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Photodynamic Therapy of Newly Implanted Glioma Cells in the Rat Brain

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Background and Objective: A syngeneic rat brain tumor model is used to investigate the effects of aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) on small clusters of tumor cells sequestered in normal brain.

Study Design/Materials and Methods: Biodistribution studies on tumor-bearing animals were undertaken in order to determine the occurrence of photosensitizer in tumor cells invading normal brain. ALA–PDT toxicity in normal brain and gross tumor were evaluated from histopathology. Effects of PDT on isolated glioma cells in normal brain were investigated by treating animals 48 hours after tumor cell implantation.

Results: Fluorescence microscopy of frozen tissue sections showed that photosensitizer content was limited and variable in tumor tissue invading normal brain. ALA–PDT with high light doses resulted in significant damage to both gross tumor and normal brain, however, the treatment failed to prolong survival of animals with newly implanted glioma cells. In contrast, animals inoculated with tumor cells pre-incubated in vitro with ALA showed a significant survival advantage in response to PDT.

Conclusion: The results show that ALA–PDT could not prevent tumors from forming if treatment was performed shortly after tumor initiation. This was likely due to inadequate levels of ALA/PpIX in the glioma cells. Lasers Surg. Med. 38:540–548, 2006. © 2006 Wiley-Liss, Inc.

Key words: aminolevulinic acid; BD-IX rat; brain tumor; glioblastoma multiforme

INTRODUCTION

The extent of surgical tumor resection is an important prognostic factor for patients with malignant gliomas such as glioblastoma multiforme (GBM) [1,2]. Unfortunately, even in cases of gross tumor resection (as determined from post-operative MRI), the tumor recurs with high frequency. This is likely due to the migratory behavior of glioma cells–a trait attributed to their developmental character within the CNS. Both individual cells and micro-colonies of tumor cells have been shown to infiltrate in or beyond a region of brain-adjacent-to-tumor (BAT)—a zone that may extend several cm from the resection margin. In approximately 80% of all cases, recurrent tumor growth occurs within a 2–3 cm margin of the surgical resection cavity [3].

Eradication of infiltrating glioma cells poses a significant clinical challenge that is unlikely to be solved using conventional treatment regimens consisting of ionizing radiation and chemotherapeutic agents. This is due to a number of reasons. First, migratory cells in and beyond the BAT are protected to varying degrees by the blood–brain barrier (BBB), which acts as a formidable barrier against systemic delivery of chemotherapeutic agents. Second, even if the BBB can be circumvented, for example, by employing implantable polymer wafers impregnated with drugs, selective uptake may not occur due to decreased proliferation rates of migrating glioma cells [4]. This characteristic, in combination with their reduced ability to undergo apoptosis, would presumably make these cells resistant, not only to chemotherapeutic agents, but also to ionizing radiation and would explain the disappointing results obtained with aggressive focal treatments including brachytherapy [5,6] and the selective delivery of chemotherapeutic agents or radiation [7–9].

Photodynamic therapy (PDT) is a local form of treatment involving the administration of a tumor-localizing photosensitizing drug that is activated by light of a specific wavelength [10]. This therapy results in a series of photochemical and photobiological events that cause

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irreversible damage to tissues. The aim of PDT in neuro-oncology is to eliminate the nests of tumor cells remaining in the BAT region following surgical removal of bulk tumor while minimizing damage to the surrounding brain tissue. A number of clinical studies have been attempted to investigate the ability of PDT with first-generation photosensitizers to eliminate malignant glioma cells beyond the resection margin [11,12]. The rather disappointing results of these studies have provided the impetus to evaluate the efficacy of several so-called second-generation photosensitizers and prodrugs, such as ALA, for use in PDT of gliomas. In ALA-induced endogenous photosensitization, the heme biosynthetic pathway is used to produce protoporphyrin IX (PpIX)—a photosensitizer that may prove effective for the treatment of brain tumors [13–16]. The primary rationale for using ALA is its superior localization to tumor tissues compared to normal white matter [17].

PDT efficacy depends on a number of factors including, oxygenation status, total light dose (fluence), the rate of light delivery (fluence rate), and photosensitizer concentration and localization. The high degree of fluorescence in both human and experimental glial tumors following ALA administration indicates that the level of PpIX in gross tumor tissue is probably sufficient for efficient PDT [18–20]. The high drug levels observed in gliomas are likely due to passive diffusion across a compromised BBB. In contrast, the concentration of photosensitizer in infiltrating cells within and beyond the BAT is contingent on the sensitizer's ability to cross the BBB and selectively accumulate in these cells. The ability of ALA to diffuse across intact BBB is somewhat uncertain, and therefore it is unclear whether sufficient levels of PpIX required for efficient PDT can be achieved in migrating glioma cells located in and beyond the BAT. The purpose of the experiments reported here was to determine the effects of ALA–PDT on small clusters of tumor cells sequestered in otherwise normal rat brain. In this study, we have employed newly implanted BT_4C tumor cells to mimic the characteristics of infiltrating glioma cells. Our results indicate that although ALA–PDT had a significant effect on developed tumors it could not prevent tumors from forming if treatment was performed shortly after tumor initiation, before BBB degradation occurred.

