Apoptosis Following Photodynamic Tumor Therapy: Induction, Mechanisms and Detection

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Abstract: As a treatment modality for malign and certain non-malignant diseases, photodynamic therapy (PDT) involves a two step protocol which consists of the (selective) uptake and accumulation of a photosensitizing agent in target cells and the subsequent irradiation with light in the visible range. Reactive oxygen species (ROS) produced during this process cause cellular damage and, depending on the treatment dose / severity of damage, lead to either cellular repair / survival, apoptotic cell death or necrosis. PDT-induced apoptosis has been focused on during the last years due to the intimate connection between ROS generation, mitochondria and apoptosis; by this PDT employs mechanisms different to those in the action of radio- and chemotherapeutics, giving rise to the chance of apoptosis induction by PDT even in cells resistant to conventional treatments. In this review, the (experimental) variables determining the cellular response after PDT and the known mechanistic details of PDT-triggered induction and execution of apoptosis are discussed. This is accompanied by a critical evaluation of wide-spread methods employed in apoptosis detection with special respect to *in vitro* / cell-based methodology.

Key Words: Photodynamic therapy, anticancer treatment, apoptosis, cell death, apoptosis detection methods

1. INTRODUCTION

Reactive oxygen species (ROS) represent a cellular stress factor that can – if produced over a certain level of quantity – effectively induce the active mode of cell death, apoptosis [1-3]. This sensitivity of cells towards ROS overproduction can be used for the removal of harmful or unwanted cells. Photodynamic therapy (PDT) utilizes this effect to treat several types of early stage tumors and other diseases [4-7]. Typically, PDT involves a two step protocol; firstly, a light-absorbing compound (the photosensitizer) is taken up (mostly) specifically by target cells [8-12], secondly, the sensitizer is activated by irradiation with visible light of the appropriate energy [13-15]. Since the light penetration depth in tissue increases with the wavelength, an absorption maximum at the highest possible wavelength (red / near infrared) is taken for activation of the sensitizer [16-20].

Upon absorption of a photon the photosensitizer is energized into a high-energy singlet state, from which it may change into the triplet state by intersystem crossing. The triplet state of these molecules is relatively long-lasting ($\geq 10^{-3}$ seconds [21]) and can exert the transfer of an electron to adjacent molecules (preferably oxygen; referred to as a type I photochemical reaction) or energy to molecular oxygen (type II photochemical reaction) [13]. The transfer of an electron to molecular oxygen produces the superoxide anion, which can form hydrogen peroxide H_2O_2. Due to its ability to diffuse through membranes, the latter might be toxic for neighbouring cells as well. By taking up another electron, H_2O_2 can split up into two hydroxyl radicals – the most dangerous member of the ROS family (E_0= +1.35V) – which can attack and oxidize any compound of biological origin; additionally, these processes are facilitated by low activation energies [22-24]. In simple chemical systems, especially if porphyrins are used as photosensitizers, PDT has been shown to predominantly exert its effect by type II photochemical reaction. The latter generates singlet oxygen, a highly reactive molecule, which reacts with many biomolecules [25-28]. The operational sequence of PDT is outlined in Fig. (**1**).

The overall response of cells to PDT depends on external parameters, such as the incubation protocol or the light dose applied and on internal parameters, subsumed under 'cellular *susceptibility*' towards PDT. The outcome of such treatment can be classified as either (i) repair and survival of target cells, (ii) apoptosis or active cell death or (iii) necrosis or passive cell death, the latter causing leakage of cell content into tissue and subsequent inflammatory processes (reviewed in [29]). Since PDT has been shown to be a potent inducer of apoptosis [26, 30-33] and in some cases can trigger active cell death even in cells, which have turned out to be unable to undergo apoptosis after chemo- or radiotherapy [34], this review shall focus on the mechanisms, which lead from the excited photosensitizer to the induction and execution of apoptosis. The last chapter shall deal with detection methods for apoptosis and – at least to a certain level – weight against their respective benefits and drawbacks.

1.1. Active Cell Death: Key Factors, Mechanisms and Implications on Cells and Tissues

The increasing interest in apoptosis is based on two facts. Firstly, in many cases, apoptosis can be found at lower doses of medical treatment than those required to elicit necrosis [35, 36]. The so-called 'apoptotic window' (we shall use this term in the following as an indicative of a certain range of treatment doses which causes apoptosis) can be found 'in

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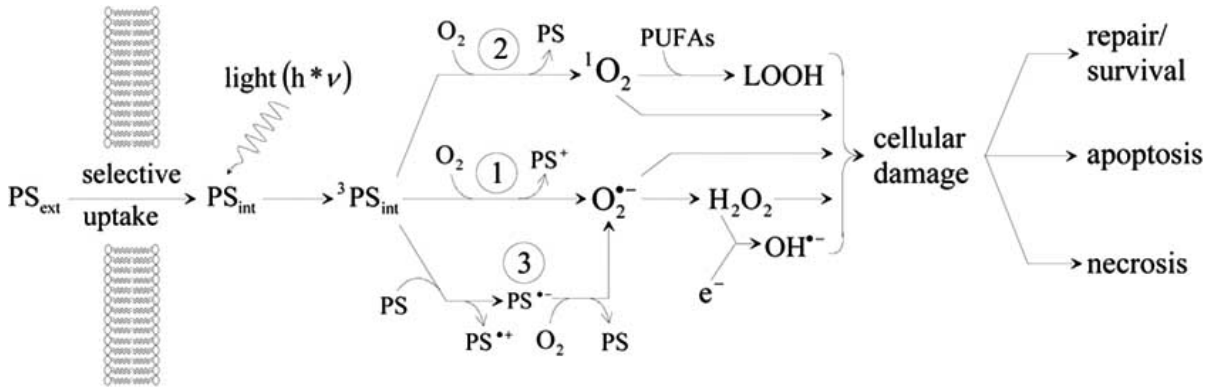

Fig. (1). Schematic representation of the principle of photodynamic treatment. PDT involves the (selective) uptake of a photosensitizing agent (PS) from the extracellular space (PS_{ext}) into target cells (PS_{int}) and the subsequent activation by irradiation with visible light (h**v*). In the following, the activated sensitizer ($^3PS_{int}$) can either (1) transfer an electron to a nearby target molecule amid formation of the superoxide anion (ROS, type I photochemical reaction) or (2) energy to adjacent molecules yielding singlet oxygen (type II photochemical reaction) or (3) activate surrounding photosensitizing molecules. Superoxide anions may be transformed into the cell permeant H_2O_2, which - in turn - can split up into hydroxyl radicals. Singlet oxygen can induce a chain reaction of lipids creating long-living peroxyl radicals and thus lead to an amplification of ROS production and subsequent photodynamic damage. Activated photosensitizing molecules form superoxide radicals as well. The cellular damage induced by these reactive species may be repaired or lead to cell death via apoptosis or necrosis.

between' survival and necrosis. This 'apoptotic window' comprises a dose range where the damage is too serious to allow repair of the cell, but not severe enough to endanger the cellular ion homeostasis, a process, which usually leads to water influx and a loss of membrane integrity, both being typical of necrosis (see Fig. (**2**)) [37, 38]. Secondly, apoptosis has been described to have a rather anti-inflammatory effect, which is advantageous for cancer patients compared to massive necrosis and subsequent severe inflammation. However, the effects of apoptosis on the immune systems are being discussed at present, and the paradigm 'necrosis causes inflammation, apoptosis is anti-inflammatory' may be modified in close future [39-43].

