Interstitial laser photochemotherapy of rhodamine-123-sensitized rat glioma

STEPHEN K. POWERS, M.D., WILLIAM C. BECKMAN, JR., PH.D., J. TONY BROWN, AND LINDA C. KOLPACK

Division of Neurosurgery, Department of Surgery, University of North Carolina School of Medicine, Chapel Hill, North Carolina

The effect of interstitial laser photochemotherapy with the mitochondrial-specific intravital dye rhodamine-123 (Rh-123) was studied using a malignant rat glioma model system (RT2). Tumors were transplanted subcutaneously into the flank of athymic mice and into the cerebrum of adult rats. The Rh-123 photosensitization was produced by direct intratumoral injection of Rh-123 into the mouse RT2 flank tumors and by intravenous Rh-123 administration to adult rats with implanted RT2 intracerebral tumors. Intratumoral irradiation with 150 mW of argon laser light for an exposure time of 15 minutes was performed using a conical sapphire-tipped quartz optical fiber. Control groups of animals received either no treatment, Rh-123 injections, or administration of 150 mW of argon laser light for 15 minutes. Both flank and intracerebral tumors showed progressive diminution in size after treatment with Rh-123 photochemotherapy. There was no evidence of tumor recurrence in 60% of Rh-123 photochemotherapy-treated tumors. Recurrences in tumors treated with Rh-123 photochemotherapy usually appeared at the periphery of the original tumor at 10 days after treatment. Histologically, photochemotherapy-treated intracerebral tumors showed progressive shrinkage with increasing tumor necrosis over time. The finding of residual or recurrent tumor at the periphery of the original tumor mass suggests that the lack of penetration of the blue-green (argon) light was responsible for preventing complete tumor ablation. Our results suggest that Rh-123 photochemotherapy can destroy malignant gliomas in vivo; however, the poor penetrability of the photoactivating blue-green light may limit the effectiveness of this treatment for large or extensively invasive tumors.

KEY WORDS • photochemotherapy • rhodamine-123 • brain neoplasm • laser • rat

One of the principal shortcomings of chemotherapy in the treatment of cancer is the indiscriminate targeting of drugs. The highly toxic agents which are usually employed are primarily effective on cells that are in a state of high metabolic activity or are rapidly dividing. The uncontrolled destruction of healthy tissues that frequently results often counterbalances any advantage derived from the killing of tumor cells.

Photochemotherapy is a relatively new technique, using a nontoxic chemical agent — a photosensitizer — that both has an affinity for tumor tissue and is converted into a cytotoxic form only in the presence of radiant energy. Selective destruction of the tumor can therefore be optimized by choosing an appropriate (tumor cell-specific) photosensitizer and also by limiting adverse side effects associated with light delivery (in general, thermal damage) to photoactivate the drug.

Hematoporphyrin derivative (HpD), other porphyrins, and phthalocyanine derivatives have been studied recently for their potential use for photochemotherapy of malignant tumors. None of these have been shown to be tumor cell-specific in vivo. In fact, it is now well recognized that the porphyrins, including HpD, are localized in or near vessels in the tumor and normal tissues such that the photodynamic effect results in ischemia due to vessel thrombosis and disruption and not due to direct cellular changes within the tumors.

The mitochondrial-specific dye rhodamine-123 (Rh-123) is selectively retained by the mitochondria of malignant gliomas both in vivo (SK Powers, K Ellington, in preparation) and in vitro. Normal brain tissue does not accumulate Rh-123 (K Ellington, SK Powers, in preparation) so that a high therapeutic ratio of Rh-123 is achieved in tumor compared to brain. In the rat,
clinical signs of toxicity are not seen following the intravenous injection of Rh-123 in doses equal to or less than 20 mg/kg of body weight. Nonthermal levels of blue-green light generated from an argon laser will photoactivate Rh-123 into a cytocidal species. The combination of Rh-123 and blue-green light results in a time-dependent killing of human malignant cells in vitro.14

We have used Rh-123 and interstitially delivered argon laser light to treat malignant rat glioma (RT2) transplanted into the subcutaneous space of the flank of athymic mice and into the cerebrum of adult rats. This report presents our observations.

Materials and Methods

Origin and Culture of RT2 Tumors

For these studies the RT2 glioma cell line was utilized. This tumor was derived from a glioma which developed after the injection of Rous sarcoma virus (Schmidt-Ruppin strain) into the brain of a weanling rat.

