequences of PDT. These changes are largely caused by the damage of adhesion molecules located in cell membranes. Several adhesion molecules have been reported to be involved in the PDT response, however specific molecular pathways depend on cell line, fluence rate and PS used [77]. BPD-PDT mediated changes in adhesive properties of cells were studied by several groups. Decreased adhesion to collagen IV, fibronectin, laminin and vitronectin, as well as loss of $\beta 1$ integrin-containing focal adhesion plaques were detected in ovarian cancer cells [78]. A decreased expression of CD44V6, its lectins (aHt1.3, PNA, SNA) and MHC-I molecules were observed in colon cancer [79]. In the later case BPD- and HPD-mediated PDT were compared, however no difference was detected [79]. Vonarx et al. [80] investigated the effect of HPD-PDT on colon cancer cells with a difference in vivo metastatic potential. HPD-PDT increased the adhesiveness rate of both cell lines

on plastic and decreased it on extracellular matrix, which correlated with decreased metastatic potential.

ALA-PDT of human adenocarcinoma (WiDr) cells inhibited the attachment of suspended cells to a plastic substratum. It also induced the increase of intracellular space in dense colonies, formation of lamellipodia between the cells and redistribution of α V β 3 integrin. The distribution of another adhesion molecule—E-cadherin was not changed under these conditions [81]. Pyridinium zinc (II) phthalocyanine and polyhaematoporphyrin were found to significantly decrease the efficiency of trypsinization of RIF-1, HT29 human colonic carcinoma, and ECV304 human umbilical vein endothelial cells adhering to plastic when PDT was carried out [77]. Meta-tetrahydroxyphenylchlorin (mTHPC, Foscan) however did not show this effect. This observation was partly explained by an increased activity of the enzyme tissue-transglutaminase in the cells. Similar results were reported by Uzdensky et al. [82] who showed that sublethal PDT of human WiDr adenocarcinoma cells and D54Mg glioblastoma cells with ALA-PPIX or disulfonated tetraphenylporphyrin (TPPS(2a)) inhibited their trypsin-induced detachment from a plastic substratum. Studies discussed above show that PDT-induced changes in adhesion could lead to a decrease of tumor metastatic potential. However, in one report BPD-mediated PDT of orthotopic rat prostate cancer increased the level of metastasis [83].

The adhesion changes induced by PDT are different for cancer and normal cells and their substrates. Neutrophil adhesion to endothelial cells is enhanced by PF-PDT with α L-, α M- or α X- β 2 receptors of the neutrophils involved in this process [84]. Platelet adhesion to the extracellular matrix and fibrinogen was significantly decreased after PDT of these substrates, however PDT of collagen resulted in significantly increased platelet adhesion, with large aggregate formation [85].

Volanti et al. [86] used pyropheophorbide-a methyl ester (PPME) to induce the expression of ICAM-1 and vascular cell adhesion molecule (VCAM)-1 via activation of NF- κ B in HMEC-1 cells. Increased ICAM-1 and VCAM-1 expression at the protein level was not observed, although IL-6 was secreted. Using specific chemical inhibitors, they showed that the lack of ICAM-1 and VCAM-1 expression was the consequence of their degradation by lysosomal proteases. The proteasome and calpain pathways were not involved. All these observations were consistent with the fact that no adhesion of granulocytes was observed in these conditions.

Cytokines

Cytokines are small-secreted proteins, which mediate and regulate immunity, inflammation, and hematopoiesis. They must be produced de novo in response to a stimulus. They generally (although not always) act over short distances and short time spans and at very low concentration. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules.

The vascular effect that causes hemorrhagic tumor necrosis after PDT, was originally thought to be mediated by TNF- α as administration of this cytokine had been shown to cause similar vascular effects and direct tumor effect in mouse models. The first description of cytokine production by PDT was reported by Evans et al., who measured the energy-dependent production of TNF- α by macrophages treated with PDT using the L929 assay, and TNF- α production was inhibited at the highest PDT doses [87]. TNF- α gene transcription increased in keratinocytes treated with Pc4 and light [88], and photodynamic activation of the monocyte cell line U937 differentiated into macrophages with mTHPC, also induced a dose-dependent production of TNF- α [89]. However, although anti-TNF- α antibodies and pentoxifylline, an inhibitor of cytokine transcription, prevented cutaneous photosensitivity in adult C3H/HeN mice injected with Pc4, none of these agents affected Pc4 PDT-induced tumor (RIF-1) regression [88].

PF can induce immune responses in the absence of light; a research group compared its effect with PPIX and showed these porphyrins produced lymphocyte proliferation and secretion of IL-2, IL-3, TNF- α and interferon γ (IFN γ), by human or murine mononuclear cells without an activating light. Combined stimulation of cells by mitogens and porphyrins maintained optimal vital ionic balance of potassium, sodium and chlorine in the lymphocytes. In the cells, thus, treated, there was a significant increase in intracellular calcium, important for lymphokine secretion. They proposed that the effect of PF on the immune system involves enhanced cytokine secretion, which may account for the subsequent tumor eradication by PDT [90].

Treating LLC cells with mono-l-aspartyl chlorine e6 (NPe6) and light increased expression of the mRNA of IL-2, IL-6, and TNF- α 6 h later. Cytokine-gene-transfected cells, namely LLC-IL-2, LLC-IL-6, and LLC-TNF- α cells were then treated with PDT. IL-

6 gene transfected LLC-IL-6 cells were significantly more sensitive and showed higher levels of apoptosis than the parent LLC cells and other cytokine gene-transfected cells, demonstrating that IL-6 expression plays a role in cellular sensitivity to PDT [91].

