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Laboratory Investigation

Repetitive 5-aminolevulinic acid-mediated photodynamic therapy on human glioma spheroids

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Summary

The response of human glioma spheroids to repetitive 5-aminolevulinic acid-mediated photodynamic therapy (PDT) was investigated. In all cases, light fluences were kept below toxic thresholds to simulate conditions typically found at 1–2 cm depths in brain adjacent to tumor. Significant inhibition of spheroid growth was observed following multiple PDT treatments at sub-threshold light fluences. The effect appears to be insensitive to the treatment intervals investigated (weekly or bi-monthly). In all cases, suppression of growth was observed for the duration of treatment. Low fluence rates ($\leq$5 mW cm^{-2}) appear to be more effective than high fluence rates (25 mW cm^{-2}). No evidence of PDT resistance was found in this investigation.

Introduction

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 [1]. This therapy results in a series of photochemical and photobiological events that cause irreversible photo-damage to tumor tissues. PDT has several features that make it an effective adjuvant therapy in the treatment of brain tumors: it is a local form of treatment in which the treated volume is limited by high attenuation of light in brain tissues and repeated applications of PDT is an option due to low long-term morbidity. The aim of PDT is to eliminate the nests of tumor cells remaining in the margins of the resection cavity following surgical removal of bulk tumor while minimizing damage to surrounding brain tissue. However, due to the limited penetrance of light in brain tissues, long treatment times will be required to deliver sufficient light doses (fluences) to depths of 1–2 cm in the resection cavity (BAT, brain adjacent to tumor) [2]. In addition, a number of *in vitro* [3–6] and *in vivo* [7–10] studies suggest that the threshold light fluence necessary for efficient elimination of tumor cells depends on the rate at which the fluence is delivered – lower fluence rates appear more efficacious.

To date, all clinical PDT trials have employed short-term intraoperative or stereotactic light delivery techniques [11,12]. This is unlikely to eradicate tumor cells deep in the BAT due to the inability to deliver toxic threshold light fluences in a reasonable time period. In addition, porphyrins (hematoporphyrin derivative and Photofrin®) have been used almost exclusively in clinical PDT trials of the brain [11–14]. These photosensitizers are not suitable for use in repetitive PDT treatment regimens due to their uncommonly long period of cutaneous photosensitization, lasting up to several weeks. Due to the drawbacks of traditional porphyrins, other photosensitizers, or prodrugs such as ALA are currently being evaluated for use in PDT of gliomas.

In ALA-induced endogenous photosensitization, the heme biosynthetic pathway is used to produce protoporphyrin IX (PpIX) – a potent photosensitizer [15–17]. Heme is synthesized from glycine and succinyl CoA. The rate-limiting step in the pathway is the conversion of glycine and succinyl CoA to ALA, which is under negative feedback control by heme. Through

the introduction of ALA, the regulatory feedback system becomes overloaded causing an accumulation of PpIX, which, when activated, causes the photosensitizing effect for PDT and porphyrin fluorescence for diagnosis.

ALA has been used primarily as a topical agent in the treatment of superficial skin lesions [18]; however, the abundance of ALA-induced PpIX in rapidly proliferating cells of many tissues provides a biologic rationale for ALA-mediated PDT in a wide variety of lesions [19]. The combination of increased tumor-to-normal brain tissue localization [20], short period of skin photosensitization (24–48 h) and oral administration, makes ALA an appealing compound for potential use in repeated PDT treatments of glioma patients. Previously published studies in both animals and humans have demonstrated significant PpIX concentrations in brain tumors and almost no accumulation in normal white matter [20–26]. Such a selectivity has been exploited for application in fluorescence guided resection of glial tumors [25,27].

In the study reported here, the response of human glioma spheroids to repetitive ALA-mediated PDT was investigated. Light fluences were purposely kept under toxic thresholds to simulate conditions typically found at 1–2 cm depths in the BAT. The development of PDT resistance was investigated by removing surviving cells from multiply-treated spheroids, growing new spheroids, and subjecting them to renewed PDT treatments. In all cases, treatment efficacy was evaluated by monitoring spheroid growth.