MATERIALS AND METHODS

Cell Cultures

The BT_4C tumor was originally derived from transformed fetal BD-IX rat brain cells after exposure to ethylnitrosourea [21]. The BT_4C cells were grown as monolayers in RPMI medium with 10% heat-inactivated newborn calf serum (FCS) at 37°C and 5% CO_2. The cell line tested negative for viral agents in a rat antibody production test according to the Federation of Laboratory Animal Association (FELASA) recommendations.

PDT on BT_4C Monolayers

BT_4C cells were incubated in 1 mM ALA (PhotoCure, ASA, Oslo, Norway and Sigma, St. Louis, MO) and serum free-medium for 4 hours. Following incubation, cells were washed, plated out in a flask and irradiated with 635 nm light from a diode laser (High Power Devices, North Brunswick, NJ). The cultures were exposed to a range of radiant exposures (2–50 J/cm^2) delivered at light irradiances of 5, 25, or 35 mW/cm^2. Following irradiation, cells were harvested with trypsin, counted using a Coulter Counter (Beckman Coulter, Model ZF, Fullerton, CA) and then incubated at various cell densities in complete media for 11–14 days to allow for colony growth. Colonies that grew from the surviving cells were stained with crystal violet and counted (>50 cells/colony). The percentage survival was calculated as 100×(number of colonies/number of experimental cells plated)/(number of control colonies formed/number of control cells plated). Each single experiment was constructed to consist of six data points for each dose examined. Each experiment was repeated four times.

Experimental Animals

Inbred BD-IX rats (Harlan Olac, Bicester, UK and Charles River Laboratories, Wilmington, MA) of both sexes weighing at least 250 g were caged in Macrolon III cages. The animal holding rooms were maintained at constant temperature and humidity on a 12-hour light and dark schedule at an air exchange rate of 18 changes per hour. Animal care and protocol were in accordance with national legislation and institutional guidelines. The animals tested negative for parasitical, bacterial, and viral agents according to the FELASA recommendations. For the surgical procedures, the rats were anesthetized either with a combination of fentanyl/fluanisone and midazolam, or pentobarbital. Buphrenorphin (0.08 mg/kg s.c.) was used as a post-operative analgesic. The animals received a total of three doses administered at 24-hour intervals. Pentobarbital (100 mg/kg i.p.) was used to euthanize all animals.

Rat Glioma Model

A complete description of this model has been provided elsewhere [22]. Briefly, to establish intracranial tumors, the anesthetized rats were fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA), the skin was incised and a 1.0-mm burr hole was made to fit the following coordinates: 3 mm posterior to and 2 mm to the right of the bregma and depth of 3 mm. The injection device consisted of a 30-G blunt cannula connected through a catheter (Small Parts, Miami Lakes, FL) to an infusion pump (Harvard Apparatus, Holliston, MA). The cannula was fixed in the electrode holder of the stereotactic frame, and then vertically introduced into the brain. A total of 10,000 cells in 5 μl of RPMI were injected into the brain during a period of 1 minute. The cannula was kept in place for 2 minutes, and slowly retracted to prevent the spread of tumor cells during retraction. Closure was done with bone wax and sutures. Tumor take was 100%.

Magnetic Resonance Imaging of Tumor-bearing Rats

In order to evaluate BBB patency, BD-IX rats were imaged in a 0.5 T open interventional MR scanner (GE Signa SP, GE Medical Systems, Waukesha, WI). Eighteen

days following tumor inoculation, animals were anesthetized, and after subcutaneous injection of gadolinium contrast (1.0 ml of 0.5 mmol/ml Magnavist: Berlex Laboratories, Wayne, NJ) were subjected to a T1-weighted 3D gradient echo pulse sequence ($TR = 34$ milliseconds; $TE = 12$ milliseconds, slice thickness = 1.3 mm) using a receiver coil designed for scanning the human eye region.

Porphyrin Biodistribution Studies

Seventeen days after tumor induction, four animals were given 60 mg/kg ALA i.p. and euthanized 4–5 hours later. In a previous study [23], this ALA concentration resulted in maximum PpIX fluorescence in gross tumor. The brains were carefully removed and sectioned in two parts along the plane of tumor cell injection. The samples were then mounted in Tissue Tek (Sakura Finetek USA, Torrance, CA), immersed in liquid nitrogen, and stored at −80°C. Sections were cut with a cryostat microtome to a thickness of 8–10 μm and mounted on clean glass slides. Images of fluorescence from the cross sections were made using a microscope with a cooled CCD-camera (C4742-98, Hamamatsu, Japan). A 390–440 nm excitation filter, 460 nm beam splitter, and 610–650 nm emission filter were used to optimize imaging of PpIX fluorescence. An exposure integration time of 15 seconds was used, resulting in less than 5% photobleaching of porphyrin fluorescence. The frozen sections were subsequently stained with hematoxylin and eosin (H&E) for histologic identification of tumor infiltration in the BAT. Image analysis was used to measure average PpIX intensity in bulk tumor, BAT and normal brain. To quantify porphyrin fluorescence, which is bleached much faster than background autofluorescence, images were acquired before and after bleaching most of the fluorescence. This was accomplished by exposing the sample to excitation light for 4 minutes. The second image was subtracted from the first to provide an image of pure bleachable porphyrin fluorescence, corrected for most autofluorescence and other background sources. In all cases, PpIX fluorescence intensities in BAT and normal brain were expressed as percentages of the average gross tumor PpIX fluorescence intensity.