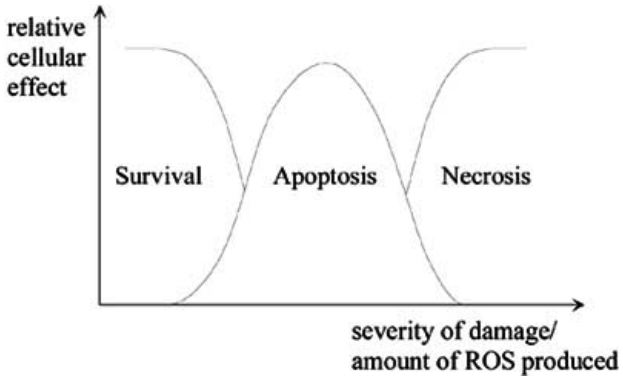

Fig. (2). Dependence of the cellular response on the increasing degree of PDT-induced damage. The basic modes of cellular response are dose-dependent and with increasing amount of ROS produced a transition from survival to apoptosis and finally to a necrotic response is usually observed.

While the mechanisms of apoptosis induction vary and are triggered via internal and external factors, the execution of apoptosis represents a typical process, which is – at least in many cases – not influenced by the initiation process itself (for a detailed review on apoptosis see e.g. [44, 45]). Apoptosis is morphologically characterized by cell shrinkage and other distinctive changes such as nuclear chromatin condensation and segregation of the cell into apoptotic bodies, the latter preventing leaking of cell content (enzymes etc.) into tissue [46]. Among the characteristics of active cells death named above, other features involve changes in cellular biochemistry (e.g. the activation of a distinct class of proteases) and membrane structure; these hallmarks shall be discussed in the following sections.

Mitochondria: Central Organelles in Active Cell Death

Research during the last decade revealed a great range of apoptotic stimuli from inside or outside the cell to converge in mitochondria for further signal processing and execution. The intermembrane space of these organelles contains several proteins, which are centrally involved in apoptosis, among them cytochrome c, the apoptosis inducing factor (AIF), Smac/Diablo (second mitochondrial activator of caspases), Omi/Htr2A (high temperature requirement A) and inactive pro-forms of caspases [43]. Pores in the inner and/or outer mitochondrial membrane (IMM and OMM) execute the release of these proteins and fire off the apoptotic process. The pore-opening is further controlled by proteins of the Bcl-2 family (B-cell lymphoma 2, see below) [47-51].

Caspases: Inducers and Executers of Active Cell Death

Cysteinyl aspartic acid specific proteases (caspases) are hydrolytic enzymes centrally involved in the apoptotic process. The whole set of caspases is present in any cell in so-called pro-forms (zymogens, procaspases). Cleavage of the latter by autocatalysis or by other caspases activates their catalytic functions. Based on the fact that caspases may activate each other by proteolysis, it stands to reason that a cascade of caspases might be engaged during apoptosis (see also Fig. (**3**)). The most common activation pathway involves the processing of caspase 9 from procaspase 9 by the apoptosome, an enzyme complex which is compiled from cytochrome c, APAF-1 (apoptosis protease activation factor-

1) and (d)ATP ((deoxy-) adenosine-5´-triphosphate). Caspase 9 activates caspases 3, 6 and 7 ('effector' caspases) which attack several key enzymes on the metabolic map and manifest the morphological, biochemical and energetic changes typical of active cell death (a comprehensive list of physiological caspase substrates can be found in [52]). Caspases 2 and 8 (among others referred to as 'initiator' caspases) are involved in signal transmission for receptor-induced apoptosis (e.g. via the Fas- (CD95) or TNF receptor (tumor necrosis factor)) and can directly activate caspase 3. However, a cross-talk exists to pro-apoptotic members of the Bcl-2 family, which renders caspase 8 mediated apoptosis sensitive to mitochondrial control: caspase 8 cleaves Bid (a proapoptotic member of the Bcl-2 family), yielding tBid which translocates to mitochondria where it is assumed to be involved in mitochondrial pore opening and apoptosis execution [53-57].

The Conflictive Bcl-2 Protein Family

The proteins of the Bcl-2 family can regulate the onset of active cell death in various ways. Most of them act at the level of mitochondria by either advantaging or hampering pore formation (or opening). Bad, Bak and Bid represent pro-apoptotic proteins and are involved in the signal transmission from caspase 8 to mitochondria. At least Bax is known to be a component in some models of mitochondrial pores [58-60].

The anti-apoptotic proteins Bcl-2 and Bcl-X_L block pore-opening and cytochrome c release. Other hypotheses suggest formation of pores by Bcl-2 and Bcl-X_L and thus a proapoptotic function [61]. The regulation of pore opening remains unclear, but the ratio of pro- to anti-apoptotic Bcl-2 family members might be involved in the decision between survival and apoptosis [61-64]. For a recent overview on functions of Bcl-2 proteins, see [65].

Cell Surface Death Receptors

Receptors on the outer surface of the plasma membrane play an important role in the initiation of active cell death, especially in the effector functions of cellular immunity [66-68]. Two well described receptors are the Fas receptor (also known as Apo-1 or CD95) and the TNF receptor family. Binding of ligands to the respective receptor activates caspase 8 through the death inducing signalling complex (DISC, comprised of several adaptor molecules such as FADD (Fas associated death domain)) and may facultative involve mitochondrial control (the Bid crosstalk, see above and Fig. (**3**)). Several (parallel?) mechanisms of signal processing and transduction seem to exist in Fas- and TNF-triggered apoptosis that have been reviewed in [69-71] and [72-74], respectively.

Apoptosis and Cellular Energetics

The execution of apoptosis is an active process *per definitionem* and thus requires energy in form of equivalents of phosphorylation power. Key steps in the apoptotic cascade directly depend on the availability of (d)ATP or GTP (guanosine-5´-triphosphate). So, for example, the formation of the apoptosome from APAF-1 and cytochrome c [75, 76], the transport of pro-apoptotic factors into the nucleus [77], the condensation of chromatin and the formation of apoptotic bodies [78] as well as morphological changes [79] require large amounts of nucleoside triphosphates. The decisive role of the intracellular ATP level was elucidated in several publications, where a minimum ATP level necessary for apoptosis execution was defined [80-82]. In accordance with these we have shown the intracellular ATP level to remain close to control levels until late in apoptosis, when active cell death was triggered by aluminium (III) phthalocyanine tetrasulfonate-based PDT ($AlPcS_4$-PDT) in A431 cells (human epidermoid carcinoma) [36]. Data from a subsequent publication of our group suggested glycolytic ATP formation to compensate for mitochondrial malfunction: the mitochondrial membrane potential – the most important driving force for ATP production – decreases early in the apoptotic process probably due to pore-opening in mitochondria. Cells grown in media without glucose, but with mitochondrial substrates (pyruvate for citric cycle / oxidative phosphorylation) failed to undergo apoptosis due to the lack of ATP; the cell death mode shifted towards necrosis [83]. This finding is substantiated by a recent publication by Kirveliene *et al*. [84]. Taken together, these results are indicative of the fact that apoptosis, as an active cellular process, not only requires minimal cellular energy levels but furthermore is characterized by active mechanisms that ensure sufficient energy supply.

