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Cell death and growth arrest in response to photodynamic therapy with membrane-bound photosensitizers

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Abstract

Photodynamic therapy (PDT) is a treatment for cancer and for certain benign conditions that is based on the use of a photosensitizer and light to produce reactive oxygen species in cells. Many of the photosensitizers currently used in PDT localize in different cell compartments such as mitochondria, lysosomes, endoplasmic reticulum and generate cell death by triggering necrosis and/or apoptosis. Efficient cell death is observed when light, oxygen and the photosensitizer are not limiting (''high dose PDT''). When one of these components is limiting (''low dose PDT''), most of the cells do not immediately undergo apoptosis or necrosis but are growth arrested with several transduction pathways activated. This commentary will review the mechanism of apoptosis and growth arrest mediated by two important PDT agents, i.e. pyropheophorbide and hypericin.

Keywords: Photodynamic therapy; Photosensitizer; Apoptosis; Mitochondria; Bcl-2

1. Introduction

Photodynamic therapy (PDT) is a treatment for cancer and certain non-malignant pathologies that are generally characterized by overgrowth of unwanted or abnormal cells [1–3]. The procedure requires exposure of cells or tissues to a photosensitizing drug followed by irradiation with visible light of the appropriate wavelength, usually in the red or near-infrared region and compatible with the absorption spectrum of the drug. Upon light absorption, the photosensitizer undergoes excitation that brings it in its excited triplet state. The triplet can participate in a one-electron oxido-reduction (Type I photochemistry) with a neighboring molecule, producing free radical intermediates that can react with oxygen to generate various reactive oxygen species (ROS). Alternatively, the triplet-state photosensitizer can transfer energy to ground state oxygen (Type II photochemistry), generating singlet molecular oxygen, a highly reactive form of oxygen that reacts with many biological molecules, including lipids, proteins, and nucleic acids [4–7] (Fig. 1).

The first drug approved by several agencies for PDT is a porphyrin oligomer (Photofrin), which is highly effective but exhibits several drawbacks such as: (i) a tendency to cause prolonged skin photosensitivity; (ii) an activation wavelength lower than that optimal for effective penetration through tissue; and (iii) a poorly defined chemical composition which makes a detailed understanding of its mode of action and pharmacokinetics difficult. These limitations have encouraged the development of many so-called second-generation photosensitizers [8,9]. Most of them are porphyrin-like molecules, such as benzoporphyrins, pheophorbides, texaphyrins, phthalocyanines, and naphthalocyanines, or endogenously generated photosensitive metabolites, such as protoporphyrin IX. Several have other types of structures, e.g. hypericin, rhodamine, methylene blue, and derivatives of these molecules. In the case of protoporphyrin IX, synthesis of this photosensitizing precursor of heme is greatly enhanced by supplying an earlier metabolic precursor, 5-aminolevulinic acid (ALA) [10]. Recently, approval has also been obtained for PDT of age-related macular degeneration with Verteporfin [3] and for treatment of actinic keratoses with Levulan (formulated ALA) [11].

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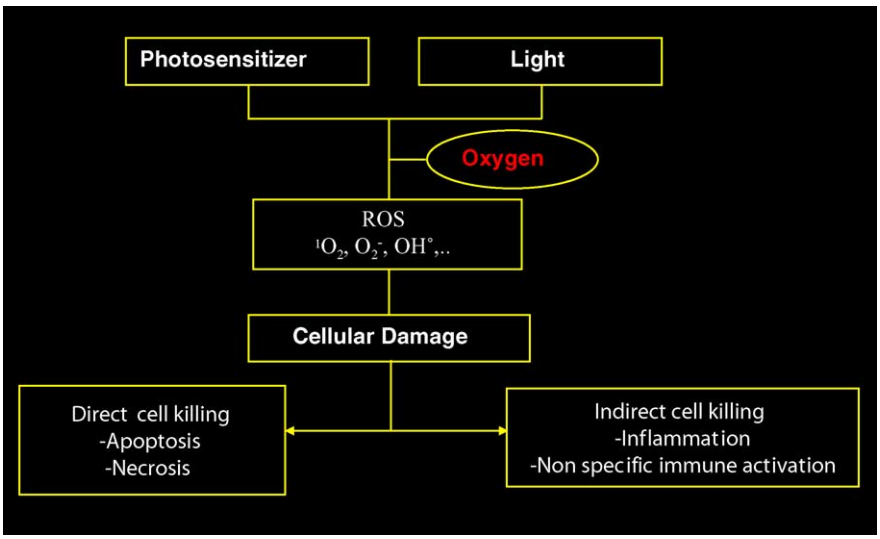

Fig. 1. Schematic representation of the various partners important for cell killing mediated by PDT. ROS is reactive oxygen species.