PDT of Normal Brain and Gross Tumor

In order to determine the effects of treatment on normal brain, 16 non-tumor-bearing animals were irradiated with increasing light doses (9–54 J, input power: 7.5–45 mW) 4 hours following ALA (250 mg/kg i.p.) administration. Four animals in the high fluence group (54 J) were given steroids (Solo-Medrol: 1 mg/kg) on the day of treatment and once per day on the 2 following days. Animals were euthanized 24 hours after treatment and their brains removed for histopathology.

To investigate the direct effects of PDT on tumor pathology, seven animals were subjected to treatment 15 days following inoculation of BT_4C cells and compared to untreated tumor-bearing controls by histopathology. In all cases, animals were administered 125 mg/kg ALA i.p. and subjected to light irradiation 4–5 hours later. Prior to irradiation, animals were anesthetized and positioned in the stereotactic frame. The skin incision was opened and the bone wax removed from the burr hole. A 400-μm bare flat-end quartz fiber with numerical aperture 0.22 was introduced directly into the brain using the same coordinates employed during tumor induction. Light from a 635 nm diode laser was delivered interstitially over a time interval of 90 minutes. Animals were subjected to radiant exposures of 26 J (fluence rate = 4.8 mW). The rats were euthanized 15–48 hours following treatment and their brains removed and prepared for histopatology. Tumor diameters were between 4 and 7 mm.

PDT of Isolated Glioma Cells

Two days following inoculation of 10,000 BT_4C cells, animals were administered either 125 mg/kg ALA i.p. or 100 mM buffered ALA (pH 7) in a 20 μl volume injected directly into the brain. The drug was injected stereotactically using identical coordinates to those used for tumor induction. In all cases, the animals were given light treatment (26 J, 4.8 mW) 4–5 hours following ALA injection. In another set of experiments, BT_4C cells were incubated in 6 mM ALA for 4 hours in serum-free RPMI in vitro. This drug concentration is well below the threshold for dark toxicity observed in this cell line (ca. 50% cell survival following 4 hour incubation in 24 mM ALA). Following incubation, cells were re-suspended in fresh medium and 10,000 were inoculated as previously described. The cannula was kept in the brain for 15 minutes in order to reduce mechanical cell migration with cannula withdrawal. After slow withdrawal of the cannula, the laser fiber was stereotactically introduced and the animals were given light treatment (13 or 26 J delivered at a fluence rate of 4.8 mW). A summary of all in vivo experiments can be found in Table 1.

RESULTS

ALA–PDT on BT_4C Monolayers

Effects of radiant exposure and irradiance on BT_4C cells in monolayer are illustrated in Figure 1. The data clearly show an increase in ALA–PDT efficacy with increasing radiant exposure, however, even at the highest exposure investigated (50 J/cm^2), approximately 5–10% of cells survived treatment. For a given exposure, lower irradiances were shown to be more effective. This was especially the case for the intermediate exposures (10–20 J/cm^2) studied. The small difference in survival between cells exposed to irradiances of 5 and 25 mW/cm^2 suggests that 25 mW/cm^2 is sufficient for effective PDT of BT_4C cells. As illustrated in Figure 1, the effect of irradiance diminishes with increasing exposure. This closely mirrors the trend observed in human glioma cells subjected to ALA–PDT [24].

Magnetic Resonance Imaging

A typical T2-weighted contrast-enhanced coronal image through an 18-day old tumor is illustrated in Figure 2. The scan shows a clear contrast enhancement indicating a defective BBB in the tumor.

