Effects of Photodynamic Therapy Using Mono-L-aspartyl Chlorin e₆ on Vessel Constriction, Vessel Leakage, and Tumor Response

Kimberly S. McMahon, T. Jeffery Wieman, Pamela H. Moore, and Victor H. Fingar

Department of Surgery, Division of Surgical Oncology, University of Louisville, Louisville, Kentucky 40292

ABSTRACT

The effect of photodynamic therapy using mono-L-aspartyl chlorin e₆ (NP6) on both direct cytotoxicity and vascular damage was examined. Sprague-Dawley rats bearing chondrosarcoma tumors were given i.v. injections of 5 or 10 mg/kg NP6 and exposed to 135-J/cm² 664-nm laser light either 4 or 24 h after NP6 injection. The percentage of viable tumor cells was estimated either immediately after the completion of light treatment or 24 h after treatment using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Measurements of arteriole constriction and venule leakage in normal cremaster tissues were made during and 1 h after the light treatment. Tumor response was evaluated for the 4 different NP6 dose and time combinations. Both direct tumor cytotoxicity and vascular stasis were observed during light treatment. Vessel leakage did not occur. Blood flow stasis was a result of platelet aggregation and the mechanical obstruction of flow rather than vessel constriction. The magnitude of direct cytotoxicity and vascular response was dependent on both the amount of NP6 delivered and the delay between injection and light treatment. Tumor cure was found in animals either when given high NP6 doses or when treated early after NP6 injection. Treatment regimens which maximized the effect of both vascular stasis and direct tumor cytotoxicity were found to produce the best tumor response. Dose combinations which produced vascular stasis with minimal early cytotoxicity did not result in cure. The combined mechanisms of damage after photodynamic therapy using NP6 suggests that this photosensitizer may have specific advantages for clinical use and provides a benchmark for the development of new photosensitizers.

INTRODUCTION

NP6 is a promising new photosensitizer for use in PDT. NP6 has many characteristics which make it an attractive photosensitizer. It is a chemically pure agent, it absorbs light at long wavelengths (664 nm) (1), and PDT using NP6 has produced tumor cure in experimental tumor models (2, 3). Furthermore, studies have demonstrated a reduced duration of cutaneous photosensitivity compared to Photofrin (4). NP6 is currently undergoing phase I clinical trials for use in photodynamic therapy.

Though preliminary data yield promising results, the mechanism of tumor destruction in vivo using NP6 has yet to be completely resolved. Damage to tissue after PDT using NP6 appears to be secondary to vascular damage and hemostasis. Nelson et al. (5) found RBC extravasation and hemorrhagic necrosis after treatment that was correlated with damage to the subendothelial zone of capillaries. This damage was shown to result from collagen disruption and fragmentation rather than the collagen coalescence observed after treatment with PDT using Photofrin. A vascular mechanism of damage is further supported by work by Gomer and Ferrario (4) and Ferrario et al. (6), who found that treatment efficacy was best when light treatment was delivered between 2 and 6 h after NP6 injection. Significant decreases in treatment efficacy occurred if light treatment was given 12–24 h after NP6 injection, even though tumor levels of photosensitizer were greatest at this time. The contribution of direct tumor cytotoxicity on tumor response in vivo is not known, but it is likely to play some role because high levels of drug are bound to tumor cells.

This study was designed to evaluate the events which lead to blood flow stasis after NP6 activation using an intravital microscopy model and compare the sequence to that observed in tissues treated with other photosensitizers. The role of direct tumor cytotoxicity in PDT using NP6 was evaluated using a modification of the MTT assay. Tumor cytotoxicity after PDT using NP6 is expected based on the tissue distribution studies done by Gomer and Ferrario (4) and Ferrario et al. (6), however, direct cytotoxicity may be inhibited as a result of tissue hypoxia. Observation of the timing required before vascular stasis may provide information regarding this inhibition.