The primary role of PDT is to kill unwanted cells, and this occurs through two major pathways: (i) a direct pathway leading either to apoptosis and/or necrosis [12]; and (ii) an indirect pathway that will cause the recruitment of inflammatory cells (neutrophils, macrophages, etc.) [13] and the nonspecific activation of the immune system [14] (Fig. 1). The balance between these two pathways is monitored by numerous parameters and among others, the intracellular localization of the drug, the fluence rate, the physico-chemical properties of the photosensitizer, the oxygen concentration and the cell type [12]. When the light fluence and/or the photosensitizer and oxygen concentrations are not limiting within a tumor (high dose PDT, Fig. 2), the direct pathway turned out to be favored. However, high dose PDT can also cause a vascular occlusion of the tumor with a subsequent ischemia-induced tumor cell death. When one of these components (light, photosensitizer or oxygen) is limiting (low dose PDT, Fig. 4), any apoptosis and/or necrosis are hardly detected, but several tumor cells transduction pathways are activated switching on gene transcription [15]. Induction of an effective inflammatory response is often seen at low dose PDT although a secondary inflammatory response is also associated with necrosis of tumor cells.

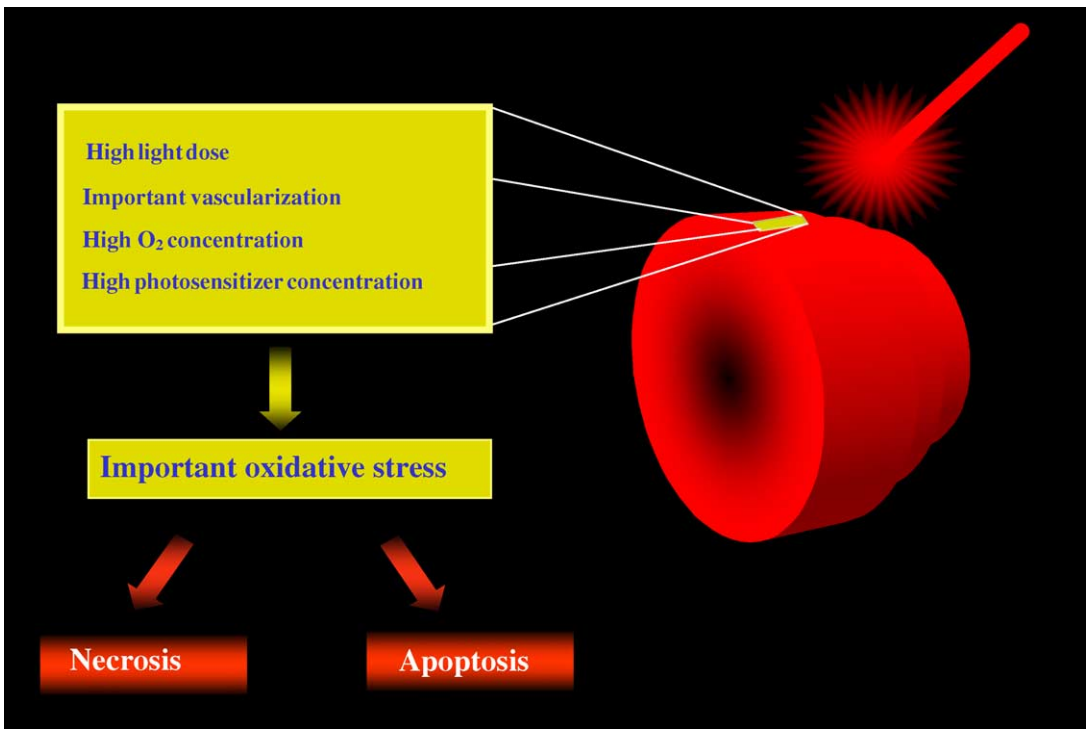

Fig. 2. Experimental conditions that are important to reach a high PDT effect at the level of a solid tumor.

In this article, we will present the molecular dissection of the cellular effects associated with high dose and low dose PDT. Due to the vast number of photosensitizers studied in the field of PDT, we will limit this article to the works done with two photosensitizers: pyropheophorbide methyl ester (PPME) and hypericin (HYP), which are hydrophobic photosensitizers that are mainly located in membranes (endoplasmic reticulum and Golgi, nuclear and plasma membranes, and lysosomes). PPME is a so-called second-generation photosensitizer that is not used in clinics yet whereas HYP is actually approved for the diagnosis of bladder tumors.

2. High dose PDT

In order to follow the mechanism of tumor cell death in the so-called high dose PDT, human colon cancer cells (HCT-116) were treated with micromolar concentrations of PPME and light fluences leading to less than 10% of cell survival after 24 hr.