The effects of PDT on the activity of the IL-10 gene promoter and on IL-10 mRNA stability was studied using the murine keratinocyte line, Pam212. In vitro PDT-induced IL-10 mRNA and protein expression from Pam212 cells, which was correlated with an increase in AP-1 DNA-binding activity and activation of the IL-10 gene promoter by PDT. Deletion of an AP-1 response element from the IL-10 gene promoter was shown to abrogate the PDT-induced promoter activity indicating that the AP-1 response element is critical to IL-10 induction by PDT. In addition, PDT resulted in an increase in IL-10 mRNA stability, which may also contribute to the increased IL-10 expression in Pam212 cells following PDT [92].

Neutrophils have become recognized as important contributors to the effectiveness of tumor eradication by PDT. The ability of PDT using the PS 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH) to induce proinflammatory cytokines and chemokines, as well as adhesion molecules known to be involved in neutrophil migration, was examined in mice. In this study the researchers found that HPPH-PDT induced neutrophil migration into the treated tumor, which was associated with a transient, local increase in the expression of macrophage inflammatory protein (MIP)-2 (the murine equivalent of IL-8). A similar increase was detected in functional expression of adhesion molecules, i.e. E-selectin and ICAM-1, and both local and systemic expression of IL-6 was detected. The kinetics of neutrophil immigration mirrored those observed for the enhanced production of chemokines, IL-6 and adhesion molecules. Subsequent studies showed that PDT-induced neutrophil recruitment is dependent upon the presence of MIP-2 and E-selectin, but not on IL-6 [93].

Cecic et al. used a mouse SCCVII squamous cell carcinoma model to investigate the activity of neutrophils in tumors treated by PDT [94]. They saw a massive and sustained sequestration of these cells in PDT-treated tumors but also revealed their activated state evidenced by the presence of released myeloperoxidase. Among the adhesion molecules expressed on tumor vascular endothelium, ICAM-1 appears to be of primary importance in the invasion of neutrophils into PDT-treated tumors, because its functional blocking with monoclonal antibodies reduced the tumor cure rate. A marked upregulation of its ligands CD11b/CD18 and CD11c/CD18

found on neutrophils associated with PDT-treated tumors supports this assumption. IL-1 β activity was critical for the therapeutic outcome, since its neutralization diminished the cure rates of PDT-treated tumors. No significant effect was observed with anti-IL-6 and anti-TNF- α treatment [95].

In a BALB/c mouse model, PDT delivered to normal and tumor tissue in vivo caused marked changes in the expression of cytokines IL-6 and IL-10 but not TNF- α . IL-6 mRNA and protein were strongly enhanced in the PDT-treated EMT6 tumor. PDT also increased IL-6 mRNA in exposed spleen and skin. These data suggest that the general inflammatory response to PDT may be mediated at least in part by IL-6. In addition, IL-6 may modulate the local antitumor immune response. In contrast, IL-10 mRNA in the tumor decreases following PDT. IL-10 is markedly induced in the skin of mice exposed to a PDT regime and it plays a role in the observed suppression of cell-mediated responses seen following PDT [96].

PF-based PDT of mouse EMT6 tumors induces neutrophilia. In addition to complement fragments (direct mediators) released as a consequence of PDT-induced complement activation, there are many secondary mediators that all arise as a result of complement activity: IL-1 β , TNF- α , IL-6, IL-10, G-CSF and KC, thromboxane, prostaglandins, leukotrienes, histamine, and coagulation factors [97].

PDT-induced cytokines have been measured in patients. IFN γ , IL-1 β , IL-2, and TNF- α were assayed for in the urine of four patients treated with PDT for bladder cancer, in seven control patients undergoing transurethral surgical procedures, and in five healthy control subjects. Quantifiable concentrations of all cytokines, except gamma interferon were measured in urine samples from the PDT patients with the highest light energies, while no urinary cytokines were found in the PDT patient who received the lowest light energy nor in any of the control subjects [98].

Serum samples from patients treated on a Phase I clinical trial of PDT for mesothelioma were examined at the maximally tolerated dose of Foscan for evidence of a cytokine-mediated inflammatory response. Patients underwent pleurectomy or extrapleural pneumonectomy followed by intraoperative PDT of the thorax. IFN γ , TNF- α and IL-12 showed no elevation, but IL-1 β , IL-6, IL-8 and IL-10 levels were elevated after surgery and PDT. IL-1 β showed a statistically significant variation from baseline after surgery and IL-6, after PDT. The results suggest a systemically mediated inflammatory response resulting from thoracic surgery followed by PDT [99].

Stress response

In response to many stresses, including heat, oxidizing conditions, and exposure to toxic compounds, all cells produce a common set of heat shock proteins (Hsps) and glucose-regulated proteins (Grps). Experiments in *E. coli*, yeast, fruit flies and mice have shown that increased expression of these proteins can protect the organism against stress-induced damage. Most, but not all, heat shock proteins are molecular chaperones that bind and stabilize proteins at intermediate stages of folding, assembly, translocation across membranes and degradation. Heat shock proteins have been classified by molecular weight, for example, Hsp70 for the 70-kDa heat shock protein. The transcription of genes belonging to the Hsp family is regulated by a mechanism involving the binding of heat-shock factors (HSFs) to specific heat-shock elements (HSEs). In nonstress conditions, the transcription factor HSF is found in the cytoplasm, in a monomeric form, associated with Hsp70 [100]. During cellular stress Hsp70 binds to denatured proteins, freeing HSF that trimerizes and migrates to the nucleus, where it binds to HSE.