MATERIALS AND METHODS

Photosensitizer NP6. NP6 was a generous gift from Beckloff Associates, Kansas City, MO, and Nippon Petrochemicals Company, Tokyo, Japan. NP6 was received in a lyophilized powder and was reconstituted using 0.9% sodium chloride injection (United States Pharmacopoeia) to a concentration of 2.0 mg/ml. NP6 was prepared at weekly intervals and stored in the dark at 4°C. NP6 was injected into the tail vein of rats at doses of 5.0 or 10.0 mg/kg, 4 or 24 h before the light treatment.

Tumor Model. A chondrosarcoma tumor line maintained in our laboratory was used in this study (7). Approximately 500 mg of nonneocritic tumor from donor animals were minced in 2 ml of Hank’s balanced salt solution containing 2000 units streptomycin and 2 mg penicillin (Sigma Chemical Co., St. Louis, MO). The suspension was passed through a tissue sieve (Collector tissue sieve, 30-mesh screen; Sigma) and then through graded needles. For tumor implantation, 0.3 ml (3 x 10⁸ cells) tumor suspension was injected s.c. into the center of the thigh on the right hind limb of Sprague-Dawley rats (100–130 g; Harlan Laboratories, Indianapolis, IN) with a 21-gauge needle. Tumors were used for experimentation when they had reached a surface diameter of 6–10 mm and a thickness of 2–3 mm. Tumors were free of evident necrosis at the time of treatment.

Animals were given injections of the photosensitizer before treatment. Prior to treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). The right hind limbs of tumor-bearing or control rats were shaved and depilated. Tumors and a surrounding area of normal skin were illuminated as described below.

Light Treatment Protocol. For photosensitizer activation, an argon dye laser (models 165 and 375B; Spectra Physics, Mountain View, CA) with a fiber optic light delivery system was used to illuminate a 2.0-cm-diameter field centered on the cremaster or the tumored right hind limb. The wavelength was adjusted to 664 nm. The wavelength was verified by a scanning monochrometer (model DMCl–02; Optometrics, Ayer, MA). The power density of light was adjusted to 75 mW/cm² as measured by a thermopile (model 210; Coherent, Auburn, CA). This power density was not sufficient to induce a significant increase in tissue temperature (8). A micro lens (gradient index lens; General Fiber Optics, Inc., Cedar Grove, NJ) was attached to the end of the fiber to ensure uniform light distribution. Treatment areas were illuminated with 135-J/cm² light (30-min treatment).

Received 12/14/93; accepted 8/16/94.

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1This investigation was supported by USPHS Grant CA51771 awarded by the National Cancer Institute, Department of Health and Human Services, by the Department of Surgery, and by the James Graham Brown Cancer Center.

2To whom requests for reprint should be addressed, at University of Louisville, Department of Surgery, James Graham Brown Cancer Center, 529 South Jackson Street, Louisville, KY 40292.

3The abbreviations used are: NP6, mono-L-aspartyl chlorin e₆; PDT, photodynamic therapy; FITC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RBCC, RBC column.
Cremaster Muscle Preparation for Microvascular Studies. Sprague-Dawley rats (100—150 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed on their backs on a temperature-controlled heating pad. Rectal temperature was maintained at 37°C and back temperature was monitored with a thermocouple to avoid local overheating of the skin. The right cremaster muscle was prepared for microvascular observations in the manner reported previously (8). The muscle was slit on the ventral midline and spread with sutures over a cover glass which was positioned in the bottom of a 60-ml capacity Plexiglas bath. This bath was filled with a modified Krebs solution. The right carotid artery was cannulated for the measurement of mean arterial blood pressure and heart rate, and for the infusion of fluorescein isothiocyanate-labeled albumin. Blood pressure and heart rate were monitored using a blood pressure analyzer (MicroMed Instruments, Louisville, KY). Arteriolo and venule pairs were chosen for the study based on their diameter (20—30 μm) and branching order (in general, fourth-order vessels were chosen although occasionally third-order vessels were studied). Arteriolo could be easily discerned from other vessels by the presence of a smooth muscle wall, rapid blood flow, and blood flow in the direction from large vessels to small vessels. Venules had no muscle wall, exhibited slower blood flow, and had flow in the direction from small vessels to larger vessels. RBCC diameter and arteriole wall diameter were measured every 5 min for the 30 min of light treatment, and every 10 min for a 1-h observation period following light therapy. The RBCC diameter is a measurement of the inner lumen of the vessel. Reductions in RBCC diameter without a change in wall diameter indicate that aggregates have formed at the inner vessel wall. Nine to 11 animals were used in each different experimental group for measurement of vessel diameters.