2.1. Apoptosis vs. necrosis

Quantification of the precise extent of apoptosis vs. necrosis induced by PPME-mediated PDT of cancer cells was approached by flow cytometry using propidium iodide (PI) that stains DNA and fluorescein immunolabeling of the protein Annexin V, which binds in a highly selective manner to phosphatidyl serine; this phospholipid flipping from the inner to the outer leaflet of the plasma membrane during apoptosis [16]. This technique allows to discriminate between necrosis, apoptosis and late apoptosis. Shortly after PDT (4 hr) carried out at 6.4 J/cm^2, less than 5% of cells undergo apoptosis, to reach about 20% after 24 hr (Fig. 3A). Although necrosis was rather stable over the time (<20%), late apoptosis–necrosis significantly increased with time (from 11 to 47%) indicating that PPME is not a strong inducer of apoptosis like other PDT drugs showing affinity for mitochondria [17,18]. As for many other photosensitizers, the ratio between necrosis and apoptosis appears to be dependent on several parameters such as light dose, post-irradiation time, and PPME concentrations.

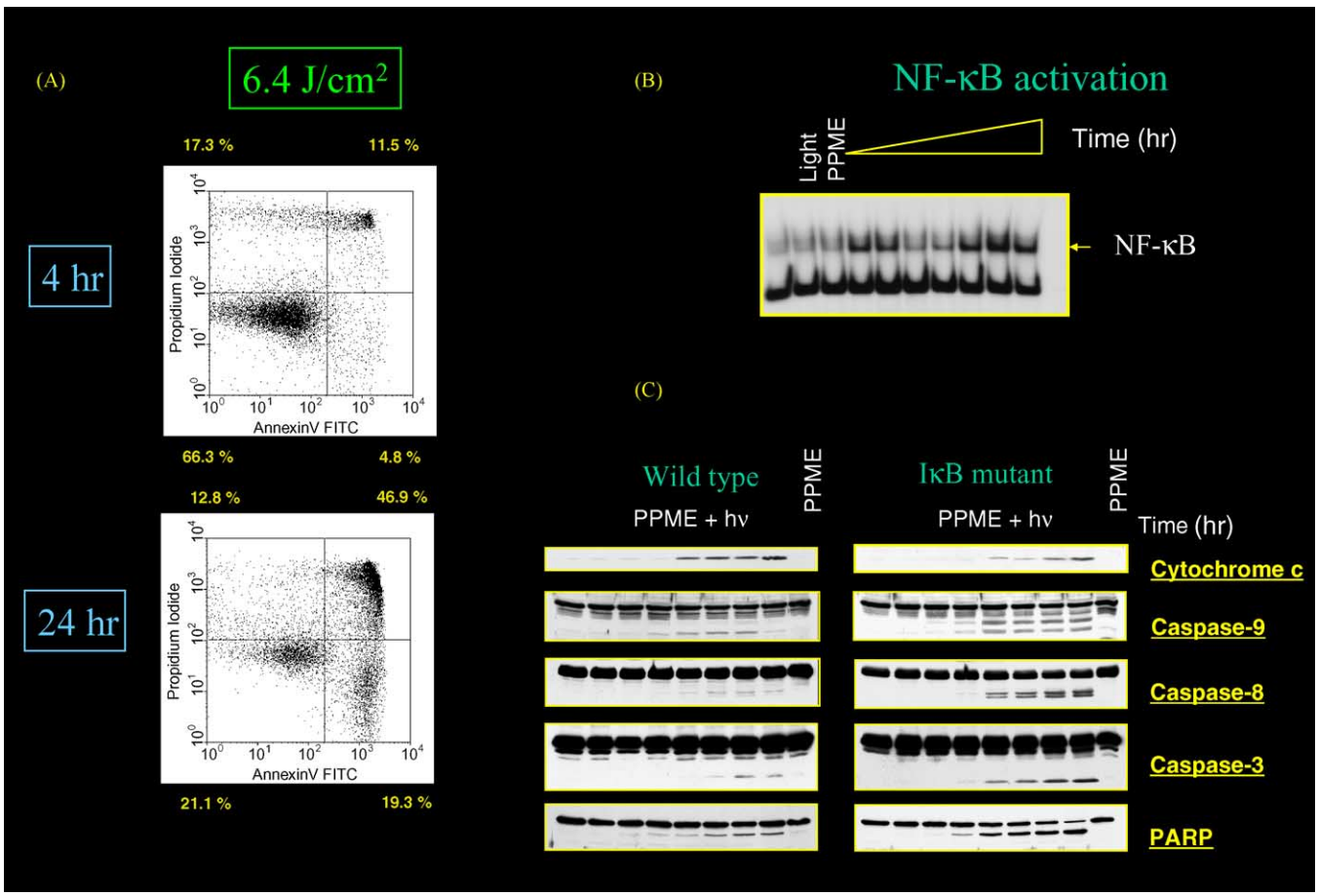

Fig. 3. (A) Determination of necrotic vs. apoptotic cells ratio using Annexin V/PI FACS analysis. HCT-116 cells were incubated with 6 μM PPME, then irradiated with 6.4 J/cm^2 of red light and harvested after either 4 or 24 hr before being stained for AnnexinV/PI and FACS analyser. (B) NF-κB analysis by band shift assay of tumor cells treated by PPME-mediated PDT. The specific NF-κB band is indicated by an arrow. The experimental conditions are the same in (A). (C) Western blot analysis of wild-type and mutated IκBα expressing HCT-116 cells treated by PPME-mediated PDT. Analysis were carried out after 10, 20, 30, 60, 120, 180, 240 and 360 min. The lane PPME refers to cells incubated with PPME but kept non-irradiated.

