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Vascular Accumulation of a Novel Photosensitizer, MV6401, Causes Selective Thrombosis in Tumor Vessels after Photodynamic Therapy[1]

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ABSTRACT

The antivascular effects of photodynamic therapy (PDT) and their mechanisms are not clearly understood. Here, we examined the effects of PDT with a novel photosensitizer MV6401 on the microvasculature in a mammary tumor (MCaIV) grown in a murine dorsal skinfold chamber and in normal tissue controls. The mice were irradiated with light 15 min after i.v. administration of MV6401 when the drug was localized only in the vascular compartment, as shown by fluorescence microscopy and immunohistochemistry. PDT with MV6401 caused a dose-dependent biphasic blood flow stasis and vascular hyperpermeability, as determined by intravital microscopy. This biphasic response was classified into two components: (*a*) an acute response observed immediately after PDT; and (*b*) a long-term response observed at times greater than 3 h after PDT. The acute temporal vascular effects were characteristic of vasoconstriction but not of thrombus formation. However, the long-term vascular shutdown was mediated by thrombus formation, as evidenced by histological evaluation and inhibition with heparin. Minimal effects were observed in normal vessels after antivascular doses used against the tumor, but there was no long-term vascular damage. In concert with the stasis, a dose-dependent tumor growth delay was observed. This study provides mechanistic insights into antitumor vascular effects of PDT and suggests novel strategies for tumor treatment with PDT.

INTRODUCTION

PDT[3] is a minimally invasive therapeutic modality that can be used as a primary therapy for early-stage disease, palliation of late-stage disease, or as a surgical adjuvant for tumors that show locoregional spread (1). PDT has been investigated as a palliative treatment for cutaneous recurrence of breast cancer and has been suggested as a potential therapy for locally invasive breast cancer (2). Three mechanisms by which PDT destroys tumors include: (*a*) direct tumor cell kill, resulting from lethal events initiated by the flux of ROS; (*b*) posttreatment immune response directed against the tumor cells; and (*c*) damage to the tumor-associated vasculature, with subsequent infarctive death of the tumor cells. Although studied in the past (3–5), the effects of PDT on tumor vasculature are not fully understood. Here we determined the vascular effects of PDT with a novel photosensitizer, as well as its mechanism of action, using a combination of intravital microscopy and immunohistochemistry.

MATERIALS AND METHODS

Photosensitizer. Fig. 1*a* shows the novel photosensitizing agent, MV6401 (Miravant Medical Technologies), a pyropheophorbide derivative with indium chelated in the center of the pyropheophorbide macrocycle. The molecular weight of MV6401 is M_r 696.9. For systemic i.v. administration, the drug was dissolved in an EYP emulsion, which was predominantly composed of cationically charged egg yolk phosphatidylcholine vesicles with an average diameter of 200 nm.

Drug Localization. Localization of MV6401 in tumors was determined by examining the fluorescence of MV6401. An excitation filter (band pass, 390–440 nm), an emission filter (band pass, 665–740 nm), and a dichroic mirror (cutoff frequency, 450 nm) were used. Using intravital microscopy, we determined MV6401 localization up to 15 min after drug administration. To quantify the drug distribution in the tumor and within the tumor vasculature, we performed vascular permeability measurements using MV6401 as the tracer and the filter set described above. A second group of tumors was harvested 15 min after MV6401 or EYP vehicle administration from the dorsal skinfold chamber of tumor-bearing mice. The tumor cell nuclei were counterstained with DAPI (Molecular Probes, Eugene, OR). The vasculature was visualized in the same sections by CD31 staining (6).

Animals and Tumor Model. The experiments were performed in severe combined immunodeficient mice of 6 months of age, bred and maintained in our gnotobiotic animal colony at Massachusetts General Hospital. Mice (males and females, 25–30 g BW) were anesthetized (90 mg ketamine HCl and 9 mg xylazine/kg BW, s.c.) and implanted with dorsal skinfold chambers. A small piece (~1 mm in diameter) of MCaIV murine mammary adenocarcinoma was implanted into the chamber, as described previously (7). All procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

PDT. The mice were treated with PDT when the surface area of the tumors was 0.25 cm^2. The photosensitizer MV6401 was injected systemically via the tail vein after anesthesia. Fifteen min after MV6401 administration, the entire window area was treated with 5 J/cm^2 of 664 nm light, delivered from a 1 W diode laser (type DD2; Miravant Medical Technologies). The light intensity incident on the treatment site was maintained at 50 mW/cm^2. We examined the PDT effects mediated by three different doses of MV6401, *i.e.*, 0.072, 0.036, and 0.018 mg/kg BW. Control group animals received EYP alone. In addition, we studied the following control groups: animals that received no drug and no light treatment; animals that received light only (up to a maximum dose of 10 J/cm^2 at a light intensity of 100 mW/cm^2); and animals that received drug alone (up to a maximum dose of 0.144 mg/kg BW). The PDT effects on both normal and tumor tissue, as well as their vasculature, were determined by intravital microscopy.