The RT2 glioma cells were cultured in Dulbecco's minimum essential medium (DMEM)/F12 base medium containing 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, 20% fetal bovine serum, 0.8 mM glutamine, and antibiotics (0.25 µg/ml amphotericin B (Fungizone), 100 U/ml penicillin, and 100 µg/ml streptomycin). Cells were grown in 60-mm sterile Corning tissue-culture dishes at a dilution of 2 x 10^4/dish and were allowed to grow to confluency (approximately 5 to 7 days). They were then passed to 100-mm dishes and again allowed to grow to confluency (approximately 2 days). The cells were then removed from the 100-mm dish with 0.2% trypsin and 0.02% ethylenediaminetetra-acetic acid (EDTA) in 0.1 M of Dulbecco's Ca ++ and Mg ++-free phosphate buffer (pH 7.25). The cells were centrifuged, resuspended, and counted on a hemacytometer using trypan blue. Cell concentration was adjusted to 1 x 10^6 cells/10 µl in culture media. Cells were then loaded into a sterile Hamilton 50-µl syringe prior to injection.

Transplantation of RT2 Flank Tumors

Culture medium containing 1 x 10^6 RT2 cells was injected subcutaneously via a 10-µl syringe into the inguinal region of the flank of each of several male athymic nude mice of the beige strain. Solid RT2 tumors developed to a size of approximately 3 to 4 mm centrally within the right cerebrum hemisphere 8 to 12 days after the injection of cells. Brain tumors were treated on either Day 9 or 10 after the injection of cells. Untreated rats generally died from their tumors within 18 days of implantation.

Laser Light Delivery

Due to the spherical or ellipsoidal shape of the tumors that grew in the flank and brain, the tumors were treated with light delivered through a quartz optical fiber with a spherical diffusing tip that was inserted into the geographic center of the tumor. We had previously studied temperature elevation effects following irradiation using a 400 µm-diameter quartz optical fiber with a 2.2 mm-diameter conical sapphire tip* placed into the center of a 1.0 cm-diameter flank tumor. It was found that tissue temperature elevation was less than 6° to 7°C at a distance of 2 mm or greater from the optical fiber tip for power outputs of 150 mW or less from the argon laser† (SK Powers, et al., unpublished data). The surface area of the sapphire tip of the optical fiber was 7.8 sq mm. Since hyperthermic damage in gliomas has been reported for absolute temperatures above 42°C15 and the normal temperature of the animals in our studies was 34° to 35°C, we used a total power output of 150 mW from the optical laser fiber.

Experimental Groups

A preliminary experiment compared the tumor growth response of flank tumors to variations in the duration of laser light exposure at a constant laser power output of 150 mW. Mice with flank tumors were divided into four groups of six each and were treated with either 0, 15, 30, or 45 minutes of interstitial laser irradiation that began 4 hours after the intratumoral injection of 0.03 ml of Rh-123 (10 mg/ml in dimethyl sulfoxide (DMSO)). Tumor response, which was determined by comparison of volumetric measurements of the tumors over time, was evident following increasing time of laser irradiation. Tumor response, however,
was not increased with laser irradiation times greater than 15 minutes (Fig. 1). Therefore, animals in the following experiments received 150 mW for 15 minutes (total energy 135 joules).

Flank Tumors. Athymic mice with RT2 subcutaneous flank tumors measuring approximately 6 to 7 mm in diameter were separated into four groups of 10 mice each. Group 1 received a direct intratumoral injection of 0.03 ml DMSO. Group 2 received 0.03 ml Rh-123 (10 mg/ml) dissolved in DMSO and injected into the tumor. Group 3 received an intratumoral injection of 0.03 ml DMSO alone and 150 mW (454 to 514.5 nm) of argon laser light for 15 minutes from an intratumorally placed 2.2 mm-diameter sapphire tip of an optical fiber. Group 4 received 0.03 ml Rh-123 in DMSO into the tumor and interstitial irradiation with 150 mW of argon laser light using the same sapphire-tipped optical fiber for 15 minutes, 4 hours after the injection of Rh-123.

Tumors were measured in three dimensions every 2 to 3 days. Precise tumor growth rates were computed from these measurements using the formula for the volume of a hemiellipsoid as follows: \( V = 0.52365 L \times W \times H \), where \( L = \) length, \( W = \) width, and \( H = \) height (this is the common morphological form of these tumors\(^\text{11}\)). Tumor growth rates were determined by comparing the slopes derived from the tumor growth curves over weekly intervals.

