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Review paper

Fundamentals of photodynamic therapy: cellular and biochemical aspects

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Photodynamic therapy of cancer is based on the photosensitizing ability of dyes which, after administration, are present in a somewhat higher concentration in tumors than in surrounding normal tissue. After light activation of the sensitizer, singlet oxygen and probably oxygen free radicals are formed and consequently all kinds of cellular components are affected. This review focuses on cellular and biochemical aspects of photodynamic therapy. Both damage to different cellular targets and cellular responses after photodynamic treatment are discussed.

Key words: Hematoporphyrin derivative, photosensitizers, photodynamic therapy, clonogenicity, bladder tumor, cell death.

Introduction

Photodynamic therapy (PDT) of cancer is based on the photosensitizing ability of dyes which, after administration, are preferentially taken up or are preferentially retained in neoplastic tissues.[1,2] Exposure of the dye-loaded tumor to (laser) light activates the sensitizer, reactive oxygen species are generated and so destruction of the tumor can be achieved.[1,2] Basically two types of reactions can occur after photoactivation of porphyrin dyes (Figure 1). One reaction involves the generation of free radicals (type I photochemical reaction) and in the other singlet oxygen ($^1O_2$) is generated (type II)[2,3]In both reactions the ground state sensitizer (^0S) is excited by the absorption of light quanta to a higher excited singlet state (^1S), followed by the intersystem crossing (ISC) to a triplet state (^3S). The metastable triplet state sensitizer can react with a suitable substrate, forming free radicals directly (type I), whereas in type II reactions, the energy is transfered to ground state (triplet) oxygen and consequently singlet oxygen ($^1O_2$) is produced. Excited porphyrins act mainly by a type II reaction.[4] Tumor necrosis is shown to be caused both by direct tumor cell injury and by indirect effects, mediated by stasis of the blood flow in the tumor.[5,6]

The basic scheme of PDT is shown in Table 1. The drug is given intravenously, although intratumoral injections are also possible. *In general*, the tumor:normal tissue concentration ratio of the photosensitizers currently used is maximal about 24–48 h after photosensitizer injection. The ratio depends on various factors including tissue type, the way of administration and the type of sensitizer.

Ideally, a photosensitizer should have the characteristics listed in Table 2. Photosensitizers should be chemically pure in order to be approved for use in PDT of cancer patients. For obvious reasons the dark toxicity should be very low and tumor cell selectivity and photochemical reactivity should be high. Because light penetration in tissue increases

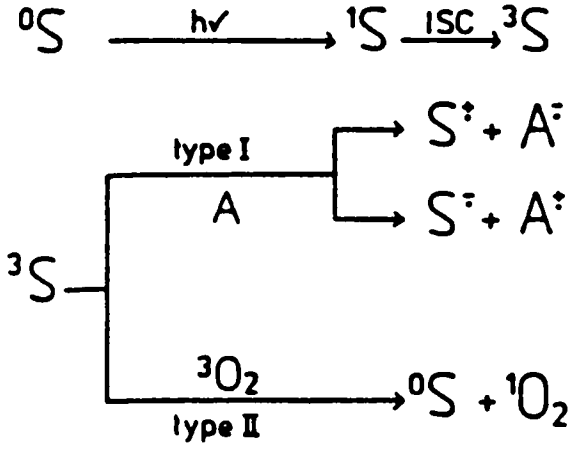

Figure 1. The main photochemical reactions after porphyrin photoactivation. ^0S, ^1S, ^3S and S· represent ground state, excited singlet state, excited triplet state and radical of the porphyrin molecule, respectively. $^3O_2$ abd $^1O_2$ represent the triplet ground state oxygen and the first excited singlet state of oxygen, respectively. 'A' means a certain substrate.

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Table 1. Basic steps of photodynamic therapy.

1. Injection of the sensitizer.
2. Accumulation retention of the sensitizer in the tumor.
   a. Transport to tumor and other tissues.
   b. Uptake by and clerance from tumor and other tissue.
3. Photoexcitation: penetration and absorption of light.
4. Intersystem crossing to triplet state of sensitizer.
5. Generation of reactive intermediates.
6. Photooxidizable cellular constituents.
7. Functional and structural alterations.
8. Cell damage/death and tumor necrosis.

