APOPTOSIS INDUCTION BY PHOTODYNAMIC TREATMENT WITH ENDOGENOUS PROTOPORPHYRIN IX?

Short title: Apoptosis by ALA-PDT?

B. KRAMMER, T. VERWANGER, G. SCHNITZHOFER, K. ÜBERRIEGLER, I. REITER

Institute of Physics and Biophysics, University of Salzburg, Hellbrunnerstr. 34, A-5020 Salzburg, Austria

Kurzfassung

Apoptose kann in mehreren Zell-Linen durch photodynamische Behandlung mit verschiedenen Photosensibilisatoren, einschließlich dem mit Aminolevulin-Säure-stimulierten endogenen Protoporphyrin IX (PpIX) induziert werden. Dieses PpIX wird erfolgreich als Tumormarker in der Diagnose verwendet und besitzt gute Eigenschaften für die Tumortherapie (PDT).

Ziel der Studie war es, die Zelltodmechanismen und deren Konsequenzen für die Tumorbehandlung mit PpIX zu untersuchen, da die Art des Zelltodes die Wirksamkeit der Therapie zu beeinflussen scheint. Das Fehlen einer Reaktion auf einen therapeutischen Ansatz wird oft durch Fehlen einer Apoptose-Induktion verursacht. Auf der anderen Seite scheint die Art des Zelltodes während der primären Tumorbehandlung einen starken Einfluss auf jene Immunreaktionen zu haben, welche benötigt werden, um ein Nachwachsen des Tumors zu verhindern: das Immunsystems wird effektiver durch nekrotischen Zelltod als durch Apoptose aktiviert.

Wir konnten in Studien über Nachweismethoden für Apoptose - unterstützt durch Zellzyklus- und Genexpressions-Analysen - zeigen, daß lichtaktiviertes, endogenes PpIX hauptsächlich Nekrosen und nur eine kleinere apoptotische Fraktion in Fibroblasten-Kulturen verursacht. Dieser Unterschied zu Studien in der Literatur, die hauptsächlich Apoptose-Induktion beschreiben, könnte teilweise durch ein Umschalten zwischen Apoptose und Nekrose, abhängig vom intrazellulären ATP-Gehalt, erklärt werden. Sehr wahrscheinlich wird in durch ALA-PDT geschädigten Zellen, die ihr zelluläres Energieniveau noch über einem kritischen Wert halten können, ein apoptotisches Programm in den Mitochondrien eingeleitet. Die Ausführung verläuft über Schädigung mitochondrialer Funktionen, Cytochrome c-Freisetzung und Caspasen-Aktivierung zum morphologischen Erscheinungsbild der Apoptose.

Abstract

Apoptosis can be induced in several cell lines by photodynamic treatment with different photosensitizers including aminolevulinic acid (ALA)-stimulated endogenous protoporphyrin IX (PpIX). This PpIX is successfully used as tumor marker in diagnosis, and owns good sensitizing properties for tumor therapy (PDT). The aim of the study was to investigate cell death mechanisms and its consequences for tumor treatment with PpIX, since the mode of cell death seems to influence the efficiency of the therapy. The no-response to a therapeutical approach is often caused by lack of apoptosis induction. On the other hand, the mode of cell death during primary tumor eradication seems to have a strong impact on those immune reactions, which are required in order to prevent tumor recurrence: the immune system is more effectively activated by necrotic cell death than by apoptosis.

We could show in apoptosis detection studies - supported by cell cycle and gene expression analysis   that light-activated, endogenous PpIX induces mainly necroses and only a smaller apoptotic fraction in fibroblast cultures. This difference to studies in the literature describing mainly apoptosis induction could be partly explained by switching between apoptosis and necrosis, dependent on the intracellular amount of ATP concentration. It is very likely that in ALA-PDT-damaged cells maintaining a cellular energy level above a threshold, an apoptotic program is induced in mitochondria. The execution proceeds via impairment of the mitochondrial functions, cytochrome c release and caspases activation to the morphological appearance of apoptosis.