Intracerebral Tumors. Adult Fischer 344 rats harboring intracerebral RT2 tumors were divided into a treatment and two nontreatment groups. At 9 to 10 days after RT2 cell implantation into the right cerebral hemisphere, treated rats were anesthetized with intraperitoneal acepromazine (1 mg/kg) and ketamine HCl (10 mg/kg). The rats then received Rh-123, 10 mg/kg, injected over 30 seconds into the femoral vein. Approximately 4 hours after Rh-123 injection, the treated rats were reanesthetized with acepromazine and ketamine, the scalp was opened in the sagittal midline, and a small craniectomy (3 mm in diameter) was made in the right frontoparietal area centered over the cortical site of the RT2 cell injection. A 2.2 mm-diameter conical sapphire tip attached to a 400-\( \mu \)m quartz optical fiber was then introduced stereotaxically to a depth of 3 mm below the cortical surface at the center of the craniectomy. The argon laser was set to deliver 150 mW of total power output from the fiber tip for a duration of 15 minutes. After laser application, the laser fiber probe was removed. The craniectomy was covered with bone wax, and the scalp was sutured closed. The rats were allowed to recover and then were returned to their cages where food and water were given ad libitum.

Untreated rats either received an intravenous injection of Rh-123 (10 mg/kg) or laser irradiation with 150 mW of argon light for 15 minutes only. These rats were compared with treated rats for histological changes in the tumor and brain at various times after treatment in order to assess tumor response and changes in the normal surrounding brain tissue that might suggest treatment-associated toxicity.

Results

Mouse Flank Tumors

The results of the in vivo study comparing the effects of Rh-123 alone, laser light alone, and Rh-123 plus laser light versus no treatment on RT2 flank tumors are shown in Fig. 2. Comparison of mean tumor volume measurements indicated that at all time points the photochemotherapy group (Group 4) had smaller tumors than Group 1 with no treatment (\( p < 0.005 \)) and Group 2 with Rh123 only (\( p < 0.05 \)). However, Group 4 tumors (Rh-123 plus laser) did not differ significantly in size from Group 3 tumors (laser treatment alone) during the first 7 to 10 days after treatment, but then showed a greater delay in tumor growth in comparison to Group 3 after 10 days that became more apparent with an increasing follow-up period. This suggests that the laser light alone may have exerted a slight tumoricidal effect (possibly hyperthermia near the optical fiber tip) that could account for some of the early effects on tumor size and growth.

Six of the 10 mice in Group 4 showed no regrowth of their tumor masses during the study and could be considered cured. The other four tumors recurred af-

![Fig. 1. Plots of mean volumes of RT2 flank tumor versus time after treatment with intratumoral injection of Rh-123 (0.3 mg) and with variable periods (0, 15, 30, and 45 minutes) of interstitial argon laser light exposure. There was no significant difference in tumor size following treatment for lengths of laser exposure greater than 15 minutes. Vertical lines represent standard errors.](image-url)
ter 10 days and appeared as doughnut-shaped lesions. There was no statistically significant difference between the original tumor size in these four rats in comparison to the six rats without recurrence. It is suspected that regrowth that occurred at the tumor margin of these animals reflects inadequate penetration of the blue-green light from the interstitial laser fiber located centrally in the tumor.

Rat Intracerebral Tumors

Difficulty was encountered in assessing tumor response on the basis of animal survival. It became apparent early in this study that intracerebral tumors often varied in size and that other factors, such as pulmonary infections (the incidence of which was increased due to anesthetizing these animals on two separate occasions), often complicated the posttreatment course. Therefore, we did not believe that interpretation of survival studies after treatment of the rat intracerebral tumors would yield significant information relevant to tumor response. However, distinct histological changes were noted in the tumors following Rh-123 and laser treatment in comparison to tumors that received either Rh-123 or laser light alone.

During the first 10 days after treatment, the tumors that received Rh-123 plus laser treatment were smaller at any given time point in comparison to the other groups. No tumor was seen in six of 10 rats that were sacrificed and evaluated histologically at 3 weeks after treatment. Three of 10 rats evaluated 3 weeks after Rh-123 photochemotherapy had epidural tumors. The one other rat had a 5 mm-diameter subgaleal tumor that did not extend intracranially. Tumor necrosis became increasingly greater in terms of relative percentage of tumor volume with increasing length of time after treatment in the Rh-123 plus laser-treated tumors (Fig. 3). At 2 weeks after treatment, the rats treated with laser plus Rh-123 were observed to have residual tumors, mostly at the periphery of the original tumor site. This again suggested that the lack of penetration of the blue-green light was responsible for preventing complete tumor ablation. Except for mechanical injury to the brain at the site of insertion of the optical fiber, the surrounding brain tissue in all three groups was unaffected by this treatment. Ischemic or thermal changes were not seen either in the tumor or surrounding brain.

Discussion

Rhodamine-123 (Rh-123) has been shown to preferentially accumulate in the mitochondria of carcinoma cells, glioma cells, and muscle cells. Studies have shown that Rh-123 does not bind to normal brain parenchyma even in the presence of a blood-brain barrier (BBB) defect at the periphery of an intracerebral tumor (SK Powers, K Ellington, in preparation). Human glioma cells treated with Rh-123 and argon laser light demonstrated increasing tumor cell killing with increasing time of exposure to laser light. The current study also shows that photochemotherapy using intratumorally or intravenously administered Rh-123 and blue-green light from an argon laser results in the destruction and slowed growth of glial tumors.

