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The Effects of Ultra Low Fluence Rate Single and Repetitive Photodynamic Therapy on Glioma Spheroids

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Abstract

Background and Objective—Achieving local control of gliomas with photodynamic therapy (PDT) requires the delivery of adequate light fluences to depths of 1–2 cm in the resection margin where the majority of local recurrences originate. This is clinically impractical with current single-shot, intraoperative PDT treatments due to the length of time required to deliver adequate fluences. Multiple or extended treatment protocols would therefore seem to be required. The response of human glioma spheroids to 5-aminolevulinic acid (ALA)-mediated PDT using single or, repetitive light delivery protocols was investigated at both low and ultra low fluence rates.

Study Design/Materials and Methods—Human glioma spheroids (400 μm diameter) were subjected to sub-threshold light fluence (1.5, 3, or 6 J cm^{-2}) ALA-PDT consisting of four light delivery schemes: single treatment given over either 1 or 24 hours, repetitive treatment given either as four 1 hour light treatments separated by a 4 day interval, or 24 hours light delivery, consisting of four 24 hours treatments separated by a 3 day interval. Treatment efficacy was evaluated using a growth assay. In some cases, confocal microscopy was used to image cell viability.

Results—The repetitive and single light treatment protocols were most effective when delivered at ultra low ($\mu\text{W cm}^{-2}$) fluence rates. In all cases, growth inhibition was light dose-dependent. The repetitive ultra low fluence rate treatment (1.5 J cm^{-2} ; irradiance = 17 $\mu\text{W cm}^{-2}$) light delivery protocol was the most effective resulting in total growth inhibition during the 2-week observation period.

Conclusion—Ultra low light fluence rate ALA-PDT results in significant spheroid growth inhibition. Repeated administration of ALA was required during repetitive and/or protracted single PDT treatment protocols. The existence of a lower fluence rate limit, below which the efficacy of threshold light fluences diminish was not found in these studies.

Keywords

photodynamic therapy; 5-aminolevulinic acid; human glioma spheroids; fluence; fluence rate; repetitive PDT; chronic PDT; malignant glioma

Introduction

The therapeutic goal following gross surgical resection of brain tumors is to eliminate infiltrating tumor cells remaining in the margins of the resection cavity while minimizing damage to normal brain. Presently, there is no single treatment modality that can accomplish this goal since infiltrating tumor cells can be found in otherwise normal brain up to several cm from the resection site. A number of novel therapies, including photodynamic therapy (PDT), are currently under active investigation. PDT is a local treatment involving the administration of a tumor-localizing photosensitizing drug that is activated by light of a specific wavelength [1]. In 5-aminolevulinic acid (ALA)-induced endogenous photosensitization, the heme biosynthetic pathway is used to produce a potent photosensitizer—protoporphyrin IX (PpIX) [2–5]. The combination of increased tumor-to-normal brain tissue localization [6] short period of skin photosensitization (24–48 hours) and oral administration, makes ALA an appealing compound for potential use in repeated or extended low fluence rate PDT treatments of glioma patients. Previously published studies in both animals and humans have demonstrated significant PpIX concentrations in brain tumors with little accumulation in normal white matter [6–12]. Such selectivity has been exploited for fluorescence-guided resection of glial tumors [11,13].

A number of in vitro [14–17] and in vivo [18–22] studies suggest that the threshold light fluence necessary for efficient elimination of tumor cells depends on the rate at which the fluence is delivered. To date, most clinical PDT trials have employed short-term high fluence rate intraoperative or stereotactic light delivery techniques [23,24]. This is unlikely to eradicate tumor cells that have infiltrated into surrounding brain due to the inability to deliver toxic threshold light fluences in a reasonable time period. Furthermore, due to the short doubling time of malignant glioma cells, the kill rate per cell doubling indicates that multiple repetitive and/or extended time treatment protocols will likely be required. The concept of repetitive PDT involves the renewed application of both drug and light with relatively long treatment free intervals and has been studied both in vitro and in vivo [25–27]. The concept of extended interval low fluence rate PDT, in which both the photosensitizer and light are delivered continuously at low rates for extended periods of time to increase selective tumor cell kill has also been investigated [2]. Both conventional and extended PDT delivers the same light energy over the course of treatment and they share a similar mechanism of action, that is, the generation of singlet molecular oxygen leading to apoptotic and/or necrotic cell death. It is not known whether the lower concentration of singlet oxygen produced over extended periods (hours to days) has the same therapeutic effect as the higher concentrations produced acutely in conventional PDT. The existence of a lower fluence rate limit, below which the efficacy of threshold light fluences diminishes, is presently unknown and is an important question addressed in these experiments. In the study reported here, human glioma spheroids were subjected to ALA-mediated PDT in either

single or repetitive treatment protocols. Light was delivered over either a 1 or 24 hours period for both, single, or repetitive treatments. The response to the treatment regimens was evaluated using a simple growth assay. In all cases, low light fluences (1.5–6 J) were used to simulate conditions that would typically be found at 1–2 cm depths in the resection cavity wall.