Apoptosis was also followed by two most common endpoints analysis such as morphological changes (cell shrinkage, condensation of nuclear chromatin, formation of apoptotic bodies) and DNA fragmentation into large fragments (300 and 50 kbp) and then to oligonucleosome-sized fragments (multiples of ~200 bp), which appear as a ''ladder'' of DNA bands upon agarose gel electrophoresis. Although observation of these endpoints is an indicator of apoptosis, quantification of the percentage of apoptotic cells in a population by such an assay is impossible. For this purpose, we also used the TUNEL assay during which fluorescently-biotinylated nucleotides are added to the ends of DNA fragments within fixed cells.

2.2. Apoptosis induced by high dose PDT is mediated by the mitochondrial pathway of caspase activation

The Western blot analysis of pro-caspase levels and their proteolytic processing together with other markers such as the cytoplasmic release of cytochrome *c* and the PARP cleavage has been carried out (Fig. 3C). PPME-mediated PDT gave rise to the appearance of cytochrome *c* in the cytosol. This event occurred 2 hr after PDT and was accompanied by pro-caspase-9 cleavage into active caspase-9. Activation of pro-caspase-3, PARP cleavage and DNA fragmentation occurred in temporal proximity. Pro-caspase-8 cleavage into its active form was observed concomitant with the activation of the other pro-caspases. However, caspase-8 did not appear to have a primary role in the initiation of the apoptotic signal because pro-caspase-8 activation was completely blocked by the caspase-3 specific inhibitor z-DEVD-fmk. Pro-caspase-8 is therefore likely activated downstream of caspase-3 and therefore contributes in a feedback mechanism to further enhance the release of cytochrome *c*. This suggests that PPME-mediated PDT does not lead to apoptosis through the recruitment of the Fas, TNFα or related death receptors, cell surface proteins known to induce apoptosis through mobilization and processing of pro-caspase-8. Moreover, pre-treatment of cells with 10 μM specific caspase-9 inhibitor (LEHD-CHO) completely abrogated caspase-3-like cleavage activity thereby indicating that caspase-9 is likely an initiator caspase for PDT-induced apoptosis.

Model fluorogenic peptide substrates were also used to follow pro-caspase-3 and pro-caspase-9 activation together with inhibitors based on active site peptide motifs. Both a dose- and time-dependence was observed in pro-caspase-3 and -9 activation after PPME-mediated PDT confirming the data generated by Western blot analysis.

2.3. High dose PDT activates the anti-apoptotic NF-κB-dependent pathway

Among the transcription factors that can interfere with apoptosis, NF-κB is one of the most important. NF-κB is known to control the expression of genes involved in inflammation, immune regulation, cell proliferation and apoptosis [19,20]. NF-κB exists as a dimer residing in the cytoplasm in association with an inhibitory subunit, one of several IκB factors. Stimulation by pro-inflammatory cytokines [21] and by PPME-mediated PDT ([22], Fig. 3B) leads to NF-κB activation that is dependent on phosphorylation of two specific serine residues (S32, S36) of IκB, ubiquitination of specific lysine residues (K21, K22), and targeting of the protein for degradation by the 26S proteasome. Upon release of the inhibitory subunit, the remaining dimer can enter the nucleus to promote transcription of genes containing NF-κB binding motifs. The most common NF-κB dimer is composed of a p50 and a p65 (RelA) species, of which there are several variants. Recently, DNA microarray screens reproducibly identified more than 400 NF-κB-dependent target genes [23]. Most of them belong to the following categories: inflammation and immune-like responses, growth and development, growth arrest and apoptosis, proliferation and survival, signal transduction and cell cycle, adhesion and extracellular matrix, metabolic pathways. Since NF-κB appears as a major regulator of cell survival, it was therefore of interest to know whether induction of NF-κB after PPME-mediated PDT exert real anti-apoptotic functions. The key role of Ser 32 and Ser 36 residues in IκBα degradation during the process of NF-κB activation was demonstrated with pro-inflammatory cytokines such as TNF and IL-1 but also in the case of PPME-mediated PDT [22]. This was revealed using a HCT-116 cell line stably overexpressing a dominant negative IκBα mutant form (S32, S36A). In this line, there is no NF-κB activation after PPME photosensitization [22]. Wild-type and mutated HCT-116 cell lines have then been compared for their sensitivity to PPME-mediated apoptosis [24]. The wild-type IκBα expressing cells did not exhibit any difference from wild-type HCT-116 cells in terms of proliferation rate or PPME uptake. However, survival rate was slightly reduced in S32, S36A cells compared to wild-type cells. *In situ* TUNEL labeling performed on both treated cells supported the latter observation. PDT-treated HCT-116 S32, S36A cells displayed a much larger number of TUNEL positive cells as compared to wild-type cells. Similarly, measurement of caspase-3 activity clearly revealed a higher inducibility in the S32, S36A cell line as compared to wild-type cells. When other intermediates involved in PPME-mediated apoptosis were followed, cleavage of pro-caspase-3, -8, -9 and PARP was more pronounced for the HCT-116 S32, S36A cells despite similar cytochrome *c* release kinetics (Fig. 3C). It is possible that caspase activation may be hindered by the NF-κB-encoded inhibitor of apoptosis proteins (IAP) that act downstream of cytochrome *c* release. Similarly, under experimental conditions that led to a lower level of apoptotic cell death (i.e. 3.2 J/cm^2 gave rise to a 50% cell death after 24 hr), differences in apoptosis parameters between

