The Role of Microvascular Damage in Photodynamic Therapy: The Effect of Treatment on Vessel Constriction, Permeability, and Leukocyte Adhesion

Victor H. Fingar, T. Jeffery Wieman, Sandra A. Wiehle, and Patricia B. Cerrito

ABSTRACT

Intravital microscopy of the rat cremaster muscle was used to evaluate changes in vessel constriction, vessel permeability, and leukocyte adhesion during and after photodynamic therapy (PDT). Animals were given Photofrin doses of 0–25 mg/kg i.v. 24 h before treatment. Cremaster muscles were exposed to 135 J/cm² light at 630 nm. Animals given 5 mg/kg Photofrin showed no vessel constriction or increase in vessel permeability to albumin. Doses of 10 and 25 mg/kg Photofrin caused a dose-related constriction of arterioles which was observed within the first minutes of illumination at the higher drug dose. After the initial constriction, arteriole response to PDT was biphasic in nature, with some vessels relaxing to nearly control levels while others remained fully constricted. Constriction of venules occurred only at the highest porphyrin dose studied (25 mg/kg) and was delayed in comparison to arteriole constriction. Photofrin doses which produced arteriole constriction also caused an increase in venule permeability to albumin, which occurred shortly after the start of light treatment and was progressive with time. Leakage began at specific sites along the venule wall but became uniform along the entire length of the venule by 1 h after treatment. Changes in the adherence of polymorphonuclear leukocytes to venule endothelium were also observed with PDT. Photofrin doses of 25 mg/kg and 45 J/cm² light were sufficient to cause polymorphonuclear leukocytes to become adherent to the vessel wall. A second group of animals was given indomethacin trihydrate to examine the involvement of cyclooxygenase products such as thromboxane in vessel response to PDT. Animals given 5 mg/kg indomethacin intraarterially 1 h before light treatment showed no constriction of arterioles or venules at all Photofrin and light doses studied. No increases in venule permeability to albumin were seen in this group of animals. This suggests that cyclooxygenase products including thromboxane are important in causing vessel constriction and changes in permeability during PDT. The initiating event which causes the release of these vasoactive agents remains unknown.

INTRODUCTION

Tumor destruction following PDT is a consequence of specific damage by reactive oxygen species resulting in both direct tumor cell death and blood vessel stasis. Experiments using rats and mice indicate that blood flow stasis is largely reversible for some time. Blood flow stasis and thrombus formation were first described in 1911 by Hausmann and later verified by McGovern. Vessel constriction and stasis were observed by Star et al. within the first minutes of phototherapy using transparent window chambers. Star speculated that tumor cure was dependent on vascular damage, rather than on direct tumor cell death. These findings confirmed similar conclusions by Henderson et al., who quantitated tumor cell clonogenicity at various times after PDT and by Selman et al., who demonstrated reductions in blood flow after PDT using a radioactive microsphere method. Reed et al. studied vessel constriction using an intravital microscopy model and found results similar to those of these investigators. This study clearly identifies vessel constriction and platelet aggregation as early events in PDT.

Fluid leakage from vessels has been studied by Kerdel et al. and Lim et al., who demonstrated increased edema after PDT. Mast cells, macrophages, and endothelial cells have been shown to release specific mediators which produce tissue edema. Edema is often observed in the treated areas of patients given PDT, a phenomenon first described by Meyer-Betz. Increases in tumor interstitial pressure, an alternative measure of vessel permeability, have been recently observed after PDT. The administration of cyclooxygenase inhibitors or serotonin antagonists has been shown to reduce the effects of PDT on edema. The detailed events that lead to increases in vessel permeability or leakage during PDT have not been fully investigated.

This study describes experiments in which blood vessel response to PDT is evaluated in detail. Arteriole and venule diameter changes and vessel leakage were measured during and after PDT using Photofrin and 630-nm light. The studies were repeated in the presence of a cyclooxygenase inhibitor, indomethacin, to determine the involvement of cyclooxygenase products, such as thromboxane, in vessel damage.