Materials and Methods

Cell Culture

The grade IV GBM cell line (ACBT) used in this study was a generous gift of G. Granger at the University of California, Irvine. The cells were cultured in Advanced DMEM (Invitrogen, Carlsbad, CA) with high glucose and supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 $\mu\text{g ml}^{-1}$), and 4% heat inactivated fetal bovine serum (Invitrogen). Cells were maintained at 37°C in a 7.5% CO₂ 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 approximately 400 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 400 μm . The spheroid culture medium was changed twice weekly.

PDT Treatments

ALA (Sigma) was prepared in growth medium. Petri dishes with spheroid cultures were exposed to 635 nm light from a diode laser (Intense-HPD, North Brunswick, NJ). The light was coupled into an optical fiber terminating in a frontal distributor (FD, Medlight, Ecublens, Switzerland) providing a circular uniform light field. The spheroids were grown as bulk cultures in Petri dishes, each containing 15–25 spheroids. Four different treatment protocols were investigated (Fig. 1). Spheroids were initially incubated in 100 $\mu\text{g ml}^{-1}$ ALA for approximately 4 hours, prior to light irradiation. Spheroids in the single treatment PDT groups were subjected to radiant exposures of 1.5, 3, or 6 J cm⁻² for either 1 hour (group 1, irradiances of 0.42, 0.83, 1.7 mW cm⁻²) or for a 24 hours period at ultra low irradiance (group 2, irradiances of 17, 35, 70 $\mu\text{W cm}^{-2}$). Spheroids in the repetitive treatment groups were treated with illumination of 1.5, 3, or 6 J cm⁻² for either 1 hour (group 3) or 24 hours (group 4) at the same irradiances used for the single treatment groups. The spheroids received four light treatments with a 4-day interval for the low fluence group (group 3) or a 3-day interval for the ultra low fluence rate group (group 4). In the case of the long-term irradiations (groups 2 and 4), an additional 100 $\mu\text{g ml}^{-1}$ ALA dose was administered at the midpoint (12 hours) of the 24 hours light exposures. The illumination was performed at 37°C and with buffered medium (CO₂-Independent Medium, Invitrogen) in order to maintain physiological conditions during the long irradiation times. Control cultures consisted of light-only (no ALA) for the three fluence levels. At the conclusion of each irradiation, 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.

ALA-Administration Protocol—In order to separate light effects from photosensitizer pharmacokinetics, one group of spheroids was subjected to chronic 24 hours PDT (radiant exposure = 6 J cm^{-2}) with ALA administered as a single $200 \mu\text{g ml}^{-1}$ dose (4 hours incubation) while another group was subjected to the standard two $100 \mu\text{g ml}^{-1}$ doses, with the last dose given 12 hours into the light exposure. Control spheroids were subjected to the standard two $100 \mu\text{g ml}^{-1}$ doses, with the last dose given 12 hours into the 24 hours incubation but in the absence of light. One hour light treatment was then given to a fluence level of 6 J cm^{-2} .

Cell Viability Imaging—To determine spheroid viability after PDT, cultures were co-stained with Hoechst 33342 (Invitrogen) and Ethidium Homodimer 1 (E1169, Invitrogen) at 5 and $1 \mu\text{g ml}^{-1}$ in culture medium, respectively. The viability assays were performed 48 hours following the beginning of the PDT light exposure. The spheroid cultures were incubated with the dye mixture for 1 hour at 37°C . After incubation, the cultures were washed three times with Hank's Buffered Saline Solution (HBSS). The cultures were then examined using an inverted laser scanning microscope (LSM 510 META, Zeiss, Oberkochen, Germany) with a $20\times/0.5$ lens (EC Plan-Neofluar, Zeiss). The blue Hoechst fluorescence from all cell nuclei was imaged using 800 nm two-photon excitation and 390–465 nm detection. The red Ethidium Homodimer fluorescence from necrotic nuclei was imaged confocally with 543 nm excitation and 576–704 nm detection. Scans were made at planes $100 \mu\text{m}$ into the spheroids with simultaneous excitation of the two dyes, and the images were pseudo-colored blue and red. Vertical distances were corrected for spherical aberrations.

