Targeting Tumor Vasculature and Cancer Cells in Orthotopic Breast Tumor by Fractionated Photosensitizer Dosing Photodynamic Therapy


ABSTRACT

Photodynamic therapy (PDT) is a locally administered therapy currently being investigated in various clinical and preclinical settings. Tumor-host interaction is an important determinant of tumor biology and response to treatments. Here we report for the first time the effects of PDT on an orthotopic, murine mammary tumor model. PDT utilizes two individually nontoxic components: (a) the localization in the target site of a photosensitizing drug; and (b) the activation of the photosensitizer by light of an appropriate wavelength and energy. PDT after a single dose of the photosensitizer MV6401 induced drug dose-dependent, long-term blood flow shut down and tumor growth delay in the MCAIV tumor, grown in the mammary fat pad. The plasma half-life of MV6401 was 20 min, and the drug was confined to the vascular compartment shortly after administration. However, it accumulated in the interstitial compartment at 2–6 h after the administration. Two equal MV6401 doses injected 4 h and 15 min before the light administration allowed the photosensitizer to localize in both vascular and tumor cell compartments. The fractionated drug dose PDT more effectively induced tumor growth delay than the same total dose given as a single dose either at 4 h or at 15 min before light administration. The long-term effect of the fractionated drug PDT on blood flow was also more extensive than single-dose PDT. Fractionated photosensitizer dosing PDT offers a new strategy to optimize PDT therapy.

INTRODUCTION

PDT1 is a locally administered therapy that can be used as a primary therapy for early-stage disease, palliation of late-stage disease, or as a surgical adjunct for tumors that show locoregional spread (1). In several countries, including Japan, the Netherlands and Canada, PDT is approved as a treatment for lung cancer, esophageal cancer, gastric cancer, and bladder cancer. In the United States, PDT has been approved as a primary treatment for both late-stage and early-stage lung cancer (not amenable to surgery) and for obstructive esophageal cancer, actinic keratoses of the skin, and also for the treatment of the proliferative neovascular disease of the eye, age-related macular degeneration. PDT has also been investigated as a palliative treatment for cutaneous recurrence of breast cancer (2, 3) and has been suggested as a potential therapy for locally invasive breast cancer (3, 4).

PDT is a binary therapy in which two individually nontoxic components are combined to mediate cell and tissue death. The first component is the localization of a photosensitizing molecule in the target tissue, and the second component is the activation of the photosensitizer by light. In the light activation process, the photosensitizer acts as an energy transducer and transfers energy to molecular oxygen, resulting in the generation of a series of highly ROS, particularly singlet oxygen. These ROS have been shown to cause oxidative damage to a number of molecules including lipids, proteins, and glycoproteins, and as a consequence, damage to cellular membranes, organelles, and protein complexes. There are three major mechanisms by which PDT may act to mediate tumor destruction: (a) by direct tumor cell kill resulting from lethal events initiated by the flux of ROS; (b) by causing damage to the tumor-associated vasculature, with subsequent infarctive death of the tumor cells; and (c) by initiating a post-treatment immune response directed against tumor cells. Each of these processes has been shown to occur after PDT (1, 5); however, the precise mechanisms that are responsible in a tumor depend on the type of photosensitizing molecule, method, and/or schedule of PDT and other factors associated with the tumor phenotype.

There is a growing body of evidence that tumor-host interaction regulates biology and treatment response of tumors (6). Thus, orthotopic tumor models provide clinically relevant information. However, to our knowledge, there has been no report of PDT on an orthotopic mammary tumor in a preclinical animal model. Prior orthotopic models are limited to prostate (7), ovarian (8), and brain cancer (9). Therefore, we determined the effects of PDT in an orthotopic breast cancer model, and we found that PDT with the photosensitizer MV6401 (10) causes drug dose-dependent tumor vascular stasis and tumor growth delay. We hypothesized that a long interval between i.v. drug administration and light would result in MV6401 localization in the tissue compartment of the tumor after extravasation from the vasculature, with subsequent light activation resulting in direct tumor cell kill. Conversely, a short interval between drug administration and light activation results in direct effects on the vascular compartment, as described previously (10). Thus, we proposed that PDT after multiple administrations of sensitizer at appropriate times prior to a single activating light dose would target both the vascular and tissue compartments of the tumor. To test this hypothesis, two doses of MV6401 were administered at 4 h and 15 min before a single light treatment. These studies demonstrated that a regimen of fractionated dosing of the photosensitizer was superior to single dosage in mediating long-term vascular and tumor growth control. To our knowledge, this is the first study to report fractionated drug dose PDT.

MATERIALS AND METHODS

Photosensitizer. The photosensitizing agent, MV6401 (Miravant Medical Technologies, Santa Barbara, CA), is a pyropheophorbide derivative with indium chelated in the center of the pyropheophorbide macrocycle, as reported previously (10). The molecular weight of MV6401 is M_r 696.9. For systemic i.v. administration, the drug was dissolved in an EYP emulsion, which was predominantly composed of cationically charged egg yolk phosphatidyl cholines vesicles with an average diameter of 200 nm.