2. PARAMETERS INFLUENCING THE CELL DEATH MODE AFTER PDT

As PDT is known to effectively induce apoptosis, the occurrence of this mode of cell death, however, depends crucially on the severity of damage set in the cell and on the primary target of the photosensitizing agent. Apoptosis represents, as remarked above, a 'window' phenomenon: for any photosensitizer / cell type combination, a certain range of treatment parameters might exist, where cells die mainly by active cell death (according to Fig. (**2**)). This 'apoptotic window' may differ in its dimension, but it is to be found at treatment conditions in between survival and necrosis of the target cells, the latter causing an irreversible damage of cells [85]. The 'apoptotic window' for a given model system depends on several parameters. Among these, the treatment protocol, namely the incubation period and the concentration of the photosensitizing agent as well as the irradiation parameters represent factors, which can be influenced by the experimenter. Both account for the severity of damage set in the target cells. The incubation protocol determines the effective intracellular concentration of the photosensitizing agent; longer incubation periods and / or higher sensitizer concentrations result, in most cases, in higher intracellular levels up to a saturation value [85-87]. The dynamics of ROS produced (and therefore the damage) is further influenced by the irradiation parameters: light fluence [light energy per area] and light power density [light power per area]. Some authors report the oxygen available for ROS formation to be a limiting factor for ROS production at high power densities; accordingly, this limits the damage as well [88-90].

Not only the effective concentration and the irradiation influence the cell death mode, but also the chemical properties of the photosensitizing agent, since it determines both, the quantum yield and therefore the amount of ROS produced per mole photosensitizer and photons, but also the

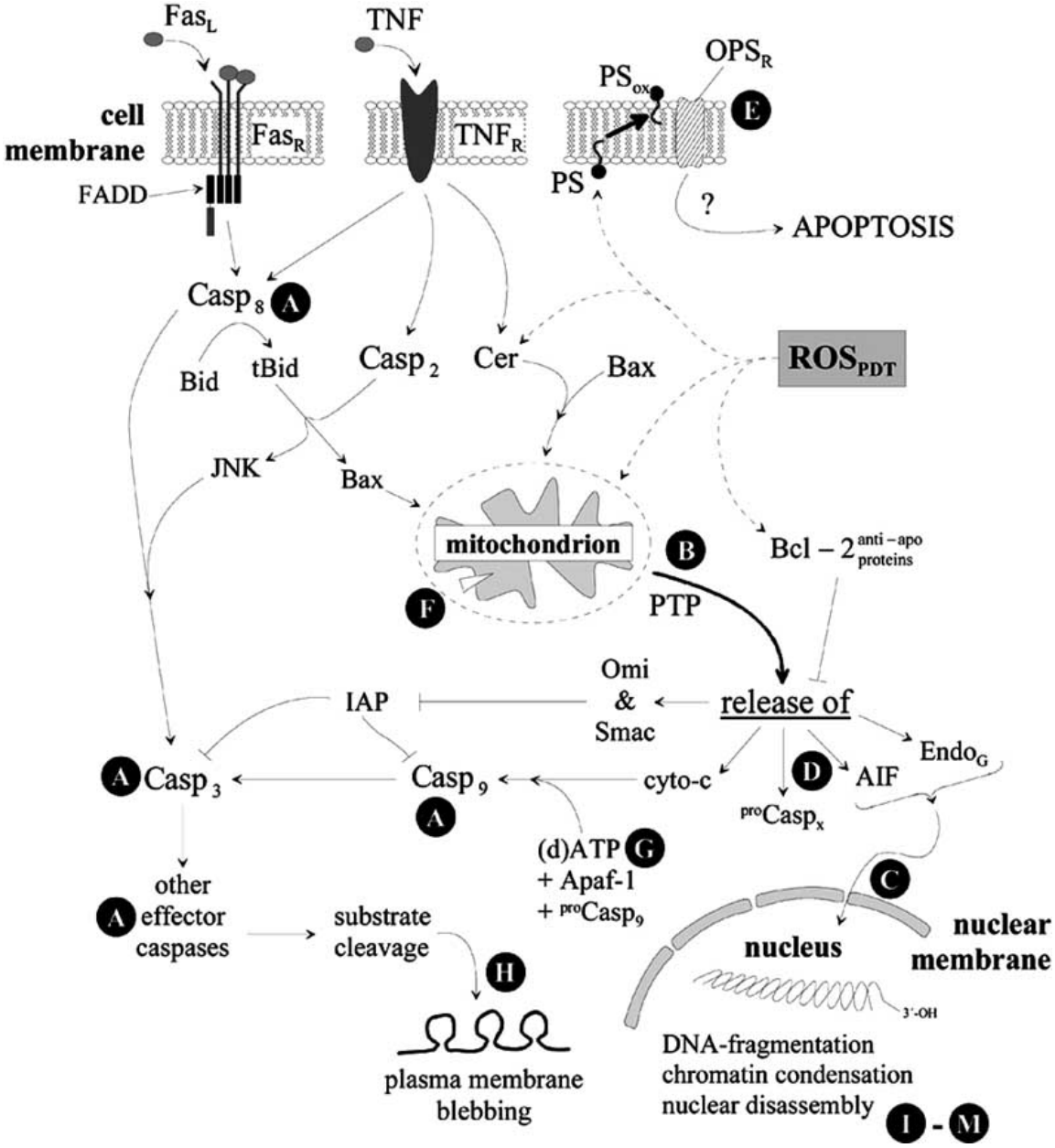

Fig. (3). Apoptotic pathways and -detection methods.

In the frame of the models of apoptosis induction (by PDT) the following methods can be applied for characterization and / or quantification of apoptotic cell death: the activity of several caspases is independently assessed by the use of synthetic fluorigenic (or colorimetric) caspase substrates (A, *fluorescence measurement* of reactions based on cell lysates or *flow cytometry*); mitochondrial membrane permeabilization may be visualized by calcein-AM stainings (B, *fluorescence microscopy*); translocation of EndoG and AIF can be demonstrated by immunochemical staining (C, visualization of protein localization by specific (fluorescent) antibodies – *fluorescence microscopy*); the latter technique is also applicable with proteins released from mitochondria (D; alternatively, subcellular fractions may be probed with antibodies by *Western blotting*); exposure of (oxidized) phosphatidyl serine (PS) is determined by staining with fluorescence-labelled Annexin-V antibodies (E, *fluorescence microscopy* or *flow cytometry*); energetic changes during apoptotic cell death such as breakdown of the mitochondrial membrane potential ($\Delta\psi$) and the intracellular ATP level are characterized by fluorescent stains (e.g. JC-1) and luciferase-based luminometric methods, respectively (F and G / *fluorescence microscopy* or *flow cytometry* and *luminometry*, respectively); formation of characteristic blebs (apoptotic bodies) of the cell membrane can be verified by *light* or *electron microscopy* (H).