Results

The data presented in Figures 2 and 3 clearly show that the ultra low fluence rate treatment protocols (single and repetitive) were the most effective. The results are particularly striking in the case of repetitive treatment at the lowest fluence rate (Fig. 3) which resulted in 100% spheroid growth inhibition. This was considered to be a real effect since all control spheroids demonstrated growth over the observation period. As illustrated in Figure 2, growth inhibition was clearly light dose-dependent: ultra low light PDT (irradiance = $35 \mu\text{W cm}^{-2}$), with 3 J cm^{-2} of light, was sufficient to achieve statistically significant growth suppression compared to the controls, whereas 6 J cm^{-2} was necessary for single and repetitive PDT, respectively given at the higher fluence rate (1.7 mW cm^{-2}). Not surprisingly, the rate of spheroid growth was sensitively dependent on both the treatment protocol and radiant exposure (Fig. 4). The data are consistent with the results presented in Figures 2 and 3, that is, ultra low fluence rate PDT was found to be the most effective at delaying spheroid growth and higher radiant exposures resulted in slower growth for each of the three treatment protocols. Complete growth inhibition throughout the 30-day observation period was achieved only for spheroids subjected to 1.5 J cm^{-2} repetitive ultra low fluence rate PDT (Fig. 3) or for 12 J cm^{-2} given in a single treatment (data not shown).

The necessity of repeated ALA administration for extended light exposure (24 hours) PDT was investigated by comparing the administration of a single $200 \mu\text{g ml}^{-1}$ dose given 4 hours prior to light treatment to a regimen consisting of two $100 \mu\text{g ml}^{-1}$ doses: one given 4 hours prior to light treatment and the second 12 hours later. As shown in Figure 5, the rate of spheroid growth was reduced to almost zero with a second ALA dose. A significant reduction in the proportion of spheroids growing after PDT was only achieved when ALA administration was repeated during the light exposure as seen in Figure 6e. The result shows that continued presence of light and photosensitizer is necessary for ultra low fluence rate PDT to be effective. The effect was not related to prolonged illumination alone (Fig. 6c) nor to prolonged exposure to ALA (Fig. 6d).

When spheroid cultures were stained with the dead cell marker Ethidium Homodimer 2 days after the start of PDT and imaged by laser scanning microscopy, there was a striking difference between acute and chronic treatments. Representative sections through spheroids from treated and control cultures are shown in Figure 7. In the case of acute PDT, none of the three radiant exposures resulted in significant necrosis as evidenced by the lack of red signal. Conversely, high densities of necrotic cells were observed in cultures treated with ultra low fluence rate PDT. The extent of necrosis depended on the light dose. For 6 J cm^{-2} , a high density of necrotic cells was observed in both the outer spheroid layer as well as deeper in the spheroid.

Discussion

The photodynamic effect depends on a number of factors including light fluence and fluence rate, photosensitizer concentration, tissue oxygenation status, and intrinsic tissue sensitivity to the PDT effect. The high fluorescence observed in glial tumors following oral administration of 20 mg kg^{-1} of ALA suggests that PpIX levels in gross tumor tissue are sufficient for effective PDT [11–13]. In contrast, the main limiting factors for successful postoperative PDT include the inability to attain: (1) threshold light fluences in the wall of the resection cavity due to the high attenuation of light in the brain, and (2) adequate levels of PpIX throughout the volume of brain tissue harboring infiltrating tumor cells due to the blood–brain barrier. While ALA–PDT has been shown useful for localized and transitory opening of the blood–brain barrier, allowing the passage of photosensitizer as well as anti-cancer agents [28], the delivery of adequate light fluences to centimeter depths from the resection cavity wall, remains a significant challenge due to the excessive treatment times required to deliver adequate light doses.

The key finding of this study was that extremely low light fluence rates resulted in significant spheroid growth inhibition. It was shown that both single (6 J cm^{-2} delivered over 24 hours: irradiance= 0.14 mW cm^{-2} ; Fig. 2) and repetitive (1.5 J cm^{-2} delivered over 24 hours: irradiance= 17 mW cm^{-2} ; Fig. 3) PDT regimens resulted in complete growth inhibition. Based on simple diffusion theory calculations for a 2 cm diameter spherical light applicator positioned in the resection cavity, a $17 \mu\text{W cm}^{-2}$ fluence rate is achievable at a depth of approximately 2.8 cm in brain tissue [29]. The calculations assume an input power of 1W ($\lambda = 630\text{nm}$), an optical penetration depth of 3.2 mm [30–33] and a diffusion constant of $5.4 \times 10^{10} \text{ mm}^2 \text{ s}^{-1}$ [30–33]. From a clinical perspective, these calculations suggest that

fluence rates adequate for effective PDT can be delivered to depths exceeding 2 cm in the resection margin.

