Photoinduced Photodynamic Therapy Induces Vascular Occlusion and Apoptosis in a Human Sarcoma Xenograft Model

Brett W. Engbrecht, Chandrakala Menon, Alexander V. Kachur, Stephen M. Hahn, and Douglas L. Fraker

Harrison Department of Surgical Research [B. W. E., C. M., D. L. F.] and Department of Radiation Oncology [A. V. K., S. M. H.], School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104.

ABSTRACT

Photodynamic therapy (PDT) involves light activation of a photosensitizer, resulting in oxygen-dependent, free radical-mediated cell death. Little is known about the efficacy of PDT in treating human sarcomas, despite an ongoing clinical trial treating i.p. sarcomatosis. The present study evaluates PDT treatment of a human sarcoma xenograft in nude mice and explores the mechanism of PDT-mediated antitumor effect. Athymic nude mice, 6–8 weeks of age, were s.c. injected with 5 × 10⁶ cells of the A673 human sarcoma cell line. Tumors were allowed to grow to a diameter of about 10 mm. Photofrin (PF), 10 mg/kg, was injected by tail vein, and 24 h later, 630 nm light was delivered to the tumor with fluences of 50, 100, 150, or 300 J/cm² at a fluence rate of 250 mW/cm². To assess the efficacy of PDT in the treatment of sarcomas, photosensitizer uptake/retnention studies and dose-response studies were performed. Studies carried out to determine the mechanism of tumor response included tumor temperature measurements before, during, and after treatment; tumor vascular perfusion studies with laser Doppler; electron microscopic analysis of tumor sections for vascular occlusion; and analysis of tumor cryosections for endothelial cell damage, apoptosis, and necrosis. At all time points of analysis, photosensitizer levels were greater in tumor than in muscle. Dose-response studies showed that at 100 J/cm², five of six mice had a complete response to treatment, one of six had a partial response, and no deaths occurred. Temperature measurements indicated that thermal injury did not contribute to tumor response. Vascular perfusion studies demonstrated a significant reduction in blood flow as early as 6 h after PDT. Electron micrographs revealed erythrocyte aggregation in tumor microvessels starting as early as 2 h after treatment and complete occlusion of blood vessels by 12 h. Starting as early as 4 h after PDT, apoptosis first appeared in endothelial cells lining the occluded blood vessels and became more widespread at later time points. PDT is an effective treatment for this human sarcoma xenograft in nude mice. The mechanism of tumor destruction in this model appears to be vascular damage with initial apoptosis in tumor endothelial cells and delayed tumor cell apoptosis. This therapy may be valuable in the treatment of patients with sarcomatosis.

INTRODUCTION

PDT is an antineoplastic treatment that involves the use of a photosensitizer, such as PF, the application of visible light of the wavelength specific for the photosensitizer, and the presence of oxygen leading to reactive oxygen species-mediated cytotoxicity to the treated cell. PF, the photosensitizer used in this study, is a partially purified derivative of hematoporphyrin that is activated by light at 630 nm. On absorbing a photon, PF is excited to a high energy singlet state. By an intersystem cross-over, the excited photosensitizer can undergo an electron spin conversion and yield a triplet sensitizer that can then be involved in a type I or type II photochemical reaction to produce damaging reactive oxygen species. (1) The photochemical reaction that occurs in the presence of oxygen is preferably the type II reaction.

There has been increasing enthusiasm for the use of PDT as a cancer treatment for surface malignancies with reports that hematoporphyrin derivative (2) and some second-generation photosensitizers (3, 4) are selectively retained in malignant tissues. The mechanism for tumor responses to PDT in vivo has been studied (4–10). Direct tumor cell kill, vascular damage, and inflammatory responses have all been shown to contribute to tumor destruction. A large portion of PF-mediated PDT damage to tumors in vivo has been shown to be related to vascular damage (5, 9, 11). PF-mediated PDT causes a reduction in blood flow in tumors and normal tissue that is associated with vasoconstriction of arterioles and thrombosis of venules (12), as well as erythrocyte aggregation and stasis (13–16).

Studies evaluating the mode of cell death after PDT in vivo have shown evidence of both apoptosis and necrosis (3, 17, 18). The interplay of the direct tumor cell cytotoxicity and vascular effects of PDT and the induction of either necrosis or apoptosis within a tumor is complicated and under active investigation (19, 20). Insights into the mechanisms of tumor response and into the mode of cell death after PDT, especially in human tumors, may lead to ways to maximize the efficacy of this treatment.

