Photoirradiation therapy of experimental malignant glioma with 5-aminolevulinc acid

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Object. Accumulation of protoporphyrin IX (PPIX) in malignant gliomas is induced by 5-aminolevulenic acid (5-ALA). Because PPIX is a potent photosensitizer, the authors sought to discover whether its accumulation might be exploited for use in photoirradiation therapy of experimental brain tumors, without injuring normal or edematous brain.

Methods. Thirty rats underwent craniotomy and were randomized to the following groups: 1) photoirradiation of cortex (200 J/cm², 635-nm argon-dye laser); 2) photoirradiation of cortex (200 J/cm²) 6 hours after intravenous administration of 5-ALA (100 mg/kg body weight); 3) cortical cold injury for edema induction; 4) cortical cold injury with simultaneous administration of 5-ALA (100 mg/kg body weight) and photoirradiation of cortex (200 J/cm²) 6 hours later; or 5) irradiation of cortex (200 J/cm²) 6 hours after intravenous administration of Photofrin II (5 mg/kg body weight). Tumors were induced by cortical inoculation of C6 cells and 9 days later, magnetic resonance (MR) images were obtained. On Day 10, animals were given 5-ALA (100 mg/kg body weight) and their brains were irradiated (100 J/cm²) 3 or 6 hours later. Seventy-two hours after irradiation, the brains were removed for histological examination.

Irradiation of brains after administration of 5-ALA resulted in superficial cortical damage, the effects of which were not different from those of the irradiation alone. Induction of cold injury in combination with 5-ALA and irradiation slightly increased the depth of damage. In the group that received irradiation after intravenous administration of Photofrin II the depth of damage inflicted was significantly greater. The extent of damage in response to 5-ALA and irradiation in brains harboring C6 tumors corresponded to the extent of tumor determined from pretreatment MR images.

Conclusions. Photoirradiation therapy in combination with 5-ALA appears to damage experimental brain tumors selectively, with negligible damage to normal or perifocal edematous tissue.

KEY WORDS • glioma • 5-aminolevulinc acid • photodynamic therapy • porphyrin

The median survival time in patients with malignant gliomas is limited to little more than 12 months and with tumor progression usually occurring at the margins of the former resection cavity. Taking recurrence patterns into account, patients might benefit from more aggressive local therapy. Photodynamic therapy, a form of photoradiation therapy, is a cancer treatment based on the apparently selective accumulation of photosensitizing drugs in malignant tissue. When activated by light of an appropriate wavelength, the photosensitizer exerts tumor-toxic properties. Photodynamic therapy has proven useful in the treatment of a number of different neoplastic lesions and has also been used for local adjuvant therapy of malignant gliomas.

Currently, HpD, and its purified versions, porfimer sodium (Photofrin) and dihematoporphyrin ether (Photofrin II) are used for photoradiation therapy in combination with laser light in the range of 630 to 635 nm. These sensitizers, however, have the profound disadvantage of causing prolonged skin sensitization. Patients treated with these drugs are required to stay out of direct and indirect sunlight for as long as 2 months. Furthermore, damage to normal brain tissue has been reported in preclinical studies and local treatment selectivity may be limited, because these sensitizers appear to participate in edema bulk flow and thus may be transported into brain regions devoid of tumor.

Recently, we investigated a novel substance, 5-ALA, for use in intraoperative fluorescence-guided resection of malignant gliomas. The substance 5-ALA is a naturally occurring metabolite in the heme biosynthesis pathway. Excess exogenous 5-ALA leads to accumulation of highly fluorescent heme precursor porphyrins, such as PPIX, in a number of malignant tissues, including malignant gliomas. Accumulation is highly specific and does not appear to occur in normal brain; however, apart from their strong fluorescence, endogenous porphyrins such as PPIX are also efficient photosensitizers. In contrast to hematoporphyrin derivatives, side-effects of 5-ALA in patients are negligible and include mild elevation of liver enzymes and a period of skin sensitization limited to fewer than 48 hours. Consequently, photoirradiation therapy with 5-ALA–induced porphyrins may be a modality worth investigating, especially as an adjuvant treatment after 5-ALA fluorescence-guided resection of malignant gliomas and for direct treatment of residual tumor areas for which...
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the use of conventional surgical techniques may prove too dangerous.

To qualify as a photosensitizer, 5-ALA must fulfill a number of prerequisites. Its accumulation should be specific for malignant glioma tissue. It should not propagate or diffuse with cerebral edema to sensitize adjacent normal brain tissue while exerting sufficient phototoxic effects on tumor tissue.

