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Review article

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PHOTODYNAMIC THERAPY IN MELANOMA - AN UPDATE

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Melanoma, a cancer that arises from melanocytes is one of the most unresponsive cancers to known therapies. Several studies showed encouraging results of the efficacy of photodynamic therapy (PDT) using different experimental settings *in vitro* and *in vivo* as well as a few clinical reports, suggesting a possible role as an adjuvant therapy in the management of advanced melanoma (stage III and IV). In experimental settings, PDT using different protocols on human and mice melanoma cells induced significant apoptosis, necrosis, tumor growth arrest and prolonged the survival of the animals, but seldom achieved complete remission and/or was followed by recurrence and side effects. Clinical reports showed regression of choroidal melanoma and skin melanoma metastasis following PDT. PDT consists in administration of a photosensitizer, which undergoes excitation after suitable irradiation emitted from a light source and generates singlet oxygen ($^1O_2$) and other cytotoxic oxygen species such as superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$). The antitumor effects result from the combination of direct tumor cell photodamage, destruction of tumor vasculature and activation of an immune response. To increase the effectiveness of PDT in melanoma, the therapy has to overcome the protective mechanisms like pigmentation and increased oxidative stress defense, possibly through inhibition of melanogenesis and melanosome targeted photosensitizers. The optimal protocols for tumor and vascular targeted PDT could destroy melanoma and endothelial tumor cells and activate the immune response, thus increasing the overall efficacy. Combination of PDT with immune stimulation therapies might increase the efficiency in destroying the initial tumor as well as micro metastases and decrease the melanoma relapses.

Key words: *apoptosis, immune response, melanin, melanoma, photodynamic therapy, superoxide anion, caspase*

INTRODUCTION

Malignant melanoma is a cancer that arises from melanocytes, pigmentary cells found predominantly in the skin. Although melanoma accounts for only 4% of skin cancer cases, it causes 79% of all skin cancer related deaths. Diagnosis and staging is based on histopathology examination of the tumor following surgical procedures and sentinel lymph node biopsy if histopathology staging, based on primary tumor thickness, ulceration and mitosis surpasses T1b or T2 (1). If diagnosed early, melanoma can be cured by surgical resection with almost 80% effectiveness for thin lesions. However, once metastases occur, it is largely refractory to existing therapies (1, 2). Median survival time for patients with stage IV melanoma is approximately 9 months, and 3-year survival rates are less than 15% (1). The National Comprehensive Cancer Network (NCCN) recommends for stage III and local recurrence of melanoma: intralesional injection of BCG or interferon, local ablation or radiation therapy (2). Other potential therapies include interferon α-2b, vaccines, high-dose bolus interleukin-2 alone or in combination with chemotherapy (3). The agents used in the treatment of stage IV melanoma are dacarbazine, temozolomide, high-dose interleukin-2, and paclitaxel (with or without carboplatin) (2).

Topical 5% imiquimod cream and irradiation of skin metastases with a continuous-wave 810 nm laser was used to widen the response to distant nonirradiated lesions (4). There are studies that show encouraging results of the efficacy of photodynamic therapy (PDT) in melanoma, in different experimental settings *in vitro* and *in vivo* as well as several clinical reports. However, there is a very limited experience of the PDT in melanoma and these sporadic reports are to be followed by extensive clinical studies, using selected photosensitizers and standard irradiation protocols in order for PDT to be accepted as an effective procedure in melanoma therapy. Taken these into consideration, the role of PDT in melanoma remains to be established. For the time being, PDT can be considered as an alternative adjuvant therapy in selected cases of advanced melanoma (stages III and IV).

PDT has been approved in many countries for the treatment of lung, esophageal, bladder, skin and head and neck cancers. It is a minimally invasive two-stage procedure that requires administration of a photosensitizing agent (PS) followed by illumination of the tumor with visible light, usually laser generated (5, 6). This leads to subsequent biochemical events that cause destruction of selected cells (7). PS absorbs a photon of an appropriate wavelength of light to form an excited triplet state (8). The excited molecule can then transfer energy to the (triplet) ground state of molecular oxygen to produce the highly cytotoxic singlet oxygen ($^1O_2$) -type II reaction or undergo electron transfer (type I reaction) with the ultimate formation of superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$).