Porphyrin Distribution in Tumor-bearing Rats

Porphyrin fluorescence in the tumor border area and the BAT was evaluated 4–5 hours after ALA injection, and

TABLE 1. Summary of In vivo Studies

Study	Group	*n*	Tumor age (days)	ALA dose	Light dose/ dose rate
PpIX biodistribution	1	4	17	60 mg/kg i.p.$\Delta t = 4–5$ hours	n/a
PDT–normal brain	1	4	16	250 mg/kg i.p.$\Delta t = 4–5$ hours	9 J/7.5 mW
	2	4	16	same as Group 1	18 J/15 mW
	3	4	16	same as Group 1	54 J/45 mW
	4	4	16	same as Group 1	54 J/45 mW + steroids
PDT–bulk tumor	1	7	15	125 mg/kg i.p.$\Delta t = 4–5$ hours	26 J/4.8 mW
PDT–isolated cells	1	6	Controls—no PDT	0	0/0
	2	6	2	125 mg/kg i.p.$\Delta t = 4–5$ hours	26 J/4.8 mW
	3	7	2	100 mM direct $\Delta t = 4–5$ hours	26 J/4.8 mW
	4	4	0	6 mM in vitro incubation	26 J/4.8 mW
	5	4	0	6 mM in vitro incubation	13 J/4.8 mW

compared to the fluorescence in normal brain (greater than 3 mm from the tumor border) and gross tumor. Selective porphyrin accumulation in bulk tumor tissue is clearly apparent in the fluorescence micrograph presented in Figure 3a. For comparative purposes, an H&E section of the same region is illustrated in Figure 3b. Fluorescence was also observed in the BAT up to 2–3 mm from the tumor border (Fig. 3a), with intensities of up to 20% of that in bulk tumor tissue. No significant porphyrin fluorescence (i.e., <1% of that found in bulk tumor) was detected more than 3 mm from the tumor border. The infiltrating nature of BT_4C tumors is evident from the lack of tumor encapsulation, the finger-like projections extending into normal brain as well as small clusters of cells independent of the main tumor. Of particular interest is the observation that many of the sequestered clusters of cells in close proximity to, but separate from the main tumor bulk, appeared to show no increase in fluorescence compared to the surrounding BAT, indicating a relatively low level of porphyrin production (Fig. 3a).

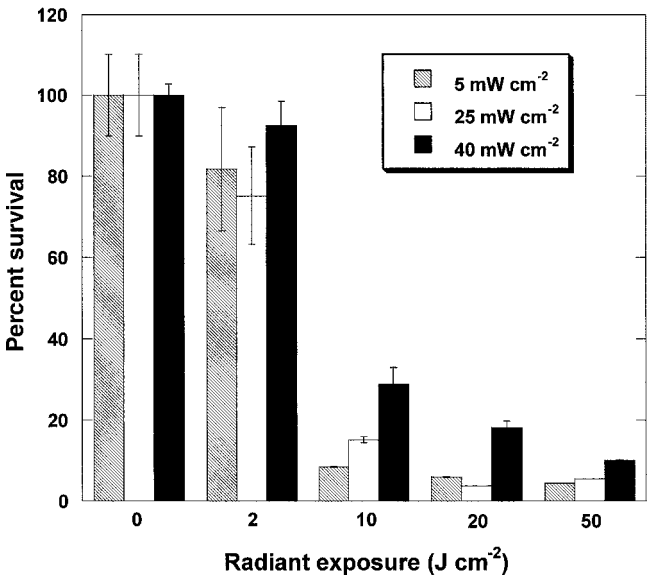

Fig. 1. Effects of radiant exposure and irradiance on BT_4C cells in monolayer. Cells were incubated in 1 mM ALA for 4 hours prior to light exposure ($\lambda = 635$ nm). Each data point represents the mean of four experiments. The error bars denote standard deviations.

Effects of PDT on Bulk Tumor, Isolated Glioma Cells, and Normal Brain

In order to determine the toxicity of PDT on normal brain, non-tumor-bearing animals were subjected to ALA–PDT at increasing light fluences ranging from 9 to 54 J (Fig. 4). No morbidity was apparent up to 18 J. Fifty percent mortality was observed at 54 J, but the addition of steroid treatment completely protected the animals at this fluence (Fig. 4). Histological examination of sections from a surviving rat

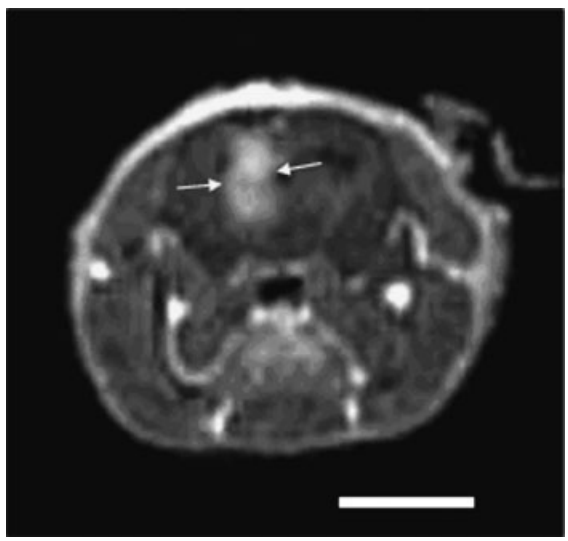

Fig. 2. T1-weighted post-contrast coronal image taken of a BD-IX rat on the 18th day post-tumor cell implantation. The hyperintense region denotes the tumor (arrows). Scale bar = 5 mm.