The following apoptotic changes in the nucleus are accessible experimentally i) chromatin condensation and nuclear fragmentation are visualized by DNA-staining fluorescent dyes (I, *fluorescence microscopy*), ii) reduction of nuclear DNA content during fragmentation is shown by flow cytometry analysis (J, subG_1-assay), iii) DNA fragmentation can be further demonstrated by gel electrophoresis (K) or the COMET assay (L), and iv) the occurrence of free 3´-OH groups (a consequence of DNA fragmentation) can be detected using the TUNEL assay (M). For methodological details and appropriate references see text.

cellular localization and thus the primary target of PDT [18, 19, 91]. The threshold concentration for complete cell killing therefore depends on the chemical properties as well. So, for example, we could find 100% cell kill of A431 cells by apoptosis / necrosis with 10 μM $AlPcS_4$ (a hydrophilic sensitizer) and fluences >4 $J.cm^{-2}$ (660 nm). At similar fluences (at 610 nm), comparable cell kill could be found at concentrations of 0.09 μM hypericin (a lipophilic sensitizer from extracts of *hypericum perforatum*), about three orders of magnitude lower concentration than $AlPcS_4$ (unpublished results). In order to increase the efficiency of PDT, a wide spectrum of photosensitizing agents with different chemical properties has been successfully established during the last 25 years. They range from mainly lipophilic (such as hypericin, Photofrin and meso-tetrahydroxy phenylchlorine (mTHPC)) to hydrophilic (for example $AlPcS_4$). Due to their chemical structure, lipophilic dyes preferably localize in membrane systems, such as the plasma membrane, the ER (endoplasmatic reticulum) and Golgi systems, but also in mitochondria. On the contrary, hydrophilic dyes accumulate in the cytoplasm and in lysosomes [16-19, 91]. Among all photosensitizers, δ-aminolevulinic acid- (ALA) induced endogenous protoporphyrin IX (PPIX, precursor in biosynthesis of heme) should be mentioned: an excess of ALA abolishes the negative feedback regulation of heme on its biosynthesis pathway and produces high concentrations of PPIX that can be employed as an endogenous photosensitizing agent [92-94]. The synthesis of PPIX runs in mitochondria and, as a consequence, ALA-derived PPIX will be selectively found in mitochondria, at least after short incubation periods [95]. The intimate connection between sensitizer localization and location of the primary damage is based on the short lifetime of ROS, which are responsible for the short diffusion lengths of these reactive molecules. Accordingly, Moan *et al*. have estimated the intracellular diffusion distance to be smaller than 20 nm [96]. Thus, the chemical properties clearly determine the area of primary damage: dyes localizing in the plasma membrane will therefore more likely cause a loss in membrane integrity (and subsequent necrosis) than dyes localizing in the cytoplasm, in mitochondria or other organelles [30, 97-100]. In the special case of ALA-induced PPIX, ROS formed in mitochondria have been shown to effectively induce apoptosis [101-104]. Consequently, in some cases, ROS formation by PDT in or in proximity of mitochondria has been shown to induce apoptosis even in cells, which are otherwise resistant to apoptosis induction by conventional tumor treatment [34].

3. MECHANISMS OF PDT-INDUCED APOPTOSIS

The phenomenon of apoptosis induction by PDT has been depicted in an excellent review by Oleinick *et al*. [33]. Some possible mechanisms of how damage set by PDT triggers apoptosis (illustrated in Fig. (**4**)) shall be discussed in the following chapters.

3.1. Mitochondria as Primary PDT-Targets

Overproduction of ROS in Mitochondria and Its Direct Effects on Mitochondrial Pore Opening

Following a theory of Skulachev, which has been suggested in three outstanding review articles [3, 24, 105], apoptosis represents an evolutionary old mechanism to eliminate ROS-overproducing cells, which pose a threat to the cellular community (tissue). Indeed, some of the key proteins (cytochrome c in the cytoplasm or the AIF) seem to play an important role in the cellular antioxidant defence. The primary role of these proteins might be to quench ROS in the cytoplasm. If the radicals exceed a certain level of quantity, the same proteins in the cytoplasm fire off active cell death [3, 24, 105].

PDT-produced ROS are likely to cause the same effect. For detailed mechanisms interrelating ROS production and the mitochondrial pore opening, several models have been suggested up to now (see Fig. (**4**)): oxidation of a critical cystein residue (cys^{56}) under conversion of sulfhydryl groups into disulfide bridges has been shown to transform the specific adenine nucleotide transporter (ANT, which imports ADP and exports ATP to the cytosol) into an unspecific pore permeable for solutes with less than 1.5 kDa [106, 107]. As a result, gradients of low molecular weight molecules between the matrix and the cytosol disappear. Normally, mainly concentration gradients of K^+ and Cl^- regulate the osmotic pressure in the matrix, which are lost during pore opening. Now the matrix protein concentration, which is supposed to be higher than in the cytosol, is responsible for matrix osmolarity which, in turn, causes water influx and matrix swelling. The inner mitochondrial membrane is folded (cristae structure) and unfolds as a consequence of volume increase. Since the area of the outer membrane is smaller, its rupture leads to the release of pro-apoptotic proteins (e.g. cytochrome c and the AIF) from the intermembrane space into the cytoplasm which start the apoptotic cascade [108-111]. The effect of ROS-induced pore formation involving the ANT could be inhibited by dithiotreitol (DTT), a disulfide reductant [112]. Peroxidation of lipids surrounding the ANT is suggested to bring about the same effect [113, 114]. Damage of lipids in the mitochondrial membranes might cause an initial drop of the mitochondrial membrane potential (Δψ). This primary reduction in Δψ increases the probability of opening of the voltage dependent anion channel (VDAC) in the outer mitochondrial membrane due to the voltage-sensitivity of this channel [115-117]. Pore-opening via the VDAC allows escape of pro-apoptotic proteins and initiates the apoptotic cascade.

The essential participation of mitochondrial pores in PDT-induced apoptosis is also substantiated by findings which indicate that inhibitors of pore opening and -formation, such as cyclosporine A (CsA) and bongkrekic acid (BA) can cancel out apoptosis triggered by compounds that accumulate in mitochondria. So, for example, verteporfin has been shown to target the ANT and set off pore formation and Δψ loss in isolated mitochondria. This effect could be inhibited by CsA [118]. Singlet oxygen generated in mitochondria by Pc 4 PDT (phthalocyanine 4) on A431 cells caused a rapid permeabilization of the inner mitochondrial membrane, mitochondrial depolarization and cytochrome c release, as shown in a study by Lam *et al.* [117]. However, in clear contrast to these studies, irradiation of mitochondria loaded with hematoporphyrin rather prevented mitochondrial pore opening by a mechanism of site-selective inactivation of discrete pore functional domains [119].