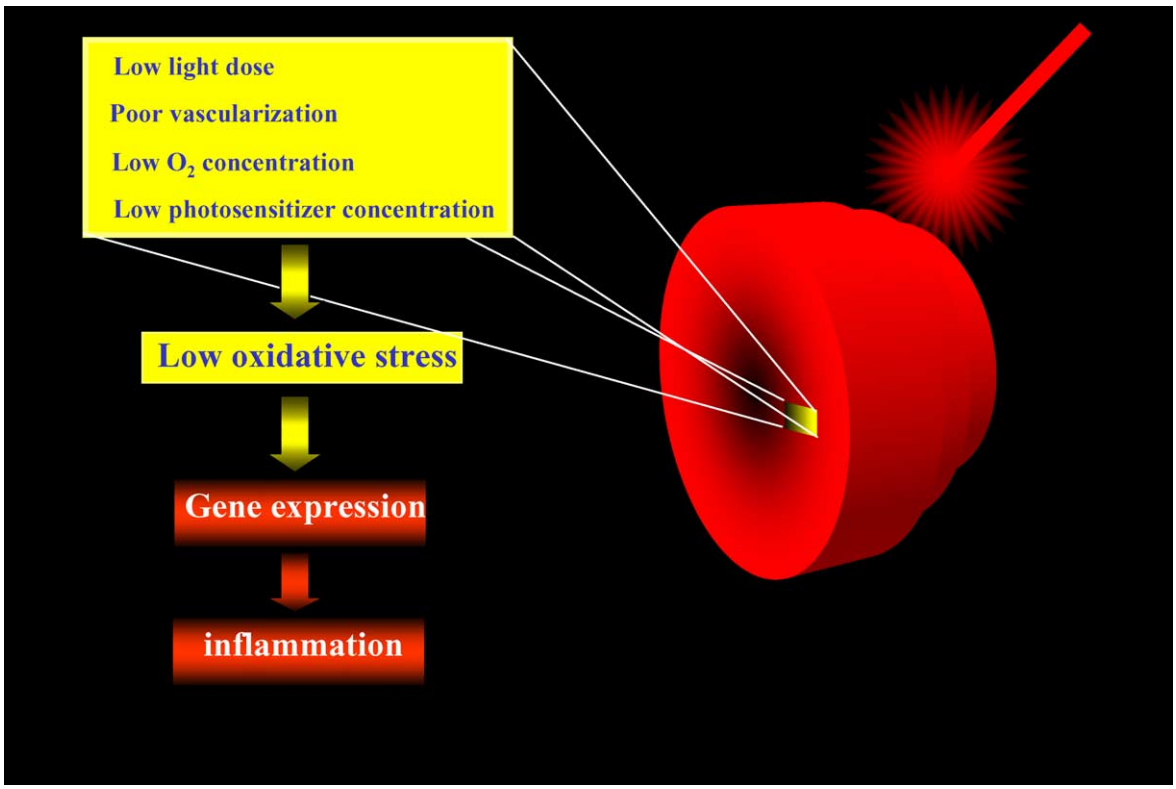

Fig. 4. Experimental conditions where a low dose PDT effect can be observed at the level of a tumor.

these cell lines were also evident. Taken together, these results reinforce the hypothesis for a protective role of NF-κB against PDT-induced apoptosis through a likely activation of anti-apoptotic genes.

3. Low dose PDT

The study of the cellular effect of low dose PDT was carried out on HeLa cells treated with nanomolar concentrations of HYP and fluences that lead to a cell survival higher than 80% (Fig. 4).