One potential application of PDT is to treat the lining of the peritoneal cavity in patients with diffuse intraabdominal malignancies. Patients with intraabdominal and retroperitoneal sarcomas often develop diffuse i.p. spread or sarcomatosis without any evidence of distant spread. There is no effective treatment for sarcomatosis using standard surgical techniques, radiation therapy, or chemotherapy. Even when all gross disease is excised, these patients are destined to have recurrent tumor due to i.p. spreading of disease. Although preclinical studies have demonstrated the effectiveness of PDT in treating a variety of murine tumors including sarcomas (21–23), little is known about the treatment of human sarcomas in preclinical models, despite the fact that the results of two clinical trials have been reported treating sarcomas (24, 25). Also, in our institution, there is an ongoing clinical trial to treat patients with i.p. sarcomatosis (4). Preclinical models assessing the response of human sarcomas to PDT could potentially be used to optimize treatment protocols.

The present study, therefore, focuses on the effectiveness of PF-mediated PDT in treating human A673 sarcoma xenografts in nude mice. The selectivity of PF in tumor tissue compared with normal tissues, the mechanism of tumor response, and the mode of tumor cell death are also explored.

MATERIALS AND METHODS

Animals, Tumor Production, and PF Injection. Athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN), 6–8 weeks of age, received s.c. injections of 5 × 10⁶ cells of the human A673 sarcoma cell line (American Type Culture Collection, Bethesda, MD). The tumors were placed in the flank and were allowed to grow until they were ~10 mm in diameter. PF (a generous gift from QLT Phototherapeutics, Inc., Vancouver, British Columbia, Canada) was dissolved in 5% dextrose at a concentration of 1 mg/ml and injected by tail vein at a dose of 10 mg/kg.

Photosensitizer Uptake/Retention Measurements. Mice were anesthetized, and tumor, muscle, skin, and serum were harvested at 12, 24, and 48 h...
after injection of PF. Samples from three mice were analyzed for PF levels per time point using an HPLC assay (26) that was modified in our laboratory. Briefly, tissue was kept in the dark at −80°C until analysis of drug concentration. Tissue, 50–100 mg, was dissolved in 1 ml of Solvable (DuPont, Boston, MA) and left overnight in the dark at 50°C. The samples were neutralized to pH 8 with acetic acid. One hundred μl of the sample were analyzed for each tissue. The intensity of the signal of PpIX was measured by HPLC using a C18 reverse phase column. The PpIX signal was chosen due to its higher retention time on the column. Also, a complete separation of PpIX signal from other peaks made it relatively easy to compare with the standard. Seventy % methanol in 10 mM sodium phosphate (pH 7.4) was used as the buffer. Concentrations of PpIX were determined from a standard curve and expressed as μg/g tissue weight. The intensity of PpIX signal was found to be linear with respect to PF concentration within a relatively wide range (1 ng/g tissue to 100 μg/g tissue), with a high correlation coefficient of 0.999. Because the levels of endogenous PpIX was below the limit of detection of the assay (<0.05 μg/g tissue), the values obtained for PpIX levels from the PF-containing tissues were used directly without subtraction of background. The tissues from one mouse that did not receive PF were used as negative controls. To determine whether the PF selectivity in tumor tissue was characteristic of the A673 tumor alone, PF levels were similarly measured in two other human sarcoma tumors, SK-ES-1 and SK-LMS-1.

**PDT Efficacy Studies.** Nude mice received 3 Gy γ-irradiation to improve tumor take 1 to 3 days before injection of A673 cells (5 × 10⁶ cells) into the s.c. space in the flank. Mice were injected with PF (10 mg/kg) when tumors were −10 mm in diameter. Control mice were injected with 5% dextrose using a similar protocol. Light was delivered 24 h after drug injection using a KTP-Yag pumped PDT dye laser (Laserscope model XP, San Jose, CA) at 630 nm with a fluence rate of 250 mW/cm² and a Microlens optical fiber (RARE Earth Medical, West Yarmouth, MA). Mice used for dose-response studies received 50, 100, 150, or 300 J/cm² (n = 6 for each group except 300 J/cm², where n = 5). There were three control groups: (a) no PF; no light; (b) PF; no light; and (c) no PF, light at either 50, 100, 150, or 300 J/cm² (n = 3 for each group).

**Mechanism Studies.** Mice were used for temperature measurements. Doppler flow studies, histological analyses, and apoptosis assays received PDT with 100 or 150 J/cm², or light only at 100 or 150 J/cm². Tumor response to PDT was categorized as “no response” if tumors continued to grow posttreatment, as “partial response” if tumors were less than 75% of pretreatment size, and as “complete response” if no tumor was visible. Tumors were categorized as a “recurrence” if they regrew after an initial response to PDT, which lasted for a minimum of 10 days after treatment.