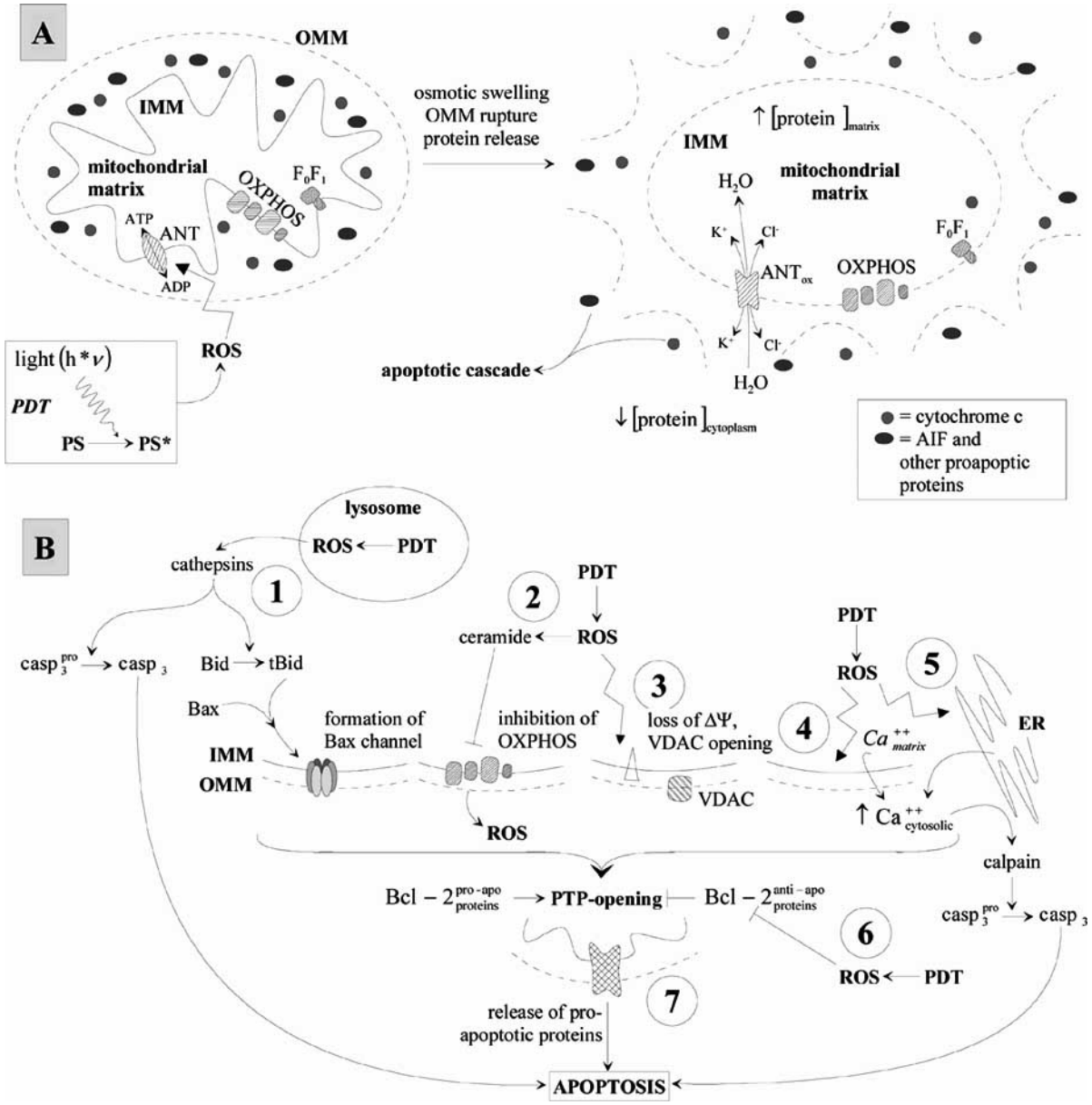

Fig. (4). Possible mechanisms of apoptosis induction by PDT.

A) PDT-generated ROS cause oxidation of a critical cystein residue the adenine nucleotide translocator (ANT) which thereby is transformed into an unspecific pore permeant for solutes less than 1.5 kDa (e.g. equilibration of the membrane gradient of chlorine and potassium ions). This causes osmotic water uptake due to the higher concentration of high molecular mass compounds in the mitochondrial matrix. As a result, the inner mitochondrial membrane (IMM) stretches causing rupture of the outer membrane (OMM). Pro-apoptotic proteins (cytochrome c and the apoptosis inducing factor (AIF) for example) are being released into the cytoplasm and initiate the apoptotic cascade. Oxidation of lipids surrounding the ANT might exert the same effect.

B) Detailed models of mitochondrial pore opening via PDT-produced ROS involve the release of cathepsins from lysosomes; the latter can either directly activate caspase 3 or cleave Bid (tBid) and induce mitochondrial pore opening via Bax (pathway indicated by 1). In the proximity of mitochondria, ceramide can be formed by ROS (pathway 2). This leads to inhibition of the oxidative phosphorylation, single electron transfer to oxygen and further (amplificated) formation of superoxide anions. The latter are potent inducers of mitochondrial pore opening. A decrease of the mitochondrial membrane potential due an ROS-induced loss of membrane integrity of the inner membrane leads to opening of the VDAC due to the voltage-sensitivity of this channel (pathway 3). An increase of mitochondrial Ca^{2+}as a consequence of ROS formation causes membrane destabilization, pore opening and apoptosis (pathway 4). Calcium released from the ER following PDT can either directly diffuse into mitochondria and induce pore opening via pathway 4 or directly activate caspase 3 through the calpain pathway (pathway 5). The anti-apoptotic protein Bcl-2 has been shown to be a target of PDT-formed ROS. Oxidation of Bcl-2 leads to pore opening (pathway 6). All these pathways converge into opening of more or less well defined mitochondrial pores which, in turn, allow escape of pro-apoptotic proteins (7). For references see text.

Effects on Ceramide as Inducers of Mitochondrial Apoptosis

Ceramide (a sphingolipid) is known to play an important role in many initiation pathways of active cell death [120-122]. Separovic *et al*. reported PDT with Pc 4 to result in elevated ceramide levels in L5178Y-R cells and subsequent onset of active cell death [123, 124]. These findings were substantiated by Nagy *et al*. in A431 cells using the same photosensitizing agent [125, 126]. Wispriyono *et al*. also provided biochemical and genetic evidence of the role of the de novo synthesis of sphingolipids in apoptosis post Pc 4 PDT [127]. Pc 4 photodynamic therapy could even induce ceramide generation and apoptosis in acid sphingomyelinase-deficient mouse embryonic fibroblasts [128]. Production of ceramide could therefore represent a widespread mechanism of apoptosis induction by PDT.

Effects of Elevated Calcium Levels on Mitochondria

Calcium is a second messenger ion associated with a huge functional variety of regulatory processes in cells. Elevated calcium levels also accompany PDT-triggered apoptosis in many model systems (see below). It has been shown that mitochondrial Ca^{2+} overload leads to pore opening and onset of apoptosis [129-132]. Kowaltowsky *et al*. suggested Ca^{2+} ions to amplify the membrane depolarization induced by ROS by binding and destabilization of lipids on the inner surface of the mitochondrial membrane, thus leading to conformational changes in membrane proteins and pore opening [110, 133-135].