Gomer et al. first showed that elevated levels of mRNA encoding Grps as well as increases in Grp protein synthesis after mouse RIF-1 cells were incubated with PF for 16-h (but not 1 h) and illuminated [101]. In separate experiments, a transient elevation of Grp mRNA levels was observed in transplanted mouse mammary carcinomas following in vivo PDT treatments. They went on to show that in vitro PDT with mono-aspartyl chlorine e6 or tin etiopurpurin but not with PF increased both Hsp70 mRNA and protein levels in RIF-1 cells [102]. PDT of RIF-1 tumors in mice gave an increased expression of Hsp70 using all three PS. In vitro PDT of RIF cells with Npe6-induced HSF-1 binding to HSE, and transiently induced the expression of a reporter gene containing an inducible Hsp70 promoter [103]. This led to a proposal that PDT could be a light activated targeted inducer of specific gene expression (for instance suicide genes) if the gene of interest could be linked to the heat shock or Grp promoters [104]. Hanlon et al. reported that PF-PDT of HT29 and RIF-1 cells and their PDT resistant sublines led to increase of the mitochondrial Hsp60 and suggested that this protein may contribute to PDT resistance [105]. The same group subsequently implicated Hsp27 in mediating this PDT-resistance as shown by the creation of cells stably overexpressing Hsp27 [106]. In vivo and in vitro studies, using a mouse mammary sarcoma (EMT6) cell line stably transfected with a plasmid consisting of the gene for green fluorescent protein (GFP) under

the control of an Hsp70 promoter, showed that sublethal doses of mTHPC-PDT-induced Hsp70-driven GFP expression [107]. Recently Jalil and a group from Poland demonstrated upregulation of Hsp27, Hsp60, Hsp72/73, Hsp90, and Grp78 after PF-PDT of mouse C26 cells and linked this protein expression to the effectiveness of immature dendritic cell mediated immunotherapy [108].

Heme oxygenases (HO) degrade heme to carbon monoxide, iron and biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase. Not only does HO catalyze the removal of the dangerous heme molecules, which can generate harmful radicals when in the free form, but also the products of HO activity can act as neurotransmitters, regulate vascular tone and protect cells from various insults. The HO gene contains binding sites for several transcription factors, including an AP-1 consensus sequence [109] that may contribute to an up-regulation of gene expression since this transcription factor may be activated in PDT (see above). PDT of Chinese hamster fibroblast cells (V-79) with PF or with RB increased HO protein levels [110]. Other workers have shown that HO can be induced in the dark after incubation of cells with HPD or zinc phthalocyanine (ZnPC) [111]. Lin and Girotti showed that pre-incubation of cells with hemin could cause resistance to PDT by inducing expression of HO1 [112].

Increases in expression of anti-oxidant enzymes may also be caused by PDT. Studies using human adenocarcinoma HeLa cells showed induction of manganese superoxide dismutase (MnSOD) mRNA following photosensitization with PF [113]. Recent studies using murine colon-26 (C26) cells showed that PF-PDT increased the protein levels of MnSOD, but not of Cu,Zn-SOD [114]. Transient transfection of the T24 bladder cancer cell line with the MnSOD gene, but not with the Cu,Zn-SOD gene, or pretreatment of C26 and T24 cells with a cell permeable SOD mimetic, resulted in a considerable decrease in the effectiveness of PDT with PF. These results suggest that inhibition of SOD activity may be effective in potentiating the antitumor effectiveness of PDT [114]. This was shown by a study using 2-methoxyestradiol (2ME), a SOD-inhibitor capable of potentiating the antitumor effects of PDT. The combination produced retardation of tumor growth and prolongation of the survival of tumor-bearing mice. A recent study [115] showed that human glutathione S-transferase (GST) isoforms GSTP1-1 (P1-1) and GSTA1-1 (A1-1) bind with high affinity to hypericin (HYP) and differentially quench its PDT properties and this antioxidant effect was attributed to classic ligand activity of

GSTs, wherein nonsubstrate planar aromatic anions are sequestered on, and inhibit, the enzyme.

Hypoxia and angiogenesis

Tumor hypoxia is associated with malignant progression; resistance to chemotherapy, PDT and radiotherapy; increased metastasis and poor prognosis. Many of these effects in hypoxic tumor cells are mediated by oxygen-regulated transcriptional activation of a specific set of genes whose relation to therapy resistance is only poorly understood. The hypoxia-inducible factor (HIF)-1 is a master transcriptional activator of oxygen-regulated genes, which are involved in anaerobic energy metabolism, angiogenesis, cell survival, cell invasion, and drug resistance. HIF-1 is a heterodimer composed two sub-units HIF-1 α bound to HIF-1 β , but whereas the latter sub-unit is constitutively expressed, HIF-1 α is rapidly degraded under normoxic conditions [116]. Hypoxia stabilizes HIF-1 α , allowing the formation of the transcriptionally active HIF-1 complex, which binds to the hypoxia response element found in the promoter region of specific genes, including the vascular endothelial growth factor (VEGF) gene [117]. HIF-1 is a positive factor for tumor growth and is constitutively upregulated in several tumor types [118]. Because PDT is capable of rapidly consuming significant amounts of tissue oxygen and also shutting down the blood supply to the tumor that delivers oxygen, the treatment may itself produce severe levels of hypoxia.