Key Words: apoptosis, photodynamic, protoporphyrin, fibroblasts, oncogenes, immune reaction

1 Introduction

Photodynamic tumor therapy (PDT) represents a rapidly developing modality for cancer treatment, which is based on a two components: 1) preferential uptake and retention of a photosensitizer by tumors; and 2) irradiation of the tumor area with light wavelengths specifically absorbed by the photosensitizer. The photoexcited sensitizer generates short-lived and highly reactive oxygen species (ROS) which induce irreversible damage to cell targets located in its microenvironment. The resulting tumor damage occurs by at least two mechanisms, namely reduction or cessation of the blood flow due to vascular damage and direct killing of the neoplastic cells. PDT is world-wide clinically applied and now approved in several countries with Photofrin (a porphyrin derivative absorbing red light which is characterized by a good penetration power into mammalian tissues) as the phototherapeutic agent (1).

Several approaches are being devised in order to optimize the efficiency of PDT and minimize its side effects, such as general skin photosensitivity. Optimization could be reached by manipulating processes and pathways on the subcellular level. The long-lasting success of the therapy is determined by the mode of the cell death, by the proliferative activity and mutations of the cells escaping the treatment, and finally by the action of the immune system, which in turn is influenced by the mode of cell death. The relatively high efficiency of the photodynamic tumor therapy might be based on the induction of both, apoptosis as well as necrosis, and could be further optimized by directed application of both cell death modes.

A promising new approach to PDT is the employment of the endogenously produced photosensitizer, protoporphyrin IX (PpIX). This is an intermediate in the haem biosynthetic pathway which is overproduced after external administration of the early precursor 5-aminolevulinic acid (ALA). ALA-induced endogenous PpIX is already used since several years as fluorescence tumor marker with great success for photodiagnosis (PD) of superficial tumors and dysplasias. Due to the increased ALA uptake or PpIX synthesis by tumor cells, the reliability of tumor detection is high.

For therapeutic purposes, too, application areas of ALA have continued to increase and ALA-PDT has been used successfully to treat superficial head and neck cancer, various gastrointestinal cancers, Barrett's oesophagus (2), as well as a variety of dermatological non-neoplastic (3) and neoplastic (2) skin diseases.

The superiority of ALA as a phototherapeutic prodrug is mainly due to the fact that it is an endogenous substance with a fast pharmcokinetic and low side effects. It is a non-invasive procedure which produces excellent cosmetic results. ALA may be administered either systemically or topically and it is well tolerated by patients and can be applied repeatedly without cumulative toxicity (3).

Apoptosis (programmed or physiological cell death), detected by Kerr et al. (4) already 1972, describes a suicide program, which the cells use to respond to unrepairable damage induced by e.g.UV or ionizing irradiation, to stimuli such as Fas binding, or to lack of essential stimuli such as growth factors. Apoptosis is an active process, which requires in general the coordinated action of proteins. Unlike necrosis, it needs a functional energy-producing system at the beginning. Microscopically detectable indications for apoptotic cell death are morphological changes such as chromatin condensation and alterations of the cell surface characterized by the occurrence of blebs, followed by DNA fragmentation by endonucleases, cell shrinking and packing of the cell content into apoptotic bodies. These bodies are phagocytized by neighbouring cells and macrophages or left to secondary necrosis in *in-vitro* situations. However, the hypothesis is generally accepted that many pathways lead to apoptosis induction, but only one pathway, namely the apoptotic program, is responsible for apoptosis execution.

Based on the knowledge that in radiation- and chemotherapy tumor destruction by apoptosis or by removing barriers which prevent the induction of apoptotic processes plays a crucial role (5), this cell death mode is already used deliberately for increasing the therapeutical efficiency for two reasons: 1) The therapeutical doses required are lower for apoptosis than for necrosis generation; 2) resistance against a therapeutical approach manifests itself often in the lack of apoptosis induction.   In contrast to this it could be proved that not apoptosis, but necrosis stimulates the immune system, an important component in tumor eradication (6, 7).

