

# Photodynamic Therapy: Combined Modality Approaches Targeting the Tumor Microenvironment

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**Background and Objectives:** Photodynamic therapy causes direct cytotoxicity to malignant cells within a tumor. Photodynamic therapy (PDT) can also have both direct and indirect effects upon various non-malignant components of the tumor microenvironment. This action can lead to PDT-mediated angiogenesis and inflammation, which are emerging as important determinants of PDT responsiveness.

**Study Design/Materials and Methods:** Preclinical studies have been performed to document how PDT modulates the tumor microenvironment. The expression, function, and treatment relevance of angiogenic growth factors, proteinases, and inflammatory molecules have been monitored following PDT using mouse tumor models.

**Results:** Photofrin-mediated PDT was shown to be a strong activator of VEGF, MMPs, and COX-2 derived prostaglandins within the tumor microenvironment. Inhibitors that target these angiogenic and pro-survival molecules can enhance the effectiveness of PDT.

**Conclusions:** Improvements in PDT tumor responsiveness may be achieved by employing combined modality regimens targeting malignant cells as well as treatment-induced angiogenesis and/or inflammation. *Lasers Surg. Med.* 38:516–521, 2006. © 2006 Wiley-Liss, Inc.

**Key words:** photodynamic therapy; angiogenesis; inflammation; tumor microenvironment

## INTRODUCTION

Many of the initial investigations examining photodynamic therapy (PDT) in the context of a cancer treatment focused on the preferential uptake of porphyrin derivatives in tumors and the destruction of malignant cells following the photochemical generation of singlet oxygen [1–3]. PDT was observed to produce rapid and localized tumor tissue destruction while limiting toxicity to normal tissue exposed to both the photosensitizer and the activating light source. During the early development of PDT many investigators attempted to either improve the biological, chemical, and physical properties of photosensitizers or increase the knowledge of how PDT-induced tumor cell destruction [1–3]. A variety of photosensitizers were synthesized possessing properties of increased absorption of tissue penetrating near infrared light, improved quantum yields of reactive oxygen species, increased drug accumulation in

tumor tissue and decreased drug retention within normal tissue. At the same time, important information was obtained regarding cell sensitivity to PDT as well as apoptotic and necrotic pathways associated with PDT-mediated cytotoxicity. Positive clinical results have encouraged an expanded mechanistic analysis of cellular and tissue responses associated with PDT [4]. Mitochondria, lysosomes, and plasma membranes are all identified as subcellular PDT targets [5]. Preclinical studies from a number of laboratories show that PDT induces direct tumor cell kill as well as localized inflammation and acute vascular injury within treated tumors leading to increased expression of angiogenic and pro-survival molecules [6]. More recently, a variety of molecular, immunological and biochemical studies have expanded our understanding of the complex nature of the cytotoxic responses associated with PDT.

Growing evidence indicates that various components of the tumor microenvironment play significant roles in determining the efficacy of chemotherapy and radiation therapy [7,8]. Findings from these studies also appear to be relevant to PDT. The tumor microenvironment is made up of malignant cancer cells and connective tissue as well as a myriad of host cells including endothelial cells, pericytes, and inflammatory leukocytes (macrophages and neutrophils) [7,8]. Leukocytes are recruited into tumors, stimulate the endothelium, and indirectly activate tumor vascularization. These inflammatory leukocytes release a number of factors that modulate endothelial cell activity and angiogenesis. Neutrophil recruitment in tumors can be followed by VEGF and MMP-9 release with associated angiogenesis and invasion [8,9]. Tumor associated macrophages exhibit a phenotype that favors tissue growth,

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angiogenesis, and tissue remodeling. Aggressive tumors are associated with an activated environment rich in growth factors, chemokines, and proteolytic enzymes which convert stromal cells into promoters of tumor growth by enhancing angiogenesis, tissue breakdown, and tissue remodeling [10]. Inflammation-dependent angiogenesis is an important determinant of tumor growth and has led to the targeted chemoprevention of inflammatory angiogenesis as a method of both treating and preventing cancer.