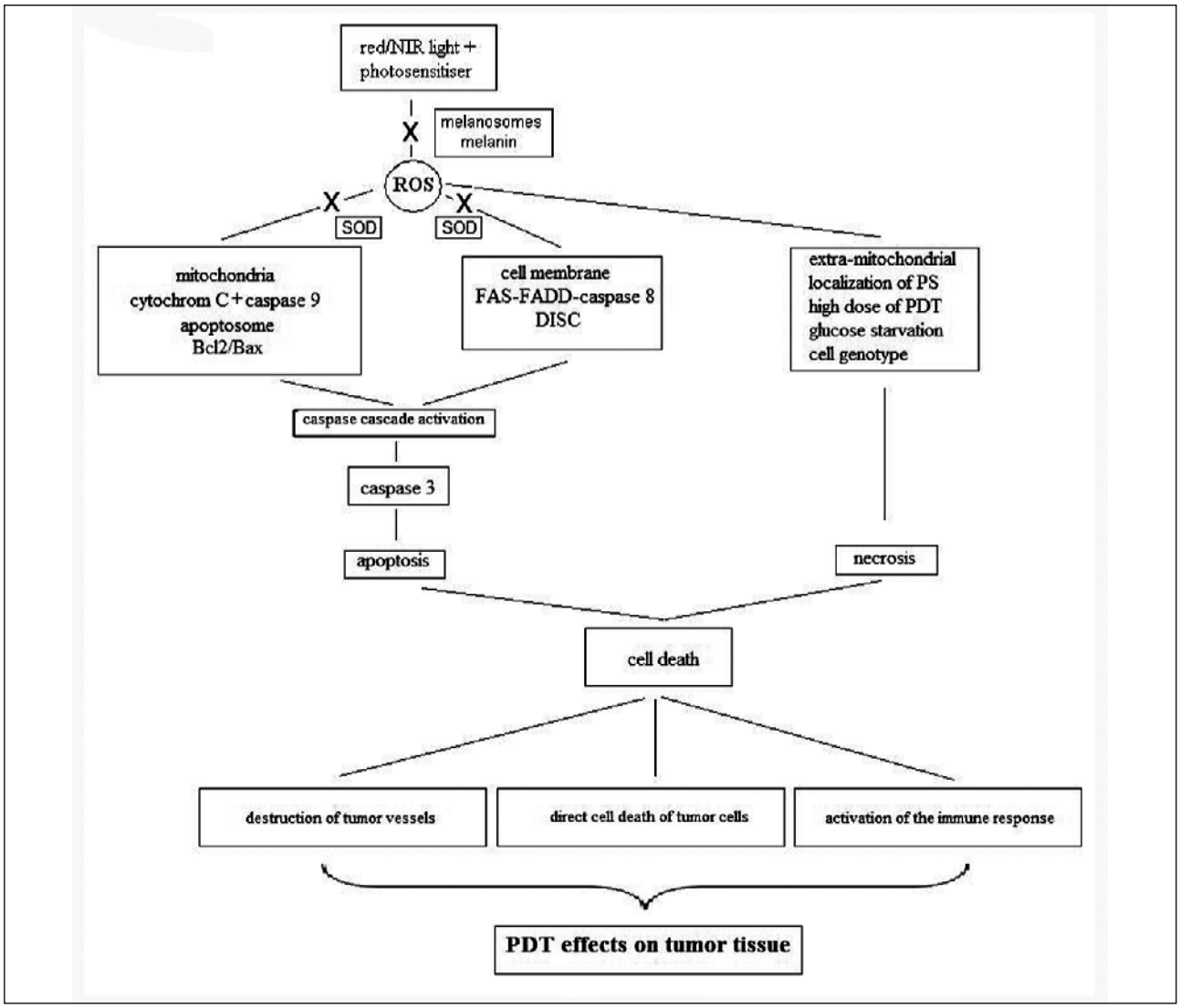

Fig. 1. Mechanisms of action of PDT in melanoma. PDT requires administration of a photosensitizer, followed by it's activation through irradiation with light of suitable length (red or near infrared - NIR). This generates reactive oxygen species, especially superoxide anion ($O_2^{\bullet-}$). These highly cytotoxic molecules induce direct tumor cell photodamage, destruction of tumor vasculature and activation of an immune response. In melanoma, PDT has to overcome the antioxidant defense mechanisms, represented by enhanced melanogenesis and superoxide dismutase activity. Direct tumor cell death (melanoma and endothelial cells) may be a result of apoptosis through mitochondria or membrane mediated pathway and/or necrosis depending on the accumulation of the photosensitizer, light dose, cell type. Necrosis can be beneficial for the final outcome of the therapy since it enhances the immune response following PDT and can lead to further destruction of local and distant melanoma cells.

These reactive oxygen species can oxidize important biological molecules such as proteins, lipids, and nucleic acids (9).