MATERIALS AND METHODS

Animal Model. Male 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 previously reported. The muscle was silt on the ventral midline and spread with sutures over a cover glass which was positioned in the bottom of a 60-ml capacity Plexiglass bath. This bath was filled with a...
modified Krebs solution (15). The bath was constantly mixed by aeration with CO₂ and N₂ gases. The pH of the Krebs solution was maintained at 7.40 ± 0.02 (SD) by adjusting the flow of CO₂ into the bath. An indwelling heater maintained the bath temperature at 35°C. The left carotid artery was cannulated for the measurement of mean arterial pressure and heart rate and for the infusion of fluorescein-labeled albumin and indomethacin. Blood pressure and heart rate were monitored using a blood pressure analyzer (MicroMed Instruments, Louisville, KY). Arteriole and venule pairs were chosen for 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). Arterioles 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.

Photosensitizers. Photofrin (Photofrin II) was a generous gift of Quadra Logics Laboratories (Vancouver, BC, Canada). Photofrin was injected into the tail vein of rats 24 h before light treatment. Photofrin was administered at a concentration of 2.5 mg/ml and was stored in the dark at –20°C until used.

Cyclooxygenase Inhibitor. A water-soluble form of indomethacin (sodium indomethacin hydrate) was kindly provided by Merck, Sharp and Dohme Research Laboratories (Rahway, NJ). Indomethacin was diluted to 1 mg/ml in saline and injected intraarterially at a dose of 5 mg/kg.

Light Treatment Protocol. For photosensitizer activation, an argon dye laser (Spectra Physics 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. The wavelength was adjusted to 630 nm and verified by a scanning monochromator (Optometrics model DMC1-02). The power density of light was adjusted to 75 mW/cm² as measured by a thermopile (Coherent model 210). A microlens (gradient index lens; General Fiber Optics, Inc., Cedar Grove, NJ) was attached to the end of the fiber to ensure uniform light distribution to the cremaster preparation.

Production of Labeled Fluorescent Materials. FITC was conjugated with bovine serum albumin by a modification of the method of Chadwick (16). Bovine serum albumin (1.0 g; Sigma Chemical Co., St. Louis, MO) and fluorescein isothiocyanate on celite (170 mg of 10% FITC on celite; Sigma Chemical Co.) were mixed with 10 ml of freshly prepared bicarbonate buffer (0.6 g/100 ml Na₂CO₃ anhydrous, 3.7 g/100 ml NaHCO₃, adjusted to pH 9.0) and allowed to conjugate overnight at 4°C. The conjugation mixture was centrifuged for 5–10 min at 1750 x g to sediment the celite particles. The supernatant was dialyzed at 4°C in cellulose tubing (M.W. 12,000 cutoff size) in fresh Nairn's solution (8.5 g/liter NaCl, 346 mg/liter NaH₂PO₄, H₂O 2,0.17 g/liter Na₂HPO₄ anhydrous, adjusted to pH 7.4) to separate unbound dye from the conjugated FITC-albumin. Aliquots of the diazoyed compound were stored in the dark at –20°C in sealed vials.

Light and Fluorescent Microscopy and Image Analysis. Transmitted light and fluorescence microscopy was used for visualization of the rat cremaster (15, 17). For fluorescent microscopy, 0.5 ml/kg body weight of FITC-albumin was injected into the carotid artery via the previously implanted cannula. An incident-light illumination system (epiillumination) with a 460–490-nm band from a mercury arc lamp was used to stimulate the FITC for brief periods (< 5 s) at specified times during and after PDT. These short durations of illumination did not induce any vascular changes (17). In the absence of macromolecules (FITC-albumin) in the interstitium, the microscopic images of the blood vessels appear as bright white columns on an otherwise dark background.