2 Aim of the Study

The aim of the current study was to answer the questions, whether in human fibroblasts apoptosis is induced by ALA-PDT under the applied conditions, over which signalling pathway (mitochondria, nucleus, ..) this happens, and with which consequence for the immune sytem. One result should be to set up a working hypothesis - based on the literature and own results - about start and execution of ALA-PDT induced apoptosis.

3 Apoptosis in PDT: Results from the Literature

Also PDT induces apoptosis beside necrosis, *in vitro* as well as *in vivo*. While most former studies describe tumor necrotization due to lack of discrimination, increasingly more recent papers report about PDT-induced apoptosis, starting 1991 with a study by Agarwal et al. (8) about apoptosis in mouse lymphoma cells following photodynamic treatment with chloroaluminum phthalocyanine. Induction of apoptosis can be caused by many photosensitizers, 1) dependent on the cell line used 2) dependent on the incubation protocol: longer incubation times combined with lower drug doses seem to promote apoptosis, when irradiation is performed within a lethal dose (LD) range for 50% - 99% of the cells (LD_{50} -LD_{99}), and 3) dependent on the charge of the sensitizers and connected with this on intracellular distribution and localisation. Kessel et al. (9) demonstrated that subcellular localisation of the sensitizer, which is generally dependent on its properties and application mode, is crucial for apoptosis induction. Membrane photodamage e.g. seems to delay or prevent an apoptotic response to PDT and leads mainly to necrosis (10). Mechanisms and signal transduction processes in PDT-induced apoptosis are currently under investigation. A recent paper describes e.g. the stimulation of ceramide synthesis by PDT (11) and another caspase-3 activation by PDT (12).

Main sources for photodynamic induction of apoptosis in-vitro might be the intracellularly generated reactive oxygen species (ROI's), which influence signal transduction pathways including the expression of genes, preventing (mainly *bcl-2*-family) or promoting apoptosis (e.g. p53 and c-*myc*). ROI production by PDT

during irradiation or by long-persisting photoproducts in the post-irradiation interval can be recognized as apoptosis trigger (13). Cytochrome c release and decrease of the mitochondrial membrane potential in context with PDT-induced apoptosis could be observed (14). - However, if cytoplasmatic or mitochondrial damage e.g. by ROI production or hypoxia increases and the cellular antioxidant defense systems cannot compensate this, apoptosis is the way out to eliminate functionally disturbed cells (15).

Apoptosis induction following ALA-PDT comprises only a few studies (16-19). While in V79 cells maximum apoptosis (75-85%) was detected 3 - 4 hrs after ALA-PDT with a LD_{85}, WiDr adenocarcinoma cells died only by necrosis (16). V79 target mitochondria at LD_{85}, inhibiting respiration by 90%, but reducing ATP only by 15% (17). Another study found that in lymphocytes PpIX triggers mitochondrial permeability transition, disrupts the mitochondrial membrane potential and induces apoptosis (18). Webber (19) detected an apoptotic fraction in ALA-PDT treated colon-26 tumors.

