Tumor Vascular Permeabilization by Vascular-Targeting Photosensitization: Effects, Mechanism, and Therapeutic Implications

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Abstract

Purpose: Loss of vascular barrier function has been observed shortly following vascular-targeting photodynamic therapy. However, the mechanism involved in this event is still not clear, and the therapeutic implications associated with this pathophysiologic change have not been fully explored.

Experimental Design: The effect of vascular-targeting photodynamic therapy on vascular barrier function was examined in both s.c. and orthotopic MatLyLu rat prostate tumor models and endothelial cells in vitro, using photosensitizer verteporfin. Vascular permeability to macromolecules (Evans blue-albumin and high molecular weight dextran) was assessed with dye extraction (ex vivo) and intravital microscopy (in vivo) methods. Intravital microscopy was also used to monitor tumor vascular functional changes after vascular-targeting photodynamic therapy. The effects of photosensitization on monolayer endothelial cell morphology and cytoskeleton structures were studied with immunofluorescence staining.

Results: Vascular-targeting photodynamic therapy induced vascular barrier dysfunction in the MatLyLu tumors. Thus, tumor uptake of macromolecules was significantly increased following photodynamic therapy treatments. In addition to vascular permeability increase, blood cell adherence to vessel wall was observed shortly after treatment, further suggesting the loss of endothelial integrity. Blood cell adhesion led to the formation of thrombi that can occlude blood vessels, causing vascular shutdown. However, viable tumor cells were often detected at tumor periphery after vascular-targeting photodynamic therapy. Endothelial cell barrier dysfunction following photodynamic therapy treatment was also observed in vitro by culturing monolayer endothelial cells on Transwell inserts. Immunofluorescence study revealed microtubule depolymerization shortly after photosensitization treatment and stress actin fiber formation thereafter. Consequently, endothelial cells were found to retracted, and this endothelial morphologic change led to the formation of intercellular gaps.

Conclusions: Vascular-targeting photodynamic therapy permeabilizes blood vessels through the formation of endothelial intercellular gaps, which are likely induced via endothelial cell microtubule depolymerization following vascular photosensitization. Loss of endothelial barrier function can ultimately lead to tumor vascular shutdown and has significant implications in drug transport and tumor cell metastasis.

Photodynamic therapy is a modality in which a photosensitizer is administered systemically or locally and subsequently activated by illumination with visible light, leading to the generation of cytotoxic reactive oxygen species in the presence of oxygen (1). Photodynamic therapy is currently used for the treatment of various types of cancer, including lung, skin, gastrointestinal tract, the head and neck, and urological cancers (2). It has also been used as a treatment for noncancer diseases such as age-related muscular degeneration (AMD), atherosclerosis, and viral or bacterial infections (3).

Verteporfin (the lipid formulation of benzoporphyrin derivative monoacid ring) is a photosensitizer that has been approved for the treatment of AMD (4). Compared with Photofrin (the first photosensitizer with the Food and Drug Administration approval for cancer treatment), the advantages of verteporfin include a strong absorption at longer wavelengths, leading
to deeper tissue penetration and a fast pharmacokinetic behavior in vivo, resulting in a reduced skin photosensitivity. Because photosensitizing targets closely depend on the localization of photosensitizers, it is therefore important to determine the temporal and spatial changes of the photosensitizer localization. In the previous studies, we have found that the distribution of verteporfin changes dynamically as a function of time after administration. It is predominantly retained in the tumor vasculature within the first few minutes after i.v. injection (e.g., within 15 minutes) and then systematically extravasates into the tumor interstitial and cellular compartments over longer times (e.g., over a few hours) after administration (5–7). Based on this pharmacokinetic pattern, maximal tumor vascular or cellular targeting can be effectively achieved by illumination at a short or a long time point after drug administration, respectively. Light treatment typically starts at 5 to 15 minutes after administration of verteporfin to selectively target blood vessels. This vascular targeting regimen is currently used for AMD treatment in clinic (4) and experimentally for tumor destruction (5, 6, 8, 9).