The present investigation seeks to elucidate whether the photosensitizing effects of 5-ALA–induced porphyrins are sufficient for photoirradiation therapy in an animal model of glioma. Magnetic resonance imaging was used to determine tumor size prior to therapy and to compare it with the extent of phototoxic tumor damage. To determine the degree of unwanted sensitization of perifocal edematous tissue, a brain edema model was used.17,18 This edema model allowed assessment of any damage related to edema alone, thus allowing us to rule out interference from sensitized infiltrating tumor cells. Finally, 5-ALA was compared with a traditional sensitizer, Photofrin II, with regard to unwanted sensitization of normal brain.

Materials and Methods

General Procedures

All procedures were performed in accordance with German animal protection laws after review of the experimental protocol by the Bavarian government. Spontaneously breathing male Wistar rats weighing 240 to 260 g were anesthetized with isoflurane (1–2%) in oxygen during tumor implantation, cold lesioning, and photoirradiation. The animals were maintained on temperature-controlled feedback heating pads at 37˚C. Their heads were immobilized in a stereotactic head holder and a right parietal craniotomy (4 mm length × 3 mm breadth) was performed using a high-speed drill, bordering to the midline and the coronal suture for all irradiation experiments. Care was taken not to injure the dura mater to avoid cortical damage.

The 5-ALA (Medac GmbH, Hamburg, Germany) was obtained as a hydrochloride powder. For intravenous administration, 5-ALA was dissolved in phosphate-buffered saline at a concentration of 30 mg/ml immediately before use and adjusted to a pH of 6.5. Photofrin II (Lederle Parenterals, Puerto Rico, USA) was dissolved in a 5% glucose solution at a concentration of 2.5 mg/ml immediately before use and adjusted to a pH of 6. The 5-ALA was used for photoinjection at 635 nm of red light. The power density was adjusted to 100 mW/cm² and an energy density of many J/cm² was used for photolumination at 635 nm of red light. The exposed dura for 15 seconds with the aid of a micromanipulator. In six animals, 5-ALA was administered intravenously at a dose of 100 mg/kg body weight 3 hours prior to cortical cold injury.

Six hours later, that is, 3 hours after cold injury, the region of the lesion and adjacent exposed cortex were irradiated with 200 J/cm². In six additional animals, cold injury was induced without subsequent irradiation to quantify the depth of tissue damage resulting from cold injury alone.

Effect of Photoirradiation on Edematous Brain Tissue

Systemic administration of 5-ALA leads to elevated serum porphyrins,30,31 which might participate in tumor edema propagation, thus unspecifically sensitizing edematous perifocal brain tissue. To test this hypothesis with regard to 5-ALA–induced serum porphyrins, the cold injury model17 was used, with modifications.32 Briefly, cold injury to the cortex was performed by placing a 1-mm-diameter copper stamp, cooled to −68˚C by a mixture of dry ice and acetone, on the exposed dura for 15 seconds with the aid of a micromanipulator. In six animals, 5-ALA was administered intravenously at a dose of 100 mg/kg body weight 3 hours prior to cortical cold injury. Six hours later, that is, 3 hours after cold injury, the region of the lesion and adjacent exposed cortex were irradiated with 200 J/cm². In six additional animals, cold injury was induced without subsequent irradiation to quantify the depth of tissue damage resulting from cold injury alone.

Effect of Photoirradiation on C6 Gliomas

For the present experiments, the C6 model of malignant glioma was used as previously described, with minor modifications.33 The C6 glioma cells were grown in monolayer tissue cultures in Dulbecco minimal essential medium supplemented with 10% fetal calf serum and sodium pyruvate (1 mM) at 37˚C in a 5% carbon dioxide atmosphere. Penicillin G (300 IU/ml) and streptomycin (300 µg/ml) were added to the medium to prevent bacterial infection. Cultures between passages 72 and 88 were harvested by trypsinization, and the cells were suspended in saline at a concentration of 2 × 10⁶ cells/ml. Five µl (10⁶ cells) was stereotactically implanted using a 10-µl syringe via a small burr hole 2 mm lateral and 2 mm caudal to the bregma, that is, the region designated for later craniotomy. Cells were implanted at a depth of 2 mm from the skull surface, within the animals’ cortices. A superficial location was chosen to ensure reliable access to evolving tumors for photodiode irradiation via the planned craniotomy and to prevent the syringe needle from traversing the ventricle, potentially resulting in disseminated intrathecal rather than circumscripted tumor growth. The location was also chosen to ensure the detection of even superficial phototoxic tumor lesions that might be missed if the tumor cells were implanted more deeply.

