Antiangiogenic Treatment Enhances Photodynamic Therapy Responsiveness in a Mouse Mammary Carcinoma

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Abstract

Photodynamic therapy (PDT) is a promising cancer treatment that induces localized tumor destruction via the photochemical generation of cytotoxic singlet oxygen. PDT-mediated oxidative stress elicits direct tumor cell damage as well as microvascular injury within exposed tumors. Reduction in vascular perfusion associated with PDT-mediated microvascular injury produces tumor tissue hypoxia. Using a transplantable BA mouse mammary carcinoma, we show that Photofrin-mediated PDT induced expression of the hypoxia-inducible factor-1α (HIF-1α) subunit of the heterodimeric HIF-1 transcription factor and also increased protein levels of the HIF-1 target gene, vascular endothelial growth factor (VEGF), within treated tumors. HIF-1α and VEGF expression were also observed following tumor clamping, which was used as a positive control for inducing tissue hypoxia. PDT treatment of BA tumor cells grown in culture resulted in a small increase in VEGF expression above basal levels, indicating that PDT-mediated hypoxia and oxidative stress could both be involved in the upregulation of VEGF. PDT-bearing mice treated with combined antiangiogenic therapy (IM862 or EMAP-II) and PDT had improved tumoricidal responses compared with individual treatments. We also demonstrated that PDT-induced VEGF expression in tumors decreased when either IM862 or EMAP-II was included in the PDT treatment protocol. Our results indicate that combination procedures using antiangiogenic treatments can improve the therapeutic effectiveness of PDT.

Introduction

PDT involves treating solid malignancies with tissue-penetrating laser light following the systemic administration of a tumor localizing photosensitizer (1). Properties of photosensitizer localization in tumor tissue and photochemical generation of reactive oxygen species are combined with precise delivery of laser-generated light to produce a treatment offering local tumoricidal activity (2, 3). The porphyrin photosensitizer PH recently received Food and Drug Administration approval for PDT treatment of esophageal and endobronchial carcinomas (1). PDT is also undergoing clinical evaluation for the treatment of bladder, head and neck, brain, intrathoracic, and skin malignancies (1). PDT targets include tumor cells, tumor microvasculature, inflammatory cells, and immune host cells (1–3). Vascular effects induced by PH-mediated PDT include perfusion changes, vessel constriction, macromolecular vessel leakage, leukocyte adhesion, and thrombus formation (1, 4). These effects appear to be linked to platelet activation and release of thromboxane (5). Microvasculature damage is readily observed histologically following PDT and leads to a significant decrease in blood flow as well as severe and persistent tumor tissue hypoxia (6, 7). Rapid and substantial reductions in tissue oxygenation can also occur during illumination by direct utilization of oxygen during the photochemical generation of reactive oxygen species (7, 8).

Tissue hypoxia induces a plethora of molecular and physiological responses, including an adaptive response associated with gene activation (9). A primary step in hypoxia-mediated gene activation is the formation of the HIF-1 transcription factor complex (9, 10). HIF-1 is a heterodimeric complex of two helix-loop-helix proteins, HIF-1β (ARNT) and HIF-1α (11). ARNT is constitutively expressed, whereas HIF-1α is rapidly degraded under normoxic conditions. Hypoxia induces the stabilization of the HIF-1α subunit, which in turn allows for the formation of the transcriptionally active protein complex (11, 12). A number of HIF-1-responsive genes have been identified, including VEGF, erythropoietin, and glucose transporter-1 (11). VEGF, also called vascular permeability factor, is an endothelial cell-specific mitogen involved in the induction and maintenance of the neovascularure in solid tumors (11, 13). VEGF expression increases in tumor tissue under hypoxia as a result of both transcriptional activation and increased stabilization (11, 14).