The ideal photosensitizer should meet several criteria: chemical purity, preferential tumor retention, fast tumor accumulation and rapid clearance, activation by light with good tissue penetration, high absorption coefficient, no dark toxicity, minimal or absent skin photosensitivity (10). The most extensively studied PS are porphyrins (11-13), as they are the closest to meet these requirements; other classes of PS include different chlorines (14), bacteriochlorins (15-17), phthalocyanines (18, 19), porphycens (20), hypericin (21, 22), chlorophyll derivatives (23), texapyrins, antracens, purpurins and hypocrellins. ALA (5-aminolevulinic acid, Levulan), its methylester (Metvix), Photofrin and *m*-THCP (*meta*-tetrahydroxyphenylchlorin, Foscan) have been approved for use in clinical oncology (10). PDT with ALA and Metvix is effective in keratinocyte derived malignancies and premalignant lesions (24), but not in melanoma (25, 26). The antitumor effects of photodynamic therapy result from the combination of direct tumor cell photodamage, destruction of tumor vasculature and activation of an immune response (10). In order to achieve optimal tumor damage and to overcome the melanoma resistance, PDT has to act through all three mechanisms (*Fig. 1*).

MECHANISMS OF DIRECT TUMOUR CELL DEATH IN PHOTODYNAMIC THERAPY

Most studies on PDT effects were focused on those that lead to cell death, through the generation of reactive oxygen species (ROS) (10). Superoxide ion $O_2^{\bullet-}$ is one of the main products of phototoxic reactions. Due to the very short half-life of $O_2^{\bullet-}$, measured in nanoseconds, this cytotoxic molecule can diffuse only a very limited range up to 20 nm in cells (27). Therefore,

the subcellular localization of the PS determines which organelles are primarily damaged. Superoxide dismutase (SOD) both the constitutive (Cu, Zn-SOD) as well as an inducible (Mn-SOD) isoform is engaged in the $O_2^{\bullet-}$ scavenging. PDT increases the expression of the Mn-SOD but not of the Cu, Zn-SOD (28). The experimental data suggest an important protective role of the inducible Mn-SOD isoform of superoxide dismutase in tumor cells exposed to PDT (10, 29, 30). PDT has been also shown to induce DNA damage; however the mechanisms are not well understood. PDT can cause base oxidation, cross-linking of DNA strands or sister chromatid exchange (31). Moreover, DNA damage response in PDT can affect both the tumor cells and the surrounding normal cells (31). Cell death following PDT can occur either by apoptosis or by necrosis, depending on the cell type, oxygen level, concentration and intracellular localization of the PS as well as the light dose (32-34).

Apoptosis is a tightly controlled, energy-consuming process of suicidal cell death involving activation of hydrolytic enzymes such as proteases and nucleases leading to DNA fragmentation and degradation of intracellular structures (35). Apoptosis has been shown to be a rapid and dominant form of cell death following PDT in multiple experimental settings, using various PS and cell types (36, 37).

However, since apoptosis was the only form of cell death investigated in the majority of studies it is likely that some cells may also undergo necrosis after PDT. Factors that promote necrosis include extra-mitochondrial localization of PS, high dose of PDT, glucose starvation (32, 38) and cell genotype (39).

In the case of necrosis, cytosolic constituents spill into the extracellular space through the damaged plasma membrane and provoke a robust inflammatory response. These products are safely isolated by the intact membranes that initially persist in apoptotic cells, which are phagocytosed by macrophages. The acute inflammation that is caused by PDT-induced necrosis might potentiate immunity by attracting host leukocytes into the tumor and increasing antigen presentation (8).

Necrosis leads to a more important immunological activation (40) thus could be beneficial for the final outcome of PDT, especially in melanoma where the presence of a high frequency of tumor-infiltrating lymphocytes is associated with better outcomes in patients receiving treatment for melanoma (41).

PDT induces apoptosis *via* two major pathways: mitochondria-mediated or intrinsic pathway, and death receptor-mediated or extrinsic pathway (38). The mitochondrial apoptosis pathway occurs mainly when PS accumulate inside these organelles. Presumed PDT effects are: disruption of mitochondrial transmembrane potential and release of cytochrome C into the cytosol, leading to the formation of a complex called apoptosome and initiates the cascade activation of caspases (10). Caspases 2, -3, -6, -7, and -8 have been shown to be activated following PDT in various experimental models (42, 43). The mitochondrial apoptosis pathway is strongly influenced by balance between the pro- and antiapoptotic members of the Bcl2 family of proteins (36), containing two groups, depending on the influence on apoptotic pathways: Bcl-2, Bcl-XL, Bcl-w, Mcl and A1 are antiapoptotic; while Bax, Bok, Bfm, Bcl-XS and others promote apoptosis (44).