Light and Fluorescent Microscopy and Image Analysis. In order to visualize the cremaster muscle under the intravital microscope, transmitted light and fluorescent microscopy were used (8, 9). For the fluorescence study, 0.5 ml/kg FITC-labeled albumin (10) was injected into a cannula leading into the right carotid artery (equivalent to 26 μg/kg fluorescein and 10 mg/kg albumin). Epifluorescence using blue light (450—490 nm) was used to stimulate the FITC for brief periods (≤ 5 s) every 5 min during light therapy, and every 10 min for a 1-h observation period after light treatment. These short durations of illumination did not induce any vascular changes (11). In the absence of FITC-albumin in the intestinum, the images of the blood vessels appear bright white on a dark background. The images were recorded on videotape with a closed-circuit television system. A Cohu silicon intensifying target television camera (Cohu Electronics, San Diego, CA) was used to work with very low fluorescent light intensities. The camera voltage was set at 0.10 V using a 10-ng/ml fluorescein diacetate standard. A 1-h equilibration period preceded each experiment. Examination of macromolecular leakage was done using digital image analysis as described previously (8). Changes in FITC-albumin leakage from vessels were quantitated by selecting areas of interest adjacent to vessels and by measuring the alterations in the gray level of pixels in this area. Each set of experimental data was corrected for any fluctuations in the intensity of the fluorescence light source or in the camera sensitivity that may have occurred. Eight to 10 animals were used in each different experimental group.

Assessment of Tumor Response. Rats were examined for tumor regrowth daily for the first 14 days after treatment, and weekly thereafter for a total of 42 days. Treatment areas lacking a macroscopically visible and palpable tumor were considered flat. Animals free of tumor after this time were considered cured. Twenty to 35 animals were used in each treatment group.

MTT Assay. Tumor cell viability was assessed after treatment using a variation of a colorimetric MTT assay (12, 13). Animals were prepared with chondrosarcoma tumor in both the right and left hind limbs for these studies. The tumor in the right limb was given treatment as necessary and the tumor in the left limb served as an additional control. Animals were given injections of NP66 at either 5 or 10 mg/kg, and light treatment was administered at either 4 or 24 h after NP66 injection. Groups of 9 animals were used. Additional control groups consisted of animals given NP66 alone, animals given light alone, or animals given neither light nor drug. In addition, a group of 5 animals were given no photosensitizer and sacrificed without light treatment. Tumor tissue was prepared for the assay immediately or 24 h after sacrificing the animal. This data established tumor cell kill due to hypoxia and nutrient deprivation.

MTT (Sigma) was prepared by adding sodium bicarbonate buffer (pH adjusted to 7.4) with a final concentration of 5.0 mg/ml. The solution was filtered through a 0.2-μm syringe filter (sterile Millex-GS; Millipore, Bedford, MA), and stored until used.