**Temperature Measurements.** Physitemp Thermalert (model TH-8, Clifton, NJ) was used to make tumor temperature measurements. The thermal probe was inserted just off-center and mid-depth into each of the tumors, with the tip of the probe halfway into the tumor. Measurements in triplicate were made before treatment, during treatment at 5- and 10-min time points, and at 1 min after the completion of treatment (n = 3 in control and treatment groups). Control tumors were given dextrose and light at 150 J/cm². Photofrin II (10 mg/kg) dissolved in 0.05% methanol in 10 mM sodium phosphate (pH 7.4) was used as the vehicle. Control mice were injected with 5% dextrose using a similar protocol.

**Tissue Harvesting and Storage for Electron Microscopy.** After treatment and at 2, 4, 8, 12, 18, and 24 h after treatment (light-only group, n = 1; PDT group, n = 2; for each time point). Tissue for electron microscopy was fixed in 2% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and kept at 4°C for 18 h. Tissue for H&E staining, blood vessel immunostaining, and apoptosis assays was frozen in OCT (Tissue-Tek) and kept at −80°C until analysis.

**Electron Microscopy.** Fixed tissues were embedded in Epon 812 (EM Sciences, Fort Washington, PA), sectioned, and stained by standard techniques carried out at the Biomedical Imaging Core facility, Department of Pathology and Laboratory Medicine, at the University of Pennsylvania.

**Blood Vessel Immunostaining.** Cryopreserved tumor tissue sections were immunostained with rat anti-murine PECAM antibody (a generous gift from Dr. Steven Albelda, University of Pennsylvania, Philadelphia, PA) using the ABC Elite kit and Vector VIP Peroxidase Substrate kit (Vector Labs, Burlingame, CA). The slides were viewed and photographed under a light microscope at ×100.

**Apoptosis Assays.** Apoptosis assays were performed by the TUNEL assay using the In Situ Cell Death Detection kit (Boehringer Mannheim, Indianapolis, IN). The slides were viewed and photographed under a fluorescence microscope at ×200.

**Double Labeling for Blood Vessels and Apoptosis.** Cryopreserved tissue sections were fixed in cold acetone for 5 min and permeabilized using cold 0.1% Triton X-100 in 0.1% sodium acetate for 2 min. Labeling for blood vessels was carried out using the above-mentioned anti-PECAM antibody in combination with anti-rat IgG Texas red for blood vessel staining. Apoptosis assays were carried out using the In Situ Cell Death Detection kit (Boehringer Mannheim). The sections were viewed and photographed under a fluorescence microscope at ×100 using appropriate filters for Texas red or FITC.

**H&E Staining of Tissue Sections for Necrosis.** H&E staining was carried out by standard methods at the Cell Morphology Core, Institute for Human Gene Therapy, University of Pennsylvania. The sections were visually scored for extent of necrosis in the tumors and photographed by bright field microscopy at ×150.

**Statistics.** Statistical analysis of PF uptake/retention data, tumor temperature measurements, and laser Doppler flow measurements was performed using the Graphpad Instat computer software (version 3.0 for Windows NT). Values were compared using the unpaired Student’s t test with Welch correction. Statistical significance was defined as P < 0.05.

**RESULTS**

**Photosensitizer Measurements.** PF levels were greater in tumor than in muscle at all time points studied (Fig. 1). This difference was statistically significant (P = 0.01) at 12 h. The ratio of mean tumor PF levels to mean muscle levels at 12 h was 1.12±0.26. PF levels in skin were greater than in tumor at all time points studied (data not shown),
PHOTODYNAMIC THERAPY FOR HUMAN SARCOMA

Table 1 Summary of gross tumor and whole animal responses to PDT at 50, 100, 150, and 300 J/cm² light doses

<table>
<thead>
<tr>
<th>Light dose (J/cm²)</th>
<th>n</th>
<th>No response</th>
<th>Partial response</th>
<th>Complete response</th>
<th>Recurrence</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>4/5</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>2/5</td>
<td>0</td>
</tr>
<tr>
<td>150</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1/3</td>
<td>3</td>
</tr>
<tr>
<td>300</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

* Tumors continued to grow.
* Tumor <75% of pretreatment size.
* No tumor visible or palpable.
* Tumor grew back after a period of complete response.

There was increasing mortality observed with increasing fluence (Table 1). Death associated with PDT occurred within 1–5 days after treatment. Necropsies were performed on mice that suffered treatment-related death. In some of these cases, death appeared to be due to necrosis of bowel deep to the site of light treatment. In other cases, no cause of death could be identified. Of the three mice that survived a dose of 150 J/cm², all showed a complete response to treatment (no tumor visible or palpable).