The possibility of PDT producing an angiogenic response in which new blood vessel formation occurs was first observed in 1989 [119]. The tails of mice injected with meso-tetra (sulphonatophenyl) porphine and illuminated showed an increase in number of blood vessels at a low light dose but not at a dose that led to widespread necrosis. Deininger et al. [120] used Western blotting to analyze secretion of regulators of angiogenesis to the supernatants of cell lines following Hypocrellin-A and -B PDT. Both proangiogenic factors (VEGF) and antiangiogenic factors (sFlt-1, angiostatin, p43, allograft inflammatory factor-1, and connective tissue growth factor) were detected.

PF-mediated PDT-induced expression of the HIF-1 α sub-unit of the heterodimeric HIF-1 transcription factor and also increased protein levels of the HIF-1 target gene, VEGF within treated transplantable BA mouse mammary carcinoma [121]. PDT treatment of BA tumor cells grown in culture resulted in a small increase in VEGF expression above basal levels, indicating that PDT-mediated hypoxia and oxidative stress could both be involved in the overexpression of VEGF. Tumor-bearing mice treated with

combined antiangiogenic therapy (IM862 or EMAP-II) and PDT had improved tumoricidal responses compared with individual treatments. This study showed that PDT-induced VEGF expression in tumors decrease when either the antiangiogenic compounds IM862 or EMAP-II was included in the PDT treatment protocol and suggested that combination procedures using antiangiogenic treatments could improve the therapeutic effectiveness of PDT. A synthetic inhibitor of matrix metalloproteinases (MMP), Prinomastat, also increased the percentage of long-term cures after PDT with PF [122]. PDT increased the expression and activation of MMP-1, -3, -8 and -9 in BA tumors subjected to PDT with PF, and also changed the expression of MMP regulators.

Jiang et al. [123] studied angiogenesis produced in normal rat brains by PF PDT. Angiogenesis induced by PDT in normal rat brains. VEGF expression increased within the PDT-treated hemisphere 1 week after treatment and remained elevated for 6 weeks. Three-dimensional morphologic analysis of vasculature within PDT-treated and contralateral brain demonstrated PDT-induced angiogenesis that continued for 4 weeks after PDT. Similar studies found increased VEGF expression also of PCNA (a marker of proliferation) in tumor vessels of mice bearing NR-S1 squamous cell carcinomas up to 2 days post-PDT [124].

Hypoxia-induced genes have been studied in human tumors treated with PDT. PF-mediated PDT-induced expression of HIF-1 α sub-unit of the heterodimeric HIF-1 transcription factor and its target gene, VEGF, within tumors from patients receiving PF-PDT for early-stage esophageal cancers. High HIF-1 α expression was associated with a poor response to treatment [125].

Mechanisms of cell death in PDT

Although PDT can mediate many signaling events in cells, its main purpose is nevertheless generally to kill cells. Recent research has elucidated many pathways whereby mammalian cells can die, and some of the ways that PDT can initiate these processes. The concentration, physicochemical properties and subcellular location of the PS, the concentration of oxygen [126–128], the appropriate wavelength and intensity of the light, and the cell-type specific properties may all influence the mode and extent of cell death.

Modes of cell death

Kerr was the first to provide evidence [129] that cells may undergo at least two distinct types of

cell death: The first type is known as necrosis, a violent and quick form of degeneration affecting extensive cell populations, characterized by cytoplasm swelling, destruction of organelles and disruption of the plasma membrane, leading to the release of intracellular contents and inflammation. Necrosis has been referred to as accidental cell death, caused by physical or chemical damage and has generally been considered an unprogrammed process. It is characterized by a pyknotic nucleus, cytoplasmic swelling, and progressive disintegration of cytoplasmic membranes, all of which lead to cellular fragmentation and release of material into the extracellular compartment. In necrosis, decomposition is principally mediated by proteolytic activity, but the precise identities of proteases and their substrates are poorly known.

A different type of cell death was termed apoptosis, identified in single cells usually surrounded by healthy-looking neighbors, and characterized by cell shrinkage, blebbing of the plasma membrane, the organelles and plasma membrane retain their integrity for quite a long period. In vitro, apoptotic cells are ultimately fragmented into multiple membrane-enclosed spherical vesicles. In vivo, these apoptotic bodies are scavenged by phagocytes, inflammation is prevented, and cells die in 'immunological control' of necrosis resides within or outside cells. Apoptosis, requires transcriptional activation of specific genes, include the activation of endonucleases, consequent DNA degradation into oligonucleosomal fragments, and activation of caspases.