Vascular-targeting therapy is a promising strategy in cancer treatment that has received considerable attention in recent years (10, 11). Compared with conventional cancer cell–targeting approaches, targeting tumor vasculature is easier to access, more efficient in cancer cell killing, and has a lower likelihood of developing drug resistance. Although vascular damage has long been known to contribute to the overall photodynamic therapy treatment effect, intentional use of this mechanism based on the photosensitizer pharmacokinetic distribution to maximize clinical effect is a more recent technique, beginning with the implementation of verteporfin for AMD treatment. Following the success of verteporfin, the photodynamic therapy vascular targeting regimen with a short drug-light interval has been used for another photosensitizing agent Tookad, which is currently in clinical trials for prostate cancer treatment (12).

Tumor vasculature is not only a pipeline for the supply of nutrients and removal of metabolic wastes but also a common route for the delivery of anticancer agents to tumor tissues and dissemination of tumor cells to distant organs. The circulatory function of vasculature is largely maintained by the endothelial barrier that tightly controls the substance exchange between blood plasma and interstitial fluids (13). The goal of vascular targeting is to induce vascular shutdown. One of the earliest events following vascular photosensitization is, however, the loss of vascular barrier function (14, 15). Indeed, increase in vascular permeability has been documented after photodynamic therapy treatment with several photosensitizers (14). In AMD patients treated with verteporfin-photodynamic therapy, vascular leakage is observed shortly after treatment and lasts even for days before vessel occlusion (16). Given the critical role of vasculature in tumor cell survival, metastasis, and anticancer drug delivery, it is important to study the effects and mechanisms of verteporfin photosensitization on vascular barrier function. A fundamental understanding of photosensitization-induced vascular permeabilization is necessary for using this modality to target blood vessels for the treatment of cancer, AMD, and other diseases. In this article, we studied tumor vascular barrier function alteration and its mechanisms in response to photodynamic therapy with verteporfin, as used in a vascular-targeting approach.

Materials and Methods

Photosensitizer. Verteporfin (benzoporphyrin derivative in a lipid formulation) was obtained from QLT, Inc., as a gift (Vancouver, Canada). A stock saline solution of verteporfin was reconstituted according to the manufacturer’s instructions and stored at 4°C in the dark.

Cell culture. Mouse endothelial cells SVEC4-10 (American Type Culture Collection, Manassas, VA) and R3327-MatLyLu rat prostate cancer cells were maintained in RPMI 1640 with glutamine (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 100 units/ml penicillin/streptomycin (Mediatech) at 37°C in a 5% CO2 incubator.

Animals and tumor model. Male Copenhagen rats (6-8 weeks old) obtained from Charles River Laboratories (Wilmington, MA) were used throughout the study. The R3327-MatLyLu Dunning prostate tumor is an androgen-independent carcinoma, syngeneic to the Copenhagen rats, and highly metastatic to both lymph nodes and lungs (MatLyLu; ref. 17). This Dunning tumor was shown to be similar to human prostate cancer in the response to hormone therapy, chemotherapy, and radiation therapy (18). Cells used in this experiment were no more than 10 passages from the original stock in liquid nitrogen. The s.c. and orthotopic MatLyLu rat prostate cancer models were reproduced as previously described (9). Tumors were used for experiments when reaching a size of 6 to 10 mm in diameter. All animal procedures were done according to a protocol approved by the Dartmouth College Animal Care and Use Committee.

Photodynamic therapy treatments. A diode laser system (Applied Optronics, South Plainfield, NJ) with 690-nm wavelength was used throughout this study for the irradiation of in vitro cultured cells and MatLyLu tumors. The light was delivered through an optical fiber (140-μm core diameter). For the in vitro study, SVEC4-10 cells were incubated with 200 ng/ml verteporfin for 15 minutes. After removing the drug-containing medium, cells were washed with PBS and exposed to 5 mW/cm2 intensity of light for 100 or 200 seconds. Light intensity was measured by an optical power meter (Thorlabs, Inc., North Newton, NJ). For photodynamic therapy treatment of MatLyLu tumors, animals were anesthetized with an injection (i.p.) of ketamine (90 mg/kg) and xylazine (9 mg/kg) and placed on a heated blanket throughout the light treatment. The MatLyLu tumors were treated with external light illumination for 1,000 seconds at an incident fluence rate of 50 mW/cm2. Verteporfin was injected i.v. at a dose of 0.25 mg/kg at 15 minutes or 1.0 mg/kg at 3 hours before light irradiation.