**Mechanism of Tumor Cell Kill.** To determine the mechanism of tumor response, tumor temperature measurements and tissue perfusion studies were performed before treatment, during PDT, and after treatment. Electron micrographs of PDT-treated and control tumor sections were analyzed for vascular occlusion, and photomicrographs of cryopreserved tissue sections were immunostained for blood vessels and evaluated for apoptosis histologically. PDT-treated and control tumor sections were also scored for necrosis.

A slight temperature rise of ~4°C was measured after the initiation of PDT (Fig. 3). No significant difference (P = 0.974) in tumor temperature between control and treatment groups was noted before, during, or after treatment at a light fluence of 150 J/cm². Maximal
Tumor temperature was 37°C in all mice during light treatment, and the temperature decreased rapidly after light treatment was complete. These results suggest that the tumor response was not due to thermal injury.

Laser-Doppler studies of tumor vascular blood flow demonstrated a very significant reduction ($P = 0.0074$) in blood flow as early as 6 h after PDT compared with control groups (Fig. 4) at a fluence of 150 J/cm$^2$. Perfusion remained decreased up to 60 h after treatment (data not shown), consistent with the gross appearance of tumors (Fig. 2). Blood flow in the control tumors was generally stable over time.

Electron micrographs of tumor sections revealed erythrostasis starting as early as 2 h after light treatment in mice with PDT-treated tumors (Fig. 5). This change in vascular appearance was a consistent histological finding in PDT-treated tumors and persisted through all later time points studied. Two h after PDT, tumor cells and endothelial cells appeared normal and viable. After 12 h, however, the tumor cell nuclei in the immediate vicinity of the blood vessels showed condensed and abnormally fragmented chromatin. Tumor cell nuclei at a greater distance from the blood vessel appeared normal at this time point. Most tumor cells were clearly pyknotic in all locations by 36 h.
Anti-PECAM staining of control and PDT-treated tumor sections revealed that PDT caused a disruption of the endothelial lining of blood vessels (Fig. 6). Tumor sections labeled for apoptotic cells using the TUNEL assay (Fig. 7) demonstrated little or no apoptosis in the control specimens receiving no treatment, light only, or sensitizer. However, in specimens from PDT-treated tumors, progressively increasing numbers of apoptotic cells were visible starting from 4 h after PDT. At this early time point, apoptotic cells were patchy and few in number, but by 36 h there was widespread apoptosis. In PDT-treated tumor sections that were double-labeled for blood vessels and apoptosis, it appeared that the initial apoptotic cells were tumor endothelial cells (Fig. 8).

Histological analysis of tissue sections by H&E staining (data not shown) revealed patchy areas of necrosis in all sections (control and treatment) interspersed within viable tumor cell areas. Although beginning at 12 h after PDT, more abundant patches of necrosis appeared within the tumors of the PDT-treated mice; the patches were small, unconnected, and did not appear to increase in size. Necrosis, although certainly contributing to some tumor destruction, does not appear to be the primary cause of cell death after PDT in this model.

**DISCUSSION**

PDT therapy is an approved treatment or under experimental clinical investigation for a variety of malignancies and premalignant conditions including head and neck cancers (27, 28), lung cancer (29, [Figures 6-8])
mesothelioma (31, 32), and esophageal cancer (33). Clinically, there has been some application in the treatment of intraabdominal sarcomas (24, 25). However, little preclinical data exist using PDT to treat this histology. Although the demonstration of efficacy of a cancer treatment in preclinical models does not translate into clinical efficacy, most investigators would agree that the absence of such preclinical efficacy studies casts serious doubts about a treatment for a particular cancer.

In an ongoing Phase II clinical trial of PDT for diffuse i.p. malignancies at the University of Pennsylvania, approximately one-half of the patients treated have sarcomatosis. Because of these referral patterns and the need to develop new approaches for this intractable condition, we evaluated the efficacy of PDT against human sarcoma for the first time in a preclinical model.