Death receptor-mediated apoptosis occurs preferentially when PS target the cell membrane. It is triggered by multimerization of cell membrane receptors belonging to the tumor necrosis factor receptor (TNF- R) superfamily, and especially Fas receptor. The death inducing signaling complex (DISC) formed by Fas receptor, FADD (Fas-associated death domain protein) and caspase 8 is a pivotal trigger of apoptosis (45).

The role of p53 in PDT induced apoptosis in tumor cells is not understood. p53 plays an important role in the response of tumor cells to chemo- and radiotherapy (46). Although, PDT of tumor cells results in over expression of p53, the cell death might be p53-independent (38).

PHOTODYNAMIC THERAPY IN MELANOMA – EXPERIMENTAL AND CLINICAL STUDIES

There have been reports of PDT applications for melanoma treatment in both animals and humans. In animals, both amelanotic cell lines and pigmented melanoma cell lines have been used to grow tumors. In several *in vivo* experimental settings PDT using different PS and irradiation protocols on human and mice melanoma cells induced significant apoptosis (19, 24, 47-51), regression (25, 52-55), tumor growth arrest (51, 56, 57), tumor necrosis (52, 53, 58) and prolonged survival of the animals (53, 54, 58) (*Table 2*).

PDT destruction of melanoma-associated intralymphatic tumour cells and lymphatic vessels prevented metastasis and relapse in a mouse model (59).

However, PDT has seldom achieved complete remission (54) of the experimental melanoma *in vivo;* most of the reports showed partial remission (51-57) and/or were followed by recurrence of melanoma (55). Moreover, administration of the PS systemically provoked side effects in animals like: photosensitizing effect on both tumor and normal tissue (60) liver and kidney toxicity (52, 53).

In human oncology, PDT was used to treat ocular melanomas. PDT with verteporfin was partially effective as a second line of therapy (61) or effective as a first intention treatment of choroidal melanoma (62, 63) (*Table 1*). PDT using clorin e injected intravenously at 5 mg/kg and irradiation with 660-nm light was well tolerated and effective on skin melanoma metastases (64). However, there is a very limited experience of the PDT in melanoma and these sporadic reports are to be followed by extensive clinical studies, in order for PDT to be accepted as an effective adjuvant procedure in choroidal or advanced cutaneous melanoma.

MELANOMA MELANOGENESIS AND PHOTODYNAMIC THERAPY

Melanins are the main surface pigments that play a major role in photoprotection. In melanocytes, ultraviolet radiation initiates pigment synthesis in melanocytes *via* the p53- cAMP-MITF tanning pathway (65) and increases SOD activity (66). MITF (microphthalmia-associated transcription factor) is the essential regulator of protein expression involved in melanin production (such as tyrosinase, the key melanogenesis enzyme). MITF also controls the genes involved in melanocyte development, proliferation, survival and malignant transformation (67). Melanin synthesis is initiated by the enzymatic hydroxylation of the L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) and oxidation of L-DOPA to DOPA quinone. DOPA quinone is subsequently transformed to melanin in a series of reactions. All these reactions take place in the melanosomes, in order to prevent leakage of the cytotoxic byproducts of melanogenesis into the cytoplasm (68). Numerous stimuli are able to alter melanogenesis in cultured melanocytes (69).

The type of melanin produced depends on the cellular genotype and environmental factors, resulting in the black pigment eumelanin, the reddish to yellow pigment pheomelanin, or the mixed melanin that contains both components (70). Consequently, melanomas can vary from nonpigmented tumors that have no melanin whatsoever, through moderately pigmented to highly pigmented tumors, and their pigmentation level is proportional to the degree of differentiation and inversely proportional to the growth rate (71).

Table 1. Experimental photodynamic therapy studies in melanoma.