Cellular factors associated with PDT, such as necrosis, apoptosis, and hypoxia, can function as stimuli within the tumor microenvironment. Apoptotic and necrotic cells can be sensed and cleared by rapidly infiltrating tumor granulocytes and macrophages [8]. Likewise, PDT-induced hypoxia can lead to the transcriptional activation of VEGF via the HIF-1 $\alpha$  pathway [11]. Inflammation is also emerging as a primary target for antiangiogenic therapy of tumors. Nonsteroidal anti-inflammatory drugs (NSAIDs) exhibit antiangiogenic properties by blocking cyclooxygenase (COX) pathways that lead to the production of prostaglandins, which are powerful angiogenic mediators [12,13]. Data are emerging that PDT-mediated changes to the tumor microenvironment can modulate treatment responsiveness. Preclinical investigations indicate that combining PDT with targeted therapies directed at attenuating the pro-survival actions of the tumor microenvironment can enhance the therapeutic potential of PDT [11–15]. Several laboratories have also shown that PDT can induce expression and/or activation of additional proangiogenic molecules including COX-2 and prostaglandins, TNF- $\alpha$ , matrix metalloproteinases (MMPs), integrins, IL-6, and IL-8 within the tumor microenvironment [11–18]. We postulate that procedures suppressing angiogenesis and inflammation should improve tumor responsiveness. The remainder of this article provides an overview of changes within the tumor microenvironment following PDT and describes preclinical data demonstrating that treatment protocols involving combined modality approaches can significantly improve PDT. Figure 1 provides a summary of PDT-mediated responses within the tumor microenvironment and the potential usefulness of targeted inhibitors to improve PDT.

### PDT AND VEGF SIGNALING

A growing number of angiogenic factors and inducers of angiogenesis have been identified. VEGF is considered one of the most important growth factors regulating physiological and pathological angiogenesis [19]. VEGF exerts its action via endothelial cell surface receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/flk-1). VEGFR-3 (flk-4) is primarily associated with the lymphatic endothelium [19–21]. VEGFR-1 binds VEGF with high affinity, may have a role in tumor metastasis, but also can act as a decoy receptor and decrease the availability of VEGF to VEGFR-2, the principle receptor for VEGF signaling via the tyrosine kinase activity of these receptors [19,21]. VEGF expression is regulated by a variety of host stimuli including cytokines and cellular or tissue stress. Hypoxia also serves as a strong

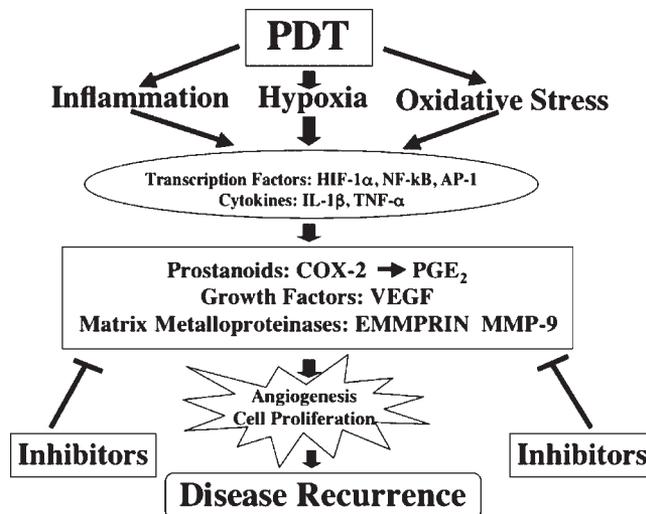


Fig. 1. Interactions between photodynamic therapy and the tumor microenvironment. PDT induces significant levels of inflammation, hypoxia, and oxidative stress in treated tumor tissue. These actions can lead to expression of a wide variety of transcription factors and cytokines directly associated with generalized stress and inflammation. Subsequent expressions of angiogenic and survival molecules are observed following PDT. These molecules may be directly involved in PDT treatment recurrences. The use of selectively targeted inhibitors to angiogenic and survival molecules may improve the therapeutic response of PDT.

stimulus for VEGF expression via activation of the HIF-1 transcription factor and is reported to upregulate VEGFR-1 and VEGFR-2 [19].

The VEGF pathway has become a primary target for antiangiogenic therapy as a result of the importance of tumor-associated angiogenesis [19,22]. The most promising applications of antiangiogenic therapy involve combined modality approaches with conventional chemotherapy or radiation therapy. Differences in the proliferation rates of endothelial cells in tumors versus normal tissue, accessibility of systemically administered therapeutic agents to endothelial cells, and the genetic stability of endothelial cells are positive reasons for using angiogenic inhibitors in combination with PDT. Roberts and Hasan [23] were the first to report on a connection between VEGF expression and photosensitizer uptake in tumors. We have shown that PDT induces expression of the transcription factor HIF-1 $\alpha$  as well as the HIF-1 $\alpha$  target gene VEGF in treated tumors [11,15]. We documented that tumor-bearing mice treated with a combination of PDT and non-specific antiangiogenic agents, IM862 and EMAP-II, improved tumoricidal response as measured by increased time to recurrence and increased cures when compared to individual treatment regimens [11]. The important question of how best to combine antiangiogenic therapy with PDT is an area that needs additional investigation. We postulate that angiogenesis associated with both the malignant lesion and the PDT treatment can lead to suboptimal therapy and tumor

recurrences. We also postulate that combining PDT with a targeted angiogenic inhibitor will enhance the therapeutic responsiveness of PDT.