3.1. Low dose PDT induces a G_2/M cell cycle arrest

The cellular responses mediated by increasing doses of PDT with HYP in HeLa cells were monitored by FACS analysis using the DNA fluorescent dye Sytox green as well as by morphological and biochemical characterization of the dying cells. Exposure of the cells to PDT with 125 nM HYP and 4 J/cm^2, promptly-induced apoptotic cell death, as reported in previous studies [25–28]. This is documented by the rapid kinetics of PARP cleavage, the time-dependent increase in cells with apoptotic morphology and by the accumulation of cells with a sub-diploid DNA content, which reached 58% 7 hr after irradiation (Fig. 5A and B). Lowering the PDT dose by 60 nM HYP and 4 J/cm^2, substantially retarded the onset of PARP cleavage and apoptosis. Only 48 hr after low dose PDT, 39% of the cells harbored a sub-G1 amount of DNA (Fig. 5A and B) whereas 24 hr after irradiation 80% of the cells survived this low dose PDT treatment and are arrested at the G_2/M border ([28], Fig. 5A).

3.2. Bcl-2 is phosphorylated during low dose PDT

Western blotting with anti-Bcl-2 antibody showed that during low dose PDT the Bcl-2 protein became phosphorylated in a time- and dose-dependent manner as revealed by the mobility shift of the Bcl-2 26 kDa band in SDS–PAGE (Fig. 5B) and by phosphatase treatment [27]. Subcellular fractionation studies indicated that this phosphorylation involves the mitochondria-associated Bcl-2 protein [28]. This transient Bcl-2 modification was specifically associated with the growth arrest imparted to the cells by low dose PDT: it occurred with kinetics matching the G_2/M cell cycle arrest and which preceded the onset of apoptosis (Fig. 5B). At the time of the induction of apoptosis during low dose PDT (48 hr), or when the apoptotic program was promptly promoted by high dose PDT (7 hr), Bcl-2 was fully dephosphorylated (Fig. 5B).

Post-translational modifications of Bcl-2 such as degradation and phosphorylation have emerged as important devices for modulating the anti-apoptotic properties of this protein [29]. In particular, some photosensitizers with a prominent mitochondrial localization have been reported to promote rapid photo-destruction of the Bcl-2 protein,