The data presented in this study demonstrate that PF-mediated PDT can lead to complete eradication of the human A673 sarcoma xenograft in nude mice. An initial complete response was achieved in five of six mice at light fluences ranging from 50 to 150 J/cm². A sustained complete response was greatest and regrowth of tumor was the lowest at the higher total light fluences, demonstrating a dose-response relationship to PF-mediated PDT. The treatment responses in this study were achieved with relatively high fluence rates (250 mW/cm²). It has been shown in some experimental tumor models that higher fluence rates lead to a greater depletion of oxygen during the

Fig. 7. Photomicrographs of control (left panels) and PDT-treated (right panels) of cryopreserved tumor sections labeled for apoptosis using the TUNEL assay. Tissue sections of control tumors at 4, 18, and 36 h (A, C, and E, respectively) show little or no apoptotic cells. PDT-treated tumor sections at 4, 18, and 36 h (B, D, and F, respectively) show a progressively increasing number of apoptotic cells with increasing time after PDT.
Fig. 8. Photomicrographs of double-labeled control and PDT-treated tumor tissue sections for blood vessels (left panels) and apoptosis (right panels). Tissue sections were immunofluorescently labeled for blood vessels using a rat anti-mouse PECAM antibody and anti-rat IgG Texas red and later labeled for apoptosis using the TUNEL assay. The double-labeled sections show that at the early time points, 4 h (A and B), 8 h (C and D), and 12 h (E and F) after PDT, apoptotic cells first appear around blood vessels.
administration of light and, therefore, to reduced PDT efficacy (33–37). Although it is encouraging that positive treatment outcomes were the results, even with the relatively high fluence rate used in this study, it is likely that better treatment outcomes are possible with lower fluence rates. The complete responses seen at 250 mW/cm² would indicate that this tumor is relatively well oxygenated such that oxygen consumption at the relatively high fluence rate used in this study did not limit the response.

One of the major reasons for enthusiasm surrounding the use of PDT in cancer treatment is the reported selectivity of many of the photosensitizers for tumor compared with normal tissues (2–4). In this study, normal tissue levels of PF were measured with an HPLC assay. This assay method allows for the determination of the concentration of individual components of PF and also allows for their separation from other porphyrin compounds present in tissue. Also, the sensitivity of the HPLC assay is very high, and the range of concentrations that can be determined by this method is higher than other known methods (from 1 ng/g tissue to 100 μg/g tissue). Moreover, the more commonly used fluorescence method requires subtraction of endogenous tissue fluorescence, which significantly reduces the ability to measure photosensitizer levels at lower concentrations. As stated in “Materials and Methods,” the level of endogenous PpIX is below the limit of detection of this assay; therefore, it does not need to be subtracted from the measured value. In this study, a greater concentration of photosensitizer was measured in the A673 tumor compared with the surrounding muscle tissue. These results are similar to earlier findings (2, 4, 19). The mechanism of preferential retention of photosensitizers in tumor compared with other tissues has been a topic of much study (4, 38). Many factors have been explored, including increased lipoprotein receptors (especially low-density lipoprotein) on tumors, uptake of sensitizers by tumor-associated macrophages, and an altered tumor microenvironment.

Mechanisms of PDT-mediated cytotoxicity in the A673 tumor were investigated by various functional and histological techniques over the posttreatment time period. Temperature measurements show that thermal injury is not likely to be a significant factor in tumor response. Both flow studies and a variety of histological studies suggest that the primary effect of PDT in this model appears to be vascular damage. Damage to the vasculature with PF-mediated PDT is known to be an important component of tumor response in other preclinical models (13, 20), and our results confirm this. The kinetics of the vascular response in the present model of a human sarcoma xenograft agree with the previous reports on tumors of different histologies. Electron micrographs of tissue sections demonstrate occlusion of the tumor blood vessels with associated erythrocytosis as early as 2 h after tumor illumination. The initial laser Doppler studies were performed 6 h after PDT and, at that early time point, showed significant loss of blood flow. Decrease in blood flow after PF-mediated PDT has also been shown in other tumor systems (39). On the basis of earlier electron microscopy results, flow is likely to be decreased and should be one of the initial events in this model. PF-mediated PDT also progressively affected the integrity of the endothelial lining of blood vessels, as shown by photomicrographs of tumor tissue sections stained with an anti-PECAM antibody. The precise molecular reasons for these findings are not clear, but all results point to an occlusion of tumor vasculature at the initiation of the tumor response.

With increasing time after tumor illumination, endothelial cell and tumor cell apoptosis was demonstrated in tissue sections. One potential explanation for this is that blood vessel occlusion and subsequent endothelial damage lead to tumor hypoxia. Hypoxia has been shown to lead to apoptosis through a variety of mechanisms, including changes in p53 protein level and caspase activation associated with cytochrome c release from mitochondria (40–42). The electron mi-

REFERENCES


Photofrin-mediated Photodynamic Therapy Induces Vascular Occlusion and Apoptosis in a Human Sarcoma Xenograft Model

Brett W. Engbrecht, Chandrakala Menon, Alexander V. Kachur, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/17/4334

Cited articles  This article cites 39 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/17/4334.full.html#ref-list-1

Citing articles  This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/17/4334.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.