There are unique therapeutic targets and corresponding therapeutic drugs associated with the VEGF-signaling pathway. Soluble VEGF can be targeted using Avastin (bevacizumab), VEGFR-2 can be targeted using DC101, and the VEGF receptor tyrosine kinase activity can be targeted using ZD6474 [19–22,24,25]. Avastin is a humanized monoclonal antibody against VEGF that has gained FDA approval for treatment of colorectal carcinoma and shows promise when combined with chemotherapy for treating tumors. Avastin binds free VEGF and thereby blocks the activation of VEGF receptors and the initiation of an angiogenic signaling cascade [19,21,22]. DC101, a mouse monoclonal antibody that binds to and blocks VEGFR-2 receptor signaling, is a primary downstream target of VEGF [20]. ZD6474 is a heteroaromatic-substituted anilinoquinazoline inhibitor of VEGF receptor-2 tyrosine kinase activity that inhibits both VEGF signaling in endothelial cells and tumor induced angiogenesis [24,25]. ZD6474 also inhibits the epidermal growth factor receptor (EGFR) tyrosine kinase and can impart direct inhibitory effect on tumor cell growth and survival. ZD6474 is in clinical trials and there are reports that this inhibitor has activity against human cancers.

In one of the first preclinical studies combining PDT and a clinically approved angiogenic inhibitor, we have examined whether Avastin could improve the effectiveness of PDT in a xenograft model of Kaposi's sarcoma (KS) [15]. Human KS-Imm tumors transplanted in nude mice were treated with PDT, PDT plus Avastin, or Avastin alone. Expression profiles of pro-angiogenic molecules were documented and the tumoricidal effectiveness of PDT combined with Avastin was determined using long-term tumor cure analysis. PDT-induced expression of HIF-1 $\alpha$ , VEGF, PGE<sub>2</sub>, TNF- $\alpha$ , and IL-1 $\beta$  within the KS tumor tissue. Significant overexpression of KS cell-derived human VEGF and to a lesser extent over expression of host cell-derived mouse VEGF were detected within the treated tumors. Combining PDT with Avastin resulted in a significant increase in the long-term responsiveness of treated KS tumors when compared to individual treatments. These results demonstrate for the first time that Avastin can improve PDT treatment effectiveness and suggest that VEGF inhibitors may ameliorate the clinical efficacy of PDT. The relevance of PDT-induced expression of PGE<sub>2</sub>, TNF- $\alpha$ , and IL-1 $\beta$  within KS lesions still needs to be examined [15].

The fluence rate of delivered light can impact PDT responses in treated tumors [26,27]. High fluence rates can deplete tumor oxygenation and decrease the effectiveness of PDT. Spatially dependent depletion of oxygen can occur during PDT and is a function of fluence rate [28,29]. In addition, tumor microenvironment changes associated with inflammation can also vary as a function of the dose rate of delivered light. We have data showing that HIF-1 $\alpha$  and VEGF expression within the tumor microenvironment are modulated by PDT fluence rate. We observe basal levels of HIF-1 $\alpha$  in control tumors and tumors treated with PDT

using a dose rate of 14 mW/cm<sup>2</sup>. However, we observe a significant increase in HIF-1 $\alpha$  protein expression in tumors treated with PDT using a dose rate of 112 mW/cm<sup>2</sup>. In this experiment all tumors were treated with a total PDT dose of 48 J/cm<sup>2</sup>. VEGF levels detected in tumors treated with the high-dose rate were double the levels detected in tumors treated with the low-dose rate of delivered light. Currently, we do not know if these changes actually play a role in PDT responsiveness. Experiments using selective inhibitors to VEGF are underway to determine if changes in PDT-mediated tumor response associated with light fluence rates actually involve alterations in VEGF directed angiogenesis.