Immediately following or 24 h after treatment, tumor cells were excised, weighed, and carefully minced under subbed lighting. Tumor was transferred to a 15-ml polypropylene centrifuge tube containing a 0.1-mL MTT solution and 1.0 mL culture medium (RPMI 1640; Sigma). Cells were incubated in the dark for 4 h at 37.5°C. After incubation, cells were centrifuged at 2500 rpm for 10 min. The supernatant was poured off and 5.0 ml dimethyl sulfoxide were added. The cells were allowed to stand for 10 min with intermittent vortexing, followed by sonication for 1 min (Sonic Disembrator model 150; Fisher Scientific, Pittsburgh, PA). Samples were centrifuged at 10,000 × g for 15 min following the addition of 15 ml distilled water. Sample absorbance at 570 nm was measured in the supernatant using a spectrophotometer (DU-62 Spectrophotometer; Beckman Instruments, Inc., Palo Alto, CA). Cells with intact, viable mitochondria turn the solution from yellow to purple. Sample absorbance was compared to a standard curve in order to calculate percent cell viability. The standard curve was prepared by measuring MTT conversion from different weights of viable tumor and verified with tumor standards containing known percentages of viable and dead cells (different amounts of tumor killed by heat sterilization were mixed into minced suspensions of viable cells to produce standards containing viable/nonviable ratios of 20:80, 50:50, and 80:20). The reproducibility and sensitivity of the assay was increased by dividing the viability data from the treated tumor by its untreated internal control.

Statistical Analysis. Data from experiments were grouped and the means calculated. Data are presented as the mean ± 2 × SEM. The experimental data set was compared using one-way analysis of variance. Dunnett’s test was used to compare the results of experiments to the controls and a multiple comparisons test was done to evaluate data groupings (14, 15). Type II error was held to 0.10 for the multiple comparisons testing. Differences between groups were considered statistically significant if P < 0.05.

RESULTS

Alterations in Vessel Diameter and Vessel Lumen Diameter. Profound changes were observed in normal cremasteric vessels treated with NP66 and light. For each of the experimental treatment groups, significant reductions in the diameter of the lumen of arterioles (RBCC diameter) were observed 1 h after treatment compared to the initial value before treatment or to the controls (P < 0.0001). Complete blood flow stasis was observed in 20—30-μm arterioles in animals given 5 mg/kg NP66, 4 h before the light treatment. Stasis occurred by the end of the light treatment and blood flow did not resume during the observation period (Fig. 1a). Blood flow stasis was a result of aggregation and coagulation of platelets and other blood constituents. Vessel constriction was not observed in animals given NP66 and light. Animals given either NP66 alone (5.0 mg/kg i.v., 4 h), or light treatment (135 J/cm²) alone showed no apparent change in vessel diameter or change in the RBCC diameter within arterioles. In animals given 10 mg/kg, i.v., 4 h before the light treatment, the vessel response was more dramatic. Blood flow ceased in arterioles within the first one-third of the light treatment (45 J/cm²) (Fig. 1b). The mechanism of stasis was similar to animals treated with 5 mg/kg NP66, i.e., stasis resulted from platelet aggregation and coagulation of blood elements rather than by vessel constriction or a combination of these effects. Platelet aggregation and thrombi formation were confirmed by electron microscopic study of treated tissues (data not shown).

The vascular response to PDT using NP66 was diminished when rats were treated 24 h after injection of 5 mg/kg NP66 (Fig. 1c). Reductions in the diameter of the RBC column were observed in vessels, but stasis was a rare event. By the completion of the light...
Fig. 1. Change in the RBCC diameter within 20–30-μm arterioles during and after PDT. Animals were given injections of 5 or 10 mg/kg NPe6 at either 4 or 24 h before light treatment. Light treatment was 135 J/cm², delivered during the first 30 min of the experiment. Vessel measurements were continued for an additional 1 h after the completion of the light treatment. a, animals given 5 mg/kg NPe6, 4 h before light treatment; b, animals given 10 mg/kg NPe6, 4 h before light treatment; c, animals given 5 mg/kg NPe6, 24 h before light treatment; d, animals given 10 mg/kg NPe6, 24 h before light treatment. Points, means of data from experiments in 9–11 animals, calculated as a percentage of the initial RBCC diameter. Bars, mean ± 2 SEM. Bars for later time points which cannot be seen fall within the size of the points.