Since these first descriptions of necrosis and apoptosis, it has become evident that the situation is somewhat more complicated with alternative modes of cell death being described. These include mitotic cell death [130], cells exhibit multiple aberrations including retardation at G (2)-M, increased cell volume, and multinucleation; programmed necrosis [131], involving the processes of induction, commitment, and execution of necrosis triggered solely by the biological stimulus induced by three-dimensional arrangement of the culture, the cysteine cathepsin-(cathepsins B or L) mediated lysosomal death pathway [132], and autophagic cell death [133], in which a normal function used to degrade components of the cytoplasm is involved and which is characterized by autophagosomes, autolysosomes, electron-dense membranous autophagic vacuoles, myelin whorls, multivesicular bodies, as well as engulfment of entire organelles. These last cell death mechanisms have been not described with PDT (autophagy and programmed necrosis).

Caspases are intracellular endopeptidases that employ cysteine at the active site and cleave their targets at aspartic acid residues [134]. The caspases are synthesized as zymogens and these precursors are converted into active enzymes via oligomerization-induced autoprocessing for initiator caspases (nos. 1, 2, 4, 5, 8, 9, 10 and 14) while effector caspases (nos. 3, 6 and 7) are activated by other proteases, including initiator caspases and enzyme B [135]. Proteolytic cleavage of cellular substrates by effector caspases determines the features of apoptotic cell death [136] and can be initiated by three different pathways involving caspase 8 (death receptor activation), the endoplasmic reticulum stress pathway involving activation of caspase 12, and the mitochondrial pathway, in which release of proteins (including cytochrome c) by mitochondria into the cytoplasm leads to activation of caspase 9 and downstream cleavage of caspase 3, 7 or 6.

Effector caspases cleave and inactivate proteins that protect living cells from apoptosis, such as the DNA repair protein, poly(ADP-ribose) polymerase, ICAD/DFF45 (inhibitor of caspase-activated DNase), the nuclease responsible for DNA fragmentation), or the anti-apoptotic Bcl-2 or Bid (a Bcl-2 homolog), which then promotes apoptosis via the mitochondria. At least 18 Bcl-2 proteins have been isolated with either pro- or anti-apoptotic activity [137]. The antiapoptotic members (such as Bcl-2 and Bcl-xL) prevent the release of cyto c from the mitochondria and the subsequent procaspase activation.

Caspases can be activated by an intrinsic pathway, triggered by various environmental insults and developmental programs, which occurs in mitochondria [138]. It involves release of cytochrome c and other apoptogenic proteins from the mitochondrial intermembrane space into the cytosol. Cytosolic cyto c acts as a cofactor in the formation of a complex with Apaf-1, procaspase 9, dATP/ATP termed the apoptosome that leads to the activation of caspase 9 and subsequent activation of executioner caspases and cell death commitment. A mechanism has been described in which caspase 8 (activated by ligation of death receptors) cleaves Bid (a BH3 only member of the Bcl-2 family) leading to mitochondrial release of cytochrome c and thence to activation of procaspase 9 and thereby amplifying apoptotic signaling [139,140]. There have been recent suggestions that non-caspase proteases such as leucocyte elastase inhibitor (LEI)-DNase II [141–143] can trigger a form of programmed cell death different from the traditional pathways of apoptosis.

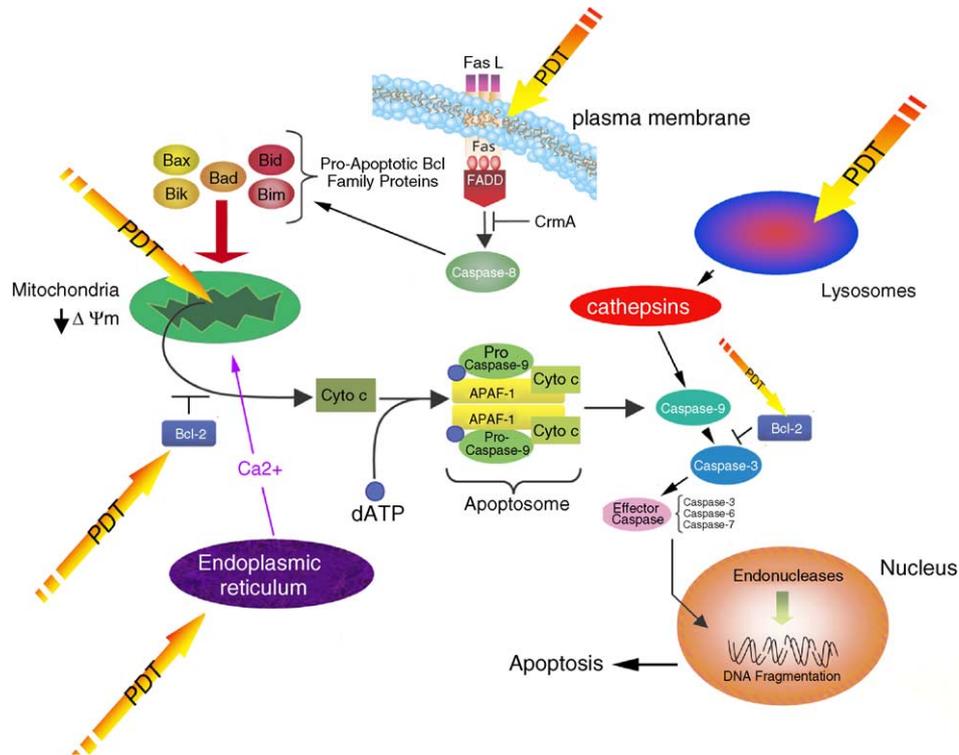


Figure 2 Cellular signaling pathways leading to apoptosis in cells after PDT. Initial targets of PDT-generated ROS depend on PS localization and include mitochondria, lysosomes, endoplasmic reticulum, plasma membrane and PS binding to Bcl-2.

