Tumor Destruction and Kinetics of Tumor Cell Death in Two Experimental Mouse Tumors following Photodynamic Therapy


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ABSTRACT

The effect of photodynamic therapy (PDT) on tumor growth as well as on tumor cell survival in vitro and in vivo was studied in the EMT-6 and RIF experimental mouse tumor systems. In vitro, RIF cells were more sensitive towards PDT than were EMT-6 cells when incubated with porphyrin (25 μg/ml, dihematoporphyrin ether) and subsequently given graded doses of light. In vivo, both tumor types responded to PDT (EMT-6, dihematoporphyrin ether, 7.5 mg/kg; RIF, dihematoporphyrin ether, 10 mg/kg; both followed 24 hr later by 135 J of light at 630 nm/sq cm) with severe vascular disruption and subsequent disappearance of tumor bulk. However, whereas the cure rate for EMT-6 tumors was 90%, it was 0% for RIF tumors. Raising the light dose to 200 J/sq cm resulted in 100% cures for EMT-6 tumors accompanied by damage to the surrounding tissues and 13% cures for RIF tumors. Tumor cell clonogenicity following PDT in vivo was assessed using the in vitro colony formation assay. In both tumors, it was found to be nearly unaffected by PDT if the tumor tissue was excised and explanted immediately following completion of treatment. This indicates that the effect of PDT on tumor destruction may be damage of the tumor circulation and not as a result of direct damage to the tumor cells.

INTRODUCTION

PDT is a new modality for the treatment of cancer through the combined use of systemically administered photosensitizing porphyrins and local applications of light (11). Its therapeutic usefulness is based on the preferential accumulation of porphyrin in tumor tissue (5, 7). The illumination of the porphyrin-containing tumor with visible light (wavelength, 630 nm) leads to selective, rapid tumor necrosis (8, 11). Preceding tumor necrosis are pronounced changes in the vascular system of the tumor (3, 5, 23).

In vitro, dose-dependent (photosensitizer + light) photodynamic cell killing is characterized by cell lysis within 1 hr after a lethal PDT dose (1) and can be achieved in all cell types studied thus far, normal as well as malignant (2, 6, 14).

Despite extensive in vivo and in vitro studies, it has not yet been established whether tumor cell killing in vivo is governed by the same mechanism as is cell killing in vitro. To answer this question, we studied the relationship between tumor destruction, tumor cure, and tumor cell survival kinetics in 2 experimental mouse systems.

MATERIALS AND METHODS

Tumor Systems. The EMT-6 and RIF tumor systems were obtained from the University of Rochester Medical Center and were maintained according to established protocols (22, 24). In our hands, the viable cell inoculum for 50% tumor takes for EMT-6 tumors was 100 to 200 cells, that for RIF tumors 10 to 20 cells. Tumors were propagated by i.d. inoculation of 1 to 2 × 10⁵ cells, harvested from exponentially growing cultures, into the right flank of BALB/c and C3H/HeJ mice, respectively. Tumors were used for treatment and/or explantation 6 to 10 days after inoculation at which point they were hemiellipsoids with a major axis of 5 to 6 mm and a minor axis (thickness) of 2 to 4 mm.