To examine the tumor configuration and accurately assess the extent of phototoxic tumor damage, MR images were obtained in all animals on Day 9 after tumor implantation. Animals received an intraperitoneal injection of 3.6% chloral hydrate anesthetic (1.2 ml/100 g body weight). All animals received 0.45 ml Gd–diethylenetriamine pentaacetic acid (Magnevist; Schering AG Pharma, Berlin, Germany) intravenously immediately prior to undergoing T₁-weighted MR imaging (slice thickness 2 mm; TR 480 msec; TE 14 msec) for which a 1.5-tesla unit was used (Magneton Vision; Siemens, Munich, Germany). Images were printed on Dry Star TM 1b films (Agfa, Cologne, Germany) for determining the depth of contrast-enhancing tumor and the coronal dimension of the brain.

Animals were reanesthetized the following day for preparation for craniotomy and photoirradiation therapy (100 J/cm²) either 3 or 6 hours after intravenous administration of 5-ALA 100 mg/kg body weight. The lower energy density in these experiments compared with the control experiments in animals without tumors was used to
test for tumor toxicity at energy levels expected to be more commonly achieved clinically. The time points were chosen in accordance with previous experiments measuring porphyrin accumulation in this model. The brains were removed after perfusion-fixation 72 hours later, embedded, cut, and stained. Lesion depths perpendicular to the surface were measured, along with the coronal diameter of the brain in the histological section. To account for specimen shrinkage associated with the embedding procedure, this value was compared with the corresponding measurement available from the MR image. The ratio of histological neuroimaging to measurements for coronal brain diameters was then used to correct the value for the depth of the histological lesion.

To test the accuracy of predicting histological tumor size from tumor dimensions obtained on MR imaging, a separate group of five animals underwent implantation of C6 gliomas and were subjected to the same protocol; however, this group did not undergo photoradiation therapy. In these animals, the size of contrast-enhancing tumor was unequivocally measured in two dimensions: its greatest diameter and the size perpendicular to its greatest diameter. These values were compared with the respective measurements obtained histologically, which were corrected for specimen shrinkage.

**Statistical Analysis**

All data are reported as the means ± SDs. Group differences were tested by analysis of variance with the post hoc Scheffé F-test.

**Results**

**Photoirradiation Therapy of Normal and Edematous Brain Tissue**

Each experimental group was composed of six animals. The results obtained in these groups were assessed for effects of laser irradiation of normal or edematous cortex with or without pretreatment with 5-ALA and for irradiation of normal cortex after Photofrin II pretreatment. Solitary laser irradiation of normal cortex without prior administration of 5-ALA resulted in a superficial layer of tissue changes characterized by karyopyknosis and perinuclear vacuolization at a mean depth of 0.44 ± 0.07 mm (Fig. 1). The same type and depth of damage was observed when 5-ALA was given 6 hours before laser irradiation (0.41 ± 0.08 mm). Cold lesioning alone resulted in a sharply demarcated area of coagulative necrosis with destruction of all cellular elements. The mean lesion depth was 0.44 ± 0.11 mm; however,
subsequent photoirradiation therapy, after administration of 5-ALA, aggravated the existing damage (0.83 ± 0.31 mm). The resulting lesions exhibited superficial coagulative necrosis in the area closer to the light source, with selective neuronal damage beyond this area. Laser irradiation of normal cortex after pretreatment of animals with Photofrin II produced sharply demarcated coagulative necrosis with a mean depth of 1.77 ± 0.22 mm, which was significantly greater than the depth of damage observed in the other groups. Figure 2 shows an example of the different qualities of cortical damage found in an animal after simultaneous cold injury for induction of vasogenic edema and treatment with 5-ALA 6 hours before photoirradiation.

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Comparison of Tumor Size. To determine whether it was possible to predict histological tumor size accurately based on MR imaging, the greatest tumor diameter and the respective diameter perpendicular to the greatest diameter were measured on the images (Fig. 3 upper right) and on the corresponding histological section (Fig. 3 upper left). The mean of the greatest diameters of tumors in the histological sections was 3.72 ± 2.31 mm; the corresponding extent of contrast enhancement on the MR image was 3.78 ± 2.38 mm (Fig. 3 lower). The respective perpendicular measurements were 1.95 ± 1.49 mm and 1.88 ± 1.54 mm, respectively. The mean differences between MR imaging–derived and histologically derived diameters were 0.06 ± 0.24 mm (p = 0.61) for the greatest diameter and 0.07 ± 0.29 mm (p = 0.63) for the perpendicular diameter. Histologically derived tumor diameters were accurately demonstrated on MR images.