Animals and Tumor Model. The experiments were performed in female severe combined immunodeficient mice of 8–10 weeks of age, bred and
maintained in our gnotobiotic animal colony at Massachusetts General Hospital. MCAIV murine mammary adenocarcinoma cells, derived from sequential passage (limited to four passages) of tumors in mice in our animal colony were used. A single cell suspension was prepared from minced tumor slurry suspended in a mixture (1:3) of trypsin 0.25% (Life Technologies, Inc., 150-065) and Hanks (Sigma H9269), filtered through Swinex style filters (Millipore, 13 mm) and centrifuged for 5 min. Mice were anesthetized (9 mg of ketamine HCl and 0.9 mg of xylazine/100 g of body weight, s.c.), and 0.03 ml of the cell suspension was injected into the mammary fat pad inferior to the nipple using a 28-gauge needle, as described previously (11). Care was taken to avoid leakage of cells to the s.c. space. All procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital.

RESULTS

PDT with MV6401 Induced Dose-dependent Tumor Blood Flow Stasis. Regardless of the drug dose, the tumor blood flow stasis was observed in all regions examined during and immediately after PDT (Fig. 3). Stasis persisted for 2 days in all treatment groups, and complete reperfusion was not observed in any regions of the tumors in the 0.12 mg/kg body weight group up to 21 days after PDT. At time points longer than 2 days after treatment, there was resumption of flow in tumor vessels treated at the lower doses, as observed by intravital microscopy. The rate of recovery in the 0.06 mg/kg body weight group was slower than in the 0.03 mg/kg body weight group. Analysis undertaken 3 weeks after treatment showed that 68, 24, and 0% of the
regions were perfused in the 0.03, 0.06, and 0.12 mg/kg body weight treated animals, respectively. Animals that received drug alone or light alone did not exhibit altered blood flow compared with the control animals.

**MV6401 Localization Depends on the Time after Drug Administration.** Because of the high reactivity and short half life of ROS, only cells proximal to the area of ROS production (i.e., photosensitizer localization) will be directly affected by PDT (16). Plasma half life of MV6401 in SCID mice was 19.5 ± 3.1 min. Using intravital fluorescence microscopy, we determined MV6401 accumulation before, immediately after drug administration, and 30, 60, 120, 240, or 360 min after drug administration in the tumor interstitial tissues, which were devoid of any blood vessels (Fig. 4). Between 60 and 120 min, we observed a significant increase in MV6401 accumulation in the interstitium. At 120, 240, and 360 min, no statistically significant change in photosensitizer signal was observed. The degree, duration, and peak time of the drug accumulation were heterogeneous within the tumor as well as among tumors. Thus, 2–6 h after the injection of MV6401 would be the window for targeting the tumor interstitium. We chose the 4-h time point to cover most of the areas with relatively high accumulation of the drug.

Subsequently, we determined the sites of localization of the photosensitizer in the orthotopic tumor vasculature and tissue at 15 min and 4 h after MV6401 administration (10, 17). There was no detectable fluorescence corresponding to the wavelength of emission from MV6401 in cryosections of tumors from animals that received the EYP vehicle alone (Fig. 5a). Sequential immunohistochemical staining with antibody to CD31 (PECAM) showed that MV6401 colocalized with CD31-positive structures 15 min after administration, indicating that MV6401 was confined to the vascular compartment at this
time and seemed to be associated with the endothelial cells lining the vessels (Fig. 5b). When the drug distribution images were superimposed with DAPI-stained nuclear images, MV6401 was observed only in the vascular space and/or associated with the vascular wall. No drug was detected in the surrounding tumor tissue. Similar analysis of tumor sections 4 h after drug administration showed that the drug was mainly localized outside the vascular compartment, with some residual drug associated with the vessel wall (Fig. 5c). Analysis of the drug distribution of tumors with fractionated drug dose showed that MV6401 was localized both to the interstitial and vascular compartments. Vessel walls, identified as CD31-positive structures, were surrounded by MV6401 from the luminal as well as from the abluminal side (Fig. 5d).

**Fractionated Dose Is More Effective Than Single Dose.** To determine the efficacy of fractionated photosensitizer dosing, we used a total dose of 0.03 mg/kg body weight, which was suboptimal as a single dose (Fig. 1). When the total drug dose of 0.03 mg/kg body weight was fractionated into two equal drug doses and the fractions were administered at 4 h and 15 min before the light exposure, a significant tumor growth delay was observed compared with single full drug dosing at either 4 h or 15 min before light (Fig. 6). The mean (50%) survival times in the fractionated dose group, the single dose 15 min group, and the single dose 4 h group were 38, 24, and 16 days, respectively (Fig. 7). Statistical analysis showed that these survival data for the fractionated dose of drug were significantly different ($P < 0.05$) from the data from either of the single drug dose groups. The fractionated drug dose PDT did not cause adverse effects in normal tissue, as is seen with the single injection of the 0.12 mg/kg body weight drug dose PDT.