The microscopic images were recorded on videotape with a closed-circuit television system which utilizes a Cohu silicon intensifying target television camera (Cohu Electronics, San Diego, CA) for work with very low fluorescent light intensities. Camera voltage was set at 0.15 V using a 10 ng/ml fluorescein diacetate standard. In each experiment, there was a 1-h equilibration period before light treatment.

Examination of macromolecular leakage was done using digital image analysis. Video images were taken from the microscope camera and were digitized at 512 x 512 pixel resolution for each video frame and stored in computer memory (PC Vision Plus; Imaging Technology, Inc., Woburn, MA). Each pixel contained grey level information (0–255 levels) from the original image. An image analysis software package (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to evaluate changes in the video images. Spatial averages of tissue fluorescence for specified areas were measured. Changes in FITC-albumin leakage from vessels were quantitated by selecting areas of interest adjacent to vessels and by measuring the alterations in the grey level of pixels in this area over a given time period. Each set of experimental data was corrected for alterations in the intensity of the fluorescence light source or in camera sensitivity that may have occurred from day to day. The correction was made as follows. Before each experiment, a cuvette filled with a 10 ng/ml sodium fluorescein standard was placed under the microscope, and fluorescence images were recorded both with and without a 25% neutral density filter in front of the camera. An average grey level of the pixels within the fluorescence images was obtained from the image analysis system. Alterations in fluorescence light intensity (offset) were discerned by changes in the magnitude of either of the fluorescence readings as compared to average values. Alterations in camera sensitivity (gain) were differentiated by a change in the numerical interval between fluorescence values with and without the neutral density filter in place. Analysis of these two variables was based on theoretical considerations and was verified experimentally by purposely altering fluorescence lamp intensity and camera gain and evaluating how these factors modify the grey level data of our fluorescence standards. Data sets were corrected for differences in gain and offset using the equation

Corrected grey level

\[ F_{\text{High Ave}} - F_{\text{Low Ave}} = \left( F_{\text{High}} - F_{\text{Low}} \right) \times \left( \text{Uncorrected grey level} - F_{\text{Low Ave}} \right) + F_{\text{Low Ave}} \]

where \( F_{\text{High Ave}} \) is the grey level for the average fluorescence of the standard without the neutral density filter in place, \( F_{\text{Low Ave}} \) is the grey level for the average fluorescence of the standard with the filter in place, \( F_{\text{High}} \) is the grey level for the daily fluorescence of the standard without the neutral density filter in place, and \( F_{\text{Low}} \) is the grey level for the daily fluorescence of the standard with the filter in place. An average of the first 50 experiments using the system gave values of \( F_{\text{High Ave}} = 150 \) and \( F_{\text{Low Ave}} = 45 \).

These calculations ensured that data from experiments performed at different times could be compared properly. Specific grey levels were assigned color equivalents in certain experiments to produce a "false color" display of macromolecular leakage from vessels. These "false color" images were found to be useful in locating specific areas along vessels from which leakage of macromolecules may have originated.

PMN Counting. PMNs in cremaster muscle blood vessels were identified by their morphology during intravital microscopy and verified by histological sections of treated and control cremaster muscle preparations. The number of rolling PMNs in venules was determined by counting PMNs over a 5-min period as they passed a given point in the vessel. The number of adherent PMNs was determined by counting the PMNs which remained stationary for a minimum of 15 s over a 100-μm length of vessel.