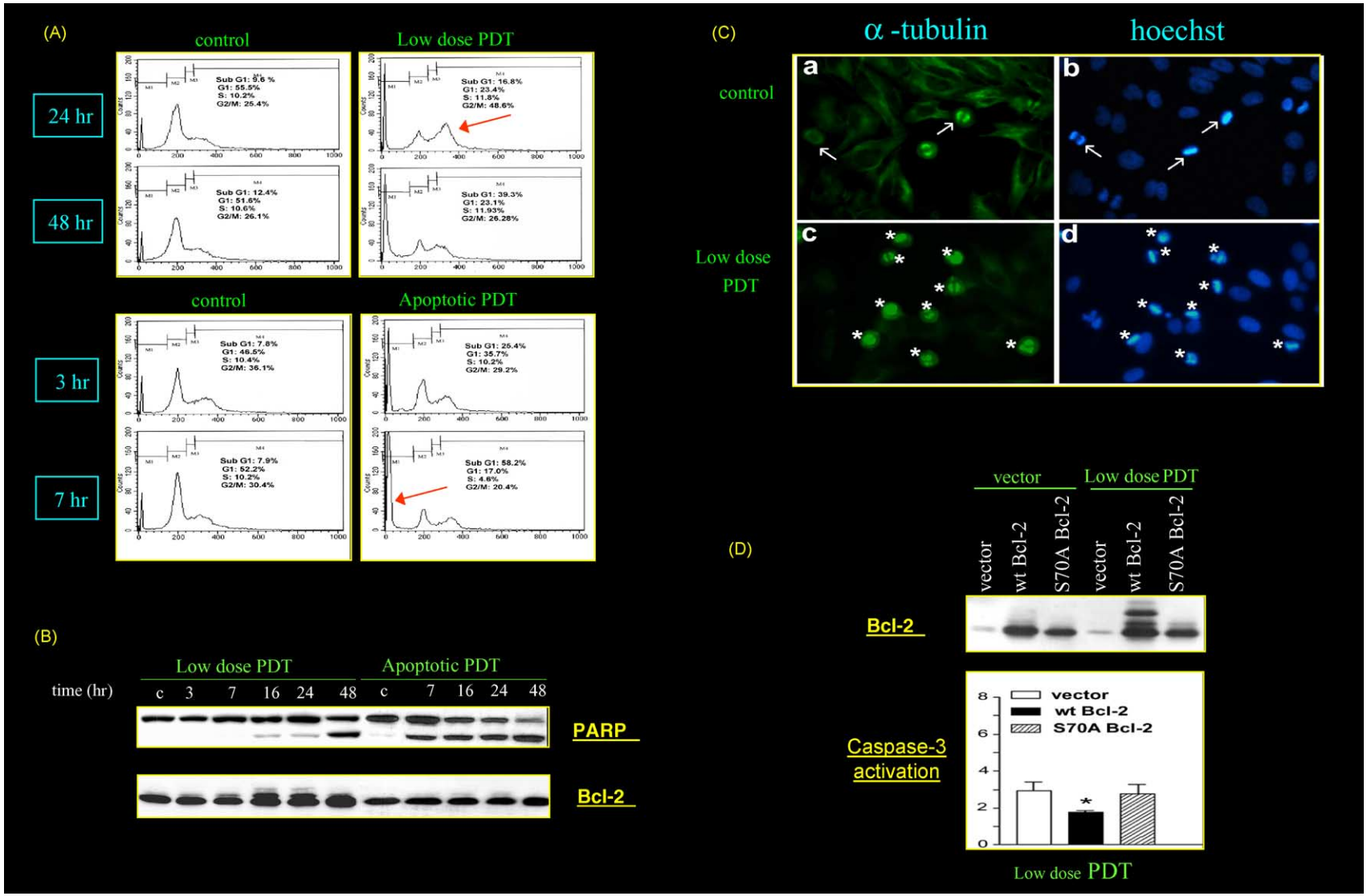

Fig. 5. (A) Low dose HYP-mediated PDT induces a time-dependent cell cycle arrest, whereas high dose PDT induces rapidly apoptosis, as measured by FACS analysis using the dye Sytox green. (B) Western blot analysis of the time-dependent Bcl-2 phosphorylation after low dose PDT. During low dose PDT, Bcl-2 phosphorylation precedes the onset of apoptosis, as monitored by PARP cleavage. In cells exposed to high dose PDT, leading to the prompt activation of the apoptotic program, Bcl-2 is fully dephosphorylated. (C) Hypericin localizes in the perinuclear area in close proximity with the microtubule network as revealed by immunostaining with the α-tubulin antibody. Low dose PDT (16 hr after irradiation) induces the formation of many abnormal mitotic spindles. (D) Serine-70 is a target site for hypericin-induced Bcl-2 phosphorylation. HeLa cells were transiently transfected with either a wt Bcl-2, a Bcl-2 mutant where the Ser^{70} was mutated in a non-phosphorylatable alanine (S70A) or with the empty vector. Caspase-3 activation, measured by DEVD-amc cleavage, was significantly reduced in wt Bcl-2 expressing cells.

with consequent loss of its anti-apoptotic function [30–32]. In our system, however, high dose HYP-mediated PDT in HeLa cells did not cause photo-destruction of Bcl-2 (Fig. 5B), thereby ruling out the possibility that loss of mitochondria-bound Bcl-2 is mechanistically involved with the initiation of the apoptotic program with this photosensitizer (Fig. 5B).

3.3. The G_2/M cell cycle arrest is caused by hypericin-mediated photodamage of the microtubules

A mitotic catastrophe is known to trigger the apoptotic program and this probably represents an in-built safety mechanism to eliminate cells with deregulated cell cycle components. Fluorescent immunostaining analysis of the cells exposed to low dose PDT revealed the presence of a dysfunctional microtubule network, aberrant mitotic spindles with unsegregated chromosomes (Fig. 5C), suggesting that photoactivated hypericin caused an irreversible impairment of microtubule structures. Furthermore, we found that hypericin co-localized with α-tubulin, a major component of the microtubule network, which is in keeping with the hypothesis that microtubules are primary targets of the ROS (e.g. singlet oxygen) locally produced during hypericin light activation.

In this context the cellular effects caused by low dose HYP-mediated PDT are similar to those induced by structurally unrelated anti-microtubule drugs, including paclitaxel, vinblastine and nocodazole [28], given that they all cause mitotic arrest accompanied by Bcl-2 phosphorylation [33,34]. Therefore, it is likely that the Bcl-2 phosphorylation represents a checkpoint for the fidelity of chromosome segregation before cell division may take place.

High dose PDT drives the photodamage over a threshold level that irreversibly impairs not only microtubules but also organelles involved in the cell death/survival decision like mitochondria, thereby bypassing the cell cycle arrest. Indeed, as shown for PPME, the release of cytochrome *c* from mitochondria and the subsequent procaspase-9/-3 activation cascade, are early events in hypericin-mediated high dose PDT in different cancer cells [25–28].

3.4. Bcl-2 phosphorylation during low dose PDT is mediated by CDK1

Bcl-2 phosphorylation has been shown to occur in a proline-rich region called the "loop region", located between the BH4 and BH3 regions (amino acids 32–80), which contains several serine and threonine residues [34–37]. Several protein kinases including Raf-1, PKCα, PKA, the MAPKs (ERKs, p38 MAPK and JNK1) as well as the cell cycle-regulated kinase cyclin-dependent protein kinase 1 (CDK1), have been implicated in these phosphorylations in different systems [34,35]. Although, the functional role of the Bcl-2 phosphorylation is still a matter of debate, this post-translational modification has been suggested to have important consequence for the anti-apoptotic function of Bcl-2. Hence, we found important to evaluate the relevance of the signal leading to Bcl-2 phosphorylation in the cellular responses induced by low dose PDT.