4 Apoptosis in ALA-PDT: Own Results

Apoptosis detection
In our studies, apoptosis could be induced in transformed human fibroblasts by 3 hrs of incubation with 200 µg/ml ALA and irradiation with a fluence of 9 J/cm^2 (Fig.1). The detection methods used were microscopical observation of morphological features such as blebbing; further fluorescence microscopy of staining with Annexin-V-FITC against the phosphatidyl-serine groups on the outer cell membrane in the case of apoptosis, and staining with propidium iodide. The latter intercalates in the DNA, when cell and nuclear membranes become leaky due to irreversible damage as it is the case in necrosis. Although apoptosis occurred, the apoptotic fraction was much smaller than the necrotic one.
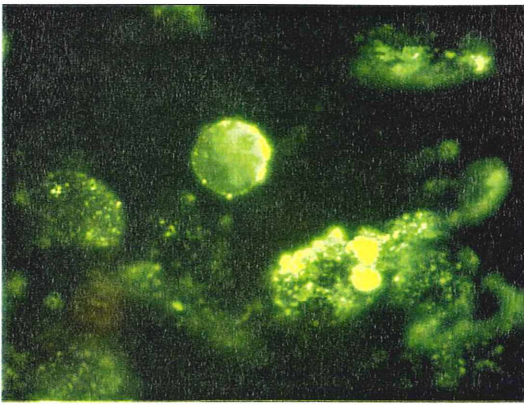
Fig. 1. Apoptoses of WI38-VA13 fibroblasts, following photodynamic treatment with endogenous PpIX (200 μg/ml ALA) and a fluence of 9 J/cm^2. Top: staining with Annexin V and propidium iodide, bottom: corresponding transmission picture

Apoptosis, intracellular localisation and targets of PpIX

Apoptosis induction is related to the intracellular sensitizer distribution. Therefore, time and site dependent formation and accumulation of endogenous PpIX and the intracellular targets for the photoinduced damage were investigated in co-cultivated normal (WI38) and transformed human fibroblasts (WI38-VA13) (20). Following results were found: Some minutes after incubation with ALA, formation of PpIX within the mitochondria could be observed, followed by relocalisation in the cytoplasm and staining of the nuclear membrane. After high ALA concentrations (1000 μg/ml), the PpIX remained longer accumulated in the mitochondria than after lower ALA concentrations (100 μg/ml), which induced within 20 mins a more homogenous distribution within the cytoplasm. Following incubation for 5 hours, fluorescence was detected in the whole cytoplasm and in the membranes. The

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nucleus was always free of PpIX. The kinetics of the PpIX production and localisation could be correlated to the ALA concentration and incubation time.

The investigation of ALA-PDT induced damage on intracellular structures by double fluorescence staining showed that mitochondria undergo a photoinduced condensation leading to destruction. In spite of no correlation to sensitizer localisation, ER fragmentation and vesiculation could be observed, followed by the appearance of a porous network with large cisternae and nucleus indentations; these might be indications for rearrangement of all membrane systems and obviously apoptotic cell death (Fig. 2). The transformed cells showed the same cell destruction pattern as normal cells but within a shorter time period.

Apoptosis, cell cycle and gene expression in PDT: background

Apoptosis is considered in many cases as cell cycle phenomenon (21), since it is used as self destruction mechanism following irrepairable damage in the proliferation. If damage cannot be repaired in the G_1-phase, apoptosis can be started in the time between late G_1- and beginning S-phase. This is partly due to the proto-oncogene *c-myc*, inducing the transcription of many genes such as cyclin A, which are active in late G_1 for transition of G_1 to S; this in turn requires cyclin dependent kinases. *c-myc* controls normal cell growth as well as apoptosis and is an immediate early growth response gene that is rapidly induced upon mitogenic stimulation of quiescent cells (22). Together with other early response genes (*c-fos, c-jun* and *egr-1*), *c-myc* has been shown to increase transiently following PDT-mediated oxidative stress (23). Investigations about *c-jun* and *c-fos* by Kick et al. (24) showed strong and prolonged induction together with expression of inflammatory cytokines.

Apoptosis induction before entrance of the S-phase is further due to the action of p53, which has the function to either repair DNA damage in G_1 or prepare apoptosis in the case of irreversible injury. The (proto) oncogene *bcl-2* promotes proliferation and prevents cells from starting apoptosis, e.g. especially when induced by *c-myc*; it is activable by cell cycle arrest at multiple points within the cell cycle. *bcl-2* expression also prevents ROI- or p53-induced apoptosis (25, 26) and can induce partial resistance to PDT with PC4 (27) (Fig. 3).