## PDT AND MATRIX METALLOPROTEINASES

Tumor growth depends in large part on the formation and maturation of new blood vessels concomitant with the degradation of the extracellular matrix. In this regard, there is growing evidence linking the expression of MMPs with these processes [30]. MMPs are members of a multi-gene family of zinc-containing enzymes that function under both physiological and pathological conditions. MMPs are endopeptidases. Several of these enzymes are found within the tumor microenvironment and are involved in tumor angiogenesis and invasion [30,31]. Cytokines, growth factors, oncogenes, and reactive oxygen species are among the stimuli that activate MMP transcription. The translated proteins are normally expressed as inactive or latent proenzymes and require the proteolytic cleavage of an NH<sub>2</sub> terminus peptide to be converted to a biologically active proteinase. In solid tumors, MMPs are often expressed by stromal cells and macrophages rather than by tumor cells. The in-vivo activity of MMPs is regulated in part by endogenous tissue inhibitors of MMPs or TIMPs. An imbalance in the expression and activation of TIMPs and MMPs leads to modifications in tumor growth.

We have obtained data suggesting that MMP-9 may play an important role in modulating PDT responsiveness [14]. MMP-9 (gelatinase B) can degrade the extracellular matrix and basement membrane as well as growth factors, cytokines, and growth factor binding proteins [31]. This suggests that MMPs such as MMP-9 can play an active role in regulating the tumor microenvironment. We have documented that treatment of mouse tumors with PDT induces strong expression of MMP-9 and the extracellular MMP inducer (EMMPRIN) along with a concomitant decrease in expression of TIMP-1 [14]. Gelatin zymography and enzyme activity assays demonstrate that PDT results in the induction of both latent and enzymatically active forms of MMP-9. MMP-9 was expressed in host cells within the tumor microenvironment but not in tumor cells. Interestingly, a broad-spectrum MMP inhibitor, Prinomastat, improved PDT tumor response without affecting normal skin photosensitization [14]. There is evidence that MMP-9 contributes to tumor angiogenesis by assisting in the formation of capillary networks and recruitment of VEGF in the tumor microenvironment as well as by promoting the invasion of the extracellular matrix by endothelial cells

[32,33]. It is currently unclear if MMP-9 activities are actually involved in modulating PDT tumor response. To address the question of MMP-9 contribution to PDT responsiveness, we plan to perform experiments using a genetic approach involving MMP-9 knockout mice [32,33].

### PDT AND CYCLOOXYGENASE-2

COX is a key enzyme involved in the conversion of arachidonic acid to bioactive lipids including prostaglandins and thromboxanes [34]. The two isoforms of COX, COX-1, and COX-2, are encoded by separate genes and play different physiological and pathological roles. COX-1 is constitutively expressed in most tissues and is involved in homeostatic functions while COX-2 is an inducible early response gene involved with inflammation and mitogenesis. A number of selective COX-2 inhibitors, including celecoxib, are highly effective in the treatment of osteoarthritis, rheumatoid arthritis, and post-surgical pain [35]. However, there are significant concerns regarding long-term use of COX-2 inhibitors and cardiovascular safety [36]. At the same time, growing evidence indicates that COX-2 activity is involved in the development and progression of a variety of cancers [37]. COX-2 inhibitors can attenuate tumor growth and this observation has led to the clinical evaluation of selective COX-2 inhibitors, including celecoxib, in chemoprevention and as an adjuvant to radiation or chemotherapy for treating solid tumors [38].

We documented that PDT induces prolonged expression of COX-2 in a mouse fibrosarcoma tumor model and that combining PDT with the COX-2 inhibitor, NS-398, enhanced tumor responsiveness without increasing normal tissue photosensitization [12]. More recently, we examined treatment efficacy when PDT was combined with celecoxib and observed that this combination increased long-term survival in a mouse mammary carcinoma model [13]. Our results also showed that COX-2 inhibition enhanced PDT-mediated apoptosis in cultured tumor cells and decreased the in-vivo expression of angiogenic and inflammatory factors in treated tumors. Our results have been confirmed by other laboratories and support the premise that protocols designed to combine PDT with a COX-2 inhibitor may be clinically beneficial [17,18]. Experiments were also performed to examine why combining PDT with specific COX-2 inhibitors improves tumor treatment responsiveness. COX-2 inhibitors induce a variety of cellular responses when used in chemoprevention or cancer therapy studies including modifications in apoptosis, cell cycle progression, invasion, and angiogenesis [39]. PDT also alters these same physiological and pathological processes [1–5]. Oxidative stress generated by PDT produces mitochondrial damage leading to rapid induction of apoptosis in cancer cells growing either in-vitro or in-vivo. We observed that celecoxib and NS-398 produced a modest increase in both cytotoxicity and apoptosis in PDT treated BA mammary carcinoma cells. PDT-induced PARP cleavage, Bcl-2 degradation, and DNA fragmentation (parameters associated with apoptosis) and all of these responses were further enhanced when PDT was combined with either

celecoxib or NS-398. These results agree with in-vitro studies examining cellular responses when COX-2 inhibitors are combined with radiation or chemotherapy [40–42].