rapidly with increasing wavelength (the light penetration is maximal at about 800 nm[7]), a large absorption coefficient at long wavelengths is advantageous. Last but not least, the photosensitizer must be able to induce tumor necrosis upon activation by light. The only photosensitizer now in phase III clinical trials is Photofrin II, an active fraction of hematoporphyrin derivative (HPD). Photofrin II is thought to be an aggregated mixture of porphyrin-oligomers linked through ester and ether bonds. The size of the oligomers ranges from two to six porphyrin rings, but mainly consists of dimers and trimers (Figure 2).[8–10] Another drawback of Photofrin II is that it has an absorption maximum at about 630 nm, where tissue penetration is not optimal. Taking into account the physicochemical properties of Photofrin II (short wavelength and unpure), it is clear that this is not the most efficient photosensitizer *theoretically* possible. Therefore, second generation sensitizers, with maximal absorption at higher wavelengths and chemically pure, are being studied. These new sensitizers include substituted porphyrins, chlorins, purpurines, phthalocyanines and bacteriochlorins.[11–14] In order to enhance the preferential uptake and retention in neoplastic tissue, the sensitizer can be bound to monoclonal antibodies,[15] latex micropsheres,[16] encapsulated in liposomes[16] or complexed with lipoproteins.[17]

Table 2. Properties of an ideal photosensitizer for tumor destruction.

1. Chemical purity.
2. Minimal dark toxicity.
3. High tumor selectivity and low toxicity in other tissues.
4. Large absorption coefficient at a high wavelength.
5. High photochemical reactivity (long lived excited triplet).
6. High ability to induce (in)direct tumor necrosis.

For an excellent recent review on mainly the clinical aspects of PDT see Pass,[10] which described clinical studies of (sub)cutaneous tumors, head/neck and ocular tumors, malignancies of the central nervous system, lung neoplasms, and gynecological, esophagal, genitourinal and gastrointestinal malignancies. By contrast, the present review focuses on the photodynamic effect on cells and cellular constituents. In order to improve PDT, knowledge of these photodynamic effects is of the utmost importance. Although a large number of cellular functions are inhibited by photodynamic treatment, the question "how does photodynamic therapy work?" is still largely unanswered.[18]

Vascular damage

By 1963, Castellani *et al.*[19] described the effects of hematoporphyrin on blood microcirculation, but this observation did not receive much attention. Originally, PDT was thought to mediate its cell killing effects primarily by direct cellular damage. The idea was that HPD was taken up selectively by tumor cells and that photoactivation of the sensitizer would therefore lead to direct cell killing. However, later it appeared that large amounts of HPD were also retained in macrophages, fibroblasts and endothelial cells.[20,21] This renewed interest in the observation by Castellani *et al.*[19] and the role for indirect tumor cell killing via cessation of the tumor

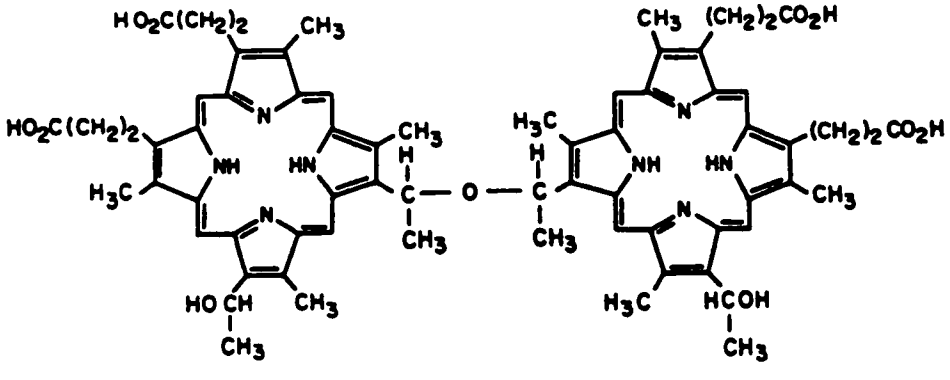

Figure 2. Chemical structure of one of the active components of Photofrin II. Bis-1-[8-(1-hydroxyethyl)deuteroporphyrin-3-yl]ethyl ether.

microvasculature or blood flow towards the tumor became a new field of research.

The role of vascular damage as a target in PDT has been deduced from two kinds of experiments. First, a clear case for this mode of PDT action was made by Henderson *et al.*[5] Using *in vivo/in vitro* protocols it was shown that the clonogenicity of EMT-6 and RIF-1 tumors, implanted in BALB/c and C3H/He mice, respectively, did not decrease if these cells were harvested immediately after PDT, despite the fact that these tumors were reduced when kept *in situ*. Obviously other factors than direct tumor cell killing were involved in PDT. Secondly, modification of vascular effects influenced photodynamically induced tumor reduction. Moreover, a reduction in blood flow was observed by Star *et al.*[6] in a 'sandwich-chamber' on the flank of a rat.