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Fig.2: Indentations of the nucleus during apoptosis following ALA-PDT

Apoptosis and the cell cycle following ALA-PDT: results (28)

Many cancer therapies including photodynamic tumor treatment (PDT) have a selective effect on cells in different phases of the cell cycle. The different sensitivity of the phases influences in turn the therapeutic success. The fact that the photosensitizer class of porphyrins cannot easily enter the nucleus, raised the question, whether photodynamic treatment with aminolevulinic acid (ALA)-induced endogenous protoporphyrin IX (PpIX) might have an effect on the cell cycle distribution of the transformed human lung fibroblast cell line WI38VA13
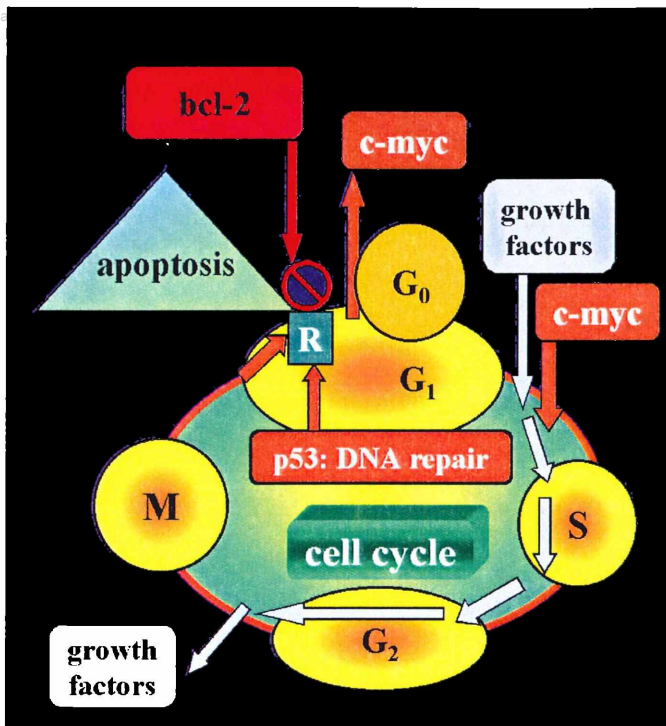
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Fig.3: Simplified schematic draft of the correlation between cell cycle, apoptosis and gene expression. Apoptosis is induced in the restriction point R of the cell cycle phase G1, when e.g. growth factors are deprived and *c-myc* is still expressed or when *p53* fails to repair DNA damage. bcl-2 prevents apoptosis induction.

A moderate cell cycle response to PDT, measured by propidium iodide (PI) and bromo-deoxyuridine (BrdU) labeling seems to be both, time and dose dependent. At 7 hours post photodynamic treatment with low doses (LD_8), a significant release of cells in G_0/G_1- and a moderate accumulation in the G_2/M-phase could be detected. This G_0/G_1-release was accompanied by a simultaneous moderate increase of the S-phase activity. Higher ALA-PDT doses (LD_{74}) induced a pronounced delay in S-phase within 4-7 h post treatment (detected by pulses of 15 mins), which was made up within 60 mins reaching BrdU incorporation rates not significantly different from the control levels (Fig.4). In spite of the high cytotoxicity, these cells were not arrested in the G_0/G_1-phase but accumulated in the G_2/M-compartment until 24 hours post treatment. We have no indication from the present study that low or