Intravital Microscopy. Intravital microscopy to measure tumor size, blood vessel perfusion, and vascular permeability was performed as described previously (7, 8). A specially designed motorized microscope stage (Optiscan Model ES102/IS102 XY Stage System; Prior Scientific, Inc., Rockland, MA) was used to observe five randomly chosen regions repeatedly during and after PDT.

Histological Examination. Tumors were harvested at various time points after PDT and fixed in 10% buffered formalin. The tumors were then embedded in paraffin, sectioned for 5-μm thickness, and stained by H&E. Vessels containing thrombi were identified under high power (×20) magnification.

Heparin Treatment. To inhibit thrombus formation, 100 units of heparin (1000 units/ml; Elkins-Sinn, Inc., Cherry Hill, NJ) were administered i.v. simultaneously with the high dose MV6401 (0.072 mg/kg BW). In another group of mice, a second dose of 100 units of heparin was given 1 h after PDT.

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[3] The abbreviations used are: PDT, photodynamic therapy; ROS, reactive oxygen species; EYP, egg yolk protein; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; BW, body weight; H&E, hematoxylin and eosin.

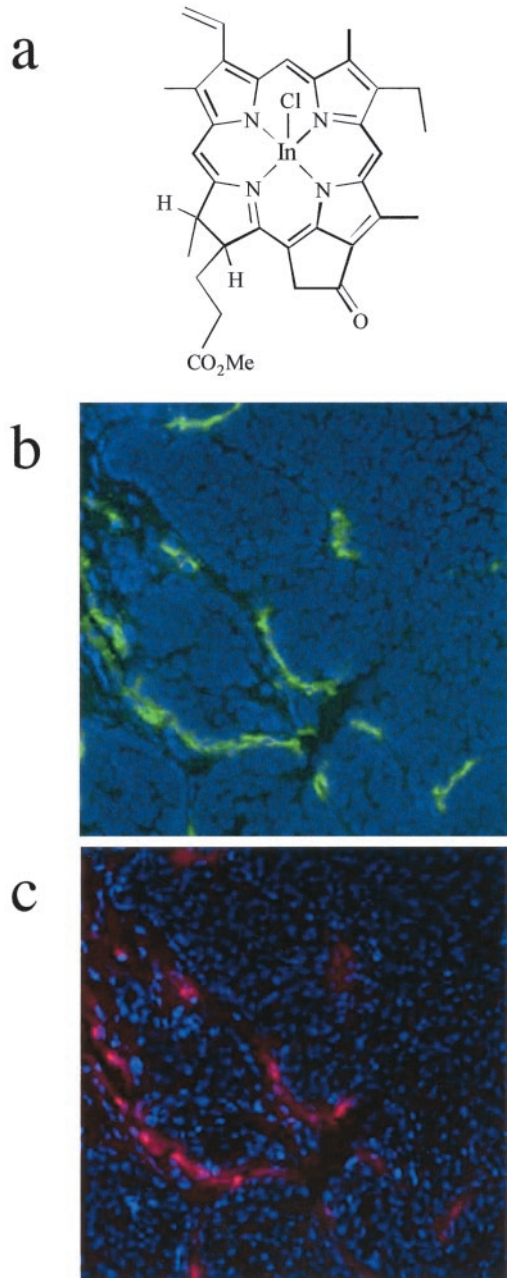

Fig. 1. Structure of MV6401 and localization of MV6401 in MCaIV tumor. *a*, indium, chloro [methyl 9-ethenyl-14-ethyl-4, 8,13,18-tetramethyl-20-oxo-3-phorbinepropanoaot (2-)-N23, N24, N25, N26], [SP-4–2-(3*S*-*trans*)]-(9 CI). This compound is also known as InCh, indium chloride methyl pyropheophorbide, indium methyl pyropheophorbide, and indium methyl pyropheophorbide-a. *b*, color composite image of CD31 staining (*green*) and DAPI staining (*blue*; ×20). *c*, color composite image of MV6401 fluorescence (*red*) and DAPI staining (*blue*) in the same region as *b* (×20).