**Blood Flow after Fractionated Dose.** Analysis of vascular perfusion of tumors treated in the fractionated dose group, the 15-min group, and the 4-h group showed that all of the treatment regimes caused blood flow stasis during and immediately after PDT (Fig. 8). Because subsequent analysis of perfusion was undertaken at later times after treatment, it was noted that tumors in the fractionated dose group showed the most extensive long-term effect on the blood flow. One week after PDT, 63, 43, and 26% of the regions were perfused in the 4-h group, 15-min group, and fractionated dose group, respectively.

**DISCUSSION**

In this study, we show for the first time that MV6401 induces vascular shutdown and long-term tumor growth delay in an orthotopic breast tumor model in a dose-dependent manner. These results are consistent with our previous study with a mouse dorsal skinfold chamber model in which we found that thrombus formation is a major cause of long-term vascular shut down (10). The highest drug dose (0.12 mg/kg body weight) used in this study induced nearly complete tumor eradication. However, we noticed severe tissue damage in the surrounding normal tissue with this dose. Although long-term vascular effects were selective to tumor vessels at low and moderate MV6401 doses, high drug dose PDT caused similar effects on normal blood vessels (10). In both orthotopic mammary fat pad and dorsal skinfold chamber models, single drug dose PDT that is tumor vessel selective could induce only moderate tumor growth control, and tumor regrowth was proportional to tissue perfusion recovery. Tissue perfusion appeared to be recovered by new vessel formation rather than by...
reperfusion of static vessels. Hypoxia and other stress induced by PDT may up-regulate angiogenic factors such as vascular endothelial growth factor (18). Thus, for better long-term tumor control with anti-vascular PDT, combination with antiangiogenic therapy and/or cytotoxic therapy may be necessary. Another strategy to improve the therapeutic response/index is fractionation. Multiple PDT light doses have been given to avoid oxygen depletion during PDT (19, 20). Similar to chemotherapy, radiation sensitizers have also been fractionated to attack tumor cells that are in different stages of the cell cycle (21). However, all of these fractionated treatments essentially target a single compartment, the tumor cells. Our approach to fractionate the photosensitizer doses offers a unique opportunity to target multiple compartments, both vascular and tumor cells.

The fact that photosensitizers are taken up by tumor cells has been exploited for decades (1), and in these previous studies the drug accumulated in the tumor tissue, allowing sufficient time between drug administration and light activation. On the other hand, we and others have demonstrated that a short interval between drug administration and light results in a vascular-specific drug localization and significant antivascular effects (10, 22, 23). Drugs should be present in both vascular and tumor cell compartments during the intermediate time points. However, it is difficult to achieve sufficient direct tumor cell killing and antivascular effects simultaneously with single dose PDT. The antivascular effects of PDT diminished rapidly with time after administration of MV6401 and may not be significant when the drug is predominantly localized in the tumor cell compartment (Fig. 8; Refs. 24, 25). Previously published studies have produced conflicting results with respect to the site of drug localization and efficacy. For example, the maximum PDT response was observed when the light treatment was carried out either at the time of highest plasma drug level (25) or at the time of highest tumor drug content (26), depending on tumors and drugs. Thus, to further optimize the therapy, we...
proposed to attack both the vasculature and the tumor cells at the same time with fractionated drug dosing. The fractionated dose used in this study exhibited greater treatment efficacy than single dose treatments, without enhancement of normal tissue damage. This is remarkable because the total amount of the sensitizer was the same, and both were treated with a single light administration. The fractionated drug dose regimen appeared to be more than additive in its efficacy. This concept may not be specific to MV6401. Appropriate fractionated drug dose may enhance the effects of PDT with other sensitizers as well.

Fractionated drug dose PDT showed more profound long-term vascular effects than single drug fraction PDT. Several explanations for this striking observation are possible: (a) both luminal and abluminal surfaces of the blood vessel wall are facing the sensitizer in the fractionated dose tumors (Fig. 5d) and thus, PDT may effectively attack both endothelial and perivascular cells; and (b) tumor blood flow is known to be temporally and spatially heterogeneous (27, 28). Fractionated dose permits more homogeneous distribution of sensitizer throughout the tumor vasculature by covering different fractions of temporally perfused vessels. If this novel finding can be translated into the clinic, it offers possibilities to optimize PDT in humans. The drug fractionation regimen tested in this study may be more practical in clinical applications because patients will only need one light irradiation. Fractionation of the light dose would require more resources and may be more invasive, depending on the application (e.g., peritoneal metastasis).

In summary, we have demonstrated that PDT is an effective approach to treat an orthotopic mammary carcinoma. Exploiting the simultaneous targeting of vasculature and tumor cells by PDT offers a new strategy, i.e., fractionating the drug dose before single light administration. Fractionated drug dose PDT is a more effective therapy on mouse mammary carcinoma than single drug dose PDT. This new strategy might lead to better PDT treatments in the clinic.

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