Histology. Cremaster muscles were dissected from their pedicle attachment immediately after the completion of examination, stretched over the surface of a polypropylene grid, and fixed in 10% buffered formalin. Specimens were embedded in paraffin after dehydration and cut parallel to the surface of the muscle into 4-μm sections. Vascular structures were stained as follows. Sections were deparaffinized, oxidized with 63.5% periodic acid for 10 min, incubated in a methenamine silver solution at 65°C for 30 min and immersed in a 0.7% gold chloride solution for 5 min and a 2% sodium thiocyanate solution for 2 min. All chemicals were obtained from Sigma Chemical Co. Sections were counterstained with Wright-Giemsa stain to identify the pattern of PMNs.
Statistical Analysis. Two nonparametric statistical techniques were used to analyze the outcome of the experiments. Multivariate cluster analysis was used to attempt to find natural groupings within the data structure (18). Five discrete variables were used to analyze the data: treatment type; duration of treatment (time); arteriole diameter; venule diameter; and macromolecular leakage. Once these natural groupings were determined, the data were pooled to find the kernel density estimation of the probability distribution (19, 20). The purpose of the kernel density estimation was to appraise the density curve of a data set which was not distributed normally. Although the animals used in the study are reasonably similar, they are not truly identical, and variability in their responses to treatment can be expected. In addition, different measurement points on vessels may not respond in the same way as other points. Based on this variability, a nonparametric approach to the statistical analysis was indicated.

RESULTS

The use of intravital microscopy of the rat cremaster muscle allowed us to measure in detail the changes in small blood vessels which occur during and 1 h after PDT. It was not possible to examine treated tissue for periods longer than this time period without adversely affecting the physiology of the cremaster muscle. The effect of PDT on arterioles of 20–30 μm diameter for different Photofrin doses is shown in Fig. 1. Only minor alterations in arteriole diameter were observed when Photofrin doses of 5 mg/kg were used. Certain vessels showed slight dilation, while others constricted slightly after 90 J/cm² light treatment. The observed constriction or dilation of arterioles remained constant for 1 h after phototherapy. Significant constriction of arterioles was observed when porphyrin doses of 10 mg/kg and above were used. At Photofrin doses of 10 and 25 mg/kg, arteriole constriction occurred rapidly, and the vessels were completely constricted and blood flow was static within 20 min of light treatment (90 J/cm²) or 15 min of light treatment (68 J/cm²), respectively. Formation of platelet thrombi was occasionally observed in animals given either 10 or 25 mg/kg Photofrin and light. Shortly after 15–20 min of therapy, two different populations of vessels could be distinguished. One set of vessels remained fully constricted throughout the completion of treatment and for at least 1 h afterward. A second population of vessels was noted which released either partially or completely from constriction to their original diameter by the completion of light treatment. No further relaxation was observed over a 1-h period after treatment. The existence of discrete populations of arterioles was verified by density estimation (Fig. 2). Two distribution modes were observed, one near 100% and the other at 0% diameter. The difference between these two populations of vessel could not be explained by their position in the vascular bed or by their location in the branching pattern.

The effect of PDT on venules within the same treatment field was considerably less than that observed for arterioles (Fig. 3). As was the case for arterioles, Photofrin doses of 5 mg/kg did not cause any change in venule diameter throughout 135 J/cm² light treatment. When Photofrin doses of 10 mg/kg were used, however, light treatment caused only minor alterations in venule diameter during and after the 135 J/cm² light treatment. Some venules showed minor dilation, while others constricted slightly. Occasional platelet thrombi were observed in venules at this and at higher Photofrin doses. The average PDT effect on venules at this porphyrin dose was not statistically different from that of controls, while increasing the porphyrin dose to 25 mg/kg did produce significant levels of constriction. Constriction of venules was noted after 45 J/cm² light treatment and continued throughout the completion of 135 J/cm² light treatment. Venule diameters constricted to 60% of their original size by the end of light treatment. No further constriction was observed after treatment. Venules did not fully constrict at any of the porphyrin doses studied.

The effect of phototherapy on capillaries and pre- and post-capillary vessels was also studied. No measurements of vessel constriction were made, since these could not be performed accurately. Blood flow stasis was observed in these vessels at the same doses and times which caused arteriole constriction and stasis.

Rats given porphyrin doses of up to 25 mg/kg Photofrin alone or light doses of 135 J/cm² alone did not show any change in the diameter of arterioles or venules.