Vessel perfusion was determined by intravital microscopy, and thrombus formation was evaluated by histological examination.

Pressure Release in the Dorsal Skinfold Chamber. To assess the effect of tumor interstitial pressure increase caused by edema formation after PDT, the coverslip of the chamber was removed, and the skin and the tumor were superfused with physiological saline in a separate set of animals. Alterations in tumor tissue perfusion by PDT were determined with the coverslip removed.

Temperature Measurements in the Dorsal Skinfold Chamber. To determine potential hyperthermic effects on the tumor caused by PDT, the glass coverslip of the chamber was removed, and the temperature of the tumor tissue was measured with a microprobe (BAT-10 Thermometer, serial number 1225; Physitemp, Inc., Clifton, NJ).

Statistical Analysis. All data are expressed as mean ± SE. The percentage of perfused regions was calculated as 100 × the number of regions with flow/number of regions examined in each tumor at each time point. χ^2 tests were performed to compare the proportions. Unpaired *t* tests were used to compare data of tumor size and vascular permeability between different treatments at a given time. Paired *t* tests were used to compare baseline and posttreatment effects within the same group.

RESULTS AND DISCUSSION

MV6401 Localized to the Vascular Compartment during PDT. Because of the extremely short life of ROS formed after PDT, only cells proximal to the area of ROS production will be damaged because of the short half-life of ROS. Hence, we first determined the localization of the sensitizer (9) during PDT by examining sections of the tissue harvested 15 min after MV6401 injection. The tumors in animals that received the EYP as a control did not exhibit detectable fluorescence using the MV6401 filter set (data not shown). Sequential immunohistochemical staining with antibody to CD31 (PECAM) showed that MV6401 always localized proximal to CD31-positive structures, indicating that MV6401 was confined to the vascular compartment (Fig. 1, *b* and *c*). When the drug distribution images were superimposed with DAPI-stained nuclear images, MV6401 was seen only in the vascular space and/or bound to the vascular wall and not in the surrounding tumor tissue. The size of the MV6401 carrier liposomes (EYP; average, 200 nm) might have hindered the extravasation within the immediate postinjection time period. In addition, it is possible that the cationic charge of the vehicle interacts with the negative charge of the glycocalyx of the vascular wall (10). Furthermore, the microvascular permeability measurements for the drug showed no appreciable extravasation within 30 min after the injection of MV6401 (data not shown), supporting the conclusion that MV6401 is localized in the vascular compartment during PDT. However, extravasation of the drug into surrounding tumor tissue was detected at later time points (45 min to 4 h; data not shown).

PDT with MV6401 Induced Tumor Blood Flow Stasis. The blood flow in MCaIV tumors treated with the low dose MV6401 (0.018 mg/kg) was minimally affected (Fig. 2*a*). In contrast, tumors treated with the intermediate (0.036 mg/kg) and high (0.072 mg/kg) drug doses exhibited blood flow stasis in all regions examined, both during and immediately after PDT (Fig. 2*a*). During light administration, vasoconstriction of the s.c. vessels feeding the tumor was observed. The vessels inside the tumors showed stasis without apparent change in their diameter during PDT. After the light administration, blood flow resumed temporarily but was sluggish and intermittent. Tumor perfusion was observed to stop a second time 3 h after PDT. At this point, vasoconstriction was not observed in either peripheral or intratumor vessels. In some cases, tumors appeared white macroscopically, and no vessels were visible microscopically (Fig. 2*c*). In other cases, intratumor hemorrhage was observed; plasma contrast agent (FITC-dextran) extravasated, and the original morphology of the vessels was not recognizable. Previous reports have suggested that vasoconstriction attributable to vasoactive cytokines such as thromboxane A2 and prostaglandins might play a role in this response (11). Nitric oxide might also be involved in this process, because superoxide rapidly interacts with nitric oxide, scavenges its vasodilatory function, and forms additional cytotoxic ROS such as peroxynitrate (12). Tumor vessels are structurally and functionally impaired and as a result do not properly respond to vasoactive stimuli. This may explain why many tumor vessels did not show appreciable change in diameter during PDT, whereas vessels in the periphery and/or feeding those tumors showed significant vasoconstriction. Nevertheless, the detected changes seemed to be sufficient to stop tumor perfusion

