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LOCAL PHOTODYNAMIC THERAPY (PDT) OF RAT C6 GLIOMA XENOGRAFTS WITH Pd-BACTERIOPHEOPHORBIDE LEADS TO DECREASED METASTASES AND INCREASE OF ANIMAL CURE COMPARED WITH SURGERY

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Photodynamic therapy (PDT), locally applied to solid C6 rat glioma tumors in the foot of CD1 nude mice, eradicated the primary tumor and also decreased the rate of groin and lung metastases. Pd-Bacteriopheophorbide (Pd-Bpheid), a novel photosensitizer synthesized in our laboratory, was used in our study. The primary lesion in the hind leg was treated by an i.v. injection of 5 mg/kg of Pd-Bpheid and immediate illumination (650–800 nm, 360 J/cm^2). This protocol and the surgical amputation of the leg were compared for local and metastasis responses. Following PDT, hemorrhage, inflammation with tumor necrosis and flattening were observed and histologically verified in the photodynamically treated tumor. Whereas local tumor control rates were up to 64% following PDT, in surgically treated animals, local tumor control was absolute. The rates of metastases in the groin and the lungs were at least 12-fold lower in the photodynamically treated animals compared with untreated or surgery-treated groups. The overall cure rates after PDT or surgery were 36% and 6%, respectively, at 8 weeks. These findings suggest that local PDT with Pd-Bpheid, which acts primarily on the tumor vasculature, efficiently eradicates the solid C6 tumors. In addition, the local PDT of the primary lesion has beneficial therapeutic effects on remote C6 metastasis, which is not obtained with surgery. It is therefore suggested, that although surgery is highly efficient for the immediate removal of the primary tumor, it lacks such systemic, therapeutic effects on distant metastases. Pd-Bpheid-PDT may thus offer a potentially superior curative therapy for C6 glioma tumors in the limb by eradicating the target tumor and by reducing the rate of metastasis in the groin and lung, possibly due to innate immunity.

Key words: *photodynamic therapy; surgery; Pd-bacteriopheophorbide; C6 glioma tumor; metastasis*

Photodynamic therapy (PDT) is based on the destruction of tumors by cytotoxic reactive oxygen species (ROS) produced upon local tumor illumination in patients administered with a photosensitizer.[1–3] Following health agency approval for photofrin- based PDT in many countries, this anti-cancer treatment modality entered clinical use for the local treatment of an increasing number of indications including skin, esophageal, lung, gastric, cervical and bladder cancers.[4]

PDT is usually considered a local anti-tumor treatment modality. However, reports from several laboratories suggest that PDT also induces beneficial systemic effects. Following *in vitro* hematoporphyrin-based PDT, adhesiveness and metastatic potential decline in DHD-K12-cultured colon carcinoma cells. Moreover, intravenous or s.c. injection of these PDT-treated cells to rats resulted in a reduced number of lung metastases compared with untreated cell injection.[5,6] Although this observation may be due to local photodynamic damage, the potential beneficial effect may be viewed as systemic. Other *in vivo* studies showed that local PDT with various photosensitizers mediates long-term tumor immunity and resistance to tumor rechallenge. It was shown that the activation of natural killer (NK) cells and T lymphocytes by PDT, along with induction of specific anti-tumor antibodies,[7] play a major role in the curative response.[8,9] Moreover, adoptive transfer of lymphocytes, which were tumor-sensitized by photofrin-based PDT in immunocompetent mice, improved the tumor response of SCID mice to PDT.[9] In addition, it was shown that neutrophils are indispensable for successful PDT. Moreover, PDT of neutropenic mice (treated with rabbit anti-rat neutrophil serum) did not retard tumor growth relative to controls.[10,11] Other studies investigated the effect of local *in vivo* PDT on distant metastasis.[12,13] One study reported a decrease and the other reported an increase in metastasis compared with surgery.

We have recently developed a new line of highly efficient photosensitizers based on bacteriochlorophyll (A. Brandis *et al.*, 2001; private communication).[14,15] The first in this series bacteriochlorophyll-Serine (Bchl-Ser) was found to be phototoxic in cultured melanoma cells with an LD_{50} value of 100 nM. This value is about 100 times lower than that of the hematoporphyrin derivative Photosan.[16,17] When tested on melanoma xenografts in mice[18–20] and DS sarcoma in rats,[21] over 80% of the tumors were cured after a single PDT session. The anti-tumor effects of Bchl-Ser using a protocol with no injection-illumination time interval were ascribed mainly to the anti-vascular action of this treatment modality.[20] When tested under the same conditions in the same cells,[16] the new derivative used here, Pd-Bacteriopheophorbide (Pd-Bpheid) (Fig. 1) shows about 1,000-fold higher phototoxicity than Photosan and greater anti-tumor efficiency than Bchl-Ser.[15,22]

The high efficiency of these new sensitizers as PDT agents prompted us to examine their local and systemic effects on distant metastases. In the present report, we investigated the local control of C6 glioma tumors and the therapeutic effects on distant metastases (groin and lung) following Pd-Bpheid based PDT.