Materials and methods

Cell cultures

The grade IV GBM cell line (ACBT) used in this study was a generous gift of G. Granger (University of California, Irvine, USA). The cells were cultured in DMEM (Gibco, Grand Island, NY) with high glucose and supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). Cells were maintained at 37°C in a 7.5% CO_2 incubator. At a density of 70% confluence, cells were removed from the incubator and left at room temperature for approximately 20 min. The resultant cell clusters (consisting of approximately 10 cells) were transferred to a petri dish and grown to tumor spheroids of varying sizes. Spheroids were grown according to standard techniques [28]. Spheroids of 250 μm diameter were selected by passage through a screen mesh (Sigma, St. Louis, MO). It took approximately 14 days for spheroids to reach a size of 250 μm. The spheroid culture medium was changed 3 times weekly.

Spheroids were also grown from surviving cells retrieved from previously treated cultures. Following four treatments over a 3-week interval, the remaining spheroids were removed from the cultures. The adherent surviving cells, generally in a nonconfluent monolayer, were released from the cultures by enzymatic action and new spheroids were established as described above. The resultant spheroids grew in an identical manner to those previously described.

PDT treatments

ALA (Sigma, St. Louis, MO) was prepared in growth medium (DMEM with 10% fetal bovine serum) and pH adjusted to 7.4. Spheroids were incubated in 100 μg mL^{-1} ALA for approximately 4 h. In all cases, spheroids were irradiated with 635 nm light from an argon ion-pumped dye laser (Coherent, Inc., Santa Clara, CA). Light was coupled into a 200 μm dia. optical fiber containing a microlens at the ouput end. Spheroids were irradiated in a petri dish. A 2 cm diameter gasket was placed in the dish to confine the spheroids to the central portion of the dish and thus limit the extent of the irradiated field. The spheroids were grown as bulk cultures in petri dishes, each containing approximately 40–50 spheroids. Since each trial was performed 2 or 3 times, a total of 80–150 spheroids were followed for a given set of parameters. One of the cultures received no treatment and acted as a control. The other cultures received PDT treatments using light fluences of either 12.5 or 25 J cm^{-2} at fluence rates of 2.5, 5 or 25 mW cm^{-2}. Some of the cultures were treated only once, while the others were treated 4 times at weekly or bi-monthly intervals. Spheroids were incubated in ALA prior to each treatment. In the case of the low fluence rate studies ($\leq$5 mW cm^{-2}), irradiation was performed in an incubator in order to maintain physiological conditions during the long irradiation times. After each treatment, spheroids were washed and re-suspended in medium. Following the last light irradiation, individual spheroids from the bulk cultures were placed into separate wells of a 48-well culture plate and monitored for growth. Determination of spheroid size was performed by measuring two perpendicular diameters of each spheroid using a microscope with

a calibrated eyepiece micrometer. Spheroids were followed for up to 12 weeks in some of the experiments.

Results

Theoretical optical distribution

Diffusion theory is commonly used to describe the propagation of light in scattering media such as brain tissue. Calculated fluence rates in brain tissue for a spherical light applicator are illustrated in Table 1. The details of this calculation have been published elsewhere [29]. The following variables were used in the calculation: a 1 W input power ($\lambda = 630$ nm), an optical penetration depth of 3.2 mm [30–32], a diffusion constant of 5.4×10^{10} mm^2 s [30–32] and an applicator diameter of 2 cm. The results shown in Table 1 illustrate one of the fundamental limitations of PDT, namely the poor penetration of light in biological tissues. For example, the calculations show a decrease in fluence rates of between 3 and 4 orders of magnitude over a tissue depth of 2 cm. The time required to deliver a sufficient optical fluence to tissues can be determined from knowledge of the fluence rate at the point of interest. Based on the data in Table 1, treatment times required to deliver a PDT threshold fluence of 50 J cm^{-2} are summarized in Table 2 for 3 different diameter applicators, including the one used for the calculations in Table 1. In all cases, an input power of 1 W is assumed.