Assessment of vascular permeability to macromolecules in the MatLyLu tumors. Effective vascular permeability in the s.c. MatLyLu tumors was determined as described (19). Immediately after photodynamic therapy treatments (both 15 minutes and 3 hours of drug-light photodynamic therapy), animals were i.v. injected with 10 mg/kg Evans blue (Sigma, St. Louis, MO) and 10 mg/kg FITC-labeled dextran (molecular weight of 2,000 kDa; Sigma). At 0.25, 0.5, 1.0, and 2.0 hours after injection, tumor-bearing animals were euthanized. After systemic perfusion with 50 mL of 0.9% saline to remove macromolecules in the circulation, tumor tissues were excised, minced, and extracted with formamide (1 mL per 100 mg tissue) for 72 hours. The absorbance of Evans blue at 620 nm was measured with a spectrophotometer (Cary 50 Bio, Varian Analytical Instruments, Walnut Creek, CA), and the fluorescence of FITC-dextran was determined with a spectrofluorometer (FluoroMax-3, Jobin Yvon, Inc., Edison, NJ) with 495-nm excitation and 518-nm emission.

Monitoring of tumor vascular function by intravital microscopy. Tumor vascular functional changes induced by vascular-targeting photodynamic therapy regimen (light treatment at 15 minutes after injection of 0.25 mg/kg verteporfin) were examined using a Zeiss fluorescence stereomicroscope (Stemi SV11) in the live animals with orthotopic MatLyLu tumors. Tumor-bearing animals were anesthetized as described above and fixed on the stereomicroscope stage. Orthotopic MatLyLu tumors were surgically exposed and treated with 50 J/cm2 dose.
were stained with rhodamine-conjugated phalloidin (Sigma; A Probes, Eugene, OR; 1:500 dilution) for 30 minutes. Actin filaments for 30 minutes at room temperature. The microtubule was stained with dye (Sigma, 5 mA, 5 mW/cm2 light for 200 seconds after incubation with 200 ng/mL endothelial cells cultured on glass coverslips were treated with -tubulin mouse monoclonal antibody (Sigma; 1:500 dilution) as described (20). Briefly, heparinized whole blood was collected from a donor rat. RBCs were isolated from the whole blood by centrifugation and washing with PBS thrice. Then, 1 mL of packed RBCs was incubated with 1 mL of Dil solution (1 mg/mL) at room temperature in dark condition for 30 minutes. After the incubation, RBC suspension was centrifuged and washed with PBS twice to remove the free dye. Then, 200 mL of Dil-labeled RBCs diluted with 800 mL PBS was i.v. injected to the animals before photodynamic therapy treatment. The movement of Dil-labeled RBCs was monitored with the stereomicroscope using a ×1 objective lens plus 6.6 zoom, and the fluorescence images were recorded with the AxioCam CCD camera. The filter set for imaging Dil dye was 530 to 550 nm for excitation and 570 to 610 nm for emission.

Assessment of monolayer endothelial permeability. In vitro endothelial permeability was measured by the diffusion of 2,000-kDa FITC-dextran through the endothelial monolayer, as described (21). SVEC-4 endothelial cells were cultured on Transwell inserts (Costar, Cambridge, MA) up to confluence. Cells were incubated with 200 ng/mL verteporfin for 15 minutes and subjected to light treatment (100 or 200 seconds of illumination at 5 mW/cm2). Immediately after light irradiation, medium containing 1 mg/mL of 2,000-kDa FITC-dextran was loaded on the upper compartment of the Transwell. The amount of FITC-dextran diffused through the endothelial monolayer into the lower compartment was measured by a SynergyHT microplate reader (Bio-Tek Instruments, Winooski, VT) with excitation at 485/20 nm and emission at 525/20 nm.