MATERIAL AND METHODS

Materials

DMEM/F12, Fetal calf serum and Glutamine were from Biological Industries (Kibbutz Beit Haemek, Israel). Phosphate-buffered saline (PBS) was from Sigma Chemical Co. (Israel). Penicil-

Abbreviations: Bchl-ser, Bacteriochlorophyll-serine; NK, natural killer cell; Pd-Bpheid, Palladium-bacteriopheophorbide; PDT, photodynamic therapy; ROS, reactive oxygen species.

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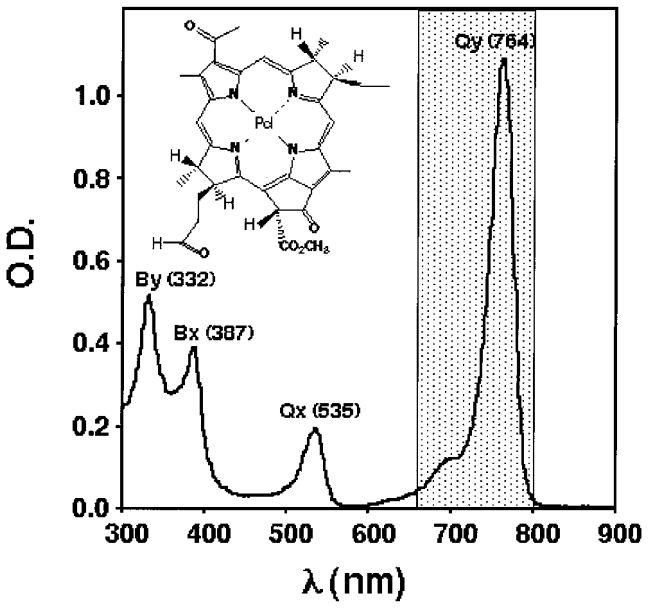

FIGURE 1 – Pd-Bpheid: Absorption spectrum, chemical formula and spectral window for PDT. The absorption spectrum (300–900 nm) in chloroform and chemical formula of Pd-Bpheid (insert) are shown. Also indicated are the major transition bands (By, Bx, Qx and Qy) and their respective maximal absorption. The spectral window used in the PDT protocol (650–800 nm) is indicated by dotted square.

lin-streptomycin was from Bio-Lab laboratories (Jerusalem, Israel). Ketamine was from Rhone Merieux (Lyon, France). Xylazine was from Bayer (Leverkusen, Germany). Oxycodone was from Rafa (Israel).

Cell culture

C6 rat glioma cells were cultured as monolayers in DMEM/F12 supplemented with glutamine, penicillin-streptomycin and 10% fetal calf serum. Cells were cultured at 37°C in an atmosphere of 5% CO_2 and passages were made every 3–4 days.

Animals

CD1 male nude mice, 6 weeks old (28–32 g), were supplied from the pathogen-free colony of the Experimental Animal Unit of the Weizmann Institute, Rehovot, Israel.

Tumor model

Cultured C6 cells (1×10^6/0.02 ml) collected in PBS were s.c.-injected in the distal dorsal foot of the hindleg of anesthetized mice. When the needle was injected below the skin, the handle of the syringe was withdrawn to verify that it had not penetrated a blood vessel. In this way, systemic dissemination of tumor cells was avoided. Tumors grew to a size of 6–8 mm (the treatment size) within 3 weeks. Tumors ($\phi = 7$ mm) located at least 3 mm distal to the ankle were used in these experiments. Animals were euthanized upon tumor burden (size $\phi > 15$ mm, ulceration and bleeding) or large groin metastasis (increasing animal weight, beyond 110% the weight at treatment). All animal procedures were approved by the Institutional Subcommittee on Research for Animal Care.

Anesthesia

Mice were anesthetized by i.p. injection of Ketamine (5 mg/kg) and Xylazine (1 mg/kg, 40 μl) 3 min before treatment. This dose was sufficient to keep the animal anesthetized for about 40 min.

Analgesia

Oxycodone (12mg/liter) in 5% sucrose was provided as drinking water immediately after treatment to the untreated control, PDT and surgery groups, for 1 week.

Euthanization

Animals were euthanized by i.p. injection of Ketamine (15 mg/kg) and Xylazine (3 mg/kg, 120 μl).

Sensitizer

Pd-Bpheid (A. Brandis *et al*., 2001; personal communication)[15] (Fig. 1), which was synthesized in our laboratory, was dissolved in 10 ml of a Cremophor based vehicle by sonication. The resulting solution was centrifuged at 13,900*g* for 1 min in an Eppendorf centrifuge and the supernatant retained. Sensitizer concentrations were calculated assuming $\varepsilon = 3.5 \times 10^4$ ($\lambda = 762$ nm) using water-diluted samples. The sensitizer solution was stored in the dark at room temperature and used over several days with occasional spectroscopic examination for purity.