Vascular changes following PDT were observed by many authors,[22–29] and have been reviewed by Zhou,[30] Ben-Hur and Orenstein[31] and Chaplin.[32] Vasoconstriction and blood cell aggregation will result in reduced blood oxygen pressure and anoxia in the tumor, finally resulting in tumor necrosis.[28,33] The biochemical mechanisms behind these phenomena are now being elucidated. Anticoagulation and coagulation are maintained by endothelial cells which produce prostacyclin and by platelets which produce thromboxane A_2. Interference in this equilibrium can play a crucial role in the reduction of the tumor blood flow, as observed by Reed *et al.*[34] Release of prostaglandin E_2 (PGE_2) after HPD photosensitization was observed by Henderson and Donavan[35] in RIF tumor cells. Fingar *et al.*[36,37] described the effects of thromboxane B_2 (TXB_2) and indomethacin (an inhibitor of an enzymatic step involved in TXB_2 synthesis) on chondrosarcoma-bearing Sprague-Dawley rats. A clear correlation between the tumor reduction and induction of TXB_2 was observed.

Histamine release was observed in mice[22,38,39] and induction of the von Willebrand factor was observed by Foster *et al.*[40] after HPD photosensitization of human umbilical vein endothelial cells. Fingar *et al.*[41] studied vessel constriction, vessel permeability and leukocyte adhesion during and after Photofrin II PDT of Sprague-Dawley rats. It was suggested that cyclooxygenase products (thromboxanes, PGE_2) were important factors in causing vessel constriction and changes in permeability. These results indicate that the therapeutic response can be enhanced by compounds that reduce blood flow and consequently reduce blood oxygen pressure. However, a side effect of reduced oxygen pressure in the tumor can be that the photochemical reactions leading to singlet oxygen generation are hampered.[4,28]

Plasma membrane damage

Binding of porphyrins to the plasma membrane was observed by fluorescence microscopy as described by Moan *et al.*[42] Thereafter light-induced membrane alterations, like ^{51}Cr or K^+ leakage or inhibition of transmembrane transport systems, were reported in CHO cells, L1210 leukemia cells and L929 fibroblasts.[43–47] Boegheim *et al.*[48–50] described the effects of HPD photosensitization on L929 fibroblasts and CHO cells with transmembrane transport systems and potassium leakage as parameters for membrane damage. Despite the large cell-type specific differences in HPD photosensitivity, a general feature that arose is that active and passive transport systems appeared to be very sensitive. However, it was suggested by Boegheim *et al.*[49,50] that damage to the plasma membrane did not contribute to the photodynamic inactivation of these cells.

Membrane damage after HPD photosensitization has been recently reviewed by Dubbelman *et al.*[51] In a systematic way they studied three cell lines, i.e. murine L929 fibroblasts, CHO epithelial cells and T24 human bladder transitional carcinoma cells, with HPD as photosensitizer. Many plasma membrane parameters could be excluded to be directly related to the loss of clonogenicity and only a few plasma membrane enzymes may be directly involved in photodynamic cell killing of these three unrelated cell lines. Among the few was the plasma membrane enzyme Na^+/K^+-ATPase, which was very sensitive, and which was not rapidly repaired. Therefore, it could not be excluded as a possible critical target in L929 fibroblasts as well as in T24 cells. However, in more detailed studies on possible mechanisms of photodynamic inhibition of Na^+/K^+-ATPase, a causal relationship between reduced enzyme activity and cell death was excluded. In L929 fibreblasts and T24 cells, glucose addition prior to light exposure reduced the enzymatic photosensitivity, but did not influence the reproductive cell death.[52]

Mitochondrial damage

Colony growth after photodynamic treatment reflects the ability of surviving cells to replicate. An increase in cell number requires energy not only for replication but also for repair processes. Cellular energy is available in the form of high energy bonds in ATP, which is produced both by anerobic

glycolysis as well as by oxidative phosphorylation. Persistent inhibition of one of these processes can ultimately lead to ATP depletion and subsequently reproductive cell death (loss of clonogenicity).