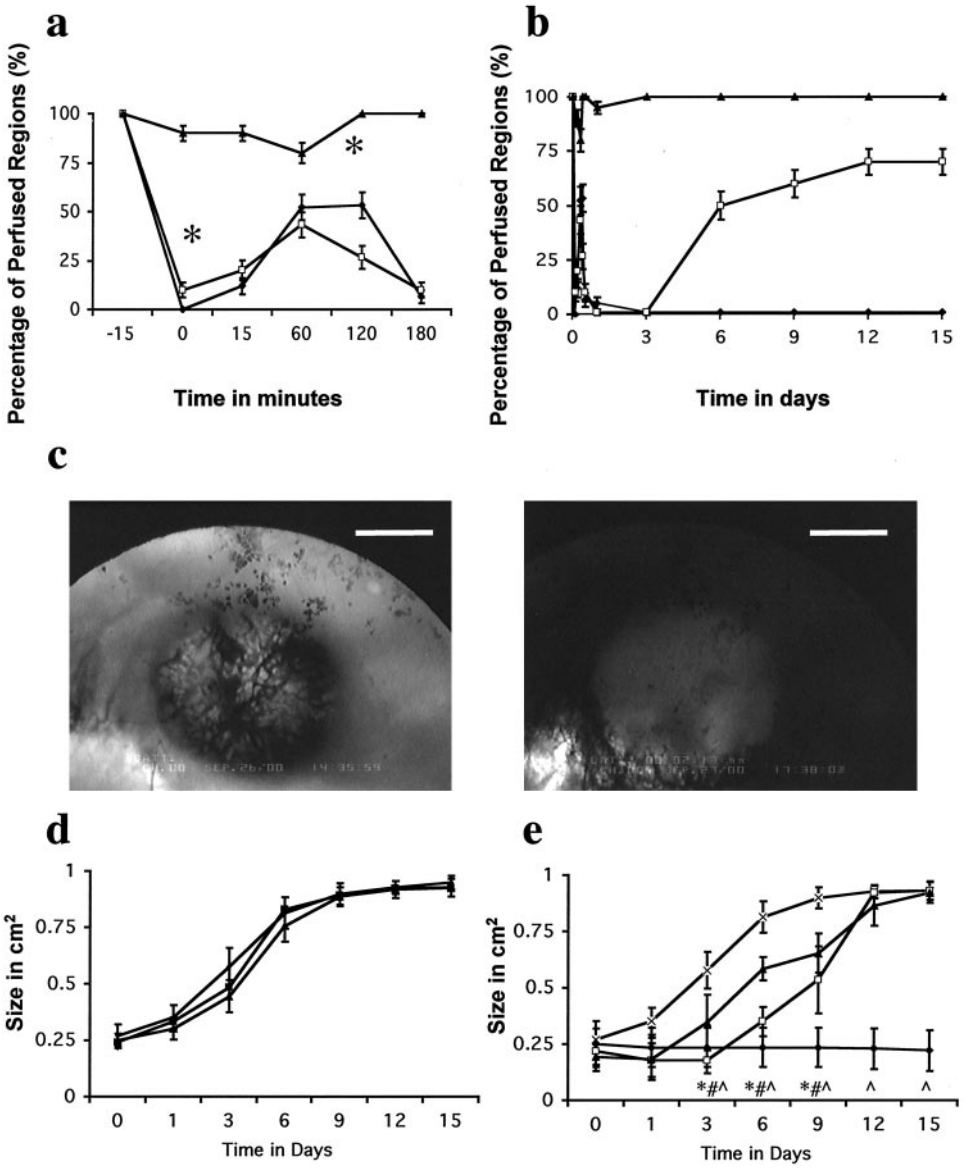

Fig. 2. Effect of PDT on blood vessel perfusion and tumor growth. *a*, acute effect of PDT on tumor vessel perfusion. Percentage of regions (five regions/animal), which exhibit blood flow as determined by intravital microscopy. Data are expressed as means; *bars*, SE. At time = 0, PDT was completed. At time = 0, 15, 60, 120, and 180 min, there is a significant difference between the lowest dose group (▲, $n = 10$) and the two other groups. *, time points with significant differences between the intermediate dose group (□, $n = 10$) and the high drug dose group (◆, $n = 12$). *b*, chronic effect of PDT on tumor vessel perfusion. Data are expressed as means; *bars*, SE. At 3 days after treatment and thereafter, there was blood flow detected in all of the regions in the tumor treated with the low drug dose group (▲, $n = 10$). Up to day 3 after PDT, there was almost no perfusion in both the high drug dose group (◆, $n = 12$) and the intermediate drug dose group (□, $n = 10$). Although the high drug dose group remained unperfused, there was new vessel formation in the intermediate drug dose group, and the tumor recovered perfusion gradually. *c*, representative transillumination images of MCaIV tumor in the dorsal skin chamber before (*left*) and 24 h after (*right*) PDT with the intermediate drug dose. *Bar*, 1 mm. Intratumoral vessels are not visible after the PDT. *d*, effects of drug or light alone on MCaIV tumor growth in the dorsal skin chamber. There is no significant difference between the control animals (◆, $n = 5$), the animals that received light alone (■, $n = 5$) at a fluence rate of 10 J/cm^2, and the animals that received MV6401 alone (▲, $n = 5$) at a dose of 0.144 mg/kg BW. The drug or light was given on day 0. The same dose of either drug alone or light alone did not affect normal dorsal skin vessels. *e*, effects of PDT with various drug doses on MCaIV