Repetitive PDT treatment

The fraction of spheroids showing growth following single or weekly repeated PDT, at a fluence rate of 25 mW cm^{-2}, is illustrated in Figure 1. As shown in the figure, all spheroids in both the control and low fluence single treatment groups were viable after 1 week in individual culture. In contrast, only 9% of the spheroids treated 4 times with 12.5 J cm^{-2} showed signs of growth. At the end of the observation period (week 8), some spheroids did show clear growth patterns, with some reaching maximum size. Spheroids treated once with 12.5 or 25 J cm^{-2} showed 100% survival measured over this extended time interval. In contrast, only 30% of the spheroids treated 4 times with 25 J cm^{-2}, or 40% treated with 12.5 J cm^{-2} demonstrated growth potential. Interestingly, no significant growth was observed in the multiply treated spheroids as long as treatment was continued.

Results of repeat PDT experiments using low fluence rates are shown in Figure 2a,b. A fluence rate of 2.5 mW cm^{-2} (Figure 2a) approximates the value expected at 1.5 cm depth in brain tissue using a 2 cm diameter spherical applicator (Table 1). In both cases, individual spheroid growth was monitored for a period of 4 weeks following the last treatment (7 weeks from culture initiation). As illustrated in Figure 2, repeated

Table 1. Fluence rate as a function of depth in brain tissue

Depth (mm)	0	5	10	15	20	25
Fluence rate (mW cm^{-2})	807	113	18	3.0	0.5	0.09

Table 2. Time required to achieve a light fluence of 50 J cm^{-2}

Depth (cm)	Time (h)		
	$D = 2.0$ cm	$D = 3$ cm	$D = 4$ cm
1.0	0.8	1.4	2.1
1.5	4.7	7.7	11.5
2.0	26.7	43.0	62.6

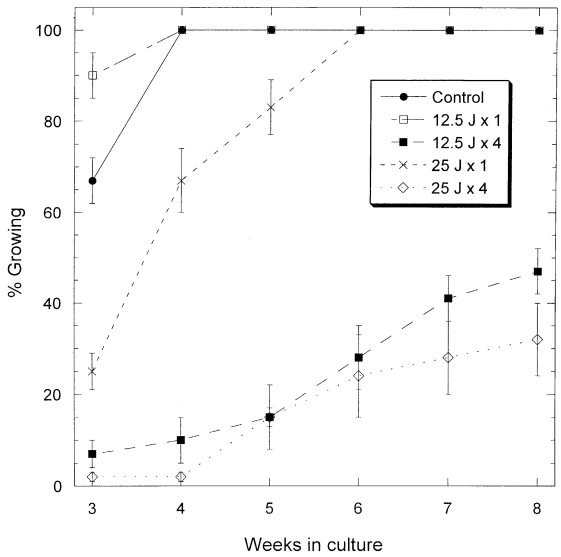

Figure 1. Spheroid growth following single or weekly repetitive PDT using high fluence rates. Spheroids were subjected to fluences of either 12.5 or 25 J cm^{-2} using a fluence rate of 25 mW cm^{-2}. Spheroids in the repeat groups were irradiated in bulk culture on day 0, and weeks 1, 2, and 3. Following the last treatment, spheroids were removed from bulk culture and plated out in individual wells. Spheroid growth was monitored for an additional 5 weeks.