Reduction in tumor progression following administration of COX-2 inhibitors may be caused in part by an apoptotic mechanism [37,39,42]. Interestingly, COX-2 inhibitors with similar IC<sub>50</sub> values can differ significantly in their ability to induce apoptosis [43]. This suggests that COX independent pathways may be implicated in the apoptotic responses observed with COX-2 inhibitors [39]. Our results demonstrate that PDT at a suboptimal dose of 100 J/cm<sup>2</sup> induced apoptosis in tumors when measured 6 hours after treatment [13]. The extensive and rapid tissue destruction associated with in-vivo PDT precluded us from examining in-vivo apoptosis at high PDT doses or at extended time intervals. Combining PDT with either celecoxib or NS-398 slightly decreased the levels of detectable apoptosis from levels observed for PDT alone. These results may be due to the difficulty in measuring differences in apoptosis when much of the tumor tissue is rapidly destroyed or that COX-2 inhibitors do not modulate in-vivo PDT-mediated apoptosis. In addition, PDT-induced inflammation can lead to the rapid accumulation of host inflammatory cells within treated tumor tissue [16]. Early on, these inflammatory cells are primarily neutrophils, which undergo constitutive apoptosis in the presence of TNF- $\alpha$  and could show up as tunnel positive cells at 6 hours post-PDT. The lower levels of apoptosis found with combined modality therapy could be due in part to the decrease in inflammation, leukocyte infiltration, and TNF- $\alpha$  within tumors following this combined modality regimen.

COX-2-mediated expression of PGE<sub>2</sub> plays a significant role in tumor angiogenesis by inducing expression of angiogenic regulatory proteins such as VEGF [44]. Interestingly, VEGF is also associated with the upregulation of COX-2 expression in endothelial cells and this involves a GATA cis-acting element in the COX-2 gene [45]. This suggests that these two genes may be mutually regulated. PDT induces a pro-inflammatory response within treated tumors associated with concomitant expression of cytokines including IL-1 $\beta$  and TNF- $\alpha$  [15,16]. Growing evidence indicates that these molecules play a significant role in angiogenesis [10]. Our results show that COX-2 inhibitors, at doses that block PGE<sub>2</sub> production, attenuate IL-1 $\beta$ , and TNF- $\alpha$  expression within PDT treated tumors. This suggests a direct association between COX-2 inhibitor-mediated downregulation of PGE<sub>2</sub> and decreased angiogenesis when PDT is combined with celecoxib or NS-398.

The applicability of long-term use of selective COX-2 inhibitors, including celecoxib, is currently under serious review due to increases in cardiovascular toxicity [36]. Nevertheless, preclinical studies and initial clinical trials indicate that celecoxib is effective as both a chemopreventive agent and as an adjuvant to radiation and chemotherapy in treating solid tumors [39]. PDT efficiently and rapidly reduces tumor burden, which would leave only minimal disease needing to be targeted with COX-2 inhibitors [1,12,13]. This suggests that the COX-2 inhibitor doses and treatment schedules may be significantly less

than those needed with standard radiation therapy or chemotherapy. Inflammation immediately following PDT, with expression of TNF- $\alpha$  and IL-1 $\beta$ , may be beneficial for treatment outcome while an extended inflammatory response would be detrimental. Therefore, treatment optimization experiments will be required in order to determine appropriate dosing and scheduling parameters. Normal skin photosensitization is not increased when PDT and COX-2 inhibitors are combined to treat solid tumors, which suggests that this combination may produce a significant therapeutic gain [13]. PDT is approved for the treatment of Barrett's esophagus and clinical studies continue to document the efficacy of PDT in treating this disorder [46]. There is also growing evidence suggesting that COX-2 levels are elevated in Barrett's esophagus and that COX-2 inhibitors may be beneficial as an adjuvant for treating this premalignant lesion [47]. Likewise, recent reports suggest that PDT combined with COX-2 selective inhibitors may be useful in the treatment of carcinomas of the skin and oral cavity [18]. Well designed clinical trials are required to determine if the use of COX-2 inhibitors for limited time periods following PDT have a role in the clinical armamentaria against solid tumors.

## SUMMARY

There is a critical need for localized cancer therapies that are effective, well tolerated and that can be repeatedly used after other procedures fail. PDT addresses each of these requirements. However, PDT has not been optimized. We postulate that PDT combined with appropriately targeted inhibitors directed at VEGF, MMPs, and/or COX-2 will further improve the therapeutic responsiveness of PDT.

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