A number of in vitro and in vivo studies have documented the enhanced cytotoxic effects of low fluence rates. PDT efficacy is diminished at high fluence rates since the consumption of molecular oxygen exceeds the rate at which oxygen can be resupplied from the capillary vasculature, leading to PDT-induced hypoxic tumor regions [34]. Theoretical modeling in multicell spheroids suggests that the spatial distribution of singlet oxygen (the primary cytotoxic agent in PDT) depends critically on the fluence rate and on the availability of ambient oxygen [15]. The central prediction of this model is that, at a particular depth, singlet oxygen concentration 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 will yield improved therapeutic responses since singlet oxygen is delivered to a larger volume of tumor cells.

The existence of a lower fluence rate limit, below which the efficacy of threshold light fluences diminishes, is not known. Some studies [35,36] suggest that very low fluence rate PDT has diminished efficacy probably due to repair of sub-lethal PDT damage: a phenomenon commonly observed with low-dose X-rays. A number of in vitro and in vivo studies have found that decreased cytotoxicity occurs at fluence rates below 5.5 mW cm^{-2} [22,36]. No evidence of a lower fluence rate limit was found in this study and the results are consistent with previous findings in an identical spheroid model which show that significant spheroid response can be obtained at fluence rates of 5 mW cm^{-2} as long as the PDT fluence threshold is exceeded [37,38].

The clinical practicality of protracted low fluence rate and/or multiple treatments is somewhat questionable using standard light delivery procedures. Clearly, more sophisticated light delivery techniques will be required to implement such PDT protocols. There are a number of methods that are particularly well suited for PDT in the brain including metronomic PDT which involves the continuous slow delivery of photosensitizer and light in the μW power range. Although tumor apoptosis has been demonstrated with this type of treatment, no increase in tumor control has been observed [39]. The use of implanted balloon applicators and portable light sources, allowing repetitive low fluence rate PDT, is an attractive alternative [29]. For example, a clinical trial employing an indwelling balloon light applicator, allowing two PDT postoperative treatments on consecutive days, has recently been presented [40].

In the experiments presented here, spheroids were subjected to radiant exposures ($1.5\text{--}6 \text{ J cm}^{-2}$) previously shown to be sub-therapeutic when delivered acutely [16]. The treatment intervals (3 or 4 days) used in the repetitive therapy groups were based primarily on the pharmacokinetics of ALA-induced PpIX. Due to its relatively rapid clearance (24–48 hours), daily fractionation will require additional ALA administration in this model system. Systemic liver toxicity, often observed with frequent ALA intake, would necessitate an extended interval of several weeks between repetitive PDT treatments in a clinical setting [41]. Repetitive ALA–PDT, separated by several weeks, have proved highly effective in a spheroid model identical to that used in the present investigation [25,26].

The increased efficacy of the repetitive PDT protocols is probably due to a number of factors. The spheroids used in this study consisted of three distinct zones: an outer layer of proliferating cells, a middle layer of viable but non-proliferating cells, and a central core of necrotic cells. Previous studies have shown that proliferating cells in the outer rim are well oxygenated and produce the highest amounts of PpIX compared to deeper layers [42]. Proliferating cells in this layer are therefore highly sensitive to PDT and slough off between treatments thus inhibiting spheroid growth. After each treatment, viable non-proliferating cells are recruited into the proliferating pool as they form a new outer layer. These surviving cells can also be utilized to form a new generation of spheroids which are highly sensitive to renewed photodynamic treatment [25]. This phenomenon probably occurs in surgically treated tumors and demonstrates the importance of repeated access to the tumor resection cavity over an extended time period, thus allowing multiple treatments. The observation that “second generation” spheroids seem even more susceptible to PDT than controls might be indicative of some cumulative cytotoxic mechanism that may also play a role in multiply treated cultures.

Since the PDT dose is dependent on both photosensitizer concentration and light dose, it is important to maintain adequate drug levels throughout the light exposure. This is particularly important for PpIX since it undergoes rapid photobleaching during ALA-PDT. This is especially relevant for the chronic treatment protocols since low fluence rates have been shown to produce higher photobleaching [43,44]. The results presented in Figures 5 and 6 illustrate the importance of maintaining adequate PpIX levels during prolonged low fluence rate treatment regimens.