tumor growth in the dorsal skin chamber. Tumor growth in cm^2 (means; *bars*, SE). Starting at 3 days after PDT, there was a significant difference between the control animals (×; $n = 5$) and the three treatment groups. The high dose group (MV6401, 0.018 mg/kg BW; ▲; $n = 6$) showed complete growth arrest. The intermediate drug dose group (MV6401, 0.036 mg/kg BW; □; $n = 6$) and the low dose group (MV6401, 0.072 mg/kg BW; ◆; $n = 6$) showed significant growth delay. *, #, and ^, time points with statistical significant differences between the control and low dose group, intermediate dose group, and high dose group, respectively ($P < 0.05$). Note that the maximum tumor surface area that can be measured was limited by the size of the dorsal skinfold chamber (0.95 cm^2), and thus, tumor size data in two-dimensions close to that value may not be an accurate representation of three-dimensional tumor volume. Data are expressed as means; *bars*, SE.

entirely. Twenty-four h after PDT, 95, 0, and 5% of the regions were perfused in the low, intermediate, and high drug dose groups, respectively.

PDT Induced Dose-dependent, Long-Term Blood Flow Stasis and Tumor Growth Delay. Vessels in the tumors treated with the low drug dose remained perfused in most of the regions studied and did not change significantly between days 1 and 15 (Fig. 2*b*). However, hemorrhage was also observed between 1 and 3 days in this group. In the intermediate drug dose group, no perfusion was evident in regions studied up to 3 days after PDT. However, active vascular growth was observed after an average of 3 days, and new blood vessels with flow became visible microsopically at the edge of the tumors. At 15 days after PDT, 80% of the examined regions in the tumor were perfused. In contrast, long-term follow-up of the high drug dose group showed complete impairment of blood flow and lack of new vessel formation up to 15 days after treatment.