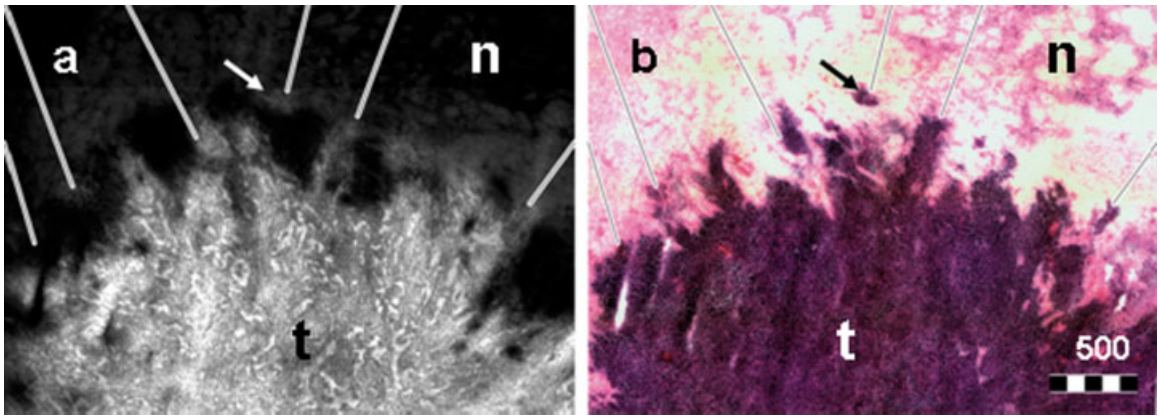

Fig. 3. PpIX fluorescence micrograph (**a**) and corresponding H&E section (**b**) illustrating the infiltrating nature of this tumor. The animal was injected with 60 mg/kg ALA (i.p.) and sacrificed 4 hours later. Normal brain and bulk tumor are denoted by n and t, respectively. The lines in each image point to corresponding areas. The arrow denotes a micro-cluster of cells that has detached from the bulk tumor. [Figure can be viewed in color online via www.interscience.wiley.com.]

receiving 54 J (Fig. 5) showed a lesion with eosinophil neuron necrosis at the coordinates where the fiber tip had been positioned during the light exposure 24 hours earlier. Edema was also apparent within the 3 mm diameter lesion.

Effects on bulk tumor tissue is illustrated in Figure 6. It is shown that ALA–PDT with 26 J of light causes extensive necrosis. Although the necrotic areas represent a large percentage of the tumor volume, viable tumor cells were nevertheless found at the tumor periphery. Central necrosis was not observed in any of the tumors in the non-treated control animals: an observation typical of this model. The effect of PDT on animals treated 48 hours following tumor cell inoculation is illustrated in Figure 7. Light treatment delivered 4 hours following systemic ALA administration (i.p.), or with direct intracranial injection, failed to produce significant prolongation of survival compared to untreated controls. In fact PDT with intracranial ALA proved lethal in 40% of the animals within 24 hours of treatment (Fig. 7). In contrast, animals inoculated with tumor cells pre-incubated in vitro with ALA showed a significant survival advantage in response to PDT. As expected, survival was dependent on light fluence—animals subjected to the higher fluence lived longer than

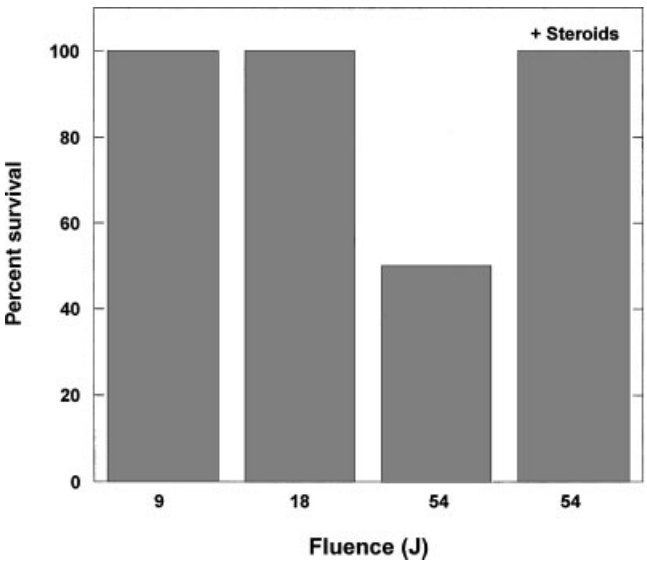

Fig. 4. Effects of ALA–PDT on normal brain of BD-IX rats. Animals were injected with 250 mg/kg ALA and subjected to light treatment 4 hours later. Each data point represents four animals. Steroids (Solo-Medrol: 1 mg/kg s.c.) were given to rats in one of the high energy groups (54 J) on the day of treatment and 2 days thereafter. A light delivery time of 20 minutes was used in all cases.