study type	authors, year of publication	photosensitizer	wave length	experimental melanoma model	effects
in vivo	Panagopoulos *et al*., 1989	chloroaluminum sulfonated phthalocyanine (CASPc)	675 nm	intraocular melanoma in rabbit	photosensitizing effect on both tumor and normal tissue
in vivo	Ozler *et al*., 1992	chloroaluminum sulfonated phthalocyanine	675 nm	Greene hamster melanoma transplanted into the subchoroidal space in rabbits	initial tumor growth arrest/ regrowth at 7 days
in vivo	Dellian *et al*., 1996	three porphycenes, photofrin	635 nm	amelanotic hamster melanoma A-Mel-3	complete local tumor remission
In vivo	Biolo *et al*., 1996	liposome-delivered Si (IV)-naphthalocyanine (SiNc)	776 nm	C57 mice bearing B16 melanoma	delay in the rate of tumor growth improved by hypertermia
in vivo	Young *et al*.,1996	liposomal benzoporphyrin derivative monoacid (BPD), verteporfin	692 nm	pigmented choroidal melanoma in albino rabbit eyes	tumor necrosis, regression, side effects
in vivo	Abels *et al*., 1997	5-aminolevulinic acid (ALA)	635 nm	amelanotic melanomas (A-Mel-3) xenografts on Syrian golden hamsters	partial remission
in vivo	Woodburn *et al*., 1998	lutetium texaphyrin	700-760 nm	C57BL/6 tumor bearing mice	double median survival time
in vitro & *in vivo*	Haddad *et al*., 1998	aluminum phthalocyanine (AlpcS4)	675 nm	B16 murine melanoma cells C57/B1 mice bearing B16 tumors	decrease in cell viability; massive tumor necrosis, prolonged mice survival, decreased tumor size, side effects
in vivo	Busetti *et al*., 1996	benzoporphyrin derivative monoacid ring A (verteporfin, BPD-MA)	690-nm	C57/BL6 mice bearing B1 melanoma	tumour necrosis, delay of tumour growth
in vitro & *in vivo*	Haddad *et al*., 2000	5-aminolevulinic acid (ALA)	635 nm	B16 melanoma cells C57/B1 mice bearing B16 tumors	tumor necrosis, prolonged survival of mice
in vivo	Hu *et al*., 2002	liposomal benzoporphyrin derivative monoacid (BPD), verteporfin	692 nm	pigmented choroidal melanoma in albino rabbit eyes	tumor growth arrest
in vitro	Szurko *et al*., 2003,	meso-tetra-4-N-methylpyridyl-porphyrin iodide and 5,10-di-(4-acetamidophenyl)-15,20-di-(4-N-methylpyridyl) porphyrin	630 nm	human malignant melanoma Me45 line	necrosis induction, inhibition of colonies growth at non-toxic concentrations
in vitro	Barge *et al*., 2004	bis (cholesteryloxy) derivate of silicon-phthalocyanine (Chol-O-SiPc)	675 nm	human-pigmented melanoma cells	cell apoptosis mediated by mitochondria
in vitro	Saczko, 2005	photofrin II	632.8 nm	human Beidegröm melanoma (BM)	induction of apoptosis dependent on photosensitiser and light doses
in vitro	Robertson *et al*., 2011	metallophthalocyanine (MPc) and 5 - ALA	680 nm 636 nm	human metastatic melanoma A375	growth inhibition, apoptosis induction, higher MPc efficiency compared to ALA
in vitro	Maduray *et al*., 2011	zinc tetrasulfophthalocyanines (ZnTSPc)	672 nm	human melanoma, keratinocytes, fibroblasts	PDT induced death in melanoma cells with minimal damage to normal cells
in vivo	Otake *et al*., 2010	D-glucose residue pendant fullerene-C(60)-(Glc)	UVA	melanoma-bearing mice	suppressed tumor growth
in vivo	Tammela *et al*., 2011	verteporfin	635 nm	mice bearing mouse melanomas; intralymphatic tumor cells	PDT destruction of tumor-associated lymphatic vessels prevented metastasis and subsequent melanoma relapse
in vitro & *in vivo*	Dabrowski *et al*., 2011a	synthetic chlorin derivative-TCPCSO(3) H	750 nm	Cloudman mouse melanoma (S91) DBA mice bearing S91 tumors	temporary or permanent tumor remission at nontoxic doses; increased survival
in vitro & *in vivo*	Dabrowski *et al*., 2011b	5,10,15,20-tetrakis(2-chloro-5-sulfophenyl) bacteriochlorin (TCPBSO3H)	750 nm	Cloudman mouse melanoma (S91) DBA mice bearing S91 tumors	initial tumor regression with recurrence at 2 months

Table 2. Clinical reports of photodynamic therapy effectiveness on patients with melanoma.

authors, year of publication	photosensitizer	PDT protocol	tumor type	effects
Barbazetto *et al*., 2003	verteporfin	1 PDT, light dose=100 J/cm^2	choroidal melanoma - 4 cases	complete regression of melanoma in two cases, the other two required enucleation
Sheleg *et al*., 2004	clorin e (6)	2 PDT, each light dose=80-120 J/ cm^2	skin metastases of pigmented melanoma - 14 patients	complete regression of skin metastasis with no recurrence, no hepatic or kidney toxicity of the drug
Donaldson *et al*., 2005	verteporfin	4 PDT; each light dose=100 J/cm^2	amelanotic choroidal melanoma - 1 case	complete regression
Soucek, Cihelkova, 2006	verteporfin	1 PDT, light dose=100 J/cm^2	amelanotic choroidal melanoma - 1 case	complete regression