Spheroids were used in this study since they mimic many of the characteristics of solid tumor nodules. For example, their three-dimensional geometry results in heterogeneous subpopulations of cells differing in proliferation, nutritional, metabolic and, most importantly, oxygenation status [28]. This rather simplistic model is somewhat limited by its inability to account for vascular responses which are important components of most PDT protocols—practically all exogenous sensitizers used in clinical PDT target primarily the tumor vasculature. In this context, it would appear that the spheroid model is ideally suited for ALA investigations since ALA-induced PpIX accumulates within cells resulting in direct tumor cell toxicity rather than vascular damage and therefore there is a high degree of confidence that the observed trends can be reproduced in animal models.

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Fig. 1.

In vitro treatment protocols. Light (635 nm) was administered once or four times. In both cases, the effects of low (1 hour exposure) and ultra low (24 hours exposure) light irradiance were investigated.

Fig. 2.

Effects on spheroid survival of single PDT treatment. Spheroids were incubated with ALA $100 \mu\text{g ml}^{-1}$ for 4 hours and then subjected to radiant exposures of 1.5, 3, or 6 J cm^{-2} for either 1 hour (irradiance of 0.42, 0.83, or 1.7 mW cm^{-2}) or for a 24 hours period at ultra low irradiance (17, 35, or $70 \mu\text{W cm}^{-2}$). Additional ALA ($100 \mu\text{g ml}^{-1}$) was added after 12 hours in the 24 hours cultures. Each bar represents the percentage of 16–24 spheroids showing growth 4 weeks following treatment. The error bars show standard deviations. The controls represent 16–24 spheroids receiving, 24 hours of light in the absence of ALA.

Fig. 3.

Effects on spheroid survival of repetitive PDT treatment. Spheroids in the repetitive treatment groups were exposed to radiant exposures of 1.5, 3, or 6 J cm⁻² for either 1 or 24 hours at the same irradiances used for the single treatment protocol. Light was given four times with a 4-day interval for the low irradiance group or a 3-day interval for the ultra low irradiance group. In the case of the long-term irradiations, an additional 100 µg ml⁻¹ ALA dose was administered at the midpoint (12 hours) of the 24 hours light exposures. Control cultures consisted of light, no ALA. Each bar represents the percentage of 15–24 spheroids showing growth 4 weeks after treatment. The error bars denote standard deviations.

Fig. 4.

Comparison of the effects of single and repetitive PDT on spheroid growth kinetics. Comparison of mean spheroid growth kinetics at 3 J cm^{-2} between controls, single and repetitive PDT with 1 hour light exposure and single PDT with 24 hours light exposure. Spheroid growth was monitored weekly over a 4-week period. Each curve represents the average of the diameter increase for 15–24 spheroids. Error bars show standard deviations.

Fig. 5.

Effects of repeated ALA administration on spheroid growth rate. The administration of a single $200 \mu\text{g ml}^{-1}$ dose given 4 hours prior to light treatment was compared to a regimen consisting of two $100 \mu\text{g ml}^{-1}$ doses one given 4 hours prior to light treatment and the second 12 hours later. Dark controls were exposed to two $100 \mu\text{g ml}^{-1}$ ALA doses, with the last dose administered 12 hours prior to termination. Error bars denote standard deviations of the mean.

Fig. 6.

Effects of ALA incubation protocol. a: Untreated control; (b) dark control, two $100 \mu\text{g ml}^{-1}$ ALA doses, with the last dose administered 12 hours prior to termination; (c) 6 J cm^{-2} PDT (24 hours) with ALA administered as a single $200 \mu\text{g ml}^{-1}$ dose 4 hours prior to light exposure; (d) two $100 \mu\text{g ml}^{-1}$ ALA doses, with the last dose given 12 hours into the 24 hours incubation but in the absence of light, followed by 1 hour light treatment (6 J cm^{-2}); (e) two $100 \mu\text{g ml}^{-1}$ doses, with the first dose administered 4 hours prior to light irradiation and the last dose given 12 hours into the 24 hours light exposure. Each bar represents the percentage of 20–25 spheroids showing growth 2 weeks after treatment. The error bars represent standard deviations.

Fig. 7.

Laser scanning fluorescence microscopy images of spheroids stained with Hoechst 33342 (blue) and Ethidium Homodimer 1 (red). All images were acquired at a depth of approximately 100 μm , 48 hours after the start of treatment. The field of view is 600 μm $\times$ 600 μm for all images.