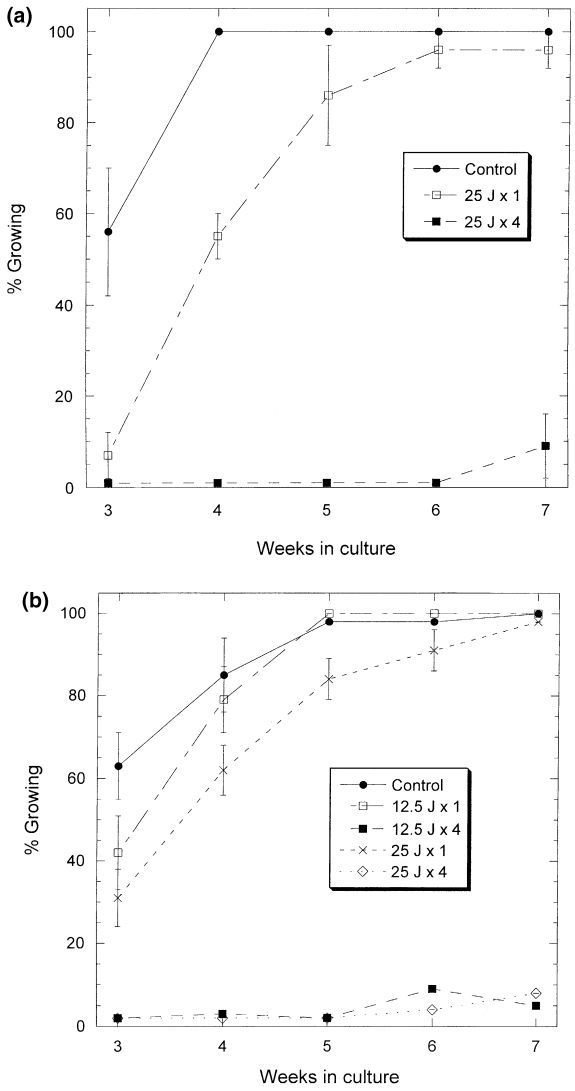

Figure 2. Spheroid growth following single or weekly repetitive PDT using low fluence rates of (a) 2.5 mW cm^{-2} or (b) 5.0 mW cm^{-2}. Spheroids were subjected to fluences of either 12.5 or 25 J cm^{-2}. Spheroids in the repeat groups were irradiated in bulk culture on day 0, and weeks 1, 2, and 3. Following the last treatment, spheroids were removed from bulk culture and plated out in individual wells. Spheroid growth was monitored for an additional 4 weeks.

PDT at fluence rates of 2.5 or 5 mW cm^{-2} appears to be more effective than high fluence rate PDT (Figure 1). For example, suppression of growth is observed over longer time intervals following low fluence rate PDT. As shown in Figure 2, there appears to be no significant difference in the growth kinetics of spheroids exposed

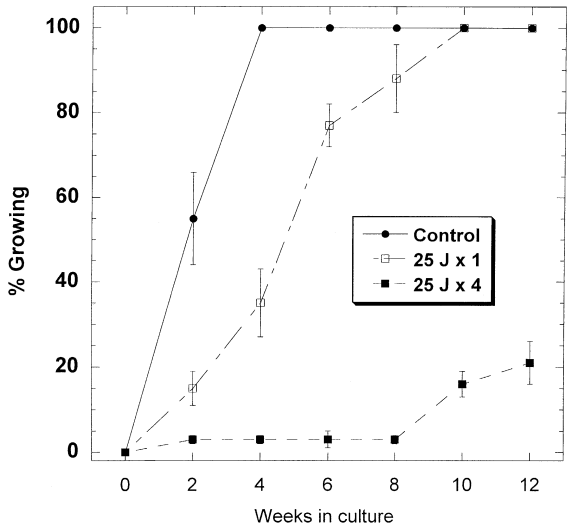

Figure 3. Spheroid growth following single or bi-monthly PDT. Spheroids were subjected to fluences of 25 J cm^{-2} delivered at a fluence rate of 25 mW cm^{-2}. Spheroids in the repeat groups were irradiated in bulk culture on day 0, and weeks 2, 4, and 6. Following the last treatment, spheroids were removed from bulk culture and plated out in individual wells. Spheroid growth was monitored for an additional 6 weeks.

to fluence rates of 2.5 or 5.0 mW cm^{-2}. In both cases, approximately 10% of spheroids exposed to fluences of 25 J cm^{-2} show growth potential at the end of the observation period (week 7).

The results of bi-monthly treatments extending over a period of 6 weeks are shown in Figure 3. As seen in the figure, the repeatedly treated cultures show minimal growth during the 6-week treatment period, however, re-growth is observed approximately 4 weeks following the last treatment (week 10). Spheroids treated once, on day 0, demonstrated significant growth after an initial delay. All spheroids in this group were observed to be growing approximately 10 weeks following initial treatment.