Effects on Anti-Apoptotic Proteins in Mitochondria

The anti-apoptotic protein Bcl-2 has been supposed to be a primary target of PDT, especially when the lipophilic photosensitizer Pc 4 is being used [136-138]. Overexpression of Bcl-2 protects from PDT-mediated apoptosis in several model systems and PDT with photosensitizers targeting Bcl-2, such as Pc 4 (which has a prevailing mitochondrial localization) can induce active cell death even in cells overexpressing Bcl-2; for details, see [33].

A recent publication reports that only membrane-inserted Bcl-2 is being photodamaged by light-excited Pc 4 since a cytosolic Bcl-2 mutant lacking the C-terminal domain for membrane insertion was not photodamaged [137, 138]. Photodamage set in the α-helices 5/6 of the region encompassing the BH1 and BH2 domains led to a crosslinking of the proteins. Photoactivated Pc 4 specifically destroyed Bcl-2, while other mitochondrial membrane proteins (such as Bak or the VDAC) or cytosolic proteins (such as Bax) were not affected [137, 138]. Treatment of HeLa cells with sublethal doses of hypericin PDT causes a phosphorylation of Bcl-2 at the Ser-70 residue [139]. The phosphorylation increased the cytoprotective role of Bcl-2, an effect, which has been proposed for other cell model systems as well [140]. However, the role of Bcl-2 seems to be dependent on the model system (photosensitizing agent and cell line used) and is hardly predictable at present.

3.2. Endoplasmatic Reticula as Primary PDT Targets

Often, PDT is accompanied by a significant increase in intracellular calcium. The latter may be released from internal stores (such as mitochondria or the ER) or come from outside the cell by influx through the plasma membrane [141]. Several authors correlated this increase in cytoplasmatic calcium with the onset of apoptosis. Tajiri *et al*. measured an strong increase of $[Ca]_i$ 1-2 hours post Photofrin-PDT applied to HSC-2 squamous cell carcinomas, which was followed by apoptosis [142]. The coincidence of increased $[Ca]_i$ and apoptosis was substantiated with PDT on V79 hamster cells [143]. Rueck *et al*. clearly identified a rise in $[Ca]_i$ as a signal for apoptosis in rat bladder RR1002 cells treated with photoactivated $AlPcS_4$ [144]. For ALA-induced PPIX-PDT on HL 60 leukemia cells, Ca^{2+} release from the ER has been assumed to represent an alternative to mitochondrial induced apoptosis [103]. In this model, elevated levels of cytoplasmatic calcium were suggested to result from photodamage set to Ca^{2+}-binding proteins in the ER. The increase in $[Ca]_i$ may contribute to apoptosis by activating the Ca^{2+}-calpain pathway, which cause PTP opening at mitochondria [133, 134] or induces activation of caspase 3 without mitochondrial involvement [145-147]. Additionally, fusion of the ER and mitochondria caused by ALA-PDT allows direct flow of calcium to mitochondria and leads to pore opening [103]. Bcl-2 has been reported to increase the emptying of ER calcium stores during apoptosis of Hela cells after verteporfin-PDT [148]. Thus, apoptosis induction after PDT using photosensitizers localizing in the ER is tightly connected with an increase of $[Ca]_i$, which either triggers mitochondrial pore opening or directly activates caspases.

3.3. Lysosomes as Primary PDT-Targets

Hydrophilic photosensitizers preferably localize in the cytoplasm and in lysosomes. Kessel *et al*. could show that lysosomal localizing photosensitizers cause the release of cathepsins from these organelles into the cytoplasm [149]. Once in the cytosol, cathepsins can either directly activate caspases [150, 151] or cleave Bid (yielding tBid) and promote this pathway leading to mitochondrial pore opening and apoptosis [152].

3.4. The Plasma Membrane as Primary PDT Target

Even photosensitizers localizing in the plasma membrane can trigger active cell death. Using evanescence illumination – which ensures photoactivation of sensitizing molecules only in the plasma membrane and a very small part of the cytoplasm – in combination with the photosensitizing drug Rose-Bengal, which localizes mainly in the plasma membrane, Lin *et al*. could observe apoptosis induction [153]; therefore, PDT-induced singlet oxygen production in the plasma membrane also can induce apoptosis. In this context, examination of the possible contribution of the outside phosphatidyl serine receptor (OPSR) to apoptotic signalling induced by ROS generated in the plasma membrane seems to be promising. The OPSR recognizes oxidized phosphatidyl serine on the outer leaflet of the plasma membrane and sends apoptotic signals to the cytoplasm [154, 155]. The contribution of the OPSR remains crucially hypothetical, but could prove worth explorating.

3.5. Other Mechanisms Leading to PDT-Induced Apoptosis

Several publications deal with the induction of expression and secretion of Fas and TNF-α by PDT. For

example, an increase of Fas in A431 cells and its appearance in the medium within one hour after PDT was measured by Ahmad *et al*. [156]. The data of Yslas *et al*. who also measured an increase of Fas antigen followed by apoptosis in PDT treated cells further substantiate the contribution of Fas to active cell death induced by PDT [157]. Pc 4 PDT applied to A431 leads to production of TNF-α, but its role in PDT-triggered apoptosis and the detailed mechanisms remain a topic of scientific discussion [158].

Also modifications of mitogen activated protein kinase (MAPK) cascades may represent a mechanism, by which PDT triggers induction of apoptosis. MAPKs are central mediators of the pathways regulating survival and cell death and have been implicated in the response of tumor cells to PDT [33]. While exposure of different cancer cell lines to PDT generally results in the activation of protein kinases JNKs and p38, which signal cellular survival, PDT causes inhibition of ERK kinases [159, 160]. The rapid down-regulation of the ERK pathway could be a necessary prerequisite for the induction of apoptosis after PDT. For details see [33].

4. APOPTOSIS DETECTION METHODS: BENEFITS AND DRAWBACKS

Several analytical methods utilize the differences in morphological, biochemical and energetic properties of (surviving,) necrotic and apoptotic cells to assess the modes of cell death. Some of these shall be described here, providing the principle of each assay and a brief evaluation of the expressiveness. In the context of the various mechanisms of apoptosis induction, these methods are outlined in Fig. (**3**). Since many assays make use of fluorescence dyes, the experimenter is advised to avoid misinterpretation of experimental data due to possible spectral interference with the photosensitizer. Since most of the mechanistic details of PDT-induced cell death have been described using *in vitro* systems we shall focus on the related methods. For a critical discussion of the methodology used with (fixed) tissue sections see [161].

4.1. Fluorescent Staining of the Nucleus

The late stages of apoptosis are characterized by several distinct changes in the cell nucleus; methods which assess these changes shall be discussed in the following chapter.

DAPI- and Hoechst-Staining for Nuclear Fragmentation

The dye 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) as well as the bisbenzimide dyes Hoechst 33258, Hoechst 33342 and Hoechst 34580 represent classical nuclear and chromosome counterstains. They can be used to stain (fragmented) nuclei and detect apoptosis via fluorescence microscopy [32, 35, 162]. This approach provides single-cell analysis which additionally allows morphological analysis of the cells by microscopy. The major disadvantage is that a large number of cells have to be analysed in order to give a representative and significant quantification and the identification of fragmented nuclei is up to the investigator and therefore linked with subjective judgement.