Treatment, vessel lumen diameters had reduced to roughly 60% of controls. The reduction in the vascular lumen was a result of adherence of platelet thrombi. Platelet aggregation and adherence to vessels continued after the completion of the light treatment until complete stasis was observed. This occurred within the 1-h observation period after the completion of PDT. A similar reduction in the vascular response to PDT and timing required before stasis did not occur in animals given 10 mg/kg NPe6 24 h before light treatment (Fig. 1d). The vascular response to PDT in these animals was nearly identical to the reaction observed in animals given 10 µg/kg only 4 h before the light treatment.

Macromolecular Leakage from Venules. Leakage of fluorescent-labeled albumin from normal cremasteric venules into the extravascular space was measured in animals given PDT using NPe6 and in controls. Leakage of the albumin macromolecule was not observed in animals given either NPe6 alone (5.0 mg/kg i.v., 4 h) or light alone (135 J/cm²). Furthermore, leakage did not occur in animals given 5 or 10 mg/kg NPe6 4 h before light treatment of 135 J/cm² (Fig. 2, a and b). Minimal values of leakage were observed in animals treated with either 5 or 10 mg/kg NPe6 when given 24 h before the light treatment (Fig. 2, c and d).

Cytotoxicity. Cell kill was estimated immediately after light treatment and 24 h after light treatment using a modification of the MTT assay. Table 1 lists the results of the cytotoxicity assay. Analysis using a multiple comparisons test revealed 4 distinct groupings of the data. No decrease in viability was observed in control tumors or in tumor from animals given drug or light treatment alone. In all cases, tumor given PDT had significantly less tumor cell viability compared to...
Table 1  Tumor cell viability after photodynamic therapy

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>Immediately after treatment</th>
<th>24 h after treatment</th>
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<tbody>
<tr>
<td>No drug, no light</td>
<td>101.5 ± 9.28</td>
<td></td>
</tr>
<tr>
<td>No drug, 135 J/cm² light</td>
<td>100.6 ± 6.56</td>
<td></td>
</tr>
<tr>
<td>10 mg/kg NPe6 24h, no light</td>
<td>100.5 ± 8.49</td>
<td></td>
</tr>
<tr>
<td>5 mg/kg NPe6 4h, 135 J/cm² light</td>
<td>76.3 ± 5.51</td>
<td>24.1 ± 5.28</td>
</tr>
<tr>
<td>5 mg/kg NPe6 24h, 135 J/cm² light</td>
<td>81.8 ± 8.6</td>
<td>21.0 ± 6.65</td>
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<tr>
<td>10 mg/kg NPe6 4h, 135 J/cm² light</td>
<td>53.3 ± 5.76</td>
<td>13.6 ± 5.91</td>
</tr>
<tr>
<td>10 mg/kg NPe6 24h, 135 J/cm² light</td>
<td>72.5 ± 5.42</td>
<td>18.3 ± 3.74</td>
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Tumor viability (percentage of controls) following PDT treatment. The magnitude of the tumor response to PDT was greater when 10 mg/kg NPe6 was administered at both 4 and 24 h before light treatment. Animals treated 4 h after injection of 10 mg/kg NPe6 showed complete tumor destruction in all cases within 48 h after PDT (Fig. 3a). Tumor regrowth was evident in selected animals by 13 days after treatment. Tumor cure was observed in 33% of these animals. Animals treated 24 h after injection of 5 mg/kg NPe6 showed initial flattening of tumor within 48 h of treatment; however, all of these animals showed tumor regrowth in the treatment area within 14–21 days of PDT (Fig. 3c). The magnitude of the tumor response to PDT was greater when 10 mg/kg NPe6 was administered at both 4 and 24 h before light treatment. Animals treated 4 h after injection of 10 mg/kg NPe6 showed complete tumor destruction as seen at the lower NPe6 dose, however, tumor regrowth was not detected until 21–28 days had passed after treatment (Fig. 3b). Tumor cure was observed in 83% of animals treated at this dose regimen. Reductions in the tumor response in animals given injections of 10 mg/kg NPe6 24 h before light treatment were noted when compared to the 4-h data. Although initial tumor destruction was observed in all animals, regrowth began at 1 week after PDT and only 12% of animals remained tumor free at the conclusion of the experiment (Fig. 3d).