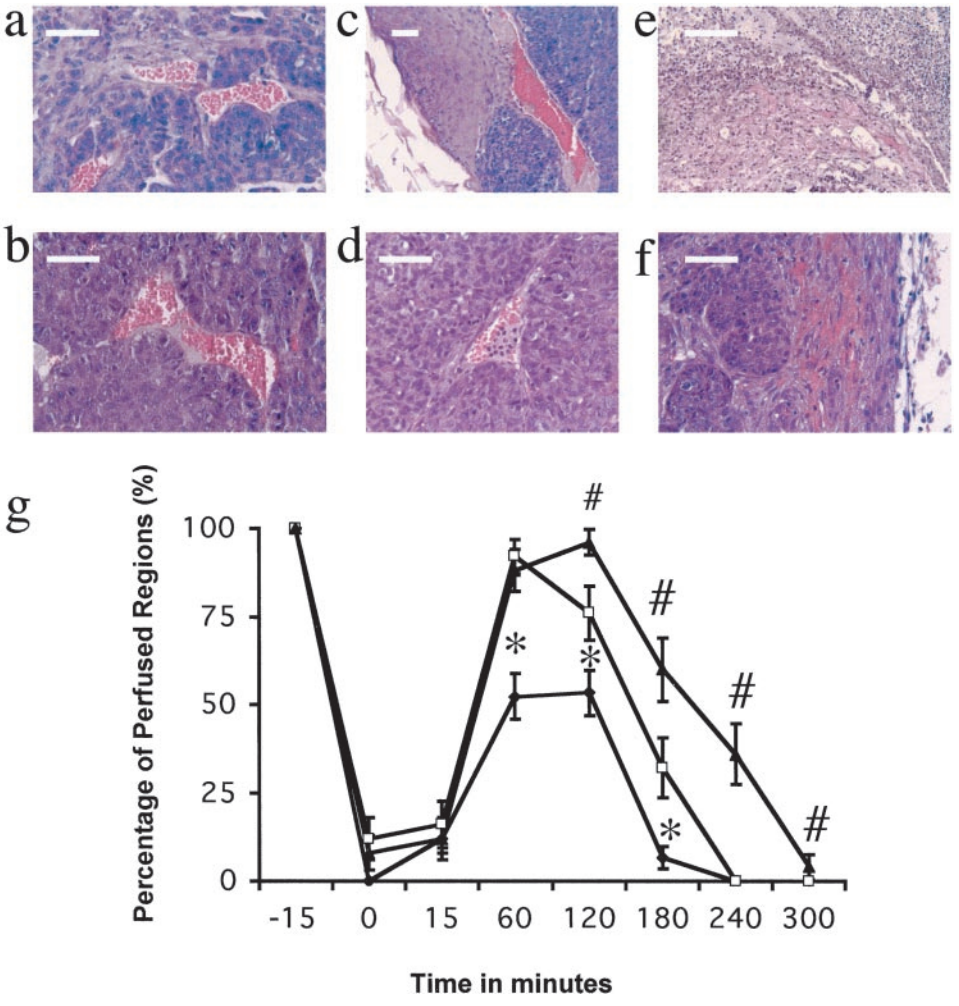

Fig. 3. Thrombus formation and effect of heparin on PDT-induced blood flow shutdown. *a–f*, H&E staining of MCaIV tumors after PDT under high power magnification. *Bar*, 10 μm. *a*, nontreated tumor vessels. *b*, tumor blood vessels 3 h after the intermediate drug dose PDT shows thrombus. *c*, tumor blood vessel 3 h after the high dose PDT shows erythrocyte stasis, which was confirmed by intravital microscopy. *d*, another tumor blood vessel 3 h after PDT with the high drug dose exhibited polymorph neutrophils in thrombus. *e*, tumor showed extensive necrosis 3 days after PDT with the intermediate drug dose. *f*, hemorrhage in the tumor 3 days after PDT with the high drug dose. *g*, effect of heparin on the high drug dose PDT-induced blood flow alterations in MCaIV tumors. All groups completed the high drug dose PDT treatment at $t = 0$. At time points 60, 120, and 180 min, there is significant difference between the MV6401-alone group (◆; $n = 12$) and MV6401 + heparin (100 units given 15 min before light treatment) group (□; $n = 6$). At time points 60, 120, 180, 240, and 300 min, there is a significant difference between the MV6401-alone group and the MV6401 + a double dose of heparin (100 units given 15 min before and 1 h after light treatment) group (▲; $n = 6$). Data are expressed as means; *bars*, SE. *, significant differences between the no-heparin and the single-dose-heparin groups ($P < 0.05$). #, significant differences between the single- and double-dose-heparin groups ($P < 0.05$).

The Duration of Tumor Growth Arrest after PDT Was Also Drug Dose-Dependent and Paralleled Blood Flow Recovery. In the group treated with the low drug dose, tumor regrowth was observed between 1 and 3 days after PDT, and treated tumors became comparable in size to the nontreated tumors by day 12 (Fig. 2*e*). The intermediate drug dose caused tumor growth arrest up to 3 days after treatment, after which growth resumed. At day 12 after PDT, no significant difference in tumor size in treated groups was observed compared with control tumors. In the high drug dose group, tumor growth arrest was observed up to 15 days after the treatment. Photosensitizer or light alone did not affect tumor blood vessels and growth (Fig. 2*d*). Note that antivascular and antitumor effects may be present with other photosensitizers (5, 13), depending on their doses and schedules. However, the effective concentrations of MV6401 were relatively low, indicating the relative potency of MV6401 as a PDT sensitizer.