Characterization of the signaling pathways involved in mediating the Bcl-2 phosphorylation, indicated that members of the MAPKs were activated, albeit to a different extent, during low dose PDT. In particular, the kinetics of JNK1 activation correlated well with the accumulation of the cell population in the G_2/M phase of the cell cycle and the timing of Bcl-2 phosphorylation. However, specific blockage of the ERK, p38 MAPK or JNK1 pathways or increased expression of p38 MAPK or JNK1, did not affect the extent of Bcl-2 phosphorylation in photosensitized cells suggesting that MAPKs were not involved in the PDT-mediated Bcl-2 phosphorylation. Also, blockage of the PI3-K-, PKCs- or TOR-mediated signals by pre-treating the cells with wortmanin, bis-indolylmaleimide or rapamycin, respectively, did not significantly affect the level of Bcl-2 phosphorylation after hypericin-mediated PDT [28].

In accordance with the PDT-induced increase in the cellular fraction with G_2/M DNA content we found a remarkable temporal correlation between Bcl-2 phosphorylation, cyclin B1 upregulation and CDK1 activation, suggesting that this protein kinase could be involved. This conclusion was strengthened by the following observations: (i) Bcl-2 phosphorylation is blocked by cell pre-treatment with inhibitors, such as roscovitine, that specifically down-regulate CDK1, without affecting other signaling pathways; (ii) CDK1 overexpression increases the level of phosphorylated Bcl-2; (iii) during high dose (apoptotic) PDT, which is associated to the sustained activation of p38 MAPK and JNK1, Bcl-2 is not modified and CDK-1 is not activated; (iv) a pool of CDK1 co-localizes with phosphorylated Bcl-2 in the mitochondrial fraction [28]. Hence, taken collectively these observations suggest that the phosphorylation of Bcl-2 results from the activation of the CDK-1/cyclin B1 pathway following low dose HYP-mediated PDT.

3.5. Bcl-2 phosphorylation at Serine-70 delays the onset of apoptosis

Identification of the phosphorylation site modified in Bcl-2 during low dose PDT was investigated by substituting Ser^{70}, one of the major Bcl-2 target [33,36–38], with a non-phosphorylatable alanine and by overexpressing this Bcl-2 mutant protein in HeLa cells. The mutation of Ser^{70} with Ala abolished Bcl-2 phosphorylation in photodamaged cells (Fig. 5D), indicating that this is the major, if not the unique, Bcl-2 phosphorylated site following low dose HYP-mediated PDT.

As suggested in two recent reviews [34,35], it is very likely that the physiological role of the Bcl-2 phosphorylation may be influenced by the phosphorylation status of additional sites, besides Ser^{70}, which are located in the ''loop-region'' of the Bcl-2 protein. This would explain why certain cellular stresses, such as interleukin-3 and growth factor withdrawal, which induces phosphorylation of Bcl-2 mostly at Ser^{70} (mono-site phosphorylation), result in an enhancement of the cytoprotective function of the protein while others, such as taxol, inducing multi-site phosphorylation of Bcl-2 (Ser^{70}, Ser^{87}, Thr^{69}), inactivate its anti-apoptotic function [37].

In our system the blockage of Bcl-2 Ser^{70} phosphorylation by the overexpression of a non-phosphorylatable Bcl-2 mutant protein, increased the activation of the executioner pro-caspase-3 in hypericin photosensitized cells (Fig. 5D). This observation suggests that the Bcl-2 Ser^{70} phosphorylation in low dose PDT increases the cytoprotective activity of the protein.

Collectively taken, these results suggest that in response to the hypericin-induced photodamage of microtubules, cells are unable to proceed into mitosis and arrest at the metaphase/anaphase checkpoint; this process is associated with a CDK1-mediated phosphorylation of the mitochondria-bound Bcl-2 protein on Ser^{70}. This Bcl-2 phosphorylation in turn would temporary increase the cytoprotective function of Bcl-2 and delay apoptotic cell death following low dose PDT, likely until a futile attempt by the cell to repair the damage has taken place.

4. Conclusions

From the data presented above, we can conclude that the cellular response to PDT vary with the overall dose of PDT (e.g. sub-lethal vs. lethal). Apoptosis and necrosis that are observed at lethal and/or supra-lethal dose of PDT are pure post-transcriptional events that occurred within a few hours after the treatment. The balance between apoptosis and necrosis appears to be dependent on the dose of PDT but also on many other features not discussed in this paper such as the cell type, its genetic and metabolic potential, the nature of the photosensitizer but also its sub-cellular localization. These considerations also apply to cell growth-arrest occurring at low dose of PDT. Importantly, signal transduction pathways involving cyclin-dependent kinase(s) plays an important role in arresting cells and delaying apoptosis. Many other signaling pathways are known to be activated by PDT; some of them providing a protective function such as the one controlled by NF-κB, others being pro-apoptotic. Because many of these signaling pathways obviously influence the cell fate after PDT, a major focus for future works will be to delineate these pathways and to understand how to modulate them with the hope to provide a better tumor cell elimination by PDT.

Acknowledgments

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