Light source

A xenon LS3-PDT lamp (Biospec, Russia) with a 650–800 nm spectral window and water filter was used. Light intensity delivered via optical fibers was measured before every experiment using a power meter (Ophir Optronics, Israel). A light power density of 150 or 200 mW/cm^2 was used. Aside from the tumor treatment site, the treated animal was protected from light with aluminum foil.

Photodynamic treatment

The anesthetized mouse was placed in a home-built chamber and the sensitizer (Pd-Bpheid, 5 mg/kg in ~0.1 ml Cremophor vehicle) was i.v. injected (bolus). The tumor ($\phi = 7$ mm) was immediately illuminated (light spot diameter 1.1 cm) for 30 min as described previously.[20] The treatment dose rates were 150–200 mW/cm^2 equivalent to 270–360 J/cm^2. During illumination the skin temperature at the illuminated site was elevated by no more than 1°C as determined with an electronic thermometer (Newtron, TM-5005-Single I/P, Taiwan).

Parameters of response to PDT

Tumor necrosis and flattening were used to indicate local response. The cure of the animals (no tumor and no metastasis) was monitored for 8 weeks. Tumors were photographed with a MX-2900 zoom Fujifilm digital camera, before and at different times after PDT.

Surgery

After anesthesia, the tumor-bearing leg of the mouse was tightly ligated with 3-0 silk, 1–2 mm above the ankle joint. Amputation through the ankle, in a mid-articular plane, was then performed. Tumor cell dissemination during surgery was avoided, by amputating only after blood vessel ligation. Subsequently, an analgesic was given for 1 week.

Groin metastases

Metastases in the groin were inspected visually and by palpation. Metastases were photographed as above and histologically confirmed as described below.

Lung metastases

At different times following treatment, the animals were euthanized and the lungs were removed. Lung metastases were macroscopically detected as white colonies, projecting from the pink lung surface. Metastases measuring over 0.5 mm in diameter were counted and considered significant. For fixation, the lungs were immersed in Bouin's solution for 4 days. Lung surface metastases were photographed and histologically confirmed as described below.

Histology

Tumors and groin metastases were dissected. The samples included the skin, subcutaneous tissue and the tumor mass. First, the samples were fixed in 4% formaldehyde in phosphate buffer, pH 7.4 and embedded in paraffin. Next, the paraffin blocks were cut (4–5 μm thick) through the skin in a midtangential plane. Finally, the specimens were stained with hematoxylin and eosin (H&E)

and micrographs were taken using an Olympus AX-70 microscope (Olympus) equipped with a Leaf MicroLumina camera (Scitex, Hertzelia, Israel).

Statistical analysis

The results were evaluated using unpaired 1-tailed t tests and 1-tailed tests of proportions. A p-value <0.05 was considered statistically significant.

RESULTS

Rat C6 glioma metastatic tumor model

Although rat C6 glioma variants are mostly nonmetastatic, it was of interest that the specific cell line that we used shows an appreciable frequency of metastases when injected into the mouse foot. Therefore, we first characterized this C6 glioma tumor model, in particular the metastasis formation. Briefly, C6 cells were injected into the hindleg of the mice with a tumor-take of 100% ($n=48$). The primary tumors reached a diameter of 6–8 mm within 3 weeks ($n=20$), 10–12 mm by 5 weeks ($n=12$) and 13–15 mm at 7 weeks ($n=16$). Macroscopic metastases to the groin appeared by 3 weeks as a protruding mass that reached a diameter of 5–10 mm at 7 weeks (Fig. 2*a*). Macroscopic metastases to the lungs appeared at 5 weeks as projecting white colonies on the pink lung surface. At 7 weeks, these colonies (1–10/lung) measured 0.5–5 mm in diameter (Fig. 2*b*). The frequency of metastases at this time was 62% in the groin and 50% in the lung. All metastases were confirmed by histological examination of the groin (Fig. 2*c*) and lung (Fig. 2*d*).

Local response of C6 glioma xenograft to Pd-Bpheid-PDT

We next determined the local response of the C6 xenografts to Pd-BP-PDT. Tumor-bearing mice were injected with 5 mg/kg Pd-BP and illuminated at light doses of 270 (group 1, $n=8$) or 360 J/cm^2 (group 2, $n=11$). Tumor response was monitored for 5 weeks (Fig. 3*a*). Inflammation and necrosis developed over 2–5 days with ultimate tumor flattening by days 7–10 at the 2 light doses used. No macroscopic tumors were seen between 16–35 days in the animal group 2, where most wounds healed and the local cure rate was 64%. In contrast, recurrences developed in all animals in group 1 around day 18 (Fig. 3*a*). Tumors in this and the untreated control (group 3, $n=16$) were allowed to grow to the size limit at which time the animals were euthanized. One example of local tumor cure followed for 142 days is illustrated in Figure 3*b*. An example of a tumor (13 mm Φ) in an untreated animal at 7 weeks after C6 cell injection is shown in Figure 2*a*.