Apoptosis and necrosis after PDT

Because of the intense interest involving cell death mechanisms, workers in the field of PDT have looked at the occurrence of apoptosis and necrosis both in vitro and in vivo [3]. Fig. 2 illustrates some of the cellular and molecular signaling pathways that have been determined to occur in cells treated with PDT in vitro. Agarwal et al. were the first to report apoptosis after PDT with chloroaluminum phthalocyanine in mouse lymphoma L5178Y cells, and found a rapid induction of apoptosis mediated by phospholipase C activation [144]. The crucial factors in determining the type of cell death, e.g. apoptosis or necrosis following PDT are: cell type, the subcellular localization of the PS; and the light dose applied to activate it locally. In general, it is believed that lower dose PDT leads to more apoptosis, while higher doses lead to proportionately more necrosis [145]. Nagata et al. [146] used the amphiphilic PS ATX-S10(Na) and human malignant melanoma cells and found that light doses that led to less than 70% cytotoxicity induced mainly apoptosis; by contrast, most cells appeared necrotic with doses that induced 99% cytotoxicity. A common feature of the apoptotic program initiated by PDT is the rapid release of mitochondrial cytochrome c

into the cytosol followed by activation of the apoptosome and procaspase 3. With PS localized in the plasma membrane, the photosensitization process can rapidly switch the balance towards necrotic cell death likely due to loss of plasma membrane integrity and rapid depletion of intracellular ATP [147]. It is also possible that high doses of PDT can photochemically inactivate essential enzymes and other components of the apoptotic cascade such as caspases. For instance, Lavie et al. [148] used the perylenequinones (hypericin and dimethyl tetrahydroxyhelianthron) and found high dose PDT inhibited apoptosis by interfering with lamin phosphorylation, or by photodynamic cross-linking of lamins.

Oleinick et al. [149] compared Pc4-mediated PDT of MCF7 cells that lack caspase 3 with the same cell line with caspase 3 transfected back in. They found apoptotic indicators only in the caspase expressing cells which also showed more loss of viability by an assay involving reduction of a tetrazolium dye; however both cell lines showed an equal degree of cytotoxicity by a clonogenic assay. Pc4 is a PS that localizes in intracellular membranes, especially mitochondria. Pc4-PDT photodamages Bcl-2 and Bcl-xL that are antiapoptotic proteins interacting with the permeability

transition pore complex that forms at contact sites between the inner and outer mitochondrial membranes [150]. These complexes and the inner membrane are distinctive in containing the phospholipid, cardiolipin and it is suggested that Pc4 resides near cardiolipin-containing sites, which are primarily on the inner mitochondrial membrane [151] and the consequent photodamage of Bcl-2 and Bcl-xL explains the induction of apoptosis by this PS.

The importance of Bcl-2 as a target of PDT was emphasized in the study by Usada et al. [152], who used transfection of wild-type Bcl-2 or certain deletion mutants in either a transient or a stable mode. Overexpression of Bcl-2 decreased apoptosis and cell death, and inhibited the activation-associated conformational change of the proapoptotic protein Bax, and higher doses of Pc4 and light were required to activate Bax in cells expressing high levels of Bcl-2.

Treatment with BPD (verteporfin) and broad-spectrum fluorescent light rapidly produced apoptosis in murine P815 mastocytoma cells [153]. Fragmentation of DNA, a fundamental characteristic of cells undergoing apoptosis, was evident within 3 h following the PDT. Western immunoblot analysis using the specific antiphosphotyrosine monoclonal antibody 4G10 indicated that molecular species of >200 kDa were phosphorylated on tyrosine residues during or immediately following the irradiation of cells loaded with BPD. Increased tyrosine phosphorylation of a 15 kDa protein was evident by 15 min postirradiation. In the absence of light, BPD did not affect the status of cellular protein tyrosine phosphorylation or cause DNA fragmentation.

The status of I κ B and NF- κ B proteins was evaluated for promyelocytic leukemia HL-60 cells treated at different intensities of PDT [66]. The action of BPD-MA, and visible light irradiation were assessed. At a BPD concentration that produced the death of a high proportion of cells after illumination, evidence of caspase 3 and caspase 9 processing and of poly(ADP-ribose) polymerase cleavage was present within whole cell lysates. The general caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD.fmk) effectively blocked these apoptosis-related changes.

The participation of the mitochondrial permeability transition pore (MPT) in PDT-induced apoptosis is mainly based on the experimental evidence that pharmacological inhibitors of the MPT can counteract PDT-mediated cell death induced by compounds that preferentially accumulate in mitochondria *ref.* Certain PS, such as PPIX and other porphyrins, exhibit high affinity for some component of the MPT pore, in particular for the outer membrane

peripheral benzodiazepine receptor (PBR) that was proposed to be a crucial target for mitochondrial PDT [154]. However, subsequent studies showed that binding to the PBR was not correlated with PDT efficacy of different classes of PS [155,156]. BPD has been shown to target the adenine nucleotide translocator in the mitochondrion, and to induce in a light-dependent fashion the permeabilization of the MPT pore encapsulated into liposomes, which reconstitute the function of the MPT-pore [157].