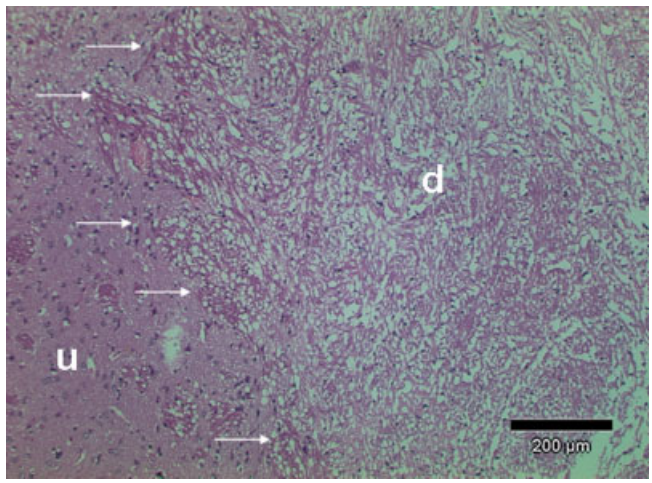

Fig. 5. Representative histology section of a non-tumor bearing animal treated with 250 mg/kg ALA (i.p.) and 54 J of light. The arrows denote the boundary between PDT-damaged (d) and unaffected (u) brain tissue. [Figure can be viewed in color online via www.interscience.wiley.com.]

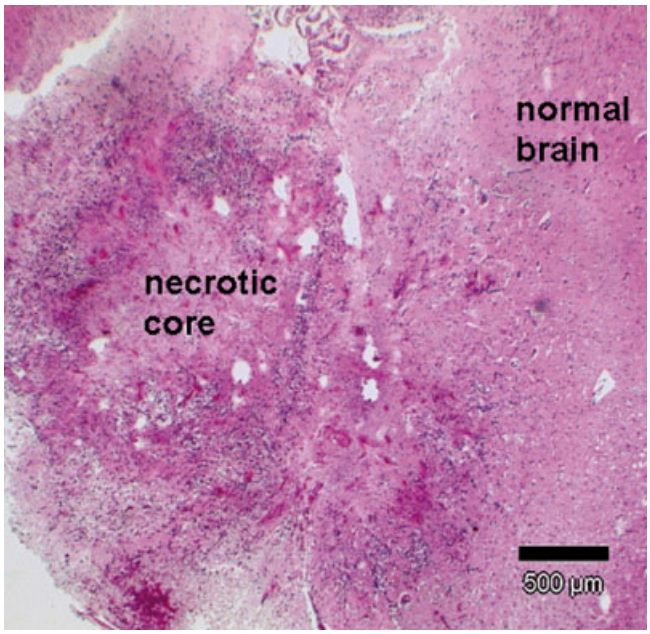

Fig. 6. Representative histology section of a tumor-bearing animal treated with 125 mg/kg ALA and 26 J of light (dose rate = 4.8 mW). Extensive necrosis can be observed in the central portion of the tumor. [Figure can be viewed in color online via www.interscience.wiley.com.]

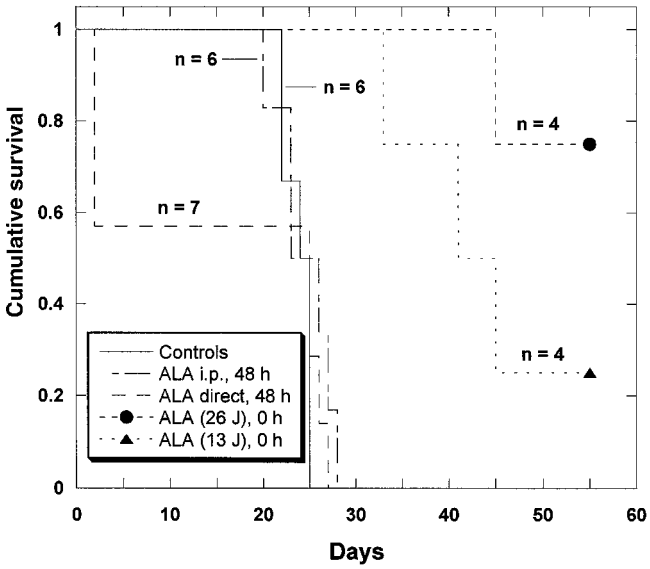

Fig. 7. Survival of animals subjected to ALA–PDT 0 or 48 hours following tumor cell implantation. Animals in the 48 hours groups received ALA i.p. (125 mg/kg) or by direct intratumoral injection (100 mM). Four to five hours later, animals were treated with 26 J of light. Animals in the 0 hour groups were subjected to 13 or 26 J of light (dose rate = 4.8 mW) immediately following injection of BT_4C cells pre-incubated in 6 mM ALA.

those irradiated with the lower fluence and were considered cured. Examination of the brains of these animals following euthanization showed no signs of tumor development.