Histology of C6 tumors after Pd-Bpheid-PDT

Pd-Bpheid-PDT-induced damage to tumor and blood vessels was examined by histology 3, 24 and 48 hr after treatment using 3

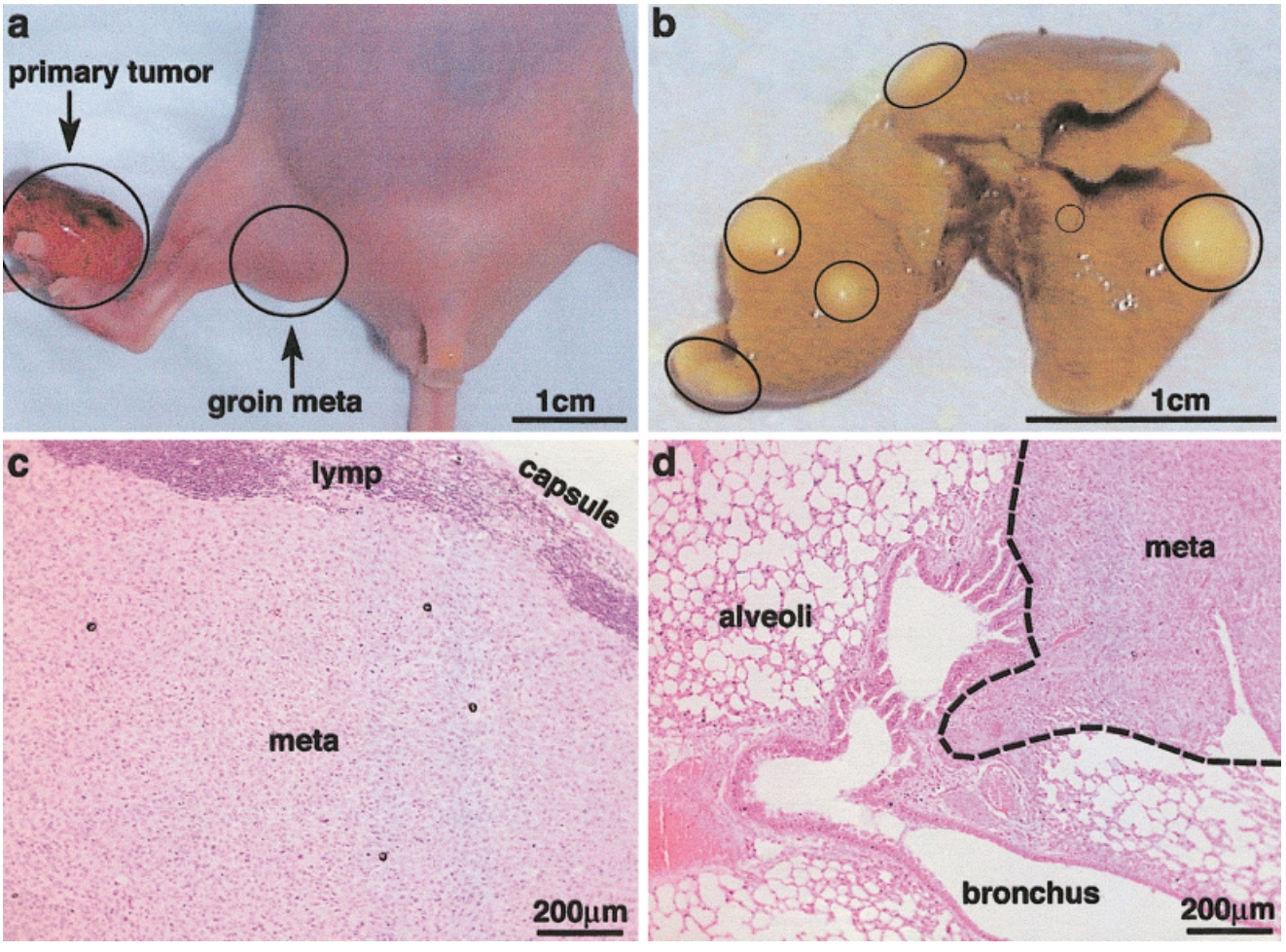

Figure 2 – C6 rat glioma xenograft: primary tumor, groin and lung metastases. The CD1 nude mouse is shown 7 weeks after C6 tumor cell injection. (*a*) The primary tumor (13 mm Φ) and the groin metastasis (10 mm Φ, encircled) are shown. (*b*) The mouse was sacrificed and the lungs removed and embedded in Bouin's solution for 4 days and then photographed. The lung surface metastases are encircled in black. (*c*) Histology: lymph node metastasis (groin). (*d*) Histology: lung metastases. Meta, metastasis; lymp, lymphocytes.