In the current study, we examined whether PDT-induced microvascular damage and the resulting hypoxia could serve as activators of molecular events leading to the increased expression of VEGF within treated tumor tissue. We also determined whether antiangiogenic compounds, which counter the actions of VEGF, could improve PDT tumor responsiveness. Our results document that PH-mediated PDT induces expression of HIF-1α and the transcription factor’s target gene, VEGF, in a transplanted mouse mammary carcinoma. We also documented enhanced tumoricidal activity when PDT is combined with antiangiogenic therapy.

Materials and Methods

Drugs and Reagents

The photosensitizer Photofrin porfimer sodium was a gift from QLT PhotoTherapeutics, Inc. (Vancouver, British Columbia, Canada) and was dissolved in 5% dextrose in water to make a 2.5 mg/ml stock solution. Reconstituted EMAP-II was prepared as described previously (15). A working solution at 10 μg/ml was prepared in PBS containing 0.1% BSA. IM862 was obtained from Cytran Inc. (Kirkland, WA) and was dissolved in saline to make a 5 mg/ml working solution (16). CoCl2 was obtained from Sigma Chemical Co. (St. Louis, MO), and a 10 mM stock solution was prepared in water.
Cells and in Vivo Tumor Model. BA mouse mammary carcinoma cells (originally obtained from the NIH tumor bank) were used in all in vitro and in vivo experiments (17). Cells were grown as a monolayer in RPMI 1640 supplemented with 10% FCS and antibiotics. The plating efficiency for the BA cells was 40–60%. s.c. BA mammary carcinomas were generated by trocar injection of 1-mm³ pieces of tumor to the hind right flank of 8- to 12-week-old female C3H/HeJ mice (17).

In Vitro and in Vivo Treatment Protocols. In vitro photosensitization protocols involved seeding cells into plastic Petri dishes and incubating overnight in complete growth medium to allow for cell attachment. PDT treatments included incubating cells in the dark at 37°C for 16 h with PH (25 µg/ml) in medium containing 5% FCS. Cells were then incubated for an additional 30 min in growth medium containing 10% FCS, rinsed in medium without serum, and exposed to red light (570–650 nm) generated by a parallel series of red Mylar-filtered 30 W fluorescent bulbs and delivered at a dose rate of 0.35 mW/cm². In specified experiments, cells were incubated with CoCl₂ (100 µM) in growth medium containing 5% FCS for 16 h. Treated cells were then re-fed with complete growth medium and incubated in the dark at 37°C until collected for analysis of VEGF secretion into the culture media. In vivo PDT tumor treatments were performed as reported previously on tumors measuring 6–7 mm in diameter (17). Briefly, PDT procedures included an i.v. injection of PH (5 mg/kg) followed 24 h later with nonthermal laser tumor irradiation using an argon-pumped dye laser emitting red light at 630 nm. A light dose rate of 75 mW/cm² and a total light dose of 200 J/cm² were used for all in vivo PDT treatments. After treatment, tumors were measured three times per week. Cures were defined as being disease free for at least 40 days after PDT (17). Antiangiogenic treatment was performed using either EMAP-II or IM862. Each compound was administered as daily i.p. injections for 10 consecutive days starting 1 h prior to PDT light treatment. Individual IM862 doses were 25 mg/kg, and individual EMAP-II doses were 50 µg/kg. Tumor tissue hypoxia was induced in selected experiments by clamping lesions for 45 min.

Western Blot Analysis. Tumors were collected at various times after treatment, homogenized with a Polytron in 1× reporter lysis buffer (Promega, Madison, WI), and evaluated for protein expression as described previously (18). Briefly, protein samples (30 µg) were size-separated on 10% (for HIF-1α) or 12.5% (for VEGF) discontinuous polyacrylamide gels and transferred overnight to nitrocellulose membranes. Filters were blocked for 1 h with 5% nonfat milk and then incubated for 2 h with either a mouse monoclonal anti-HIF-1α antibody (clone 54; Transduction Laboratories, Lexington, KY), a rabbit polyclonal anti-VEGF antibody (no. sc-507; Santa Cruz Biotechnology, Santa Cruz, CA), or a mouse monoclonal antiactin antibody (clone C-4; ICN, Aurora, OH). Filters were then incubated with either an antirabbit or antimouse peroxidase conjugate (Sigma), and the resulting complexes were visualized by enhanced chemiluminescence autoradiography (Amersham Life Science, Chicago, IL).