Immunofluorescence staining of endothelial cytoskeleton. SVEC-4 endothelial cells cultured on glass coverslips were treated with 5 mW/cm2 light for 200 seconds after incubation with 200 ng/mL verteporfin for 15 minutes. At different time points after treatment, cells were fixed and permeated with cold methanol/acetone (1:1) at −20°C for 30 minutes. Cells were subsequently washed thrice with PBS and blocked for nonspecific binding with 1% bovine serum albumin in PBS for 30 minutes at room temperature. The microtubule was stained with anti-a-tubulin mouse monoclonal antibody (Sigma: 1:500 dilution) for 1 hour at room temperature followed by incubation with Alexa 488–conjugated rabbit anti-mouse secondary antibody (Molecular Probes, Eugene, OR: 1:500 dilution) for 30 minutes. Actin filaments were stained with rhodamine-conjugated phalloidin (Sigma: 1 mg/mL) for 1 hour at room temperature. Cell nuclei were stained with Hoechst dye (Sigma, 5 μmol/L) for 15 minutes. After immunofluorescence staining, cells were imaged with a Zeiss LSM 510 confocal microscopy with appropriate filter setup for different dyes.

**Results**

Tumor vascular permeability to Evans blue and FITC-dextran (molecular weight, 2,000 kDa) was first assessed in the s.c. MatLyLu tumors. Figure 1 indicates that vascular targeting verteporfin-photodynamic therapy using a 15-minute drug-light interval increases vascular permeability; thus, tumor uptake of macromolecules is significantly increased at 2 hours after injection compared with the control tumor. In contrast, verteporfin-photodynamic therapy using a 3-hour drug-light interval does not significantly increase tumor uptake of the macromolecules. It was noted that vascular-targeting photodynamic therapy could significantly enhance tumor uptake of 2,000-kDa FITC-dextran at 0.25 and 0.5 hour after photodynamic therapy. However, the same treatment was not able to increase Evans blue tumor uptake (Fig. 1).

Vascular permeabilization induced by vascular-targeting photodynamic therapy could also be observed in the orthotopic tumor in real time with intravital microscopy. Immediately after photodynamic therapy treatment, animals were i.v. injected with 2,000-kDa FITC-dextran, and the extravasation of high-molecule dextran was monitored in live animals with a stereo fluorescence microscope. Because blood significantly quenches the fluorescence of FITC through the inner filter effects of hemoglobin (22), only a weak fluorescence signal could be observed within tumor blood vessels (Fig. 2). However, when FITC-dextran leaked out of blood vessels, its fluorescence intensity was greatly enhanced due to the loss of hemoglobin-quenching effect. As shown in Fig. 2, vascular-targeting photodynamic therapy permeabilizes tumor blood vessels, significantly increasing the extravasation of high molecule weight dextran, whereas the leakage of 2,000 kDa...
in control tumors is limited. Intravital microscope study also revealed the adhesion of fluorescence-labeled RBCs to the vessel wall shortly after vascular-targeting photodynamic therapy (Fig. 3). Blood cell adherence gradually built up and led to the formation of thrombus. Some thrombi were unstable and went into circulation, leaving blood vessels still functional, whereas other thrombi remained at where they were formed and finally occluded the blood vessels. As shown in Fig. 3, an injection of 2,000-kDa FITC-dextran highlights an apparently functional blood vessel at 120 minutes after photodynamic therapy, whereas a nearby vessel occluded by a thrombus showed no fluorescence at all. It is interesting to note that the FITC fluorescence intensity in the blood vessel in this case is much stronger than that in Fig. 2, although the drug dose injected is the same. This might suggest that although still functional at 120 minutes after photodynamic therapy, that blood vessel has low hemoglobin content. A possible explanation for this observation is that photodynamic therapy-induced thrombosis and direct photodynamic damage of RBCs causes some tumor blood vessels flowed with a lower percentage of RBC volume.

Histologic examination of H&E staining tumor sections taken from tumors at 48 hours after vascular-targeting photodynamic therapy indicated extensive vascular disruption and tumor cell death throughout tumor sections (Fig. 4). However, viable tumor cells were commonly detected at tumor periphery. Because of the existence of viable peripheral tumor cells, the vascular-targeting regimen used in this study led to no tumor cure (6).