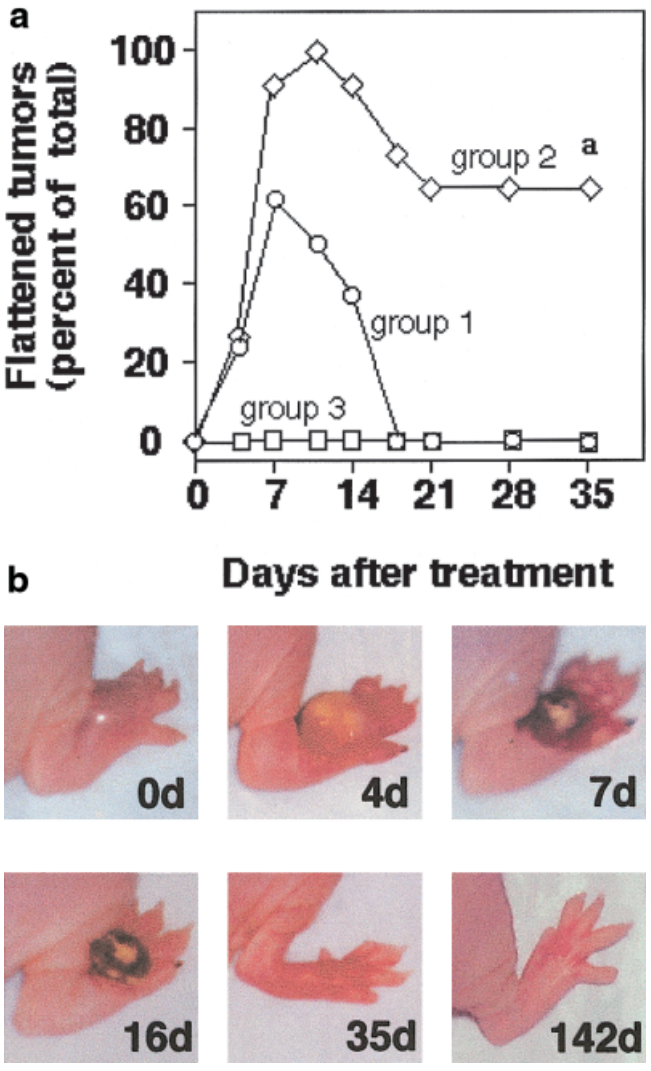

FIGURE 3 – Local response of rat C6 xenograft to PDT. (*a*) Mice with C6 glioma xenografts (ϕ =7 mm) in the hindleg were treated with 5 mg/kg Pd-Bpheid and illuminated at 270 J/cm^2 (group 1, n=8, open circles) or 360 J/cm^2 (group 2, n=11, open diamonds) or untreated (group 3, n=16, open squares). The incidence of flat tumors as a function of time after PDT is shown. $^a p<0.05$ by 1-tailed test for proportions, testing P_c= Pt *vs*. $P_c<P_t$, where P_c = proportion for untreated controls and P_t = proportion for treated subjects. All other details were as described in Material and Methods. (*b*) The clinical course of events in a tumor bearing animal (group 2) before and after PDT is presented. Photographs at day of treatment (0 day) and 4, 7, 16, 35 and 142 days after PDT are shown. By day 4, partial necrosis was seen. By day 7, tumor flattening was observed with an eschar covering the wound. By day 35, the wound healed and the animal was cured as seen by day 142.

animals per time point (Fig. 4). The histology of the C6 tumor before treatment is shown in Figure 4 (untreated). Three hours after treatment (Fig. 4, 3 hr), most tumor blood vessels were congested but intact; however, a few were mildly damaged, resulting in mild multifocal hemorrhage. Most of the tumor cells were not histologically damaged. The small number of damaged tumor cells were superficial cells adjacent to the light source. Mild, subcutaneous infiltration, including perivascular cuffing, consisted mainly of neutrophils and some round cells were seen adjacent to the tumor. Perivascular cuffing was also noticed within the tumor mass. Twenty-four hours post PDT, numerous blood vessels were damaged, causing severe hemorrhage and tumor necrosis. At this stage, the inflammatory reaction was moderate (Fig. 4, 24 hr). Forty-eight hours after PDT, necrosis was widespread and included most of the tumor (Fig. 4, 48 hr). At this time individual blood vessels, tumor and inflammatory cells were hardly discernible. These results suggest that the blood vessels are a primary target of Pd-Bpheid-PDT and that PDT initiates the inflammatory response.

The response of distant metastasis to local Pd-Bpheid-PDT of the primary tumor

To evaluate whether PDT has a systemic effect on distant metastasis, we compared the frequency of groin and lung metastases in 4 groups of animals. The experiment included 3 experimental groups described above: PDT with 5 mg/kg Pd-Bpheid using a light dose of 270 (group 1, n=8), or 360 J/cm^2 (group 2, n=11) or untreated (group 3, n=16). An additional group was surgically treated (group 4, n=16) and the tumor-bearing foot was amputated.

Groin metastases. The appearance of groin metastases was first observed on day 4 in the untreated and surgically treated animal groups. In both Pd-Bpheid-PDT groups, groin metastasis appeared by day 18 (Fig. 5). The incidence of metastasis in the untreated and surgically treated animal groups increased with time to include the majority of the animals (62%) and (88%), respectively, by day 35. In contrast, the incidence of metastases remained unchanged (25–27%) in both PDT groups from 21–35 days. These results imply that local PDT leads to a delay in the appearance of and a decrease in the incidence of groin metastasis, compared with both surgery and no treatment.