Preparation of Cell Suspensions and Colony Formation Assay. Tumors, untreated or treated, were excised under sterile conditions from mice killed by cervical dislocation. Tumors from 2 mice were pooled, weighed, and minced with scissors, and a single-cell suspension was prepared using an enzyme mixture of Pronase, DNase, and collagenase (18). Following cell dispersion, the cell suspension was strained through a wire mesh screen (Collector; E-C Apparatus, St. Petersburg, FL) to eliminate any remaining tissue clumps and cell aggregates. The cells were washed with Hanks' balanced salt solution and resuspended in the appropriate growth medium. The cell number was assessed by hemocytometer counts as well as by using a Coulter Counter excluding any erythrocytes which heavily contaminated all cell suspensions from treated tumors. Cell viability was determined by trypan blue exclusion. Smears of final cell suspensions were prepared to further identify the cell populations. The cell yield per g tissue was then calculated. Appropriate numbers of tumor cells were then plated in 100-mm culture dishes for colony formation in the following growth media. EMT-6 cells were grown in Eagle's basal medium with Hanks' salts and L-glutamine, to which were added sodium bicarbonate (1.5 g/liter), 15% FBS, and antibiotics. RIF cells were grown in minimum Eagle's alpha medium with L-glutamine and ribo- and deoxyribonucleosides supplemented with 10% FBS and antibiotics. All media and supplements were obtained from Grand Island Biological Co., Grand Island, NY. Cultures were incubated in the dark in a humidified atmosphere of 5% CO₂ in air for 7 to 9 days, at which time they were fixed and stained, and macroscopic colonies were counted. In those experiments where cells had been exposed to photosensitizer, cell isolation and/or plating were carried out in subdued light filtered.
through a sharp-cut filter (1% transmittance at 630 nm, no transmittance below that wavelength; Corning Glass Works, Corning, NY).

Photosensitizer. Photofrin II (Photofrin Medical, Inc., Cheektowaga, NY) was used in all experiments. It is derived from hematoporphyrin derivative and contains above 90% of dihematoporphyrin ether, which is the compound responsible for the in vivo tumor-photosensitizing effect (10).

In Vitro PDT. EMT-6 and RIF cells, derived from untreated tumors as described above, were grown as monolayers. At a density of about 5 x 10^5 cells/plate, they were exposed to photosensitizer (25 μg/ml) for 24 hr in growth medium containing 10% FBS. The medium was then removed, cells were detached using 0.25% trypsin, resuspended in fresh, photosensitizer-free growth medium containing 10% FBS, and replated at known cell numbers. Four hr were then allowed for cell attachment and removal of any loosely attached photosensitizer, after which the cells were exposed to graded doses of light (4 milliwatts/cm² at 590 to 640 nm; light source from GTE-Sylvania, Salem, MA). Following light exposure, plates were incubated for colony formation as described above. All manipulation of cells exposed to photosensitizer were carried out in subdued, filtered light as described before.

In Vivo PDT. Tumor-bearing mice were given injections of photosensitizer i.p. (7.5 mg/kg for EMT-6 or 10 mg/kg for RIF). Twenty-four hr later, the animals were restrained without anesthesia in specially designed holders, and tumors were given localized, external light treatment using an argon dye laser system (75 milliwatts/cm² at 630 nm; Spectra Physics Corp., Mountain View, CA). Light exposure times were 30 min (135 J/cm²) or 45 min (200 J/cm²). Light was delivered to 2 tumors simultaneously through 200-μm quartz fibers. Following light treatment, mice used for tumor explant were killed either immediately or at varying times later. Mice used to assess tumor response were observed for up to 91 days posttreatment. Tumor temperature measurements before and during light treatment were taken by means of 0.005-inch copper-constantan microthermocouples (Omega Engineering, Inc., Stamford, CT) which were inserted at the base of the tumor and were coupled with a digital readout device.

Tumor Anoxia Experiments. Anoxia was produced by killing tumor-bearing mice by cervical dislocation. For control purposes, the animals were given porphyrin at the doses used for PDT treatment 24 hr prior to sacrifice. After sacrifice, mice were kept at 37°C for varying periods of time up to 4 hr, at which time the tumor tissue was excised and prepared for colony formation assay as described above.

Measurements of Photosensitizer Uptake by Tumor Tissue. Photosensitizer uptake was compared in EMT-6 and RIF tumors by means of a 5-mwatt HeNe laser (Spectra Physics) and a photodiode (Infrared Industries, Waltham, MA), covered by an appropriate scattering material. Three measurements were carried out on each of 3 different animals of each tumor type.