Endothelial barrier function was also assessed by the diffusion of 2,000-kDa FITC-dextran through the endothelial monolayer cultured on transwell inserts. As shown in Fig. 5, photosensitization with verteporfin (200 ng/mL for 15-minute incubation) significantly increased monolayer endothelial permeability to the macromolecule 2,000-kDa FITC-dextran in a dose-dependent manner, whereas the permeability in the control endothelial cells (with 200 ng/mL verteporfin only, no light) was very limited.

Changes in endothelial cytoskeleton induced by verteporfin-photodynamic therapy were examined with immunofluorescence staining. In the control cells, microtubules extend throughout the cytoplasm to the cell periphery, whereas actin only distributes at the cell periphery (Fig. 6, top). This distribution pattern is important for maintaining endothelium integrity (23). Microtubule disassembly was noted shortly after verteporfin-photodynamic therapy followed by the formation of actin stress fibers located in the cell central region (Fig. 6, middle). Accompanying the actin stress fibers formation, endothelial cells were observed to retract and display a round morphology, leading to the formation of intercellular gaps (Fig. 6, bottom).

Discussion

The goal of tumor vascular targeting is to selectively modulate tumor vascular function for a therapeutic purpose (24). To achieve this goal, therapeutic effectors or cytotoxic agents need to be selectively delivered to the tumor vascular targets. Although there are a variety of potential tumor vascular markers that can be exploited for the selective vascular targeting through conjugating therapeutic agents with tumor vasculature homing molecules, a marker that is absolutely specific for tumor vasculature has not yet been and may never be found (25). Passive targeting of tumor vasculature based upon the temporal confinement of an i.v. injected agent might be practically the most effective approach to targeting tumor blood vessels. This is especially true for photodynamic therapy, where light needs to be applied to activate the photosensitizing compounds that are otherwise not biologically active at all. Photodynamic therapy can be developed as an effective and selective vascular-targeting modality because photosensitizers are exclusively localized within the vasculature shortly after systemic administration and, more importantly, the selectivity of action to the desired site comes through the ability to accurately deliver light provided by current laser fiber technology. Indeed, photodynamic vascular-targeting therapy has already been in clinical applications for AMD and is under clinical investigation for cancer treatments. However, in spite of extensive studies, a detailed scenario of how photodynamic therapy causes vascular shutdown remains unclear. The present study focuses on studying the effects and mechanisms of vascular permeabilization, an early event commonly observed after photodynamic vascular-targeting therapy.
Our present results show that photosensitization with verteporfin significantly increases overall vascular permeability in both s.c. and orthotopic MatLyLu rat prostate tumors. Thus, tumor uptake of macromolecules was increased after the initial photosensitization treatment. This effect seems dependent on the photodynamic therapy conditions and the size of macromolecules. A vascular-targeting photodynamic therapy regimen employing a short drug-light interval induced a stronger effect than the cellular-targeting photodynamic therapy using a long interval (Fig. 1). This is likely because vascular barrier function is maintained by the integrity of endothelial network and specific intravascular photosensitization induced by vascular-targeting photodynamic therapy is able to induce more structural and functional changes on the endothelium. Previous studies also showed that vascular targeting photodynamic therapy employing a short drug-light interval caused more reduction in blood flow (5, 8, 26). It is interesting to note that the increase in tumor uptake of 2,000 kDa dextran was more significant than that of Evans blue. This difference might be related to the size of these two macromolecules. Evans blue strongly binds to albumin in the blood. Its behavior reflects the transport of albumin (19), which is about 67 kDa with a diameter of about 7 nm. This size is similar to the effective pore size of 6 to 7 nm occurring in most normal blood vessels (27), whereas the size of 2,000-kDa dextran is estimated to be about 100 nm (28). Because tumor vessels typically have larger interendothelial junctions than normal blood vessels (29), there might be little hindrance for the transvascular transport of Evans blue-albumin complex. Therefore, further increase in vascular permeability induced by vascular photosensitization may have little influence on the extravasation of albumin that can already across tumor vessel wall. However, it can
significantly facilitate the extravasation of larger molecules, such as 2,000-kDa dextran, that are otherwise difficult to transport across the endothelial barrier.