Fig. 3. Tumor response and cure following PDT. Animals were given injections of 5 or 10 mg/kg NPe6 at either 4 or 24 h before light treatment. Light treatment was 135 J/cm² 664 nm to a 2-cm diameter spot centered over the tumor. Animals were considered cured of tumor if no regrowth was observed before 35 days after treatment. a, animals given 5 mg/kg NPe6, 4 h before light treatment (n = 12); b, animals given 10 mg/kg NPe6, 4 h before light treatment (n = 24); c, animals given 5 mg/kg NPe6, 24 h before light treatment (n = 16); d, animals given 10 mg/kg NPe6, 24 h before light (n = 17).

effect of PDT using NPe6 and light was assessed in the chondrosarcoma tumor model. Animals treated 4 h after injection of 5 mg/kg NPe6 showed complete tumor destruction in all cases within 48 h after PDT (Fig. 3a). Tumor regrowth was evident in selected animals by 13 days after treatment. Tumor cure was observed in 33% of these animals. Animals treated 24 h after injection of 5 mg/kg NPe6 showed initial flattening of tumor within 48 h of treatment; however, all of these animals showed tumor regrowth in the treatment area within 14–21 days of PDT (Fig. 3c). The magnitude of the tumor response to PDT was greater when 10 mg/kg NPe6 was administered at both 4 and 24 h before light treatment. Animals treated 4 h after injection of 10 mg/kg NPe6 showed complete tumor destruction as seen at the lower NPe6 dose, however, tumor regrowth was not detected until 21–28 days had passed after treatment (Fig. 3b). Tumor cure was observed in 83% of animals treated at this dose regimen. Reductions in the tumor response in animals given injections of 10 mg/kg NPe6 24 h before light treatment were noted when compared to the 4-h data. Although initial tumor destruction was observed in all animals, regrowth began at 1 week after PDT and only 12% of animals remained tumor free at the conclusion of the experiment (Fig. 3d).