Histology. Tumors were removed from animals before and after varying times of PDT treatment, fixed in buffered formalin, sectioned 5 μm thick, and stained with hematoxylin-eosin.

RESULTS

Effect of In Vitro PDT. Chart 1 shows the kinetics of cell death of EMT-6 and RIF cells following exposure to photosensitizer and light treatment in vitro. Both cell types could readily be inactivated by this treatment, but their survival curves showed a difference in shoulder width (quasi-threshold dose, Dq: EMT-6, 1.23 ± 0.04 (S.D.) J/cm²; RIF, 0.78 ± 0.11 J/cm²) as well as in slope (37% dose slope, D37: EMT-6, 0.23 ± 0.04 J/cm²; RIF, 0.10 ± 0.05 J/cm²). By both criteria, RIF cells proved more photosensitive in vitro than did EMT-6 cells.

Photosensitizer Uptake In Vivo. Tissue fluorescence 24 hr postdrug injection was found to be nearly identical for both tumor types at both injected porphyrin levels. Direct comparison of fluorescence and extracted porphyrin concentration was carried out at the injected porphyrin dose of 30 μg/kg because of sensitivity limitations of the extraction procedure. Again, porphyrin levels in the tissues of both tumor types were not significantly different as measured by fluorescence (EMT-6, 417 ± 49 μg/g tissue; RIF, 382 ± 39 μg/g tissue) as well as by extraction (EMT-6, 18 ± 4 μg/g tissue; RIF, 16 ± 1 μg/g tissue).

Light Transmission. Differences were found in the transmission of light in the 2 tumor systems, stemming mainly from the difference in pigmentation of the light skin of the BALB/c mouse (60 ± 8% of incident light transmitted) and the dark skin of the C3H/HeJ mouse (37 ± 6% of incident light transmitted). The base of 3-mm thick EMT-6 and RIF tumors received 25 ± 4% and 18 ± 4% of incident light, respectively. Based on these
results, it was decided to increase the injected porphyrin dose for RIF tumors over that given to EMT-6 tumors to compensate for their reduced light transmission and to achieve a more comparable total PDT dose in both tumor systems.

Tumor Response to PDT in Vivo. Tumor response data are summarized in Table 1.

Both tumor types showed gross hemorrhage within 10 min of treatment and massive necrosis within 24 hr. Tumors were usually rendered nonpalpable within 48 hr. Microscopically, the first signs of tumor damage were congestion of tumor blood vessels and extravasation of erythrocytes. Necrosis was restricted to the tumor tissue in both tumor types at doses of 135 J/sq cm and in RIF tumors at 200 J/sq cm, but the latter dose caused necrosis also of the surrounding normal skin exposed to the laser light in EMT-6 tumor-bearing mice. During the first 5 min of light treatment, tumor temperatures rose several degrees from a base temperature of 36° but stayed constant for the remainder of the treatment time (Table 1).

Tumor regrowth, if it occurred, usually started about the fifth day posttreatment but could occur as late as 28 days posttreatment for RIF tumors.

The application of porphyrin alone or light alone did not cause any measurable effects on tumor growth.

Tumor Cell Survival following PDT In Vivo. For each cell survival experiment, 3 parameters were recorded: total cell yield per g tumor tissue; plating efficiency of recovered cells; and the resulting total number of clonogenic cells per g tumor tissue. In both tumor types and under all conditions, viability of recovered cells as determined by trypan blue exclusion was above 90%. Table 2 shows these data for control tumors and tumors explanted immediately following completion of PDT-light treatment. The cell yield for control EMT-6 tumors represents 30 to 40% of the total tumor cell population per g tissue, that for control RIF tumors 40 to 60% (20, 21). Exposure in vivo of tumors of either type to porphyrin alone did not significantly influence the clonogenicity of tumors. Likewise, exposure to light alone was without effect (data not shown). Exposure to the full PDT treatment, immediately followed by excision and explantation of tumor cells, also revealed no reduction in tumor clonogenicity of EMT-6 tumors and only a slight reduction in RIF tumors.