The administration of indomethacin (5 mg/kg 1 h before treatment) was found to inhibit the constriction of both venules and arterioles after PDT (25 mg/kg Photofrin, 135 J/cm², 630 nm).
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Fig. 2. Kernel density estimates at each dose level of Photofrin. x-axis, arteriole diameter (percentage of controls); y-axis, Photofrin dose administered to rats 24 h before light treatment; z-axis, probability of occurrence. Each peak in the estimation represents a mode of the distribution; two modes in the same distribution indicate the existence of two subpopulations.

Quantitation of macromolecule leakage from the blood vessels was performed by measuring the leakage of fluorescein-labeled albumin from vessels into the interstitial space. Digital image analysis verified that macromolecular leakage occurs predominantly from venules after PDT. "False color" analysis of FITC-albumin localization suggested that macromolecular leakage occurs at a number of focal points along the venules, rather than continuously along the vessel wall. No leakage of FITC-albumin was observed in arterioles treated with PDT in this study. Animals given either light alone or 25 mg/kg Photofrin alone showed no leakage of FITC-albumin from venules into the interstitium. A threshold for the initiation of leakage and an upper limit for the Photofrin dose required to produce leakage were observed (Fig. 5). Photofrin doses of 5 mg/kg and a light dose of 135 J/cm² were not sufficient to produce macromolecular leakage in venules. Doses of 10 and 25 mg/kg Photofrin caused progressive leakage over the duration of the light treatment and for at least 1 h after treatment. The administration of 25 mg/kg Photofrin and light did not increase the magnitude of leakage from venules when compared to the 10 mg/kg photosensitizer dose.

The administration of indomethacin 1 h before light treatment completely inhibited macromolecular leakage from venules at Photofrin doses of 25 mg/kg and light doses of 135 J/cm² (Fig. 6). Data from animals given PDT and indomethacin were not significantly different from controls given drug or light treatment alone.

Changes in WBC aggregation were also observed in venules during PDT. Cells were identified as polymorphonuclear leukocytes by histological analysis of cremaster muscle sections after treatment (Fig. 7). Although the adherent cells in the histological preparations were identified as PMNs, the possibility that other types of WBC become adherent to venules during PDT cannot be excluded. In control animals, PMNs were found to roll along the inner surface of the vessel and did not stick to any given point for more than 1 s. The number of rolling PMNs could be easily counted and was found to be remarkably constant in different animals. The number of sticking or adherent PMNs was also counted. Adherent PMNs were defined as those PMNs which did not move over a time period greater than 15 s. In general, adherent PMNs did not detach themselves within 30 min of observation. In vessels exposed to light alone (no photosensitizer), 23 ± 7 (2 SEM) rolling PMNs crossed a given point on a vessel during a 1-min observation. There were at most 1 or 2 sticking PMNs along a 100-μm length of vessel in these control animals. Animals given 25 mg/kg Photofrin 24 h before light treatment had pronounced changes in the number of rolling and adhering PMNs (Fig. 8). Rolling PMNs became adherent during the first 5 min (22.5 J/cm²) of light treatment. By the completion of the light treatment, no rolling PMNs were observed, and 37 ± 5 (2 SEM) PMNs were adherent for each 100-μm length of venule. PMNs appeared to adhere to spaces between endothelial cells in certain histological preparations, but this was not necessarily the case for all sections. PMN adherence preceded or was coincident with the first observed increases in vessel permeability to FITC-albumin. No PMN adherence was observed in arterioles.

Fig. 3. Change in the diameter of 20–30-μm-diameter venules during and after PDT. Animals were given Photofrin i.v. at doses of 5 (a), 10 (b), and 25 (c) mg/kg 24 h before light treatment. Light treatment was 135 J/cm² 630 nm, delivered during the first 30 min of the experiment. Vessel measurements were continued for an additional 1 h after the completion of light treatment. Points, mean of 3–6 experiments, calculated as a percentage of initial vessel diameter; bars, 2 SEM.
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Indomethacin was given to a second group of animals to determine how cyclooxygenase inhibitors may change PMN adherence during PDT. There was no difference in the number of adherent PMNs seen after PDT in animals given indomethacin 1 h before light treatment (39 ± 5) and treated as above as compared to animals given Photofrin and light treatment.