high ALA-PDT doses lead to a significant cell cycle arrest or to apoptosis induction, which could influence the damage susceptibility of the cells during the treatment. Only transformed fibroblasts are more sensitive than their normal counterpart. Low treatment doses could even act - at least transiently - as stimulating factors, which may promote growth of tumor cells escaping the therapy.
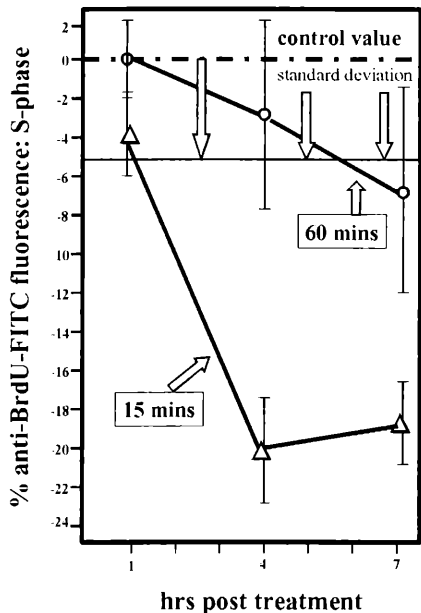

Fig.4: Measurement of the S-phase activity of transformed human fibroblasts following incubation with endogenous PpIX ($200\mu g/ml$ ALA for 3 hrs) and irradiation with 9 J/cm^2. BrdU incorporation was detected after BrdU pulses of 15 and 60 mins and related to the control values

PI fluorescence in the sub-G_0/G_1 region of the fibroblasts was slightly increased at 24 hrs post treatment with an LD of 74 %. The appearance of a DNA stainability lower than that of G_0/G_1 cells (= sub G_0/G_1 peak) is considered to be a marker of apoptotic cell death (29).

In contradiction to findings in the literature (21), apoptosis in ALA-PDT might not be seen as cell cycle phenomenon. The final decision between apoptosis and survival is often made at the restriction point in the cell cycle phase G_1, if the damage cannot be repaired by the repair gene p53. The attempt of repair would have generated a measurable G_0/G_1-arrest.

Apoptosis and gene expression following ALA-PDT(30)
As part of this study quantitative determination of the expression of the (proto)oncogenes *c-myc* and *bcl-2* in normal and transformed human fibroblasts at

different times following photodynamic treatment with 5-aminolevulinic acid-stimulated endogenous protoporphyrin IX and low dose irradiation was carried out by quantitative RT-PCR. The aim was to investigate, if irreversibly increased (proto)oncogene expression can be found, and if expression changes are involved in cell cycle alterations and in initiation of apoptotic processes. The results show the following: 1) Lack of mutagenic risk, since the overexpression of *c-myc* and *bcl-2* is transient: no constitutive overexpression of the proto-oncogenes *c-myc* and *bcl-2* could be detected 24 hrs after treatment; 2) an increase of *bcl-2* and *c-myc* associated with an increase of the DNA synthesis activity of the cell cycle of transformed cells at 7 hrs after treatment with low (LD$_8$) PDT-doses; 3) a possible role of *bcl-2* in counteracting processes which could be at least precursors for apoptosis induction: *bcl-2* follows the course of the *c-myc* expression within 24 hrs pt in transformed cells, and 4) higher constitutive expression of both genes in transformed than in normal fibroblasts (Fig. 5).
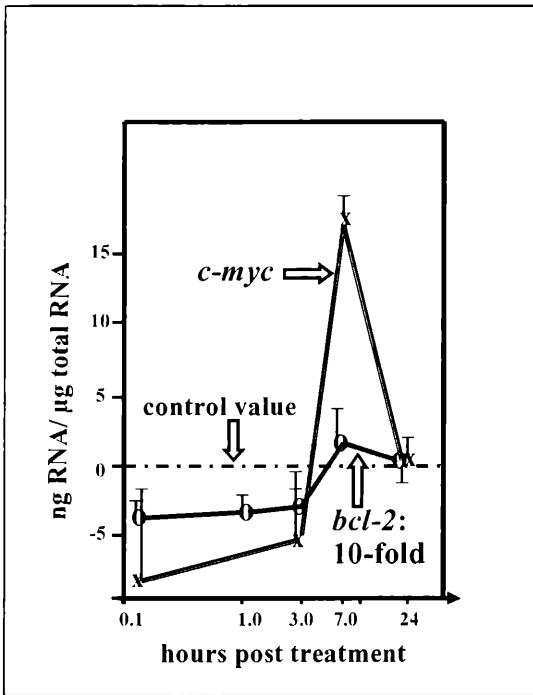

Fig.5: Expression changes of c-*myc* and *bcl-2* in transformed human fibroblasts following incubation with endogenous PpIX (200μg/ml ALA for 3 hrs) and irradiation with 4.3 J/cm^2, related to the control values (Method: quantitative RT-PCR).