The response of spheroids grown from surviving cells retrieved from previously multiply treated cultures is illustrated in Figure 4. These cells gave rise to spheroids (second generation) that grew with similar growth kinetics to spheroids derived from untreated cells (first generation). After 2 weeks in culture, both groups of spheroids grew to maximum size (Figure 4 – 0 J cm^{-2}). As shown in Figure 4, both types of spheroids were sensitive to ALA-mediated PDT. Interestingly, the second generation spheroids grown from previously treated spheroids were found to be

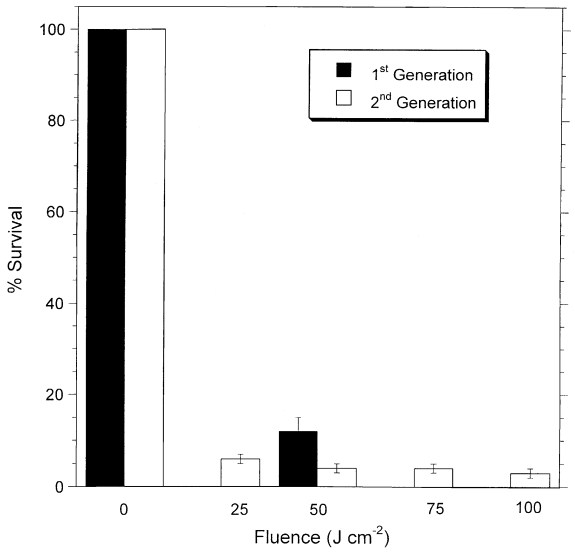

Figure 4. Response of second generation spheroids to PDT. In all cases, fluences were delivered at a fluence rate of 25 mW cm^{-2}. Spheroid survival was evaluated 4 weeks after treatment. The first generation spheroid groups consist of spheroids composed of cells not previously treated. The second generation groups consist of spheroids composed of cells previously treated 4 times. Fluences and fluence rates of 25 J cm^{-2} and 25 mW cm^{-2} were used in each of the four prior treatments.

growth inhibited at lower fluence levels then first generation spheroids, and even sub-optimal fluence levels (25 J cm^{-2}) were highly effective.

Discussion

The photodynamic effect depends on a number of factors including, light fluence, fluence rate, tissue oxygenation status, photosensitizer concentration, and intrinsic tissue sensitivity to the PDT effect. The high degree of fluorescence observed in glial tumors following oral administration of 20 mg kg^{-1} of ALA suggests that PpIX levels in tumor tissue and in BAT are sufficient for effective PDT [25,27]. In all likelihood, the limiting factor for successful PDT is the inability to attain threshold light fluences throughout the BAT. This is due to the rapid attenuation of light in brain tissue. Thus, the delivery of adequate light fluences to cm depths in the BAT requires treatment times of the order of hours (Table 2). Such long treatments are impractical with standard one-shot intraoperative procedures. Clearly, more sophisticated light delivery techniques are required for improved PDT outcome.

In this study, a simple spheroid model was used to investigate the efficacy of various light delivery schemes. Spheroids were used in this study since their three-dimensional geometry results in heterogeneous subpopulations of cells differing in proliferation, nutritional, metabolic and, most importantly, oxygenation status [28]. The local environment surrounding the various cells in the spheroid is dependent on their position, thus mimicking the gradients found in solid tumors.

In these experiments, spheroids were subjected to light fluences (12.5 or 25 J cm^{-2}) previously shown to be sub-optimal at the fluence rates used in this study [5]. The effectiveness of repetitive PDT using sub-optimal fluences and low fluence rates has significant clinical relevance since local control requires the elimination of tumor cells at depths where fluence rates are unlikely to exceed a few mW cm^{-2} (Table 2). The treatment intervals (weekly or bi-monthly) chosen in this study were based primarily on the pharmacokinetics of PpIX. Due to the relatively rapid clearance of this photosensitizer, daily fractionation will likely require additional ALA administration, however, the systemic liver toxicity often observed with frequent ALA intake would likely preclude such treatments [33]. Furthermore, due to logistical and quality-of-life issues, weekly or bi-monthly treatments are much more appealing than daily fractionation.