Flow Cytometric Determination of the DNA Content / Cell Cycle Analysis

The method is carried out by measuring the DNA content of cells after fixation and permeabilization with ethanol and subsequent staining with propidium iodide (PI) using FACS analysis (fluorescence activated cell sorting) / flow cytometry. Cells in the resting and gap-phase (G_0/G_1) contain a certain amount of DNA (diploid phase), which is characterized by a distinct peak of red fluorescence. Due to nuclear fragmentation and condensation, the DNA content decreases during apoptosis, which can be quantified by the detection of cells with lower fluorescence, the so called subG_1 fraction [163-165]. Despite the general advantages of analysis by flow cytometry (single-cell analysis, rapid assessment of large cell numbers) the quantification of apoptosis at late stages of active cell death may lead to an underestimation since secondary necrosis may occur at that time (characterized by small cell fragments that are not recorded by this method). On the other hand, an overestimation of apoptotic cell death may occur due to counting of DNA-containing particles coming from one single cell as separate events.

DNA Laddering

Agarose gel electrophoresis is a method to detect fragmentation of DNA during apoptosis. DNA extracts of apoptotic cells (yielded by standard techniques of isolation of genomic DNA involving cell lysis and protein digestion by proteinase K, phenol-chloroform extraction and DNA precipitation by ethanol) show a typical 'DNA ladder' configuration consisting of multiples of 180 – 200 bp DNA-fragments when separated by agarose gel electrophoresis and visualized by ethidium bromide staining. These fragments are generated during late stages of apoptosis by Ca^{2+}/Mg^{2+}-dependent endonucleases which cleave at the linker regions between nucleosomes [166, 167]. It should be mentioned that the detection of DNA fragmentation by gel electrophoresis is only qualitative and therefore *not* the method of choice for quantification of apoptosis in cell populations.

COMET-Assay

The single cell gel electrophoresis assay (also known as COMET assay) is a technique for analysing and measuring DNA damage/degradation in individual cells. Due to DNA fragmentation during apoptosis, this method is also applicable for detection of single apoptotic cells. In the COMET assay, cells are embedded in a thin agarose gel on a microscope slide. The cells are lysed to remove all cellular proteins and the DNA is subsequently allowed to unwind under alkaline or neutral conditions (depending on the protocol). In the following, the DNA is separated by electrophoresis, stained with a fluorescent dye (e.g. ethidium bromide) and cells are evaluated by fluorescence microscopy. During electrophoresis, DNA fragments migrate away from the nucleus resulting in a typical comet-like tail of apoptotic nuclei, while the DNA of intact cells remains in the nucleus [168]. Though allowing single-cell analysis, interpretation of the results might be difficult with respect to distinguishing between apoptosis and necrosis since (uncontrolled) DNA breaks may also occur during necrotic cell death [169].

TUNEL Assay

The TUNEL technique (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) is an enzymatic method for detection of DNA fragments generated during apoptosis. Free 3'-OH termini of DNA fragments are labelled by addition of FITC-dUTP (fluorescein isothiocyanate deoxy-uracil-5'-triphosphate) which is catalysed by terminal deoxynucleotide transferase (TdT) in a template-independent manner. FITC fluorescence is evaluated by flow cytometry analysis or fluorescence microscopy. Advantages of the TUNEL assay are the ability to reveal early DNA breaks during apoptosis and the good distinction of labelled and unlabelled cells [170-173]. According to a recent publication, care must be taken to avoid false-positive results when applying the TUNEL assay to tissue sections [174].

4.2. Determination of Biochemical Features of Apoptosis

Apoptosis, as active process, is characterized by several changes in cellular biochemistry and morphology. Some of the events typical of active cell death can be determined by enzymatic analysis; other methods deal with the change in localization of proteins relevant in the apoptotic process.

Caspase Assays

The activity of caspases, key players in apoptosis, can be analysed by the determination of cleavage of the specific fluorogenic (tetra-) peptide substrates [175, 176]. Several of these substrates, each more or less specific for one distinct caspase, are available commercially. Especially determination of caspase 3 activity is generally used as a specific marker for cells which have entered the execution phase of apoptosis. Briefly, cells are harvested, the protein content is being determined by standard methods, followed by lysis of cells. After addition of the substrate, the release and the concomitant activation of fluorochromes can be measured [57, 177, 178]. Recent developments allow flow cytometric analysis of caspase activation, thus providing single cell evaluation [179]. The activation of caspases involves the cleavage of inactive precursors (pro-caspases) and hence can be detected by western blot analysis using specific antibodies [180].

Staining of Phosphatidyl Serine on the Outer Surface of the Cell Membrane

In viable cells, phosphatidyl serine (PS) is directed to the cytoplasmatic compartment. However, in apoptotic cells, PS appears on the outer leaflet of the plasma membrane, where it can be detected by fluorescent labelled Annexin-V conjugates. Annexin-V probes are being used for flow cytometry analysis, confocal- or epifluorescence microscopy and can be combined with nucleic acid stains, such as propidium iodide (red fluorescence, permeant to necrotic cells only), to accurately assess mixed populations of living (no fluorescence), apoptotic (Annexin-V fluorescence without red nuclear staining) and necrotic cells (ideally red fluorescence from nucleic stain only; under certain circumstances, also necrotic cells can display Annexin-V stain, possibly by binding to PS at the inner membrane surface) [181-184]. The use of this method requires accurate control procedures in order to avoid misinterpretation of PI stained secondary necrotic cells.

Determination of the Translocation of Proteins from Mitochondria into the Cytoplasm

The translocation of cytochrome c from the mitochondrial intermembrane space into the cytoplasm can be monitored by several methods. One approach involves subcellular fractionation and probing of cytosolic (and mitochondrial) fractions for the presence of cytochrome c by means of western blotting and immunochemical detection. This allows sensitive detection of cytochrome c in the cytoplasm but requires careful control procedures to prevent false-positive results caused by mitochondria damaged during the fractionation procedure. Using intact cells, the release of cytochrome c alternatively can be monitored by immunochemical visualization and fluorescence microscopy. For this purpose the cells are permeabilized and probed with anti-cytochrome c antibodies which, in turn, are labelled with secondary FITC-labelled antibodies. The leakage of cytochrome c is indicated by a change in the fluorescence pattern from punctuate signals to more diffuse fluorescence distributed in the cytoplasmatic compartment (and can be verified by costaining with mitochondria-specific dyes such as MitoTracker®) [185]. This method is of special interest when the temporal dynamics of cytochrome c release shall be analysed. A similar methodology is applicable also to analysis of translocation of AIF to the nucleus.

Determination of Pore Opening by Migration of Calcein-AM

Mitochondrial pore opening can be furthermore estimated by a quick and elegant method published by Lemasters *et al*. [186]: cells are loaded with calcein-AM, which is cleaved to fluorescent calcein by esterases in the cytoplasm. Calcein cannot translocate into mitochondria and those organelles may be counterstained by dyes such as TMRM (tetramethylrhodamine, methyl ester) as long as they produce some $\Delta\psi$. Whenever pore opening occurs during apoptosis, green calcein fluorescence becomes uniform within the cells due to diffusion of the dye into mitochondria, accompanied by a loss in red TMRM-flourescence caused by a loss of $\Delta\psi$. This alteration in fluorescence characteristics is monitored by confocal microscopy and (again) is well suited for time-course studies.