The possible protective effects of constitutive and especially PDT-induced pigmentation and antioxidant defense provided by increased SOD activity in melanoma are not yet understood, but they might explain the relative resistance of pigmented melanomas to PDT. Pigmented human xenograft melanotic melanoma in mice, was far less responsive to PDT than amelanotic melanoma (72). The protecting role of melanin and melanosomes in chemotherapy was shown *in vitro* (73, 74); however, it is not currently understood in PDT. Members of the ATP-binding cassette (ABC) transporters are located in the melanosomal membrane (73). ABC transporters, reduce the effectiveness of treatment by lowering the concentration of the anticancer drug inside the cell. This is of major importance in resistance of melanoma cells to chemotherapy and may be

involved in accumulation of the PS in PDT. Inhibition of the ABC transporter system with pentagalloylglucose and procyanidins from *Oenothera paradoxa* Hudziok defatted seeds extract, increased chemotherapy effectiveness of vincristine treatment by more than 4 times in a metastatic melanoma line (HTB-140) *in vitro* (75).

The presence of intracellular melanin plays a role in rendering melanoma cells less susceptible to cell death, probably through the ability of this pigment to act as an intracellular antioxidant, thus neutralizing chemotherapeutic-induced reactive oxygen species (9). The mechanism of quenching of excited states of positively charged porphyrin molecules bound to melanin was determined by femtosecond absorption and picosecond emission spectroscopy (76).

Increased melanogenesis increases the resistance of melanoma cells to chemo-, immunotherapy and radiation therapy. The inhibition of melanogenesis significantly increased the sensitivity of cells to cyclophosphamide treatment (77) and to γ irradiation (78). Decreasing the melanin levels or application of the near-infrared (NIR) absorbing photosensitizers show considerable promise (8). Since melanin absorbs in the blue region of light, the photodynamic efficacy is significantly diminished with photosensitizers absorbing in this region. NIR absorbing sensitizers are much more suitable for PDT of melanoma because this region of light is the most transparent and the tissues are deeper penetrated by NIR photons. Depigmenting melanoma (UCT Mel-1) with a reversible tyrosinase inhibitor-phenylthiourea significantly increased the cell death after hypericin-mediated PDT compared to untreated melanomas; moreover the sensitivity of the tumor cells to treatment returned to the level of untreated melanoma cells on removing the tyrosinase inhibitor (79).

Increased responsiveness to PDT with lutetium texaphyrin was obtained in ApoE deficient mice bearing heavily pigmented B16F10 murine melanoma where the predominant subcellular site of photosensitizer binding was to melanosomes (80). The response of pigmented melanoma to PDT was enhanced by pretreatment with 1064-nm light from a pulse-operated Nd:YAG laser, which caused a selective breakdown of melanosomes (81). Phototherapy using hypericin (3 μM) activated by UVA irradiation induced a necrotic mode of cell death in pigmented melanoma cells and melanocytes and an apoptotic cell death in non-pigmented melanoma cells and keratinocytes. Necrosis of the pigmented cells could be related to increased permeability of melanosomes due to ROS formation (21).

The melanin content affected the effectiveness of PDT using three bacteriochlorins as photosensitisers, but the degree of reduction was significantly lower for bacteriochlorins than for Photofrin. All bacteriochlorins accumulated in melanosomes, leading to melanosomal damage. The least effective bacteriochlorin localized predominantly in lysosomes, while the most effective one preferentially accumulated in mitochondria and produced significantly higher levels of hydroxyl radicals (8).

Unfortunately, intracellular targeting is a challenge due to the difficulty in achieving sufficient penetration into the target cell (82). Melanosomal accumulation of the PS in the melanoma cells might trigger the destruction of these organelles, leading to cell destruction by melanogenesis cytotoxic byproducts including H_2O_2 and highly active quinones (83).

PHOTODYNAMIC THERAPY DESTRUCTION OF TUMOR VESSELS

Tumor vasculature and parenchyma cells are both potential targets of PDT damage. Vascular damage has been implicated as the primary antitumoral effect in PDT with various PS (84), through direct vascular cell killing, strong inflammatory response and hypoxia following vessels obstruction (82, 85).

PDT with various PS triggers the overexpression of angiogenic (VEGF), adhesion (ICAM-1) and inflammatory (TNF-α) molecules in different types of cancer models (85-87). VEGF expression could be upregulated by PDT induced hypoxia in the tumor cells (85). TNF-α is secreted by resident macrophages, stromal cells and tumor cells under oxidative stress (85, 88).