ELISA Assays. A Quantikine M mouse VEGF ELISA kit (R&D Systems, Minneapolis, MN) was used to quantify VEGF levels in cell culture media as well as in tumor extracts from control and treated mice. Results were normalized to protein concentrations from tumor tissue or cell lysates.

Statistics. Statistical analysis was performed using a two-tailed Student’s t test to analyze VEGF levels and the χ² test for evaluation of tumor cure rates.

Results and Discussion

PDT continues to show promise in the treatment of a variety of malignant and nonmalignant disorders (1, 19). The use of PDT for advanced esophageal tumors offers prolonged tumor responses compared with standard Nd-YAG laser ablation treatments. Extended tumor responses are also observed in advanced non-small cell lung cancer patients treated with PDT compared with Nd-YAG laser ablation. Likewise, PDT applications continue to be encouraging for early stage lung cancer, brain cancers, head and neck cancers, and for nononcological disorders such as age-related macular degeneration (1, 19). Nevertheless, recurrences are observed after PDT, and methods to improve the therapeutic efficacy of this procedure are needed. Multiple physiological, biophysical, and/or pharmacological variables may account for recurrences after PDT (2, 3). Nonuniform distribution of photosensitizers within tumor tissue, inadequate light distribution, photosensitizer photobleaching, and treatment-induced oxy-
gen deprivation may all contribute to suboptimal PDT responses. In the current study, we examined molecular events associated with PDT-induced hypoxia with an emphasis on determining whether PDT effectiveness could be enhanced with antiangiogenic therapy.

Several laboratories have shown that PDT produces microvascular damage within treated tumors and that PDT leads to tumor tissue hypoxia (4–8). Hypoxia mediates adaptive gene expression through the HIF transcription factor (9). An initial step in hypoxia-mediated gene activation is the formation of the HIF-1 heterodimeric transcription factor complex (10). One subunit, HIF-1β (ARNT), is constitutively expressed, whereas the second subunit, HIF-1α, is rapidly degraded under normoxic conditions by the ubiquitin-proteasome system (9, 10, 12). Because hypoxia induces increased expression and stabilization of the HIF-1α subunit as well as activates the HIF-1 transcription complex, it seemed likely that PDT-induced microvascular damage and resulting tumor tissue hypoxia could also stabilize HIF-1α and initiate HIF-1-mediated transcription. Fig. 1A uses Western analysis to show that PDT treatment of BA mammary carcinoma tumors growing in C3H mice induced expression of HIF-1α. This response was rapid, being observed within the first 5 min after PDT. Tumor clamping was used as a positive control and resulted in comparable HIF-1α expression. The HIF-1 complex functions via binding to a hypoxia response element found in the promoter region of the VEGF gene as well as in the 3’ flanking region of the erythropoietin gene (11). Expression of VEGF in areas around histologically documented tumor necrosis originally led to suggestions that hypoxia is a major regulator of tumor angiogenesis (13, 14). VEGF is a dimeric glycoprotein with strong mitogenic activity restricted primarily to endothelial cells (14). Fig. 1B documents VEGF expression after in vivo PDT. Western analysis was performed under reducing conditions on tumor lysates collected 24 h after PDT. PDT and tumor clamping both induced significant increases in VEGF expression within treated lesions. VEGF-induced angiogenesis plays an important role in tumor growth. Inhibition of VEGF activity with neutralizing antibodies inhibits the growth of primary and metastatic tumors, and attenuation of VEGF expression decreases tumor growth and vascularity (20). Our results suggest that PDT may be functioning as a mediator of tumor angiogenesis and tumor recurrence by enhancing expression of VEGF within the treated tumor mass (14).

