Effects of photodynamic therapy on human glioma spheroids

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ABSTRACT

The poor prognosis for patients with malignant brain neoplasm has led to a search for better treatment modalities. Although gliomas are considered to be disseminated tumors in the brain, most recur at the site of the previous tumor resection. Improved local control would thus be of clear benefit. The utility of photodynamic therapy (PDT) in the treatment of brain neoplasms is investigated using a human glioma spheroid model. Specifically, the effects of PDT on human glioma spheroids are investigated using Photofrin™ and 5δ-aminolevulinic acid (ALA). The effects of various irradiation schemes were monitored using a simple growth assay. A growth delay was observed at an optical fluence of approximately 35 J cm^{-2} for spheroids incubated in Photofrin. Spheroids incubated in ALA were unaffected by the PDT treatment regimens examined in this study. This was most likely a result of inadequate photosensitizer concentration.

Keywords: Photodynamic therapy, glioma, spheroids

1. INTRODUCTION

Primary intracranial neoplasms account for 2% of all cancer deaths[1]. Approximately half of these neoplasms are glioblastoma multiforme – the most aggressive variety of glial tumors[2]. There is no satisfactory treatment for these infiltrative neoplasms. The best available treatment using surgery, chemotherapy and radiation therapy, results in a median survival of 10 months[3]. Two-year survival rates (7.5%) are dismal. Failure of treatment is usually due to local occurrence of the tumor indicating that a more aggressive local therapy could be of benefit. Several studies have shown that photodynamic therapy (PDT) may prove to be useful in prolonging survival of glioma patients[4-9].

Multicell tumor spheroids have a complexity intermediate to standard monolayer cultures and tumors *in vivo*. The three-dimensional arrangement of multicellular spheroids results in heterogeneous subpopulations of cells differing in their proliferation, nutrition and oxygenation status[10]. Spheroids thus capture some of the characteristics of tumors *in vivo*. However, since this is accomplished *in vitro*, the spheroid model allows various tumor cell-specific phenomena to be studied in the absence of complex host-dependent factors[11].

In this study, the response of human glioma spheroids to PDT is presented. Spheroids were incubated in either Photofrin™ or ALA and subjected to various irradiation schemes. Spheroid response was evaluated using growth delay.

Part of the SPIE Conference on Optical Methods for Tumor Treatment and Detection: Mechanisms and Techniques in Photodynamic Therapy VIII • San Jose, California • January 1999
SPIE Vol. 3592 • 0277-786X/99/$10.00

2. MATERIALS AND METHODS

2.1. Cell cultures

Human glioblastoma cells were kindly supplied by Dr. Granger, Department of Biology, University of California, Irvine. The cells were grown in DMEM 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. 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 minutes. The resultant cell clusters (consisting of approximately 10 cells) were transferred to a petri dish and grown to tumor spheroids of varying sizes. The 250 μm diameter spheroids used in these experiments were selected by passage through a screen mesh. It took approximately 14 days for the spheroids to reach a size of 250 μm. The spheroid culture medium was changed three times weekly.

2.2. PDT treatments

Spheroids were incubated in either 5 μg/ml Photofrin (QLT Phototherapeutics Inc., Vancouver, BC Canada) for 24 hours, or in 100 μg/ml ALA (Sigma, St. Louis, MO) for 4 hours. Spheroids were irradiated in a petri dish. A 3 cm dia. 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. Following irradiation, individual spheroids were placed into separate wells of a 96 well culture plate and monitored for growth. A microscope with a calibrated eyepiece micrometer was used to measure spheroid diameter. Typically, spheroids were followed for 2 to 4 weeks.

In all cases, spheroids were irradiated with 630 nm light from an argon ion-pumped dye laser (Coherent, Inc., Palo Alto, CA). Light was coupled into an optical fiber containing a microlens at the output end. Photofrin-incubated spheroids were subjected to fluences of 15, 20, 30, 35, 40 and 50 Jcm^{-2} delivered at a fluence rate of 30 mW cm^{-2}, while ALA-incubated spheroids were subjected to fluences of 30, 50, 75 and 100 J cm^{-2} delivered at a fluence rate of 50 or 100 mW cm^{-2}.

3. RESULTS AND DISCUSSION

The response of Photofrin-incubated spheroids to various fluences is presented in Figures 1a and b. The data presented in Figure 1a show that, in comparison to the dark controls, there is no significant effect on the growth kinetics of spheroids exposed to fluences of up to 30 J cm^{-2}; in all cases, the shape and magnitude of the curves are similar. There appears to be a significant effect on the growth kinetics of spheroids exposed to fluences of 35 J cm^{-2}. This is manifested by a growth delay of approximately 7 days post irradiation. Furthermore, the terminal size of these spheroids is significantly smaller then those exposed to fluences of 30 J cm^{-2} and less (400 vs. 600 μm).

Figure 1b shows that spheroid viability is adversely affected by fluences of 40 and 50 J cm^{-2}. In both cases, the number of observable spheroids decreases rapidly following irradiation. Although the spheroids disintegrated, the cells appeared to be intact, i.e., no cellular debris was observed. Thus many of the individual cells are likely to retain their viability and may form the locus for renewed tumor growth.