Melanoma vasculature is involved in neo angiogenesis, inflammation, that lead to further invasion, migration and metastasis. Vascular-targeted PDT on M2R melanoma xenografts in mice with a water-soluble palladium-bacteriochlorophyll derivative (WST11) achieved tumor flattening and a 70% cure (89). Lumican, a small leucine-rich proteoglycan of the extracellular matrix decreased lung metastasis development not only by inducing tumor cell apoptosis but also by inhibiting angiogenesis in B16F1 melanoma transplanted to syngenic mice (90). An *in vitro* model of co-culture of human endothelial cells (HUVEC)-melanoma cell line has been used to study the interactions between the melanoma and tumor vasculature cells (91-94), the antitumor effects of hormonal (95) or UVB photo treatment (96), however, to our knowledge, it was not used for PDT studies.

In vitro PDT studies on co-cultures of HUVEC with other cancer cells showed that some tumor cell lines (squamous or colonic carcinoma, but not mammary carcinoma) produce angiogenic factors that induce HUVEC proliferation and subsequently enhance their sensitivity to PDT (97). This could also be the case for melanomas, since they have been shown to express high amounts of neurophylin 2. It leads to early metastasis, through alterations of the intercellular junctions between the endothelial cells and angiogenesis through VEGF signaling pathways (98, 99).

IMMUNE RESPONSE ACTIVATION FOLLOWING PHOTODYNAMIC THERAPY

Most of the commonly used cancer therapies like chemotherapy, ionizing radiation and major surgery are immunosuppressive. The ideal cancer therapy would not only destroy the primary tumor, but at the same time trigger the immune system to recognize, track down and destroy any remaining tumor cells, regardless of their localization. PDT, in common with some other local cancer therapies such as cryotherapy and hyperthermia, might have these desirable properties (8).

Melanoma is a highly immunogenic malignancy. Transformed melanocytes express tumor-specific peptides and tumor antigens (100) that can be presented to CD8+ T-cells by class I major histocompatibility complexes (MHC-1) and to CD4+ T-cells by MHC class II molecules on antigen-presenting cells (APC). To date, numerous antigens have been identified in both normal and transformed melanocytes, including MelanA/MART-I, MAGE-1, gp100, tyrosinase, and tyrosinase-related proteins (101). The binding of tumor-related antigens and peptides to the T-cell receptor triggers a sequence of events that ultimately leads to T-cell activation and expansion, including the binding of B7-1 and B7-2 to CD28 on the T-cell membrane and the stimulation of the phosphatidylinositol 3 kinase (PI3K)/ AKT pathway by activated CD28 (102). Histological examination of spontaneously regressing melanoma lesions has demonstrated that regression is associated with infiltration by T-cells and melanophages (103).

PDT induces a rapid inflammatory response whether it is delivered to normal tissue or to tumors. Inflammatory cytokines and chemokines IL6, macrophage inflammatory protein 1 (MIP1) and 2 (MIP2) (104) have been detected in the serum of mice that have received PDT directed at a subcutaneous tumor or to an area of normal skin. Increased levels of IL1β, IL6, IL8

and IL10 were detected in patients after surgery and intraoperative thorax PDT using Foscan at a dose of 0.1 mg/kg, 6 days before surgery and 652 nm red light at a dose of 10 J/cm^2 for mesothelioma (105).

PDT of tumors induces expression of stress proteins like: heat shock proteins HSP70, HSP47 (104), HSP60 (107), other stress-inducible proteins such as: glucose-regulated protein 78 (GRP78) (108), GRP94 (109) and heme oxygenase (110). The release of HSP-bound tumor antigens that can easily be taken up by APC from PDT-induced necrotic tumor cells increases the efficiency of antigen cross-presentation to form more effective tumor-specific cytotoxic T-cells (8). Prostaglandins are secreted by tumor cells, tumor endothelial cells and tumor-infiltrating leukocytes, but not by fibroblasts (111). Thromboxan released from endothelial cells after PDT causes vascular shutdown (112).

The acute inflammation that is observed after PDT is likely to be caused by the expression of two transcription factors, nuclear factor κB (NFκB) and activator protein 1 (AP1) (8).

PDT induced acute inflammation attracts leukocytes, especially neutrophils into the treated tumors. The pro-inflammatory effects of PDT might increase dendritic-cell migration, antigen uptake and maturation. PDT can produce tumor cures and long-lasting tumor-specific immunity (memory), as has been shown by the rejection of tumors on rechallenge in certain mouse and rat models (8). PDT plus intratumoral injection of naive dendritic cells eradicated both CT26 colorectal carcinoma cells and B16 melanoma BALB/c or C57Bl/6 mice in a significant proportion of animals or prolonged the survival of mice of which the tumors were not cured, whereas neither PDT nor intratumoral injection of naive dendritic cells alone were effective. Most importantly, PDT plus intratumoral injection of naive dendritic cells administered to one tumor site led to tumor regression at distant sites, including multiple lung metastases (113).