DISCUSSION

This study has investigated three of the effects that PDT has on the microvasculature: vessel constriction; macromolecular leakage; and PMN adhesion. These experiments were performed to provide a better understanding of how PDT causes microvascular stasis in animals and humans and to examine the relationship between tumor response and microvascular damage in PDT.

The initial experiments served to quantitate vessel constriction of small (20–30-μm diameter) arterioles and venules. A porphyrin dose-response relationship was observed for both of these vessel types. Constriction of arterioles occurred rapidly after the initiation of light treatment in animals given 10 mg/kg of Photofrin or more. Photofrin doses of 5 mg/kg caused no change in arteriole diameter with treatment. The time required for full constriction to occur was a function of the photosensitizer dosage; animals given higher Photofrin doses required less light treatment to produce arteriole constriction. These studies confirmed the work done by Star et al. (7) using transparent window chambers and by Selman et al. (9) using radioactive microspheres. These studies are similar to those previously reported by Reed et al. (10), who used 530–560 nm light and demonstrated that vessel response occurs at clinically applicable wavelengths. Our data differ from these other studies because we observed dilation of certain arteriole segments shortly after the initial constriction. Kernel density analysis showed two distinct populations of arteriole segments; one that remained constricted throughout the duration of the experiment and a second that dilated shortly after the initial constriction. This should not imply that there are groups of vessels which stay constricted and others that dilate. We actually observed a situation where different segments of the same arteriole stayed either constricted or dilated. This suggests that there are sensitive sites along a length of arteriole which produce this response to PDT. It remains unclear where these sites are and whether the vessel constriction induced by PDT is at specific or random sites. The sites of vessel constriction may coincide with areas of endothelial cell damage or cell retraction which exposes vessel basement membrane. Eicosanoid release by activated platelets and damaged vessel wall could cause high local

Fig. 4. Change in the diameter of 20–30-μm-diameter vessels during and after PDT. Animals were given Photofrin i.v. at a dose of 25 mg/kg 24 h before light treatment. Animals were given 5 mg/kg indomethacin trihydrate 1 h before light treatment. The light treatment was 135 J/cm² 630 nm, delivered during the first 30 min of the experiment. Vessel measurements were continued for an additional 1 h after the completion of light treatment. Points, mean of 3–6 experiments for arterioles (a) and venules (b), calculated as a percentage of initial vessel diameter; bars, 2 SEM.

Fig. 5. Alterations in venule permeability to fluorescein-labeled albumin. Animals were given Photofrin i.v. at doses of 5 (a), 10 (b), and 25 (c) mg/kg 24 h before light treatment. Light treatment was 135 J/cm² 630 nm, delivered during the first 30 min of the experiment. Venule leakage was measured for an additional 1 h after the completion of light treatment. Points, mean of the relative fluorescence for 3–6 experiments; bars, 2 SEM.
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Fig. 6. Alterations in venule permeability to fluorescein-labeled albumin. Animals were given Photofrin i.v. at a dose of 25 mg/kg 24 h before light treatment. Animals were given 5 mg/kg indomethacin trihydrate 1 h before light treatment. The light treatment was 135 J/cm² 630 nm, delivered during the first 30 min of the experiment. Venule leakage was measured for an additional 1 h after the completion of light treatment. Points, mean of the relative fluorescence for 3-6 experiments; bars, 2 SEM.