Since neither the cell cycle nor gene expression shows significant features of apoptosis, it is more likely that ALA-PDT induced apoptosis starts in the mitochondria than in the nucleus. *bcl-2* seems to prevent any apoptosis induction in those cells, which express this gene constitutively at a higher level. *bcl-2* expression

changes – as found in our studies might have the function to block apoptosis induction, mainly at the mitochondria by preventing mitochondrial permeability transition (31) and less in the nucleus, where *bcl-2* could have the function to regulate *c-myc*.

5 Conclusion and Consequences

ALA-PDT induces mainly necrosis. A smaller apoptotic fraction is caused by signal transduction, which starts more likely within the cell after light activation of the sensitizer than externally by membrane receptors, and more likely not in the nucleus, but in or close to the mitochondria - in contrast to apoptosis induction by DNA damaging agents. A reason for this is that targets for photodamage of ALA-PDT are mitochondria and ER (20), but less probable the nucleus, since even the cell cycle is not altered irreversibly by high doses. This is supported by localisation studies: PpIX is generated in the mitochondria, re-distributed over the cytoplasm and in the cytoplasmic and nuclear membranes, leaving out the nucleus as do porphyrins in general (20, 32, 33).
A similar observation was made by Luo and Kessel (34, 35), who associated mitochondrial photodamage with an apoptotic response.

The mode of the cell death influences the therapeutic success. Recent evidence suggests that the immune system plays a crucial role in tumor eradication by PDT (36). After primary tumor mass reduction by PDT, the immune system is able to prevent tumor recurrence. The stimulation of inflammation as one of the key factors in the processes is connected with necrotic events. With lethal PDT doses one could mainly induce necrosis and stimulate immune reactions against the remaining tumor, especially in deeper layers, or against tumor recurrence and metastases. Our recent in-vitro observations point to a stimulation of the cytotoxic function of macrophages by necrotized and of the growth promoting function by apoptotically killed cells (37).
The high proportion of necrosis following ALA-PDT, found in the present study, should be able to stimulate immune reactions and enhance the therapeutic effect.
It is reported on the other hand that apoptotic cell death is triggered by sublethal PDT doses resulting in reduced side effects but also in reduced inflammatory reactions. Like this Elmets et al. (38) has shown that blocking the inflammation reaction in vivo minimizes skin sensitivity without influencing the progress of tumor destruction.
Since resistance against a therapeutical approach manifests itself often in the lack of apoptosis induction, the latter is even regarded as a benefit in other tumor therapies.

6 Current Working Hypothesis for Apoptosis Induction

Based on the literature and on our studies we set up a working hypothesis about start and execution of ALA-PDT induced apoptosis. The formation of PpIX in the mitochondria is the last step in the heme biocycle before production of heme, which serves as prosthetic group for e.g. hemoglobin and cytochrome c. Yang et al. (39), Kluck et al. (40), and Kantrow and Piantadosi (41) reported that cytochrome c release induces apoptosis, which can be prevented by Bcl-2, located at the outer (39, 40) mitochondrial membrane. The proapoptotic factor cytochrome c is released from its places on the outer surface of the inner mitochondrial membrane at early steps of apoptosis (42). ALA-stimulated PpIX formation in excess could increase the amount of cytochrome c molecules and influence their release. The regulation of the membrane permeability by Bcl-2-related proteins may contribute to cell survival modulation (43) as a critical, rate limiting event of apoptosis (44). Bcl-x_L from the Bcl-2-family forms ion channels in intracellular membranes, which are pH-sensitive and cation-selective at physiological pH; it binds directly to cytochrome c and inhibits its release in the cytosol, with that protecting the cells against apoptosis (45). Hirsch et al. (46) reported about a mitochondrial megachannel by apposition of inner and outer mitochondrial membrane proteins, which induces a collapse of the mitochondrial transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix calcium and glutathione, and release of soluble intermembrane proteins. However, cytochrome c release and changes in the mitochondrial membrane permeability by PpIX-sensitization induce apoptosis (47-49). Cytosolic cytochrome c, Apaf 1 and procaspase 9 form an "apoptosome", activating a cascade of downstream caspases (50).