PDT affects monocyte/macrophage and lymphocyte cell lineages. Lymphocytes are usually killed by PDT, as shown in a report using DBA/2 mouse thymocytes treated with verteporfin PDT, and activated human T lymphocytes are especially susceptible (114). T-regulatory cells might be specifically inactivated by IL6 (115). Pretreatment of SKH-1 mice skin with antioxidants from *Calluna vulgaris* and red grape seeds (*Vitis vinifera* L, Burgund Mare variety) extracts resulted in significantly reduced levels of IL-6 and TNF-α after UVB irradiation compared to UVB alone (116). It is possible that the combination treatment of antioxidants and PDT might lead to a better result in stimulating local immunity response against the tumor cells.

There is also a reduction in NK cell activity of splenocytes obtained from photosensitizer-treated mice (117). PDT-treated macrophages secrete tumor-necrosis factor-α (TNFα) (8) and a potent macrophage-activating factor (MAF) as a consequence of peroxidation of lymphocyte membrane lipids (118). Macrophages showed preferential cytotoxicity to human lung adenocarcinoma cells A549 and murine squamous carcinoma SCCVII cells that have been treated with a sublethal dose of Photofrin-based PDT (119).

PDT induced systemic and tumor-localized increase in neutrophils is important in obtaining tumor cures. Increased IL1 and TNFα are potent stimulators of neutrophilia. PDT led to upregulation of the ICAM1 ligands CD11b and CD11c on neutrophils (120). An increase in the number of peripheral-blood neutrophils was found 4 hours after PDT treatment, and lasted for 24 hours. The increase in neutrophils was preceded by an increase in serum levels of IL1β. Anti-GCSF (granulocyte colony-stimulating factor) antibodies decreased neutrophil numbers and decreased the efficacy of PDT (121).

PDT has been combined with a range of immunostimulatory therapies, including microbial adjuvants, to increase the anti-tumor immunity produced by PDT alone. Association of PDT with intralesional γ -inulin (0.1 mg/mouse) delivered immediately after, rivaled zymosan (a potent classical complement activator) in delaying the recurrence of B16BL6 melanomas xenografts in mice. This effect of γ-inulin was further enhanced by IFN- pretreatment (122). Interferon alpha-2b might alter the expression of MHC making malignant cells more antigenic, as indicated by increased lymphocytic infiltration of melanoma lesions in interferon-treated patients (123). Tumor C3 protein levels, already elevated after individual PDT or γ -inulin treatments, increased much higher after their combination. At 3 days after PDT plus γ-inulin treatment, over 50% of cells found at the tumor site were CTLs engaged in killing specific targets *via* perforin-granzyme pathway (122). Complement system opsonises and makes PDT-treated tumor cells a target for lymphocytes. Binding of complement proteins to tumor antigens enhances their capture, processing, and presentation to T and B lymphocytes (124, 125).

The preparation of cancer vaccines using cell cultures exposed to PDT *in vitro* was studied. PDT-generated lysates were able to induce phenotypic dendritic cell maturation and IL12 expression and produced significant growth retardation, tumor regression and cures (126, 127). Importantly, vaccine cells that were retrieved from the treatment site at 1 hour after injection were intermixed with dendritic cells, HSP70 was expressed on their surface and they were opsonized by complement C3 (126).

Interestingly, PDT suppresses the contact hypersensitivity reaction in mice (126) but not delayed type hypersensitivity, involved in antitumor immunity (8). This is dependent by systemic IL10 release in cases where the PDT illumination penetrates the skin (red light), but is independent of IL10 when the PDT is confined to the skin layers (NIR light) (8).

FUTURE DIRECTIONS

The optimal PDT protocol in melanoma has to induce: direct tumor cell photodamage, destruction of tumor vasculature and activation of an immune response. PDT should overcome the melanoma protective mechanisms against therapy like pigmentation and increased oxidative stress defense, possibly through finding melanosome targeted photosensitizers. The optimal doses of light and photosensitizer for tumor and vascular targeted PDT could decrease both the tumor size and blood supply, thus increasing the overall efficacy. It is entirely possible that the optimal PDT regimen that produces local tumor cures will be different from the optimal PDT regimen that induces inflammation and increases the immune response (8). Combination of PDT with immune stimulation therapies might increase the efficiency in destroying the local tumor relapses as well as micro metastases and increase the overall efficacy of anti melanoma therapy.

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