Normal Blood Vessels Exhibited Stasis at the Highest Dose. To evaluate the effect of PDT on normal tissue, mouse dorsal skinfold chambers without tumors were treated with PDT using MV6401. The normal vessels and tissues were not affected by the low drug dose PDT. During the intermediate drug dose PDT, vasoconstriction and blood flow stasis were observed, but within 15 min after the PDT, blood flow was observed to resume. There were no late effects on blood flow. However, the high drug dose PDT caused relatively long-term blood flow stasis. Twenty-four h after treatment, the blood flow resumed in the larger s.c. vessels (~50 μm) but not in the smaller branches. Tissue edema was observed macroscopically in two of five animals after 24 h and appeared to resolve after 2–3 days, and the skin appeared viable. There was no hemorrhage in the normal tissue after the PDT.

Intravascular Thrombosis and Hemorrhage Are Associated with Vascular Stasis. To discern the mechanism of long-term vascular shutdown after PDT, we performed histological analysis in combination with intravital microscopy. H&E staining of the treated tumor sections revealed thrombus formation as early as 2–3 h after light treatment in both the high drug dose group ($n = 6$) and the intermediate drug dose group ($n = 4$; Fig. 3, *a–d*). Thrombi in the tumor vessels looked similar to prior published descriptions (14). The histological findings are in concert with blood vessel perfusion studies using intravital microscopy performed prior to tissue collection. Aggressive thrombus formation in the tumors with complete blood flow shutdown (high and intermediate drug doses), sporadic thrombus formation in the tumors with minimal blood flow change (low drug dose), and no thrombus in the tumors with no blood flow alteration (control) were observed.

Twenty-four h after PDT, histological analysis of tissue sections revealed patchy areas of tumor cell necrosis (Fig. 3*e*), areas of hemorrhage (Fig. 3*f*), and WBC infiltration. Hemorrhage and necrosis were not observed in the nontreated tumors. At later time points in the high and intermediate dose PDT group, the vasculature appeared disrupted, and fewer vessels were observed per section.

Heparin Abrogated PDT-induced Blood Flow Stasis. To clarify the causal relationship between thrombus formation and blood flow stasis induced by PDT, we prevented thrombus formation by systemic

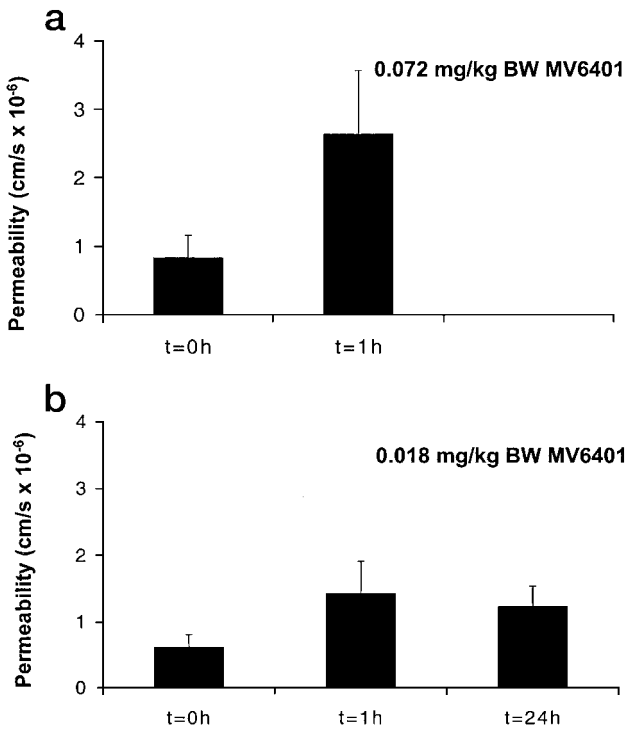

Fig. 4. Effect of PDT on vascular permeability. *a*, effect of the high drug dose PDT on vascular permeability. Vascular permeability in the same regions was measured before and 1 h after PDT using alternative color tracers ($n = 6$). There was a 3-fold increase in permeability after PDT ($P < 0.05$). *b*, effect of the low drug dose PDT on vascular permeability. Vascular permeability was elevated ~2-fold compared with the baseline value at both 1 h ($n = 3$) and 24 h ($n = 6$; $P < 0.05$) after PDT. Note that permeability in the high drug dose PDT group could not be measured at 24 h after treatment because of a lack of functioning vessels. *Bars*, SE.