In addition, the local ROI production after ALA-stimulated PpIX photosensitization depletes molecular oxygen (51), which is able to disturb the regulated processes of oxidative phosphorylation and may lead to hypoxia; this in turn can induce apoptosis. ROI´s in low concentration are able to induce formation and opening of the permeability transition pores in the inner mitochondrial membrane and subsequent release of proteins, probably proteases, from the mitochondrial inter-membrane space, which can additionally induce apoptosis (15, 52). Apoptosis induction by ROI´s is also obvious by the fact that detoxification by radical scavenger such as glutathion can inhibit apoptosis (53, 54). In correlation with ROI production and apoptosis induction, the energy metabolism becomes increasingly interesting (55-57). The intracellular ATP-concentration seems to play a crucial role in apoptosis induction as a switch in the decision between apoptosis and necrosis (57-59). Apoptotic cell death can be induced by inhibitors of energy conservation. *Bcl-2* blocks apoptosis at a point downstream of the collapse of the cellular energy homeostasis (60).

In conclusion, mitochondria appear today as the central executioner of programmed cell death (60) (Fig. 6).
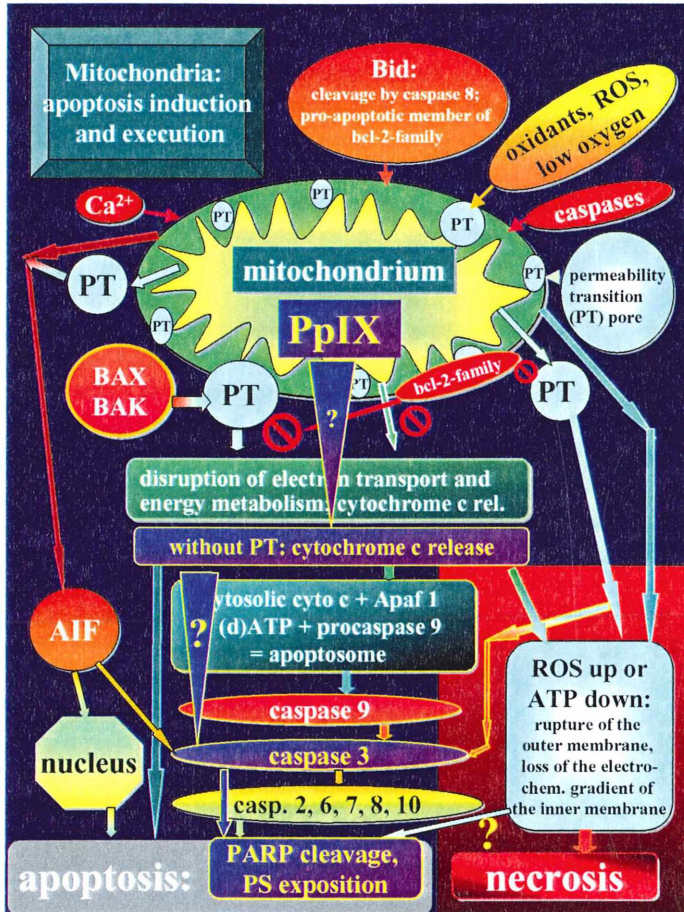
Fig. 6: Working hypothesis for apoptosis induction and execution following ALA-PDT.

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