DISCUSSION

The inherent tendency of glial tumors to infiltrate normal brain tissue significantly limits the effectiveness of conventional treatments. Although surgical resection reduces the pressure effects of the bulk tumor, it is the diffusely invading tumor cells well beyond the resection margin that are responsible for the damage to normal brain parenchyma which ultimately results in the death of the patient. The central question this work aims to answer is whether sufficient levels of ALA/PpIX and, hence efficient PDT can be achieved in micro-clusters of glioma cells residing in normal brain. In this study, we have employed newly implanted tumor cells to mimic the characteristics of infiltrating cells remaining in the resection margin following surgical removal of bulk tumor. Treatment was performed 48 hours after cell inoculation. This is an insufficient time to allow for the development of bulk tumor and BBB degradation, but long enough for the cells (doubling time = 18 hours) to form small sequestered micro-clusters which are protected by an intact BBB. In the strictest sense, the cells comprising the micro-clusters are not considered to be infiltrating since they were obtained from bulk tumor. Migrating tumor cells are thought to differ in their proliferation rate from cells in the bulk tumor [4] and would therefore be more resistant to conventional therapies such as ionizing radiation and chemotherapy. For the purposes of this work, the distinction is not important since the primary goal was to investigate the effects of PDT on cell clusters isolated by a patent BBB in an intact animal brain.

Results of in vitro studies showed that BT_4C cells are susceptible to ALA–PDT (Fig. 1) and therefore provide the rationale for further investigation in animals. Of particular importance is the observation that PDT-induced cytotoxicity is critically dependent on both radiant exposure and the rate of light delivery (irradiance)—lower irradiances are more effective, especially at low radiant exposures. The decreased efficacy of higher irradiances observed in BT_4C monolayers is in good agreement with the findings of other investigators using a variety of cell lines and photosensitizers [25,26]. Oxygen depletion, which has been shown to be important in fluence rate effects observed in vivo, is likely not involved. The observed effect may be due to different mechanisms operating under high and low fluence rates, involving oxidizing intermediates [25]. The data presented in Figure 1 give a good estimate of the light delivery conditions required for optimum PDT effect in this cell line, and therefore provide a good starting point for further study in animals.

Biodistribution studies in BD-IX rats show that bulk tumor accumulates significant levels of PpIX following i.p. administration of ALA (Fig. 3) and therefore, it is hardly surprising that ALA–PDT is capable of causing significant damage to cells in the bulk tumor (Fig. 6). Unfortunately, high fluence PDT also caused significant

damage to normal brain (Fig. 5) resulting in high mortality in non-steroid treated animals (Fig. 4). The beneficial effects of steroids suggest that mortality was likely due to treatment-induced edema and subsequent elevation of intracranial pressure. The sensitivity of normal rat brain (predominantly gray matter [27]) to ALA-induced PpIX PDT is in good agreement with the findings of Lilge and Wilson [17] who have shown that gray matter in the rabbit cortex is very susceptible to ALA–PDT-induced damage. These investigators have also found rabbit white matter to be extremely insensitive to the effects of ALA–PDT. In fact they found that light fluences in white matter must be 5,000 times higher than in tumor tissue for equivalent damage. Taken together, these results suggest that the effectiveness of ALA–PDT is critically dependent on tumor location.

The high PpIX levels observed in gross tumor is likely due to passive diffusion through a compromised BBB characteristic of the rapid angiogenesis associated with fast growing tumors. In a previous study, PpIX concentrations in the BAT were found to be 5–20% of that observed in gross tumor [23]. This is in good agreement with the findings of others [28–30] and suggests a partial BBB breakdown in this region. Additionally, it is possible that PpIX produced in the tumor could be washed out into the surrounding BAT region by the net fluid outflow associated with the edema reaction. Normal brain cells contained very low levels of PpIX (<1% of that found in bulk tumor) suggesting that only trace amounts of ALA pass across intact BBB. This implies that infiltrating micro-colonies in normal brain are protected by a patent BBB and are therefore unlikely to be more susceptible to ALA–PDT than normal brain following systemic drug delivery.

The results presented in Figure 7 clearly show that PDT performed at early stages of tumor development (48 hours) had no effect on animal survival at the light fluence (26 J) and ALA administration routes investigated. Cell titration experiments have shown that in this model at least 2,000 cells are necessary for reliable tumor induction [personal communication]. Therefore it is reasonable to assume that at least 2,000 of the original 10,000 BT_4C cells survived treatment. Increasing the light fluence does not appear to be a viable option as demonstrated by the severe damage to normal brain following PDT with 54 J (Fig. 5). These PDT experiments, performed on non-tumor-bearing animals, clearly demonstrate that trace amounts of PpIX in normal brain are sufficient to cause extensive damage at high light fluences. The severity of the effect is underscored by the fact that 50% of the animals succumbed within 48 hours of treatment in the absence of steroids (Fig. 4).