Since the tumor response data (Table 1) had shown that the administered PDT dose was sufficient to cause profound tumor destruction, experiments were carried out to follow the time course of tumor cell death by delaying tumor excision and explantation. The data from these experiments are shown in Chart 2. Both tumor types showed a decrease in tumor clonogenicity commencing shortly after completion of PDT treatment, progressing thereafter in a similar fashion, and reaching a level of 0.1% clonogenic cells per g tissue of control tumors within 24 hr. Since during this time period the total tumor weight had also decreased, the actual clonogenicity of treated tumors was approaching 0.01% of controls by 24 hr posttreatment. Whereas in EMT-6 tumors the decreased clonogenicity was due to a decrease in both cell yield and PE of recovered cells, in RIF tumors it was due mainly to a drop in cell yield with the PE of recovered cells remaining high.

Tumor Cell Survival following Tumor Anoxia. The time course of the decrease in tumor clonogenicity as a function of tumor anoxia is shown in Chart 3. With a slight shoulder of 1 hr, the number of clonogenic tumor cells decreased within 4 hr in both tumor types to below 10% of that found in controls.

Tumor response and cure following PDT

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Mouse strain</th>
<th>No. of test animals</th>
<th>Porphyrin concentration (mg/kg)</th>
<th>630-nm light power density (milliwatts/sq cm)</th>
<th>Treatment time (min)</th>
<th>Temp rise</th>
<th>% of tumor response</th>
<th>% of tumor cure</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMT-6</td>
<td>BALB/c</td>
<td>20</td>
<td>7.5</td>
<td>75</td>
<td>30</td>
<td>2.0±</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>7.5</td>
<td>75</td>
<td>45</td>
<td>2.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>RIF</td>
<td>C3H/HeJ</td>
<td>20</td>
<td>10.0</td>
<td>75</td>
<td>30</td>
<td>3.5±</td>
<td>98</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>10.0</td>
<td>75</td>
<td>45</td>
<td>3.5</td>
<td>100</td>
<td>13</td>
</tr>
</tbody>
</table>

* S.D. ± 0.3.

This treatment resulted in damage to the normal tissues surrounding the tumor.
TUMOR CELL DEATH KINETICS FOLLOWING PDT IN VIVO

Table 2
Tumor cell survival parameters in controls and tumors treated with PDT in vivo

Tumor tissue was excised and disaggregated, and clonogenicity of tumor cells was assessed by in vitro colony formation assay. Two tumors were pooled for each experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor</th>
<th>No. of experiments</th>
<th>No. of viable cells/g tumor tissue</th>
<th>% of plating efficiency</th>
<th>Clonogenicity (no. of clonogenic cells/g tissue, as cell yield x plating efficiency/100)</th>
</tr>
</thead>
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<tr>
<td>Untreated control tumors</td>
<td>EMT-6</td>
<td>15</td>
<td>3.50 ± 0.91 x 10^7</td>
<td>29 ± 5</td>
<td>9.78 ± 2.40 x 10^6</td>
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<tr>
<td></td>
<td>RIF</td>
<td>8</td>
<td>2.47 ± 0.62 x 10^8</td>
<td>39 ± 12</td>
<td>9.87 ± 4.59 x 10^7</td>
</tr>
<tr>
<td>Control tumors, porphyrin, no light^a</td>
<td>EMT-6</td>
<td>9</td>
<td>3.73 ± 1.02 x 10^7</td>
<td>26 ± 8</td>
<td>10.01 ± 4.55 x 10^6</td>
</tr>
<tr>
<td></td>
<td>RIF</td>
<td>5</td>
<td>2.35 ± 0.18 x 10^8</td>
<td>39 ± 14</td>
<td>9.21 ± 2.39 x 10^7</td>
</tr>
<tr>
<td>PDT-treated tumors^c</td>
<td>EMT-6</td>
<td>9</td>
<td>3.64 ± 1.04 x 10^7</td>
<td>25 ± 7</td>
<td>9.33 ± 4.39 x 10^6</td>
</tr>
<tr>
<td></td>
<td>RIF</td>
<td>4</td>
<td>2.35 ± 0.18 x 10^8</td>
<td>30 ± 14</td>
<td>7.00 ± 2.93 x 10^7</td>
</tr>
</tbody>
</table>