HPD was shown by fluorescence microscopy to localize in mitochondria in cultured cells.[53–55] Moreover mitochondrial disruption, such as membrane or cristae destruction or changes in membrane potential, have been observed *in vitro* by (electron) microscopy.[42,45,56] Therefore, numerous studies have focused on photodynamic damage to isolated rat liver mitochondria, as reviewed by Salet and Moreno.[57]

The photosensitivity of mitochondrial enzymes increased from intermembrane space enzymes such as adenylate kinase[48,58] via outer mitochondrial membrane enzymes like monoamine oxidase[58] to inner mitochondrial membrane enzymes such as cytochrome-*c* oxidase,[59] succinate dehydrogenase[60,50] and F_0/F_1-ATPase.[61] Impaired mitochondrial functions may lead to ATP depletion. Indeed a decrease in ATP levels immediately after light exposure has been observed both *in vivo* and *in vitro*,[60,45] Boegheim *et al.*[56] described a transient decrease in ATP levels for L929 cells, whereas in Chinese hamster ovary and T24 cells no restoration of decreased cellular ATO concentration was measured.

Nuclear damage

It is important to evaluate mutagenic side effects of new modalities to treat cancer. Not surprisingly nucleic acids and mutation/transformation have been the subject of many research papers both for HPD and phthalocyanines. At physiological pH, guanine is by far the most sensitive base for HPD photosensitization as shown by Dubbelman *et al.*[62] and by Kawanishi *et al.*[63] HPD photosensitization converts guanine into 8-OH-guanine. Alkaline-labile sites and DNA single-strand breaks have been found in NHIK 3025 cells, but it appeared that these breaks did not play a crucial role in photodynamic cell killing.[64–66] The genetic material of CHO cells is rather insensitive for HPD photosensitization. The number of induced mutants resistant to 6-thioguanine (mutation at the hypoxanthine-guanine phosphoribosyltransferase locus) observed was very low compared with equally toxic doses of X-radiation, but a comparable increase in the amount of sister chromatid exchange was mesured.[67] Combinations with X-rays or effects of 3-aminobenzamide, a potent inhibitor of poly(ADP-ribose) transferase (ADPRT), revealsed that X-rays and HPD photosensitization did not interact synergistically in inducing cell killing.[68,69]

The use of DNA repair deficient cell lines, like Ataxia telengiectasia or xeroderma pigmentosum, indicated that nuclear damage was not a dominant factor in cell death caused by Photofrin II.[70] However, Boegheim *et al.*[71] have shown that DNA excision repair is severely inhibited at a stage beyond the incision step when murine L929 fibroblasts were sensitized with HPD. Therefore, both inhibition of DNA polymerase or DNA ligase activities were likely candidates responsible for the reduced repair capacity after HPD photosensitization. The situation, however, was somewhat more complicated; neither the polymerase activity nor the ligase activity was persistently inhibited. Poly(ADP-ribose)transferase is a key enzyme system in DNA repair.[72] Neutral Ca^{2+}/Mg^{2+}-dependent endonucleases are maintained in a latent form by poly(ADP-ribose)transferase, but endonucleases are derepressed by inactivation of poly (ADP-ribose)transferase.[73] It appeared that, in photodynamically treated L929 cells, a small amount of DNA damage coincided with a severe inhibition of poly(ADP-ribosyl)ation. As a consequence, endonucleases are no longer inhibited and subsequently the DNA is broken down in random-sized small fragments.[74] In contrast to this DNA smear on agarose gels,[74] are the observations by Agarwal *et al.*[75], where a typical inter-nucleosomal DNA fragmentation (a biochemical hallmark for apoptosis) was observed after HPD photosensitization of L5178Y murine lymphoma cells.

Immunological effects

In vivo, increased amounts of cytokines in the urine of treated patients and the infiltration of lymphocytes and plasma cells into photodynamically treated tissues suggest that immune responses are triggered.[76,77] Immunosuppression has been described by Jolles *et al.*[78] Contact hypersensitivity to dinitrofluorobenzene (DNFB) was inhibited in mice after HPD photoradiation.[79] The inhibition was shown to be mediated via the development of suppressor cells. The Fc receptor for IgG on monocytes was changed upon photosensitization, resulting in decreased binding.[80] However, natural killer cell activity of splenocytes and Fc-mediated phagocytosis were only slightly inhibited by HPD photosensitization.[81] Lynch *et al.*[82] studied the HPD photosensitized induced immunosuppression in mice. It appeared that the immunosuppression was

caused by cells of the macrophage lineage. Macrophages, which take up large amounts of HPD, are reported to release TNF upon photodynamic activation.[83]

Cellular rescue responses

In general, cells respond to chemical or physical stress by changes in protein synthesis and regulation of specifically expressed proteins, so-called stress proteins. Stress proteins can be divided into three groups: heat-shock regulated proteins HHSPs), glucose regulated proteins (GRPs) and heme oxygenase stress proteins (HO).[84]