injection of heparin (100 units i.v., 15 min before high dose PDT). The single dose of heparin did not inhibit the initial blood flow stasis observed immediately after the light administration but increased blood perfusion recovery after the initial event. However, blood flow eventually shut down up to 4 h after PDT (3 h without heparin), similar to the nontreated tumor (Fig. 3*g*). A second heparin dose of 100 units, given at 1 h after PDT, further delayed the time of the vascular shutdown to 5 h. The delay in vascular shutdown with successive heparin treatments supports the hypothesis that thrombus formation plays a key role in the second phase vascular shutdown. The activity of heparin in plasma drops rapidly *in vivo* (biological half life of ~45 min; Ref. 15). In fact, a second dose of heparin given at 1 h after PDT significantly delayed the shutdown but did not completely inhibit the second phase of blood flow stasis. Repeated doses of heparin or the use of long-acting anticoagulants (16) is needed to determine whether it could be possible to further delay or prevent the long-lasting vascular effect by the blockage of thrombus formation. Nevertheless, our data suggest that vasoactive events and thrombus formation play a critical role in the initial and second long-lasting blood flow stasis, respectively.

PDT Increased Vascular Permeability. To evaluate the effect of PDT, tumor vascular permeability was measured before PDT and 1 or 24 h after PDT in the same region. One h after PDT, 2-fold and >3-fold increases in the permeability were observed in the tumors with the low and high drug dose PDT, respectively (Fig. 4). A significant increase in the permeability was present at 24 h after PDT in the low drug dose group. The group treated with the highest dose had no blood flow at that time. The relatively large increase in vascular permeability in the group treated with the high drug dose compared with the group treated with the low dose suggests a dose-response relationship. The increased vascular permeability may play a role in the process of vascular shutdown. The increased microvascular resistance caused by leukocyte-platelet aggregates and interactions with the endothelial cell, mechanical obstruction or collapse of vessels as a result of edema, and blood flow stasis secondary to increased vascular permeability, attributable to a loss of pressure gradient (17), could also lead to blood flow stasis.

Intratumoral Pressure Did Not Contribute to PDT-induced Blood Flow Stasis. Because the tumors in the chamber grew in a confined space between the dorsal skin and the coverslip, it is possible that the tumor interstitial pressure increased because of increased vascular permeability and edema formation after PDT. To test whether interstitial pressure plays a role in the impairment of blood flow, we treated tumors with and without the coverslip and monitored blood flow by intravital microscopy. There was no difference in the blood flow kinetics between the group in which the coverslip was removed ($n = 3$) compared with the group in which the coverslip was in place. These results suggest that intratumoral pressure increase did not play a significant role in vascular shutdown after PDT.

PDT Did Not Increase Temperature Appreciably. PDT may induce hyperthermia and thus affect tumor blood flow and growth. To evaluate thermal effect of our PDT protocol, we monitored tissue temperature during PDT. The tumor tissue temperature increased, ranging between 0.1°C and 0.3°C by the high drug dose PDT ($n = 3$). This increase would not be expected to affect the tumor and tumor vasculature (18).

Combination Therapy. In a clinical setting, it is difficult to perform PDT on only tumor tissue without exposing normal tissue surrounding the tumor. The intermediate dose spares normal tissue. However, neovessel formation and tumor regrowth were observed beginning 3 days after the treatment, suggesting that in the clinical setting, MV6401-mediated PDT may function best in combination with other therapies, such as chemotherapy, radiation therapy, or antiangiogenesis treatment (19). In any case, the combination regimen should be carefully designed to avoid potential adverse effects of antivascular therapy, *i.e.*, poor drug delivery (20). The best combination window with MV6401-mediated PDT would be 1 h after the PDT when the tumor blood flow recovered, allowing maximum drug delivery for a second treatment modality.

In summary, PDT-induced antivascular effects occur rapidly, are tumor vessel selective, and offer potential advantages over various angiogenesis inhibitors or chemotherapy because they produce no systemic toxicity. MV6401-mediated PDT produced selective vascular effects, including vasoconstriction, vascular shutdown and thrombus formation, and tumor growth delay. These effects appeared to be drug dose dependent. Our evidence further suggests that thrombus formation plays an important role in the process of long-term blood flow stasis induced by MV6401-mediated PDT. This study provides mechanistic insights into antitumor vascular effects of PDT and suggests novel strategies for tumor treatment with PDT.

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