We next examined whether in vitro PDT of BA mammary carcinoma cells could also induce expression of VEGF. Fig. 2 shows VEGF levels collected from culture media at various time intervals for control and treatment conditions. Exposure to CoCl2 served as a statistically significant positive control because exposure to this divalent metal induces cellular VEGF expression (11). A 210 J/m2 PDT dose resulted in a modestly increase in VEGF levels when measured 24 h after treatment. The PDT doses (210 and 420 J/m2) and CoCl2 treatment produced clonogenic survival levels ranging from 33% to 96%. The in vitro PDT conditions would be expected to involve singlet oxygen-mediated oxidative stress but not induced hypoxia. These results suggest that the increase in VEGF expression observed in tumors after in vivo PDT may be associated with treatment-induced hypoxia and to a lesser extent with treatment-induced oxidative stress. Exposure of various mouse and human tumor cells to ionizing radiation and exposure of rat endothelial cells to hydrogen peroxide can up-regulate VEGF expression (20, 21). Additional studies will be required to determine similarities and differences in VEGF induction for various types of oxidative stress.

A growing number of reports have indicated that antiangiogenic agents can enhance the tumoricidal effectiveness of chemotherapy and radiation treatments (20, 22, 23). We next examined whether antiangiogenic treatments, using either EMAP-II or IM862, could enhance the tumoricidal action of PDT. EMAP-II is a single chain polypeptide that inhibits tumor growth and has antiangiogenic activity (15). EMAP-II induces apoptosis in growing capillary endothelial cells in both a time- and dose-dependent manner. EMAP-II also prevents vessel ingrowth in experimental angiogenesis models and in primary tumors. Interestingly, EMAP-II does not induce toxicity in normal organs. IM862 is a dipeptide of l-glutamyl-l-tryptophan that was initially isolated from the thymus (16). Preclinical studies have shown that the dipeptide inhibits angiogenesis in choroidallantoic membrane assays and inhibits VEGF production in monocytic lineage cells. IM862 also inhibits tumor growth in xenograft models but has no direct cytotoxic effect on tumor cells. IM862 mediates these effects by

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**Fig. 4.** The antiangiogenic compounds IM862 and EMAP-II can decrease VEGF levels in PDT-treated tumors. Tumor-bearing mice received no treatment (Control), PDT alone, or PDT plus two injections of either EMAP-II or IM862 (1 h prior to PDT and 23 h after PDT). Tumor samples were collected 24 h after PDT and assayed for VEGF expression using a commercial ELISA assay kit. Each group represents the mean (bars, SE) of six individual tumor samples. There was a statistically significant difference in VEGF levels between PDT alone and PDT plus EMAP-II (P < 0.01).

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4 R. Masood and P. Gill, unpublished data.

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**Fig. 3.** Antiangiogenic treatments enhance the tumoricidal action of PDT. C3H mice transplanted with BA mammary carcinomas received daily injections for 10 days of either IM862 (25 mg/kg per dose; n = 9) or EMAP-II (50 mg/kg per dose; n = 9) commencing 1 h prior to a single PDT treatment (5 mg/kg PH; 200 J/cm2). Mice were monitored for tumor recurrences three times per week for 40 days. Control conditions included individual antiangiogenic treatments alone (n = 9) and PDT treatment alone (n = 18). There was a statistically significant difference in the percentage of cures between PDT alone versus PDT + EMAP-II or PDT + IM862 (P < 0.05).

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**Fig. 5.** The antiangiogenic compounds IM862 and EMAP-II can decrease VEGF levels in PDT-treated tumors. Tumor-bearing mice received no treatment (Control), PDT alone, or PDT plus two injections of either EMAP-II or IM862 (1 h prior to PDT and 23 h after PDT). Tumor samples were collected 24 h after PDT and assayed for VEGF expression using a commercial ELISA assay kit. Each group represents the mean (bars, SE) of six individual tumor samples. There was a statistically significant difference in VEGF levels between PDT alone and PDT plus EMAP-II (P < 0.01).