^a Mean ± S.D.
^b Animals were given injections of porphyrin i.p. 24 hr prior to tumor explantation: EMT-6 tumor-bearing mice, 7.5 mg/kg; RIF tumor-bearing mice, 10 mg/kg.
^c Animals received porphyrin as above 24 hr prior to localized light treatment of the tumor (630 nm; 75 mwatts/sq cm for 45 min = 200 J/sq cm). Tumors were explanted immediately following completion of light treatment.

Factors must be involved. Certain aspects of the mechanism of tumor destruction described here resemble those described for the treatment of these tumors by hyperthermia (13, 15), in particular the EMT-6 tumors. It must be emphasized, however, that PDT as applied here did not lead to tumor temperature rises high enough to cause any appreciable effect on tumor growth. Whether they could have induced some sublethal effects remains to be determined. The phenomenon of delayed cell death has been observed in EMT-6 tumors after hyperthermic treatment (44°), and its kinetics was almost identical to that observed following PDT. RIF tumors did not show delayed cell death following hyperthermia, which was believed to be the reason for the incurability of this tumor by heat. In our experiments, RIF tumors showed delayed cell death very similar to that of the EMT-6 model. Nevertheless, as with hyperthermia, we found it nearly impossible to cure RIF tumors by PDT as applied here. It is likely that because of the antigenicity of EMT-6 cells, host immune responses were able to deal with small numbers of remaining viable tumor cells, whereas the nonimmunogenic RIF cells were free to repopulate the tumor. This differing immunogenicity has been suspected to be responsible also for the differing response of these tumors to radiation as well as to hyperthermia (4, 13, 18, 22, 24). It is also possible, although unlikely, that despite our efforts to equilibrate PDT doses, RIF tumors were at a slight disadvantage in treatment delivery due to the pronounced skin pigmentation of the C3H mouse.

The delayed cell death in the RIF and EMT-6 tumor models may be related to PDT damage of the tumor vasculature and consequently the treatment-induced changes in tumor physiology. This view is consistent with reports by others (5, 23) and our own histological findings which indicate vascular collapse in tumors caused by PDT. Also, oxygen measurements had shown a sharp reduction in tumor pO2 (3). Furthermore, the observed survival kinetics of tumor cells as a function of tumor anoxia in this study are consistent with the possibility of tumor cell kill after PDT, being a consequence of vascular breakdown. The
kinetics of cell death in our anoxia experiments are in agreement with those found by Denekamp et al. (9) and Lehman et al. (16), who studied tumor cell death following vascular occlusion through tumor clamping. However, vascular destruction does not seem to be solely responsible for tumor cure, since only a small fraction of RIF tumors are cured at high PDT doses despite massive vascular damage. In EMT-6 tumors, at least 20 min of light exposure was necessary for cure, whereas vascular damage occurred as early as 10 min of light treatment. Whether some component of direct sublethal tumor cell damage is involved which is readily repaired upon immediate removal of the treated tissue from the host, whereas it progresses to tumor cell death if the cells are left exposed to the altered tumor environment remains to be determined. Furthermore, the degree of vascular destruction is likely to differ in tumors of different morphology, and there may be a critical level of vascular damage and/or sublethal tumor cell damage required for total tumor eradication. We are continuing our studies with the ultimate goal being to determine which mechanism(s) prevail(s) in the response to PDT of human neoplasms.

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