We chose to study the effect of Rh-123 and argon laser photochemotherapy on malignant gliomas by using the implantable RT2 rat glioma model in the cerebrum of rats and the subcutaneous space of the flank of athymic mice. We made this choice because both models using this autochthonous tumor cell line show local tissue invasion and minimal tumor vascularity until the tumors enlarge to a diameter of greater than 6 to 7 mm. Correspondingly, there is absence of a BBB defect as demonstrated by the absence of Evans blue dye extravasation at the periphery of the intracerebrally implanted RT2 tumors that are less than 4 to 5 mm in diameter. The rat intracerebral RT2 glioma model, therefore, mimics the human condition of tumor margin invasion into normal brain parenchyma outside the region of BBB disruption. This region remains inaccessible to treatment by other photochemotherapeutic agents such as the porphyrin analogues, which are significantly bound to serum proteins and are thus incapable of crossing an intact BBB. However, Rh-123 has been shown to be selectively taken up and retained by tumor for periods of time up to 12 hours after intravenous administration in this animal tumor model (SK Powers, K Ellington, in preparation). Intratumoral injection of Rh-123 was used for treatment of the flank tumors due to the lack of vascular supply to these relatively small lesions which decreased the reliability of Rh-123 labeling of the tumors after intraperitoneal or intravenous drug administration.
Photochemotherapy of rat glioma

There was initially a decrease in the volume of RT2 flank tumors after treatment with Rh-123 interstitial photochemotherapy and interstitial laser treatment alone. Arrested tumor growth in both groups lasted for approximately 7 to 10 days after treatment. Beyond this time, tumors that had been treated by the laser only resumed a rapid growth rate and regrew to nearly equal the size of tumors in the two other control groups at 6 weeks after treatment. However, tumors treated with Rh-123 photochemotherapy either did not recur or recurred as small ring-shaped tumors around the site of the previous subcutaneous tumor mass.

The presence of apparently viable tumor cells at the periphery of the original tumor mass was also evident on histological examination of the intracerebral RT2 tumors after Rh-123 photochemotherapy. Shrinkage and progressive necrosis of the tumors was seen following treatment with Rh-123 and the argon laser. There was an arrest in tumor growth with Rh-123 photochemotherapy of intracerebral tumors and histological evidence of progressive cellular breakdown. This suggests that a vital part of the tumor cells is irreparably injured by this treatment, leading to delayed nuclear and plasma membrane disruption and cellular dissolution. Due to its mitochondrial binding, we hypothesize that Rh-123 photochemotherapy destroys cellular mitochondria. The resultant energy deprivation results in death of the affected tumor cells.

Unlike HpD, which has been shown to require oxygen to photo-oxidize essential cellular elements through the production of singlet oxygen (Type II oxidative process), Rh-123 photo-oxidative destruction of cellular components may not require oxygen and instead may be a triplet-mediated Type I radical reaction. Thus, oxygen availability may not necessarily be a limiting feature of Rh-123 photochemotherapy as it is with photosensitization using porphyrin analogues.

Unfortunately, the radius of tumor photodestruction with Rh-123 and nonthermal levels of intratumoral argon laser light appears to be limited to within 3 to 4 mm of the optical fiber. This is undoubtedly due to the poor penetration of blue-green light in tissue. This means that Rh-123 photochemotherapy would be useful only for extremely small (diameter less than 1 cm) well-localized neoplasms or possibly for the treatment of a tumor cyst wall using a single intratumoral light-emitting optical fiber. Certainly, techniques that would allow an increased light dose to the tumor while minimizing nonspecific thermal or mechanical damage to the surrounding normal tissues would enable treatment of a larger volume of tumor through Rh-123 photosensitization.

We have chosen to pursue the study of several dye compounds that have chemical characteristics similar to those of Rh-123 (that is, a delocalized positive charge throughout a heterocyclic molecular ring structure) but which have maximal absorption of light in the near infrared spectrum for their use in photochemotherapy of malignant gliomas. Light wavelengths between 700 nm...
and 800 nm are above the absorption range for hemoglobin and are readily transmitted through brain. We expect to be able to treat tumor to a greater depth with photosensitizers that absorb light and are thus activated over this spectral range.

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References


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Address reprint requests to: Stephen K. Powers, M.D., Division of Neurosurgery, 148 Burnett Womack Building 229H, University of North Carolina, Chapel Hill, North Carolina 27514.