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**Fig. 6.** The antiangiogenic compounds IM862 and EMAP-II can decrease VEGF levels in PDT-treated tumors. Tumor-bearing mice received no treatment (Control), PDT alone, or PDT plus two injections of either EMAP-II or IM862 (1 h prior to PDT and 23 h after PDT). Tumor samples were collected 24 h after PDT and assayed for VEGF expression using a commercial ELISA assay kit. Each group represents the mean (bars, SE) of six individual tumor samples. There was a statistically significant difference in VEGF levels between PDT alone and PDT plus EMAP-II (P < 0.01).

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**Fig. 7.** The antiangiogenic compounds IM862 and EMAP-II can decrease VEGF levels in PDT-treated tumors. Tumor-bearing mice received no treatment (Control), PDT alone, or PDT plus two injections of either EMAP-II or IM862 (1 h prior to PDT and 23 h after PDT). Tumor samples were collected 24 h after PDT and assayed for VEGF expression using a commercial ELISA assay kit. Each group represents the mean (bars, SE) of six individual tumor samples. There was a statistically significant difference in VEGF levels between PDT alone and PDT plus EMAP-II (P < 0.01).
enhancing production of VEGF and by activating natural killer cells. Intranasal administration of IM862 exhibits antitumor activity in patients with AIDS-associated Kaposi’s sarcoma (16). IM862 also appears to be safe and well tolerated when delivered over prolonged time periods. A PDT procedure that produced a moderate cure rate alone was used to measure positive or negative changes in tumor response when a single PDT treatment was combined with daily injections of EMAP-II or IM862 for 10 days (17). Fig. 3 shows that antiangiogenic treatment statistically enhanced (P < 0.05) the tumoricidal action of PDT as measured by tumor cures. Specifically, the 200 J/cm² PDT dose alone produced a 39% cure rate, whereas PDT plus EMAP-II or IM862 produced tumor cures of 89 and 78%, respectively. The antiangiogenic treatments alone did not produce any tumor cures or tumor regression and only slightly modified tumor growth parameters.

Finally, we examined whether the antiangiogenic derivatives used in this study modulated PDT-induced VEGF levels in treated tumors. The in vivo PDT dose delivered to tumors (200 J/cm²) induced rapid and severe tissue necrosis. Therefore, tumor samples were only collected 24 h after PDT. This timeframe allowed for two antiangiogenic drug doses (1 h prior to light treatment and 1 h prior to sacrifice). Fig. 4 shows a decrease in VEGF levels, measured by ELISA, in tumors treated with PDT combined with EMAP-II or IM862 compared with tumors treated with PDT alone. These results were obtained after only two doses of either EMAP-II or IM862. Nevertheless, a statistically significant decrease (P < 0.01) in PDT-induced VEGF levels was observed when EMAP-II was included in the treatment protocol. It is likely the 10 daily doses of EMAP-II or IM862 used in the PDT tumor treatment experiments would further attenuate VEGF levels.

In summary, we demonstrate that antiangiogenic treatments can potentiate PDT responsiveness. This result may involve attenuating the angiogenic actions of VEGF, which was observed to increase in PDT-treated tumors. Optimization of antiangiogenic parameters as well as an examination of various methods to block angiogenesis are being performed at present. The minimal systemic toxicity associated with antiangiogenic therapy suggests that these procedures should be compatible with clinical PDT and may provide an efficient strategy for selectively enhancing PDT tumor responsiveness. It will also be of clinical interest to determine whether antiangiogenic treatments can enhance PDT procedures for age-related macular degeneration because this pathology is marked by neovascularization (19).

Acknowledgments

We thank QLT PhotoTherapeutics, Inc. (Vancouver, British Columbia, Canada) for the generous gift of Photofrin, and Cytran Inc. (Kirkland, WA) for the generous gift of IM862.

References

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*Cancer Res* 2000;60:4066-4069.

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