Studies with lutetium texaphyrin (Lutex) have shown that this PS induces apoptosis through a selective modulation of members of the Bcl-2 family [158]. In a different study it has been shown that Lutex binds to lysosomes of EMT6 cells *in vitro* and to produce apoptosis in EMT6 tumors *in vivo*, indicating that lysosomally bound PSs can induce apoptosis upon photoactivation [159].

Canete et al. [160] showed that the PS palladium(II)-tetraphenylporphycene caused only necrotic cell death in A-549 cells even at low doses despite the cells undergoing apoptosis in response to serum starvation, and that this PS could produce apoptosis in a different cell line (HeLa cells). Blank et al. demonstrated that hypericin could induce mitotic cell death by targeting Hsp90 for ubiquitinylation and thus lowering levels of its client proteins mutant p53, Cdk4, Raf-1, and Plk and disturbing multiple cellular functions [161].

Thibaut et al. [162] used apoptosis inhibitors (BAPTA-AM, Forskolin, DSF, and Z.VAD.fmk) to study PDT of murine melanoma B16-A45 cells and mTHPC. Although all inhibitors tested blocked PDT-induced apoptosis, none produced a significant modification of the phototoxic effect of mTHPC on B16 cells. It has been suggested that apoptosis and necrosis share common initiation pathways and that the final outcome is determined by the presence of an active caspase. This implies that apoptosis inhibition re-orientates cells to necrosis, i.e. those cells sufficiently damaged by PDT appear to be killed, regardless of the mechanism involved.

Mitochondria were shown to be the main targets of mTHPC in some reports [163]. PDT of mTHPC-sensitized murine leukemia cells (M1 and JCS) caused rapid appearance of the apoptogenic protein cytochrome c in the cytosol (especially pronounced in JCS cells), and well correlated with the extent of apoptotic cell death. Electron microscopy revealed the loss of integrity of the mitochondrial membrane and the appearance of chromatin condensation as early as 1 h after light irradiation.

PF, a representative of the first generation PSs, is a preparation consisting of HPD. Studies described in several reports revealed the complicated effects of HPD/PF PDT on cells. The treatment can result

in apoptosis or necrosis of target cells [164,165]. Biochemical analysis indicated that HpD/PF PDT elicited lipid peroxidation and enzyme inactivation on plasma membranes, as well as mitochondria damage and inactivation of mitochondrial enzymes [166].

The role of apoptosis-related proteins was also studied in the response of human malignancies to PDT [167]. Paraffin-embedded material from 37 patients with early esophageal cancer treated with PDT (intravenous injection of HPD) was studied immunohistochemically for p53 protein nuclear accumulation and Bcl-2 cytoplasmic expression. Positive Bcl-2 and p53 expression was noted in 27% and 39% patients, respectively. 63% of tumors with Bcl-2 expression responded completely to PDT versus 23% of cases with no Bcl-2 expression ($p = 0.02$). No association of p53, T-stage and of histology grade with response to PDT or PDT/RT was noted. Bcl-2 protein expression was proposed to be associated with favorable response to PDT and could be used as a predictor of cancer response to PDT. This finding was explained by studies showing that PDT induces selective degradation of the Bcl-2 protein, leading to apoptosis by decreasing the Bcl-2/bax ratio [152].

Bystander effect

A different mechanism of cell death was described by Dahle et al. [168], who showed that during *in vitro* PDT some cells die by direct effect, but adjacent cells suffer lethal cell damage which is propagated through a chain of adjacent cells, termed the bystander effect. Treatment of MDCK II cells with the lipophilic PS tetra(3-hydroxyphenyl)porphyrin and light was found to induce a rapid apoptotic response in a large fraction of the cells. Furthermore, the distribution of apoptotic cells in microcolonies of eight cells was found to be different from the expected binomial distribution. There was an overabundance of microcolonies that had responded to the treatment as a single unit, that is, in which either all or no cells were dead, indicating that the cells are not inactivated independently, but that the bystander effect is involved in the cell death. This observation disagrees with the common view that cells are inactivated only by direct damage and indicates that communication between cells in a colony plays a role in PDT induction of apoptosis. The degree of bystander effect was higher for cells dying by necrosis than for cell dying by apoptosis. Initially it was thought that the process was mediated through gap junctional intercellular communication during or shortly after irradiation, [169,170] but when this hypothesis was tested by

treatment of microcolonies with 30 μM dieldrin, an inhibitor of gap junctional intercellular communication, there was no reduction of the bystander effect. However, workers [171] in the field of ionizing radiation where the bystander effect is also observed, showed that it may be mediated both by gap-junctional communication and also by generation of diffusible ROS that can be released into the medium and act on neighboring cells.

DNA damage

Sunlight gives rise to DNA damage by two mechanisms. On the one hand, DNA directly absorbs light in the UVC and UVB range of the spectrum (up to ~ 320 nm). The absorption gives rise to characteristic photoproducts, especially the formation of pyrimidine dimers. Their pre-mutagenic properties have been well established. On the other hand, some so far unidentified cellular constituents (probably porphyrins or flavins) act as endogenous PS that react directly with DNA or give rise to the formation of reactive oxygen species. These reactions result in oxidative DNA damage, which is also known to be pre-mutagenic. The contribution of the indirect (PS-mediated) mechanisms to the cancer risk induced by direct sunlight is not very well known. It is anticipated that the indirect mechanisms will not be as effective as direct DNA excitation, but that they will make an important contribution to the genotoxicity of sunlight in the longer wavelength range where DNA has little or no absorption. The photodynamic alteration of DNA is a singlet oxygen process, while the ionizing radiation degradation is mediated by hydroxyl radicals generated by ionization of water.