A fluence rate of 30 mW cm^{-2} was chosen based on the findings of Foster et al[11]. They observed a strong fluence rate dependence of spheroid cell survival. Survival improved with increasing fluence rate due to the creation of hypoxic volumes as a result of therapy-induced oxygen consumption. No significant differences in spheroid cell survival were observed below 50 mW cm^{-2} [11].

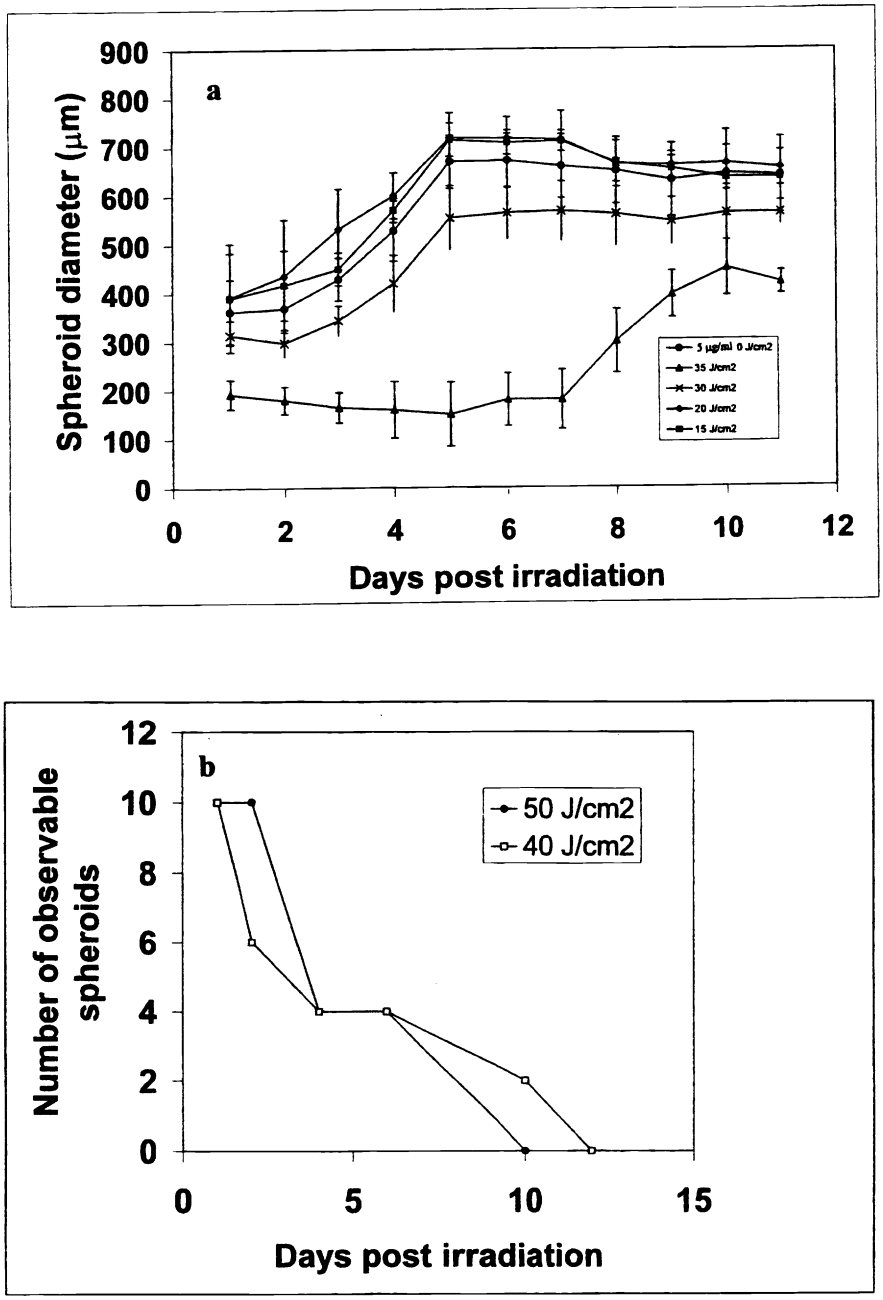


Figure 1. (a) Spheroid growth delay as a function of light fluence. Each data point corresponds to 10 spheroids. (b) Spheroid viability at high light fluences. In all cases, spheroids were incubated in Photofrin (5 μg ml^{-1}) and exposed to fluence rates of 30 mW cm^{-2}.

The results presented here are in good agreement with the findings of Terzis et al[1]. They observed an almost complete inhibition of spheroid growth for two human glioma cell lines (GaMg and U-251Mg) incubated in 5 μg ml^{-1} Photosan-3™ and exposed to fluences of 30 and 35 J cm^{-2}. These investigators noted that the remaining tumor cells were not dead and could be stimulated to invade normal tissue when exposed to a normal brain microenvironment.