Recently it became clear that also photodynamic treatment can induce stress proteins of all three main groups, i.e. HSPs, GRPs and HO.[85–87] Chinese hamster fibroblasts treated with HPD or Photofrin II were significantly more resistant to a subsequent doxorubicin treatment.[85] However, heat resistant Chinese hamster fibroblasts with increased mRNA levels of HSP70 were as sensitive to photodynamic treatment as the parental cell lines, expressing normal levels of HSP70 mRNAs.[86]

Less sensitive mutants do not have enhanced levels of glutathione (GSH), superoxide dismutase (SOD) or catalase, nor do they have enhanced expression of P170 glycoprotein.[88,89] A higher expression of certain repair enzymes has not been reported. Apparently cellular resistance or tolerance is mediated by other mechanisms.

Research with HPD-treated human bladder transitional carcinoma cells, clone T24, has revealed an insight in mechanisms of cellular resistance. It appeared that a transient increase in intracellular free calcium was involved in such a mechanism. Elevation of the intracelular free calcium concentration after HPD PDT has been shown by Specht and Rodgers[90] and after AlPc PDT by Ben-Hur *et al.*[91] In both cases no causal relationship with cell killing was established. Penning *et al.*[92] measured changes in the intracellular free calcium concentration in two cellular systems, i.e. CHO cells loaded with AlPc and T24 cells loaded with HPD. Exposure of both cells to light resulted in an increase in intracellular free calcium, reaching a peak 5–15 min after light exposure. It was shown that the calcium transient was caused by an influx of extracellular calcium rather than release from intracellular stores. Calcium chelation with Quin 2-AM or BATPA/AM did not result in reduced cell killing but rather in enhanced cell killing. This indicates that the increase in intracellular free calcium after photodynamic treatment contributes to cell survival by triggering some kind of cellular rescue response in tehse cells.

To investigate this mechanism further, HPD-induced photodynamic effects on the calcium mediated arachidonic acid cascade were studied in T24 cells.[93] Photodynamic treatment resulted in a transient release of arachidonic acid derived compounds; in particular, PGE_2 and TXB_2 were strongly increased. This release was reduced by chelation of intracellular calcium with Quin-2 or by lowering the calcium concentration in the medium by EGTA, presumably resulting in inhibition of phospholipase A_2. A similar reduction was obtained when indomethacin, as inhibitor of the cyclooxygenase pathway, was added. These three treatments enhanced the photosensitivity; on the other hand, addition of PGE_2 (but not TXB_2) protected against photodynamic treatment. The results of these experiments suggest that calcium-mediated activation of cyclooxygenase, resulting in increased levels of PGE_2, participates in a cellular defence mechanism against photodynamic cell killing. Cytoprotective effects of prostaglandins (especially PGE_2) have been described in other cell types after different forms of stress,[94,95] PGE_2 is known to increase intracellular cAMP concentration through an action on adenylyl cyclase and cAMP has the ability to stabilize plasma membrane.[96] Moreover, Bergasa *et al.*[97] demonstrated a cytoprotective effect of PGE_2 via an increase in intracellular cAMP concentration in hepatocytes exposed to hepatoxin aflatoxin B_1. Therefore, the relation between photodynamically induced PGE_2 release, changes in intracellular cAMP concentration and the involvement of AP in protection against photodynamic treatment were studied in HPD-loaded T24 cells.[98] Immediately after light exposure a light dose-dependent increase in the intracellular cAMP concentration was observed. This increase was counteracted by indomethacin. An increase in cAMP levels was also induced by PGE_2. Cell survival was enhanced by PGE_2 and reduced by indomethacin. Both direct activation of adenylyl cyclase by forskolin as well as the cAMP analogue 8′-bromo cAMP clearly inhibited photodynamically-induced cell death. Therefore, it was concluded that cytoprotection is mediated via an increase in intracellular cAMP concentration, most likely caused by PGE_2 stimulation of adenylyl cylclase.

Conclusions

Despite about 20 years of research on the biochemical background of PDT, a large number of ques-

tions remain unanswered. As is clear from this review, no critical target for reproductive cell death has been described unambiguously. What is the actual cause of the increased amounts of photosensitizer in tumor cells? Especially in the case of HPD, which porphyrin oligomer is responsible for cell killing? Do photosensitizing drugs enhance metastasis?

Obviously, these considerations vary from pure chemical origin, via *in vitro* biological problems to *in vivo* questions. To solve these questions, close cooperation between chemists, biologists and clinicians is of importance. Is the future for PDT "light at the end of a tunnel"?

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