Lung metastases. The average number of lung metastases per treated animal on day 35 is shown in Table I. The mean number of lung metastasis in untreated animals (2.12) was >2.5-fold higher than in surgically treated animals (0.81) and >10-fold higher than in animals that underwent Pd-Bpheid-PDT (0–0.18). The frequencies of lung metastases were the lowest following PDT (Table I). As with groin metastases, the lowest incidence in the number of distant lung metastasis was seen in both PDT groups, with either a partial or complete local tumor response. However, in this case surgical treatment was beneficial compared with untreated animals.

Animal cure. Spontaneous cure (no tumor and no metastasis) was never seen in untreated animals. After surgical amputation of the tumor-bearing foot there was obviously complete control of the primary tumor. However, most animals developed metastasis by 8 weeks and only 1/16 (6%) was cured. PDT with 360 J/cm^2 led to the cure of 4/11 animals (36%), whereas use of a lower light dose (270 J/cm^2) led to no cure since local tumor control did not last beyond day 18 (Fig. 3*a*). The results presented in Table I show that by providing local and distant tumor control and cure, PDT appears to be superior to surgical treatment.

DISCUSSION

Our study demonstrates that local Pd-Bpheid-based PDT is efficient for both local and systemic control of C6 glioma xenografts in mice. Using Pd-Bpheid-PDT, the success rate of tumor flattening in the foot was 64% (Fig. 3). Pd-Bpheid-PDT of the C6 glioma tumor model appears to be based on vascular destruction (Fig. 4), similar to Bchl-Ser-based PDT of mouse melanoma.[20]

PDT is generally considered a local treatment modality; however, our results demonstrate that treatment of a C6 xenograft in the foot of the mouse with Pd-Bpheid and light had a beneficial effect on distant metastases. Pd-Bpheid-PDT reduced the rate of groin metastases by approximately 2-fold (from 62 to 25–27%) and lung metastases by at least 12-fold (from 2.1 to 0–0.18/mouse) and provided an overall cure of 36% compared with untreated controls where no cure was observed. In spite of the high short-term effectiveness of surgery, the results of this treatment were inferior to PDT, with only 6% overall cure. Groin metastases after surgery were observed in most of the mice (88%) at a slightly higher rate than that seen in the untreated group (62%). This