DISCUSSION

The goal of this study was to evaluate the effects of PDT using NPe6 on vascular damage and direct tumor cytotoxicity in vivo. Evaluation of these events was found to provide important clues concerning the mechanisms of damage after PDT using NPe6.
Blood flow stasis occurred rapidly during light treatment in animals exposed to NPe6 and light. Stasis was observed in the cremaster arterioles of all animals given injections of 10 mg/kg NPe6 either 4 or 24 h before light treatment or injected with 5 mg/kg NPe6, 4 h before light treatment. Animals given 5.0 mg/kg i.v. 24 h showed a less severe arteriole response and timing before blood flow stasis was delayed. This response confirms the work by Gomer and Ferrario (4) and Nelson et al. (5), who concluded that vascular damage was responsible for tumor response. Blood flow stasis was the first morphological change that we observed during light treatment. This response is an early step in the cascade of events which may lead to the RBC extravasation and hemorrhagic necrosis found by Nelson et al. (3) in their studies using NPe6. The steps which lead to blood flow stasis were unusual compared to our studies using other photosensitizers. Blood flow stasis did not result from vessel constriction as observed in Photofrin-treated animals (7); rather, blood flow stasis was a consequence of the aggregation of blood components, namely, platelets. No vessel constriction was observed. This leads us to believe that the events incurred using NPe6 differ from those causing vessel stasis in Photofrin-treated animals. With Photofrin, large quantities of thromboxane and leukotrienes are released and are believed to cause vessel constriction. The release of these vasoactive compounds is thought to result from the early aggregation and adhesion of platelets to a damaged endothelial lining. Since no constriction is observed after PDT using NPe6, it suggests that endothelial cells are not damaged during treatment by the same mechanism observed with Photofrin (16) or that the cascade of events leading to vessel constriction is somehow blocked. In earlier studies, Nelson et al. (3) found damage to the subendothelial zone of capillaries, edema, and fragmentation of collagen and fiber elements (3). Endothelial cells along capillaries were elongated compared to controls but were otherwise normal. The platelet aggregation leading to blood flow stasis may result from damage to the platelet itself rather than the endothelial cell. Damage to platelet membranes has been shown by Zieve et al. (17) and Soloman et al. (18) for hematoporphyrin derivative and Henderson et al. (19) for Photofrin. We believe that a similar series of events may occur during PDT using NPe6. A complete lack of endothelial cell damage during PDT is unlikely, however, since platelet aggregation was found near the vessel wall as well as within the luminal space. It is more likely that damage occurs to both platelets and endothelial cells, but that the platelet response to therapy is greater. Although measurements of the release of arachidonic acid metabolites were not done in this study, it is unlikely that these metabolites play a significant role in the response. Although thromboxane will cause vessel constriction and platelet aggregation when released into the blood, these effects can be blocked or reduced by a concomitant release of prostacyclin, a product of endothelial cells (20, 21). The release of both thromboxane and prostacyclin has been observed during PDT using tin etiopurpurin, and the net result is no vessel constriction and no platelet aggregation (22). Since we observe platelet aggregation but no vessel constriction during PDT using NPe6, it suggests that neither prostacyclin nor thromboxane is released. The vascular nature of the observed tissue response to PDT using NPe6 is confirmed by the data from the MTT assay. When tumors were assayed for cell survival 24 h after PDT treatment using 5 or 10 mg/kg NPe6, the resulting cell survival was the same as that from tumors made hypoxic 24 h earlier and not given PDT treatment. Since there are significant differences in the number of tumor cures between animals given 5 and 10 mg/kg NPe6 4 h before light treatment, it suggests that the early tumor response may result from vascular stasis and the consequences of tissue anoxia and that the later response includes contributions from other factors including direct cytotoxicity and possibly immune effects.

Minimal macromolecular leakage from cremaster venules was observed during light treatment or the 1-h observation period following in animals given NPe6. This is in contrast to observations of macromolecular leakage from venules after PDT using Photofrin (8). Although leakage from venules is not a major observation in NPe6-treated animals, it does not imply that damage has not occurred. It is possible that even if venule damage occurred, vessel stasis and platelet aggregation precluded the escape of macromolecules such as albumin into the extravascular space.

Though tumor vessels are believed to be inherently more fragile and react differently to stimuli than vessels in muscle, previous studies confirm that similar microvascular damage occurs in tumor vessels (for tumors implanted in the cremaster) compared to normal cremaster microvasculature (23). One exception to this appears to involve leukocyte adhesion which is found in normal microvasculature but not within tumor microvasculature (24). Any differences between the response expected in normal or neoplastic microvasculature are likely to be minimized during PDT using NPe6 since the therapy appears to target elements in the blood rather than on the blood vessels. Vessel stasis results from platelet aggregation and thrombus formation; a response mediated by platelets and other blood elements.