Effects of Photoirradiation Therapy on Tumors. Magnetic resonance images were obtained in two groups of rats that were to undergo photoirradiation therapy, either 3 or 6 hours after administration of 100 mg/kg of 5-ALA. The mean depth of the tumor, measured perpendicular to the brain surface, was 2.7 ± 0.76 mm in the 3-hour group and 2.6 ± 1.08 mm in the 6-hour group.

Seventy-two hours after the rats underwent photoirradiation, their brains were removed subsequent to perfusion-fixation for histological assessment of damage depths. Phototoxic damage was observed in all animals at the site of tumor growth previously demonstrated on MR imaging. Histologically, damage was characterized either by coagulative or hemorrhagic necrosis (Fig. 4), sometimes surrounded by a region of perilesional pallor. Phototoxic damage was found down to a mean depth of 2.8 ± 0.16 mm in tumors in the 3-hour group and down to 2.7 ± 0.87 mm in the 6-hour group (Fig. 5). The deepest extent of damage (3.7 mm) was measured in an animal from the 6-hour group; MR imaging in this animal demonstrated the depth of damage to be 4 mm. Phototoxic damage was not always homogeneously distributed throughout the tumors. In some cases, residual nests of tumor cells, which appeared viable, were contained within apparently damaged tumor tissue. In these cases, the deepest extent of necrosis was measured.

Compared with the control groups, in which normal and edematous brain was irradiated after administration of 5-ALA, the depth of damage in tumor tissue was significantly greater (p < 0.05).

Discussion

Photoirradiation Therapy of Malignant Gliomas

Photodynamic therapy has been used as an adjuvant after surgery for malignant gliomas, with the aim of killing resid-
been exploited for enhancing their resection. Neverthe-

time of 5-ALA to label specifically malignant gliomas has

rats in each group, means ± SDs).
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of special concern for photoirradiation therapy of malignant gliomas. Edema results from tumor vessels with wide junctional clefts and endothelial fenestrations, enabling edema fluid to leave the vascular system.4 Because of its clearance by bulk flow, tumor-associated edema can be found far beyond tumor borders.28,29 Intravenously administered photosensitizers, such as Photofrin II, propagate with vasogenic edema into perifocal edematous but otherwise normal brain tissue, resulting in nonspecific phototoxicity.34,35 Experimentally, 5-ALA–induced porphyrins have been observed outside tumor tissue in regions where there is tumor-associated edema,3,38 but only to a mean distance of 1.5 mm.38 The origin of these porphyrins has not yet been elucidated. They might represent extravasated plasma porphyrins induced by 5-ALA; they might originate in tumor washed out by edema into perifocal tissue; they might result from nonspecific porphyrin synthesis in normal brain tissue that is exposed to edema-borne 5-ALA. It is also unknown whether normal neuronal or glial cells are basically capable of porphyrin synthesis after exposure to 5-ALA. It has been shown, however, that the normal BBB is virtually impermeable to hydrophilic 5-ALA40 so that any 5-ALA entering the tumor or perifocal tissue must do so through the leaky BBB within the tumor.

To test whether the concentrations of edema-associated porphyrins were great enough to nonspecifically sensitize perifocal tissue after administration of 5-ALA, a cortical cold lesion was induced in rats without tumor. The cold lesion gives rise to vasogenic edema, which is somewhat similar to tumor-related edema and has been previously used to study porphyrin kinetics in perifocal edematous tissue.5,34 In the cold injury experiments, photoillumination of edematous tissue led to a significant enlargement of necrosis, but only to a depth of 0.9 mm. This was double the lesion depth caused by the cold injury itself but was less than the depth of damage arising from irradiation of normal cortex sensitized with Photofrin II. The small zone of additional damage resulting from edema-associated porphyrins in the present experimental glioma model should not pose a concern in the human setting, where nonspecific accumulation of fluorescent porphyrins in perifocal edematous brain tissue has basically been ruled out based on 264 biopsy samples obtained from the margins of malignant gliomas in humans.38

Conclusions

To summarize, the present experiments demonstrate selective damage to experimental glioma after sensitization with 5-ALA and appropriate photoirradiation. Normal brain tissue was not susceptible to photoirradiation therapy, whereas only mild sensitization was observed in tissue harboring perifocal edema in the cold lesion model. In light of the positive experience gained from the clinical use of 5-ALA fluorescence-guided resection with regard to substance toxicity and particularly specificity of accumulation, elucidation of photoirradiation therapy with 5-ALA in the clinical setting appears warranted. Phase I/II studies in patients harboring primary or recurrent malignant gliomas will ultimately define the clinical potential of this approach, either for adjuvant therapy of tumor cavities subsequent to surgery or for treating residual fluorescent tumor tissue not amenable to surgical resection.

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References


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