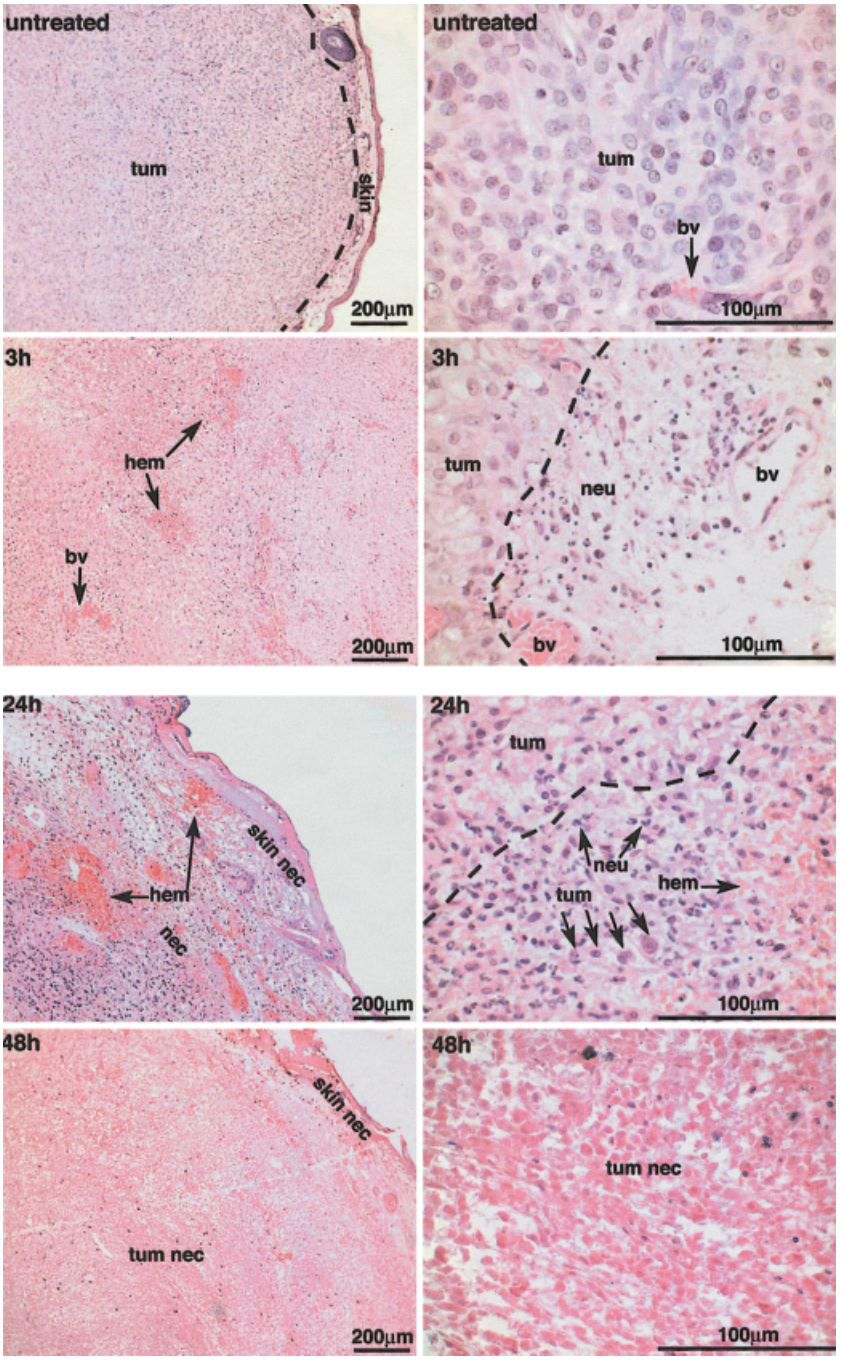

FIGURE 4 – Histology of C6 tumors after Pd-Bpheid-PDT. C6 glioma xenografts (ϕ=7 mm) in the hindleg were treated with 5 mg/kg Pd-Bpheid and illuminated at 360 J/cm^2. Tumors were harvested before and 3, 24 and 48 hr after treatment, fixed and prepared for histological examination. Micrographs at 2 magnifications of each time point are presented. bv-blood vessel, hem-hemorrhage, nec-necrosis, neu- neutrophils, tum-tumor. All other details were as described in Material and Methods.

apparent increase is however not statistically significant ($p>0.05$). In comparison to untreated control, the rate of lung metastases in the surgery group was 2.5-fold lower (0.8/mouse), but this difference was also not statistically significant ($p>0.05$). Interestingly, reducing the light intensity by 25% to 270 J/cm^2, induced a similar response in the distant metastasis (Fig. 5, Table I), whereas the local response was only temporal with 100% regrowth (Fig. 3*a*).

Changes in tumor-host relationship, including the immune state of the host, tumor angiogenesis and tumor cell adhesion, may either encourage or prevent the development of microscopic metastases to clinical manifestation.

The effect of local PDT on distant metastases was reported in only a handful of cases with quite varying results. A decreased rate of lung metastases relative to surgery or no treatment, following Photofrin II-based PDT of s.c. Lewis lung carcinoma in C57BL/6 mice was reported by Gomer.[12] Similarly, PDT with indocyanin green of s.c. DMBA-4 mammary tumors in Wistar Furth rats decreased the volume and the number of cases of axillary and inguinal metastasis, compared with no treatment.[7] In contrast, Momma and Hasan[13] showed that BPD-based PDT of R3327-MatLyLu prostate carcinoma in Dunning rats increased the number of lung metastasis (but not the frequency of animals with metastasis) compared with both untreated and surgically treated groups. The differences between the reported results and the results of our study may be due to differences in tumor type, site of implantation, animal species, PDT protocol and surgical techniques used.

Recent studies showed that PDT of sarcomas with 2-iodo-5-ethylamino-9-diethylaminobenzo(α)-phenothiazinium chloride[8] or

photofrin[9] induce long-term tumor immunity. Moreover, PDT cured the tumor only in immunocompetent but not in severely combined immunodeficient mice. It was also shown that activation of natural killer cells (NK) and lymphocytes by PDT plays a major role in the curative response. Induction of long-term tumor resistance and the production of specific tumor antibodies in response to PDT and imuno-adjuvant were also reported.[7] A rational connection between PDT and tumor immunity can be supported by fact that in order to initiate an immune response, dendritic cells, the most potent antigen-presenting cells, must be activated by endogenous signals like those expressed by stressed necrotic cells or damaged blood vessels. They then obtain the capacity to activate an antigen-specific T-cell response. Such responses are not induced by healthy or apoptotic cells. Thus, PDT of tumor tissue may create just the right set of conditions needed for the induction of tumor immunity.[23]