The mechanism of photosensitization-induced vascular permeabilization is still an unresolved issue. Because vascular barrier function critically depends on the endothelial cell integrity, which is maintained by cytoskeletal components, such as filament actin and microtubules (13), we studied the effects of verteporfin photosensitization on endothelial cell morphology, cytoskeleton, and barrier function. Our results show that photosensitization causes endothelial cell microtubule depolymerization and induces the formation of actin stress fibers (Fig. 6). Thus, endothelial cells were found to retract, leading to the formation of intercellular gaps, which result in endothelial barrier dysfunction (Fig. 5). The key question becomes how photosensitization induces the formation of intercellular gaps. Here, we found that microtubule alteration was noted before any apparent changes of actin structures and cell morphology, suggesting that microtubules play a pivotal role in photosensitization-induced endothelial gap formation. Microtubules are a cytoskeletal structure with important function in signal transduction and intracellular transport of membrane-bound organelles (23). Previous study with Photofrin also showed that microtubules were even sensitive to photodynamic therapy dose that produced little cytotoxicity (30). It is likely that direct photodynamic disruption of microtubule network triggers endothelial contraction by inducing actin cytoskeletal changes, such as the formation of actin stress fiber, a filament with contractile property. It has been shown that microtubule disruption can cause endothelial morphologic changes through the activation of Rho protein (31). We are currently investigating the involvement of Rho/Rho kinase pathway in photosensitization-induced endothelial morphologic and functional changes.

Retraction of endothelial cells not only leads to the formation of intercellular gap and therefore causes vascular barrier dysfunction but also exposes basement membrane to circulating blood cells, which triggers blood aggregation cascade and causes blood flow reduction. Our intravital microscopy study showed RBC adherence to vessel wall shortly after vascular-targeting photodynamic therapy (Fig. 3). Blood cell adherence developed into the formation of thrombi. Stable thrombi would decrease blood flow and eventually occlude blood vessels, as shown in Fig. 3. This is in agreement with electron microscopic study showing that tumor blood vessels are often congested with RBCs after photodynamic therapy treatment (32). Exposure of vessel basement membrane as a result of endothelial retraction might be only one of the mechanisms causing thrombi formation. Other mechanisms, such as release of thromboxane from platelets (33) and von Willebrand factor from damaged endothelial cells (34), could also contribute to the thrombosis process.

Because tumor vascular leakiness, on the one hand, governs the delivery of therapeutic agents into the tumor tissue and, on the other hand, facilitates tumor cell intravasation into the circulation (35), tumor vascular permeabilization induced by vascular-targeting photodynamic therapy has profound implications in cancer treatments. A therapeutic benefit of photosensitized vascular permeabilization is that it can be used to improve tumor drug delivery and enhance the therapeutic
effect. Indeed, it has been shown that photodynamic therapy regimens with low fluence and fluence rate are able to induce a significant increase in tumor vascular permeability for a sustained period of time (36). Consequently, combination of these photodynamic therapy treatments with liposomal doxorubicin led to an enhanced tumor cure. On the other hand, because tumor vasculature represents an interface between the circulation system and cancer cells, a concern of photodynamic therapy-induced vascular permeabilization is whether this can potentially induce tumor metastasis by increasing tumor cell intravasation into the circulation. There is evidence showing that sublethal photodynamic therapy damage to tumor cells indeed increases tumor metastasis (37).

Although this is considered to be related to the decrease of tumor cell adhesion to the extracellular matrix and the activation of tumor cell survival signal (such as expression of hypoxia-inducible factor-1α and vascular endothelial growth factor) following sublethal photodynamic therapy damage to tumor cells, tumor vascular permeability increase may at least contribute to the metastatic process because sublethal photodynamic therapy itself together with some tumor secreting factors (e.g., vascular endothelial growth factor) all can increase tumor vascular leakiness. Thus, our future efforts will be on exploring the mechanism and therapeutic potential of photodynamic vascular targeting in cancer therapy and anticancer drug delivery, and, importantly, addressing the concern of whether photosensitized vascular permeabilization will increase tumor metastasis.

References

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