The primary finding of this study is that multiple PDT treatments at sub-threshold light fluences result in significant inhibition of spheroid growth. The effect appears to be relatively insensitive to the treatment interval (weekly or bi-monthly). In all cases, suppression of growth is observed during the entire treatment period. Low fluence rates (Figure 2a,b) appear to be more effective than higher ones (Figure 1) in that growth suppression is observed well beyond the treatment period, however, significant re-growth at times exceeding the observation period cannot be ruled out. Fluence rates of 2.5 and 5 mW cm^{-2} appear to be equally effective in this model. It would appear that, in principle, it should be possible to treat to depths of 1.5 cm in the BAT (Table 1). It is quite possible that PDT efficacy can be extended to even lower fluence rates than those investigated in this study. This would make deeper tissues accessible to PDT treatment. This is the subject of ongoing investigations. Although a small sub-population of spheroids always appear to survive high fluence rate treatment, multiple treatments nevertheless, result in a significant reduction in survival compared to single treatments. Taken together,

the results underscore the importance of protracted, repetitive PDT.

The observation that lower fluence rates are more effective than higher ones, has been demonstrated in a number of spheroid models [4,5]. Theoretical models suggest that the spatial distribution of singlet molecular oxygen (the primary cytotoxic species in PDT) depends critically on the fluence rate and on the availability of ambient oxygen [34]. At a given spheroid depth, the concentration of singlet molecular oxygen increases as fluence rates decrease. As a result, photodynamic damage will extend further into the spheroid as the fluence rate is lowered. Thus, PDT administered at lower fluence rates yields improved therapeutic response since singlet molecular oxygen is delivered to a larger volume of tumor cells in the spheroid.

The increased efficacy of multiple PDT is probably due to a number of factors. The spheroids used in this study consist of 3 distinct zones. The outer layer or rim consists mainly of proliferating cells, the middle layer consists mainly of viable but nonproliferating cells, and the central core is composed primarily of necrotic cells. Previous studies have shown that the outer rim of proliferating cells is the best oxygenated and produce the largest amounts of PpIX compared to the other layers [35]. The outer layer of proliferating cells is therefore killed and slough off between treatments so that spheroid growth is inhibited. Some of the viable nonproliferating cells will survive a sub-threshold fluence for a given fluence rate (Figures 1 and 3) and will commence growth after treatment is curtailed when they form a new outer layer. These surviving cells can also be utilized to form a new generation of spheroids, which are still highly sensitive to renewed photodynamic treatment (Figure 4). This phenomenon probably occurs in surgically treated tumors and demonstrates the importance of repeated access to the tumor resection cavity over an extended time frame, thus allowing multiple treatments. Such schemes would be feasible in a clinical protocol employing a newly developed indwelling light applicator [36]. The observation that 'second generation' spheroids seem even more susceptible to PDT than controls (Figure 4), might be indicative of some cumulative cytotoxic mechanism that is also playing a role in multiply treated cultures.

Conclusions

Significant spheroid response is observed in the case of repetitive ALA-mediated PDT at sub-threshold light fluences. In all cases, repetitive PDT is more effective than single treatments. Lower fluence rates appear more effective – significant growth suppression is observed in response to fluence rates as low as 2.5 mW cm^{-2}. This suggests that cells residing at depths of approximately 1.5 cm in BAT are susceptible to this type of PDT treatment. In all cases investigated, spheroid growth is effectively suppressed for the duration of treatment. Nevertheless, a significant fraction of spheroids appear to be growing several weeks following termination of high fluence rate treatment regimens. The reduced effectiveness of repetitive high fluence rate PDT is attributed to inadequate oxygen concentrations rather than to the development of PDT resistance. Although the validity of extrapolating *in vitro* data to the clinic is uncertain, the results obtained in this study suggest that protracted, repetitive PDT may play a role in the local management of gliomas. It certainly bears further investigation in more sophisticated animal models.

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