Damage to DNA has been shown in several studies with *in vitro* PDT [172], however this DNA damage has not been directly linked to lethal effects. PDT has been shown to cause DNA base oxidative damage, strand breaks and cross-links. The mutagenic potential varies between cell types, possibly reflecting differences in repair capacity or damage surveillance mechanisms.

Porphyrins and/or metalloporphyrins mediated cleavage of nucleic acids occurs via oxidative attack on the sugar moiety, nucleobase modifications which lead to strand scission or by photo-induced mechanism involving either the porphyrin excited state or singlet oxygen. Free radical reactions have been suggested to be involved at several points in the multistep process of chemically induced carcinogenesis. Singlet oxygen can be readily generated inside cells and reacts efficiently with DNA causing single strand breaks. Its preferential reaction with the guanine moiety in DNA leads mainly

to one-G deletions in the DNA sequence. The mutagenicity of singlet oxygen depends on formation of lipid peroxidation products. In general, the most potent PSs are usually lipophilic and they do not accumulate in the nucleus. Therefore, despite causing mutagenic products in vitro assays this may not occur in vivo.

It has long been known that 5-ALA is capable of causing DNA damage, but it is not certain if this phenomenon is light dependent or not [173]. When ferritin or metals are present, a catalyzed oxidation of ALA produces reactive oxygen species that can damage plasmid DNA in vitro, and increases the steady-state level of 8-oxo-7,8-dihydro-2'-deoxyguanosine in liver, spleen and kidney. The DNA damage could be partially inhibited by SOD, catalase, DTPA, mannitol and melatonin. 4,5-Dioxovaleric acid, the final oxidation product of ALA, alkylates guanine moieties within both nucleoside and isolated DNA, producing two diastereoisomeric adducts [174,175]. It is possible that these mechanisms could be involved in the increase in liver cancer observed amongst sufferers of acute intermittent porphyria who have elevated levels of 5-ALA due to enzyme deficiencies [176].

Photosensitization of various types of cells by hematoporphyrin or phthalocyanines results in DNA lesions, such as single strand breaks [177,178]. The mutagenicity of PF-PDT may be related to the repair capabilities as well as to the p53 status of the cell line. Woods et al. [179] studied PDT with human HaCaT keratinocytes using the standard alkaline comet assay protocol to detect DNA strand breaks. They used PF (1 $\mu\text{g}/\text{mL}$) and 630 nm laser light and showed a dose-dependent increase in DNA migration (comet tails) starting as low as 1 J/cm^2 ; however, the breaks produced at the higher irradiation doses (10 and 25 J/cm^2) could have been caused by cell death. PF treatment in the absence of light did not result in increased DNA migration.

A similar comet assay with tail moment calculation was used to evaluate DNA damage and repair in murine glioblastoma C6 cells after PDT with m-THPC [180]. There were no changes in tail moment of C6 cells in the absence of light, whereas m-THPC-PDT (1 $\mu\text{g}/\text{mL}$) induced DNA damage immediately after irradiation. The mean value increased with the light dose (0, 10 or 25 J/cm^2) and incubation time (every hour from 1 to 4 h), but the cells were capable of significant DNA repair after 4 h, and no residual DNA damage was evident after 24-h post-treatment incubation at 37 °C. An increase in the light dose appeared to be less genotoxic than an increase in the m-THPC dose for similar toxicities.

Overall, the presently available data indicate that the risk for secondary skin carcinoma after topical ALA-PDT seems to be low, but further studies must be carried out to evaluate the carcinogenic risk of ALA-PDT in conditions predisposed to skin cancer. We can conclude that the DNA-damaging effects of PDT are dependent not only on the all variables implicit in PDT but also on the cellular mechanisms of repair and survival. For treatments giving equal levels of cell survival, DNA damage has been shown to be less for PDT-treated cells compared with those that have been X-irradiated. Although DNA, RNA and protein synthesis are affected following PDT, recovery occurs suggesting that such damage may not necessarily be lethal.

Conclusion

Increasing research is directed towards understanding the mechanisms involved in PDT at both the molecular, cellular and tissue levels. When sufficient knowledge has been accumulated, it is expected that it may be possible to more accurately predict response to treatment. Research advances in understanding cellular mechanisms of PDT will also shed light on the myriad of possible combination treatments. PDT has been combined with ionizing radiation and chemotherapy in animal models with many reports suggesting additive benefits and some demonstrating synergistic effects [181]. As yet these combinations have not been much studied in patients [182]. There is a growing range of so-called targeted pharmacological therapies becoming available to combat cancer [183,184]. These include a range of protein kinase inhibitors [185], proteasome inhibitors [186], histone deacetylase inhibitors [187] and telomerase inhibitors [188]. Whether any of these could be successfully be combined with PDT has not even begun to be explored.

Acknowledgements

Ana P. Castano was supported by a Department of Defense CDMRP Breast Cancer Research Grant. Tatiana N. Demidova was supported by a Wellman Center of Photomedicine graduate student fellowship. Michael R. Hamblin was supported by the US National Institutes of Health (R01-CA/A1838801). We are grateful to Anna Yaroslavsky and Tayyaba Hasan for help and support.

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