4.3. Determination of Energetic Parameters

Since apoptotic and necrotic cells show fundamental difference with respect to their energy metabolism, both modes of cell death can be identified by determination of some central energetic parameters.

Dynamics of Mitochondrial Metabolic Activity During Apoptosis

As a parameter for mitochondrial (metabolic) activity, the ability of the cells to convert the tetrazolium dye MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) to formazan (catalysed by mitochondrial succinate dehydrogenase) can be measured and compared to that of untreated control cells [187]. We found the temporal dynamics of the MTT activity to perfectly reflect the apoptotic fraction of cells, when cells were treated with $AlPcS_4$ PDT using different light doses: at three hours post treatment, necrotic cells had lost the ability to reduce the formazan salt while

apoptotic cells, still metabolically active at this point in time, showed significant MTT activity. Twenty-four hours post treatment these cells had terminated apoptosis, therefore the MTT signal corresponded to the surviving fraction of cells. Therefore, the difference curve between these MTT signals recorded 3 and 24 hours post treatment is indicative of the 'apoptotic window' [36]; however, it is important to note that this assay cannot *per se* identify apoptotic cell death but with respect to the temporal dynamics it allows the fast and cheap assessment of the different cellular responses after PDT since the resulting blue formazan salt can easily be measured by microplate readers. Similar expressive results indicating the dose-dependent occurrence of different cellular responses were gained with other cell lines and photosensitizing agents (unpublished data of our group).

Staining of the Mitochondrial Membrane Potential (JC-1)

Due to opening of mitochondrial pores, a reduction of the mitochondrial membrane potential is a common feature of apoptosis. The latest generation of mitochondrial membrane potential sensitive dyes, such as 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), undergoes a $\Delta\psi$-dependent accumulation in the mitochondrial matrix, which, in consequence, causes a shift in its emission maximum from green to red. Analysis can be done with flow cytometry or fluorescence microscopy [188, 189]. Untreated control cells show high red fluorescence, whereas the green signal is low. Necrotic cells are characterized by a *drastic reduction* of the red fluorescence and high green fluorescence. Apoptotic cells are characterized by a *lower*, but not completely collapsed mitochondrial membrane potential [109, 190]. The red signal is about 5 times lower than that of the untreated controls, while the green signal appears to be slightly increased [83]. As discussed by Bernardi *et al*. [191], only the red fluorescence of JC-1 (and not the ratio of green-to-red) is indicative of mitochondrial $\Delta\psi$.

Time Course of the Intracellular ATP Level

The intracellular ATP level has been reported to be a significant determinant of the cell death mode [81, 82, 192]. Apoptotic cells maintain high ATP levels for several hours, whereas necrotic cells are characterized by a rapid and complete loss of intracellular ATP [36]. For measurement, ATP is liberated from cells by either rapid lysis or extraction of the nucleotides by a chaotropic agent (perchloric or trichloroacetic acid). Once released from the cells, it can be determined by a luminescence reaction based on the firefly luciferase-catalysed ATP-dependent oxidation of D-luciferin [193]. Two methods for the rapid determination of intracellular ATP we introduced in [194] with special respect to the use of the 96-well microplate format which allows rapid and easy handling of high sample numbers.

CONCLUSIONS

(i) Photodynamic therapy is efficient in triggering apoptosis in target cells. This might be due to the main effect of PDT, the formation of ROS, either directly in mitochondria or in their proximity.

(ii) There seems to be no universal mechanism of PDT. The pathway leading to apoptosis induction depends on the model system and is tightly connected with the localization of the photosensitizing agent, which – in turn – determines the area of primary damage. As a common denominator, the involvement of mitochondria seems to be central to the apoptotic process induced by most variations of PDT.

(iii) PDT seems to be able to overcome the blocks to cell killing by induction of apoptosis in tumor cells resistant to therapies with ionizing radiation or chemotherapeutics. One might hypothesize that this is because of PDT triggers apoptosis at a 'late' stage within the apoptotic signal transduction pathway(s), in some cases by directly inducing mitochondrial pore-opening (or by photodamaging anti-apoptotic Bcl-2 proteins).

(iv) Numerous methods exist that allow the qualitative and – in some cases – also quantitative assessment of cell death. These methods are set apart from each other by their ability to accurately distinguish between the modes of cell death (apoptosis versus necrosis) and by their applicability to different kinds of samples. It is of fundamental importance for meaningful interpretation of experimental data and results published concerning the detection of cell death to be aware of the limitations and drawbacks each method is characterized by.

It can be summarized that the research on PDT-induced active cell death – as in part cited in the present review – has essentially contributed to the comprehension of the underlying cellular mechanisms. Although many of the molecular details remain unclear we are convinced that additional basic research unravelling regulation and the dynamics of the cellular response following PDT will further enhance the efficiency of photodynamic treatment.

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ABBREVIATIONS

(d)ATP	=	(Deoxy) adenosine-5'-triphosphate
AIF	=	Apoptosis inducing factor
ALA	=	δ-Aminolevulinic acid
$AlPcS_4$	=	Aluminum (III) phthalocyanine tetrasulfonate
ANT	=	Adenine nucleotide translocator
APAF-1	=	Apoptosis protease activation factor-1
BA	=	Bongkrekic acid
Bcl-2	=	B-cell lymphoma 2
caspases	=	Cysteinyl aspartic acid specific proteases
COMET	=	Single cell gel electrophoresis assay
CsA	=	Cyclosporine A
$\Delta\psi$	=	Mitochondrial membrane potential
DAPI	=	4', 6-diamidino-2-phenylindole dihydrochloride

DIABLO	=	See Smac
DISC	=	Death inducing signalling complex
DTT	=	Dithiotreitol
ER	=	Endoplasmatic reticulum
FACS	=	Fluorescence activating cell sorting, flow cytometry
FADD	=	Fas associated death domain
FITC-dUTP	=	Fluorescein isothiocyanate deoxy-uracil-5'-triphosphate
GTP	=	Guanosine-5´-triphosphate
H_2O_2	=	Hydrogen peroxide
Htr2A/Omi	=	High temperature requirement A
IAP	=	Inhibitor of apoptosis proteins
IMM	=	Inner mitochondrial membrane
JC-1	=	5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl-carbocyanine iodide
MAPK	=	Mitogen activated protein kinase
mTHPC	=	Meso-tetrahydroxy phenylchlorine
MTT	=	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
NF-κB	=	Nuclear factor κ-B
Omi	=	See Htr2A
OMM	=	Outer mitochondrial membrane
OPSR	=	Outside phosphatidyl serine receptor
Pc 4	=	Phthalocyanine 4
PDT	=	Photodynamic therapy
PI	=	Propidium iodide
PPIX	=	Protoporphyrin IX
PS	=	Phosphatidyl serine
ROS	=	Reactive oxygen species
Smac/ DIABLO	=	Second mitochondrial activator of caspases
tBid	=	Truncated Bid
TdT	=	Terminal deoxynucleotide transferase
TMRM	=	Tetramethylrhodamine, methyl ester
TNF	=	Tumor necrosis factor
TUNEL	=	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling
VDAC	=	Voltage-dependent anion channel

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