Fig. 7. Histological assessment of PMNs within 20-30 μm venules in the cremaster muscle microvasculature. Animals were given 25 mg/kg Photofrin i.v. 24 h before light treatment. a, representative section of cremaster given no light treatment; b, representative section of cremaster exposed to 135 J/cm² 630 nm. All sections were cut at a thickness of 4 μm and were stained as described in “Materials and Methods.” Bar, 20 μm.

concentrations of vasoconstrictory agents and result in the observed vessel constriction. The effects of PDT on the constriction of postcapillary venules (20–30-μm diameter) were significantly less than that observed for arterioles. Only at the highest Photofrin dose studied (25 mg/kg) did we see any reduction in the size of venules. This observation is consistent with the lack of smooth muscle in these vessels (21). The reduction in venule diameter is probably a result of platelet and leukocyte adherence to the vessel wall and external compression of the vessel by the surrounding cremaster skeletal muscle. Reductions in the velocity of flow in these venules and the appearance of platelet emboli were often observed during treatment (data not shown), but complete constriction or a reduction in the diameter of the vessel lumen was rarely seen.

Microvascular leakage of macromolecules was quantitated both during and after PDT treatment. Vessel leakage of albumin occurred slowly during light treatment and could be observed as soon as 5–10 min (23–45 J/cm²) of treatment. No leakage was observed adjacent to arterioles, regardless of the duration of treatment or the length of observation after treatment. It is possible that the smooth muscle layer surrounding these vessels prevents vessel leakage. Postcapillary venules showed the most pronounced leakage, while large venules (>80 μm) did not show much macromolecular leakage. Little or no leakage was detected from capillaries, since they usually became plugged with platelet thrombi during the course of the light treatment. Leakage in the postcapillary venules appeared to originate at specific points along these small vessels instead of over the entire vessel length. As time progressed after treatment, leakage of albumin was observed along the entire vessel.

As was observed with vessel constriction in arterioles, the extent of venule leakage of albumin was related to the Photofrin dose used in PDT. Porphyrin doses of 5 mg/kg did not result in venule leakage, while doses of 10 mg/kg and above produced nearly equivalent leakage. The increased permeability of venules measured in this study parallels measurements of tumor interstitial pressure during and after PDT (14). Although measurements of tumor vessel permeability were not performed in this study, it is conceivable that tumor vessels exhibit increases in permeability similar to that of normal vessels, and this results in increased tumor interstitial pressure.

Increased PMN adhesion to venule walls was noted in animals given Photofrin and light. Polymorphonuclear leukocyte adhesion and the release of eicosanoids from these cells are known to mediate changes in vessel constriction and vessel permeability of macromolecules (22), two events observed during PDT. These effects are similar to those seen in several injury models which involve the production of oxygen radicals (23). One example is reperfusion injury, where oxygen radicals are released after reestablishment of blood flow to ischemic areas. Studies using this model have demonstrated significant

Fig. 8. Changes in polymorphonuclear leukocyte adherence to vessel endothelium during PDT. Animals were given 25 mg/kg Photofrin i.v. 24 h before light treatment. Light treatment was 135 J/cm² delivered over 30 min. Points, number of rolling PMNs (○) and adherent PMNs (●) as defined in “Materials and Methods.” Bars, 2 SEM.

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increases in PMN adherence to venule walls after the blood flow is established, followed by increases in vascular permeability and tissue edema (24). It remains unclear if the location of PMN adherence to the endothelium correlates with the focal areas of macromolecular leakage observed early during PDT treatment. Studies are in progress to further define this relationship. Arteriole constriction during PDT is unrelated to changes in PMN adhesion, since PMNs did not become adherent to arterioles. It remains possible, however, that activation of PMNs could occur in arterioles and that these cells could release specific vasoactive eicosanoids without becoming adherent to the vessel wall.