In contrast, animals inoculated with BT_4C cells pre-incubated in ALA (denoted by Day 0 in Fig. 7), showed a significant survival advantage in response to PDT, especially at the higher light fluence investigated. The results indicate that both the oxygen concentrations in normal brain and the light fluences employed in these studies are sufficient for efficient PDT, provided that the glioma cells contain adequate levels of ALA. It is interesting to note that, unlike rats receiving ALA i.p. or intracranially, these animals tolerated PDT treatment very well.

In an attempt to circumvent the BBB and increase sensitizer levels in sequestered tumor cells, direct intracranial ALA injection was attempted (Fig. 7). Unfortunately many of the animals succumbed within 24 hours of treatment and the survival of the remaining animals was not statistically different from controls. Large craniectomies, which have been shown to be effective under other conditions [31], were ineffective at preventing early post-treatment mortality with this mode of drug delivery.

Collectively, the data suggest that the poor response of isolated cell colonies to PDT is due to the inability to deliver adequate drug doses to these cells via systemic administration. Direct intracranial injection does not appear to solve this problem as evidenced by the high toxicity and poor efficacy—likely the result of rapid ALA diffusion in the brain parenchyma. The incorporation of drugs into biodegradable polymers allowing for slow release might be a possible solution to the problem of local photosensitizer delivery [32]. Unfortunately, the hydrophilic nature of ALA, might limit its penetration in biological tissues after its slow release from the polymer. One approach to increasing ALA penetration is to attach a lipophilic ester. Lipophilic ALA esters are attractive from a clinical point of view since they would likely result in higher and more uniform photosensitizer concentrations throughout the BAT if delivered slowly and over an extended period of time. In addition, these compounds might be able to reach small nests of tumor cells protected by intact BBB.

It is unlikely that the resistance of glioma microclusters to PDT (Fig. 7) was due to high ferrochelatase activity in BT_4C cells. Ferrochelatase is an enzyme that has been shown to have lower activity in malignant tissue compared to normal tissue [33–36]. The lower activity of this enzyme in tumor tissue results in higher PpIX concentrations compared to normal tissue. The differential accumulation of PpIX in tumor tissue provides a compelling rationale for ALA–PDT. The ferrochelatase activities of rat brain tumor cells, including BT_4C cells, are unknown, however, the results presented in Figure 1 clearly demonstrate that BT_4C cells are sensitive to ALA–PDT. In fact, the response of this cell line is very similar to that of human biopsy-derived glioma cells [24] thus providing support for its use in PDT studies of brain tumors. Furthermore, the in vivo results show that the effects of ALA–PDT on bulk tumor are typical of those found in other studies, that is, ALA–PDT is capable of causing significant tumor necrosis and the treatment often results in extensive edema. Collectively, both the in vitro and in vivo data suggest that BT_4C cells are not resistant to ALA–PDT and that, under conditions of adequate light, drug, and oxygen, there is no reason to believe that BT_4C microclusters would not be susceptible to ALA–PDT.

The ability of ALA to traverse the intact rat BBB has been the subject of much debate: some studies suggest ALA is readily capable of crossing the BBB [37,38], while

others show that this barrier has limited permeability to ALA [39,40]. The results presented herein suggest that only trace amounts of ALA traverse intact BBB and are therefore in qualitative agreement with the studies suggesting limited ALA permeability. Although the trace amounts are insufficient to sensitize microclusters of BT_4C cells, they are nevertheless adequate to sensitize normal brain and cause significant damage upon exposure to high light fluences. It should be emphasized that the aim of the present work was not to provide a detailed analysis of PpIX distributions throughout the entire rat brain. The biodistribution studies were confined to the region of the cortex containing bulk tumor and normal brain within 5 mm of the tumor. From that perspective, the results are not necessarily inconsistent with the work of Olivo and Wilson [41] who investigated the microscopic distribution of ALA-induced PpIX throughout rabbit brain implanted with a VX2 tumor. In agreement with the findings of the present work, these investigators observed high tumor-to-normal cerebral cortex PpIX ratios (ca. 10:1). The higher PpIX selectivity observed in the present study (200:1) is likely due to differences in the animal models used. Although Olivo and Wilson demonstrated that ALA was able to cross the intact rabbit BBB, the implications of their findings to this work is uncertain due to the different animal models used and the relatively limited scope of the biodistribution studies presented here.

In summary, the studies presented here suggest that systemic delivery of ALA is a limiting factor of PDT efficacy in the treatment of malignant glioma cells protected by an intact BBB. The results show that systemically administered ALA accumulates in sufficient quantities in cells of the bulk tumor but not in infiltrating cells in normal brain. Of particular significance is the observation that the trace amounts of ALA-induced PpIX found in normal brain cortex is sufficient to produce severe morbidity following high light exposures. The use of ALA or ALA-ester derivatives incorporated into biodegradable polymers might be a solution to some of the problems encountered in this study. The addition of more global treatment modalities such as boron neutron capture therapy, which has the possibility of reaching infiltrating cells a considerable distance from the main tumor in combination with a local treatment modality such as PDT, is an additional avenue for further study.

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