Although vessel damage is a dominant factor in the mechanisms of tumor destruction following PDT using NPe6, other components including direct cytotoxicity were found to play a role in the observed response to therapy. Measurable estimates of direct tumor cytotoxicity were found after PDT using NPe6. Although the MTT assay used in this study does not measure tumor cell viability directly, Carmichael et al. (13) showed a good correlation between clonogenic and MTT assays when measured 24 h after treatment. Measurements of viability using MTT within the first few hours are less predictive since we cannot demonstrate in our model that a given amount of enzyme inhibition corresponds directly with a corresponding level of cytotoxicity. It is possible that PDT causes a slight reduction in mitochondrial enzyme activity in many cells, and that certain of these cells survive the initial depression of enzyme activity. We expect that inhibition of succinate dehydrogenase should significantly alter cellular respiration in affected cells and that this event is likely to precede cell death. Inhibition of succinate dehydrogenase has been shown by Hilf et al. (25) during PDT using Photofrin, and this is believed to represent an early step in cytotoxicity.

Estimates of direct cell kill were greatest when the animals were given NPe6 4 h before light treatment (47% kill at 4 h compared to 27% kill at 24 h for 10 mg/kg NPe6). The increased early cytotoxicity as a result of PDT may be one of the reasons why tumor cure is higher among animals treated at 4 h compared to 24 h after drug injection. This conclusion is supported by data from the microvascular studies. Vessel stasis was complete during the 1-h observation period for PDT using a NPe6 dose of 10 mg/kg when delivered either 4 or 24 h before treatment. Despite this, greater tumor cure was observed in the group given drug 4 h before the light treatment. The increased efficacies of treatment may be related to the greater direct tumor cytotoxicity observed at the 4-h time point.

PDT using NPe6 may have specific advantages compared to other photosensitizers since tumor regression can result both from direct cytotoxicity and from the consequences of blood flow stasis. It is not known whether the direct cytotoxicity observed at the 10-mg/kg dose of NPe6 could be increased by delaying the induction of blood flow stasis and resulting depletion of oxygen in the tissue. If direct cytotoxicity is limited at this NPe6 dose by the availability of oxygen in the tissue, it may be possible to increase the magnitude of direct cytotoxicity by delaying the vascular response (26). Certain eicosanoid inhibitors and inhibitors of platelet aggregation are currently...
under investigation to determine whether they can delay or inhibit vascular stasis after PDT using NPe6.

The vascular and direct cytotoxic response to PDT using NPe6 followed the same time dependence as tumor response previously described by Gomer and Ferrario (4) and Ferrario et al. (6). Both vascular damage and tumor response were greater when treated 4 h after administration of 5 mg/kg NPe6 compared to the response observed 24 h after injection. Differences in tumor response alone were found in animals given doses of 10 mg/kg NPe6 at 4 and 24 h before light treatment. The lack of difference in the vascular response between these time points after injection of 10 mg/kg NPe6 is likely due to the maintenance of sufficient levels of NPe6 in plasma to evoke blood flow stasis. The increased cure observed in animals treated 4 h after injection of 10 mg/kg NPe6 reflects the greater direct cytotoxicity detected with this dose regimen. This confirms previous studies which found that the tissue response to PDT using NPe6 is best when the plasma levels of the photosensitizer are high (between 2 and 4 h after injection) (4, 6, 26).

This study has served to further investigate and define the mechanisms of damage after PDT using NPe6 in vivo. Vascular stasis was observed early in the light treatment and was a result of platelet aggregation and thrombus formation. Arteriole constriction did not occur during treatment. PDT using NPe6 also produced significant direct tumor cytotoxicity as measured by a MTT assay. This effect of PDT is similar to that observed after treatment using the aluminum and zinc phthalocyanines (27, 28). Both vascular stasis and direct cytotoxicity were found to play roles in the resulting tumor response, and treatment regimens which maximized the effect from both of these mechanisms of damage produced a superior tumor response. These observations may benefit the development of new photosensitizers for use in PDT.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Patricia Cerrito for her expert assistance with the statistical analysis of the data. We also acknowledge the efforts of Nippon Petrochemicals and Beckloff Associates for providing the NPe6 used in this study.

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