The use of indomethacin, a nonsteroidal antiinflammatory agent, was found to profoundly inhibit many of the observed vascular responses to PDT. Administration of indomethacin completely blocked arteriole and venule constriction at the highest Photofrin dose studied and completely blocked venule leakage to albumin. Several studies demonstrate that indomethacin and similar agents can alter biological responses to PDT (1, 25). The administration of indomethacin effectively blocks PDT-induced increases in tumor interstitial pressure and blocks the release of thromboxane into blood (1, 14). Reed et al. (26) recently showed that topical administration of indomethacin on PDT suggest that prostaglandin and thromboxane initiate vessel constriction and vessel leakage. The use of indomethacin, a nonsteroidal antiinflammatory drug, which blocks the release of thromboxane into blood (1, 14) and blocks the release of thromboxane into blood (1, 14), was found to profoundly inhibit many of the observed vascular responses to PDT. Administration of indomethacin completely blocked arteriole and venule constriction at the highest Photofrin dose studied and completely blocked venule leakage to albumin. Several studies demonstrate that indomethacin and similar agents can alter biological responses to PDT (1, 25). The administration of indomethacin effectively blocks PDT-induced increases in tumor interstitial pressure and blocks the release of thromboxane into blood (1, 14).

Indomethacin pretreatment of animals inhibited vessel leakage but did not inhibit the adhesion of PMNs to the endothelium. This provides direct evidence that the effects of PDT which cause PMN adhesion are separate from those which cause the release of prostaglandins and thromboxanes. Polymorphonuclear leukocyte adherence to the endothelium in the presence of indomethacin may be associated with the release of leukotriene B4 and leukotriene C4, two eicosanoids previously found to be released during PDT (27). The leukotrienes are lipooxygenase products of arachidonic acid, and the production of these eicosanoids is not inhibited by indomethacin (28). While these two leukotrienes are also involved in changing vascular permeability to macromolecules in other models (29, 30), they did not appear to produce vessel leakage here. This investigation has defined the effects of PDT on microvasculature with regard to changes in vessel constriction, vessel leakage, and leukocyte adherence. These changes result in blood flow stasis within the tissue, an event associated with tumor destruction in a wide range of animal models. The effects of indomethacin on PDT suggest that prostaglandin and thromboxane initiate vessel constriction and vessel leakage. The events which precede eicosanoid release and vessel constriction remain unclear. One of two plausible hypotheses may explain the initial effects of PDT. First, the generation of singlet oxygen from excited porphyrin molecules in the outer membrane of leukocytes, platelets, or endothelial cells may directly liberate stored supplies of arachidonic acid without otherwise damaging the cells (23). Arachidonic acid can then be metabolized to the prostaglandins and thromboxanes starting the cascade. Alternatively, PDT treatment may directly injure endothelial cells by membrane disruption or mitochondrial damage (3, 4). These perturbations may cause endothelial cell rounding with exposure of the vascular basement mem-

brane (31). Platelets and leukocytes will adhere to damaged endothelial cells and/or to exposed areas between cells and release eicosanoids (32). This hypothesis may explain our observations of specified areas of constriction and focal areas of leakage.

This latter hypothesis is supported by the work of Chaudhuri et al. (33), who observed damage to intracellular organelles of vascular endothelial cells and loss of the endocapillary layer immediately after PDT (33), and by Zhou et al. (34), who demonstrated similar changes in endothelial cells immediately after PDT using hematoporphyrin derivative (34). In both of these studies, damage to the vascular endothelial cells was rapid and preceded damage to tumor cells. Damage to the vascular endothelium was progressive with time, with nearly complete denudation of endothelial cells by 24 h after PDT. These late effects on the vascular endothelium both in vivo and in vitro have been observed recently by He et al. (35) using tin etiopurpurin. Direct damage to endothelial cells early in PDT may trigger the eicosanoid release and vascular changes observed in the present study. In order to fully explain the early events which lead to vascular stasis, a comprehensive study of the endothelial cell, leukocyte, and platelet response to PDT is necessary. Investigation of the interactions between these cellular elements may provide insights into the early mechanisms of vessel damage. Continued studies are necessary to address the effects of PDT on tumor microvasculature. This investigation has examined normal microvasculature as a model system to observe the physiological changes which occur with PDT. The relative contribution of the observed effects to damage to tumor microvasculature remains to be found and is the subject of current work in our laboratory.

REFERENCES

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The Role of Microvascular Damage in Photodynamic Therapy:
The Effect of Treatment on Vessel Constriction, Permeability, and Leukocyte Adhesion


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