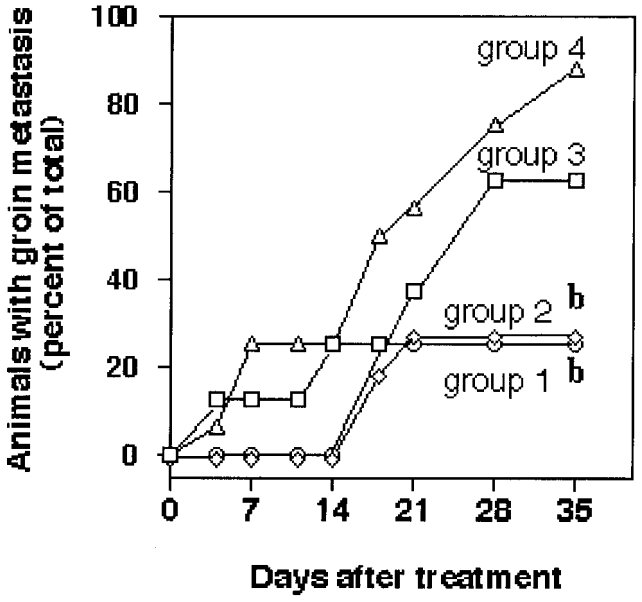

FIGURE 5 – Time course of the appearance of C6 groin metastasis after local PDT or surgical amputation of the tumor bearing leg. The appearance of groin metastases in the animal groups described in Figure 3*a* was monitored at different times after beginning of the experiment. The experimental groups were either treated with Pd-Bpheid and illuminated at 270 J/cm^2 (group 1, $n=8$ open circle), or 360 J/cm^2 (group 2, $n=11$, open diamond) or untreated controls (group 3, $n=16$, open squares) or surgically treated (group 4, $n=16$, triangles). Groin metastases were visually inspected and then verified by pathological examination and the percentage of animals, out of the total treated animals, with metastasis in the groin was calculated. Control untreated tumors continued to grow and the animals had to be sacrificed 5 weeks after beginning of the experiment according to animal welfare regulations. $^b p<0.05$ by 1-tailed test for proportions, testing $P_c = P_t$ *vs.* $P_c > P_t$, where P_c = proportion for untreated controls and P_t = proportion for treated subjects. Using the same test for comparing surgery to untreated resulted in $p>0.05$. All other details were as described in Material and Methods.

Although CD1 nude mice are deficient in T cells, NK activation against C6 tumors and the induction of tumor-specific antibodies by Pd-Bpheid-PDT are possible explanations for the observed decrease in metastasis development (Fig. 5, Table I).

Still another explanation to the lower metastasis incidence after PDT may be related to changes in tumor cell adhesion. Colon cancer cells treated *in vitro* by PDT loosely adhere to endothelial cells and induce a smaller number of metastasis when injected i.v.[5] or s.c.,[6] as compared with untreated cells.

The fact that C6 metastasis appeared above the tumor-bearing foot, following amputation, suggests that spontaneous microscopic dissemination had occurred, before surgery. It has been reported in the literature that surgery may cause immunosuppression,[24,25] consistent with an increase in the rate of metastases. However, following surgery in this model, the rates of metastases in the lung and groin were lower and higher, respectively, compared with untreated control but these differences were not statistically significant. We therefore conclude that immunosuppression seems not to play an important role in this model (Fig. 5, Table I). The methodological approach of simultaneously recording metastatic rates in 2 anatomical locations that represent different target tissues further strengthens this claim.

The development of tumors and metastases depends on angiogenesis.[26–28] Angiostatic substances (like endostatin or angiostatin) circulate in tumor-bearing animals and suppress metastasis.[26,29–31] Eradication of the primary tumor may therefore change the balance between angiostatic and angiogenic substances. Consequently, after surgical removal of the primary tumor, when the levels of such putative substances abruptly fall, metastasis can grow and become apparent. However, in this C6 model, the arguments that stand against the significance of immunosuppression also put in question the importance of the removal of putative angiostatic factors by surgery. The possible influence of PDT on the balance of angiostatic and angiogenic substances has, to the best of our knowledge, not been reported. In this respect, it is interesting to note that local X-ray irradiation of FSA-II tumors is followed by an increase in endostatin[32] and the suppression of angiogenesis at a distal site.[33]

Although systemic effects may be engraved in clinically used PDT, the basis of these observations and their generality with respect to sensitizer type, treatment protocol and the tumor type are not clear. Future experiments with immunocompetent animals will enable the elucidation of the mechanistic basis and possible clinical relevance of the described observations.

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TABLE I – LOCAL AND DISTANT TUMOR RESPONSE TO PDT AND SURGICAL AMPUTATION

Treatment	Number of mice, *n*	Local tumor control (day 35, %)	Frequency groin metastasis (day 35, %)	Frequency lung metastasis (day 35, %)	Mean lung metastasis per animal, $n \pm$ SE (day 35, %)	Cure (8 weeks, %)
None	16	0	62	50	2.12 ± 0.72	0
Amputation	16	100^1	88	25	0.81 ± 0.43	6
Pd-Bpheid 270 J/cm^2	8	0	25^2	0^2	0^3	0
Pd-Bpheid 360 J/cm^2	11	64^1	27^2	9^2	0.18 ± 0.18^3	36^1

$^1p < 0.05$ by 1-tailed test for proportions, testing $P_c = P_t$ *vs.* $P_c < P_t$, where P_c = proportion for untreated controls and P_t = proportion for treated subjects.–$^2p < 0.05$ by one tailed test for proportions, testing $P_c = P_t$ *vs.* $P_c > P_t$, where P_c = proportion for untreated controls and P_t = proportion for treated subjects.–$^3p < 0.05$ by an unpaired one tailed t test for $M_c = M_t$ *vs.* $M_c > M_t$, where M_c, M_t represent the expected number of lung metastasis per animal in control and treated groups, respectively.

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