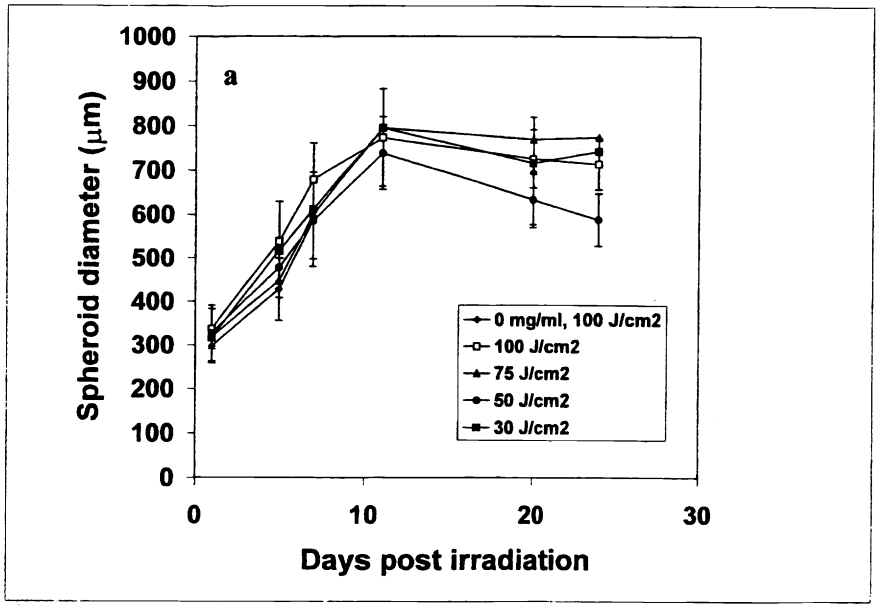
Results of the ALA-incubated spheroids are summarized in Figures 2a and b. The plots show that there is no effect on spheroid growth for the doses and dose rates examined here. This is most likely due to the low ALA concentration (100 μg ml^{-1}) used in these experiments. This was confirmed in confocal microscopy studies showing weak protoporphyrin IX (Pp IX) fluorescence from spheroids incubated in 100 μg ml^{-1} ALA. The optimal concentration for glioma spheroid cells is unknown, however, based on dark toxicity studies of monolayer cells, it is likely to be of the order of a few mg ml^{-1} [12]. The lack of response to treatment due to inappropriate dose rates cannot be ruled out. The likelihood of this being a contributing factor to the failure of treatment was minimized through judicious choice of dose rates based on the clinical experience of others[13].

Two-photon microscopy studies performed in this lab (data not shown) show significant Pp IX fluorescence to spheroid depths of approximately 100 μm. This suggests that the lack of response was not due to inadequate diffusion of ALA into the spheroids.

The rationale for the ALA studies presented here is that ALA-induced Pp IX may have significant advantages over Photofrin in PDT of advanced brain tumors. These include: (1) improved tumor-to-normal cell uptake[14], and (2) reduced normal cell sensitivity[14]. Normal brain structures express different specific uptakes for different photosensitizers and different sensitivities towards PDT. Thus, even though Pp IX is less phototoxic than Photofrin (at comparable concentrations and light doses), it may be the preferred drug due to limited photodamage to surrounding normal tissue. For example, Lilge and Wilson[14] have observed very little white matter damage for ALA-mediated PDT. This is important since most adult tumors arise in white matter.

It has been suggested that porphyrin PDT is mediated by mitochondrial benzodiazepine receptors (MBR) and that the therapeutic potencies of porphyrins correlate closely with their affinity for MBRs[15]. This would suggest a prominent role for ALA-induced Pp IX since it is one of the most potent binding agents to the MBR – its binding affinity is approximately five times higher than that of Photofrin[15]. The observation of minimal white matter damage in ALA-mediated PDT may be due to the fact that MBRs are sparse in normal neuronal tissue[16]. Interestingly, a 20-fold increase in the number of MBRs in human gliomas compared with normal brain has been observed[16]. Normal tissue damage observed in Photofrin-mediated PDT may be due to plasma membrane damage. In preliminary two-photon microscopy studies performed in this lab, strong fluorescence signals have been observed from both plasma and mitochondrial membranes of Photofrin-incubated glioma spheroid cells. In contrast, fluorescence signals from ALA-incubated spheroid cells were confined almost exclusively to mitochondrial membranes. Thus, the reduced normal cell sensitivity observed in ALA PDT may be due to the localization of damage to sites containing MBRs, i.e., the mitochondrial membrane. Normal neuronal tissue contains few of these receptors and is thus spared. Such tissue may not be spared during Photofrin-mediated PDT since damage is not specific to the mitochondrial membrane – the plasma membrane seems to be an important target as well.

The greater phototoxicity of Photofrin may be due to the fact that the MBR recognition sites for some of the porphyrin constituents are located on the inner mitochondrial membrane[17]. In contrast, the MBR recognition site for Pp IX is located on the outer membrane. The location of the porphyrin binding site is important since the diffusion distance in cells of singlet molecular oxygen is very small (<0.1 μm[18]). Thus, submitochondrial localization is important in determining phototoxicity.



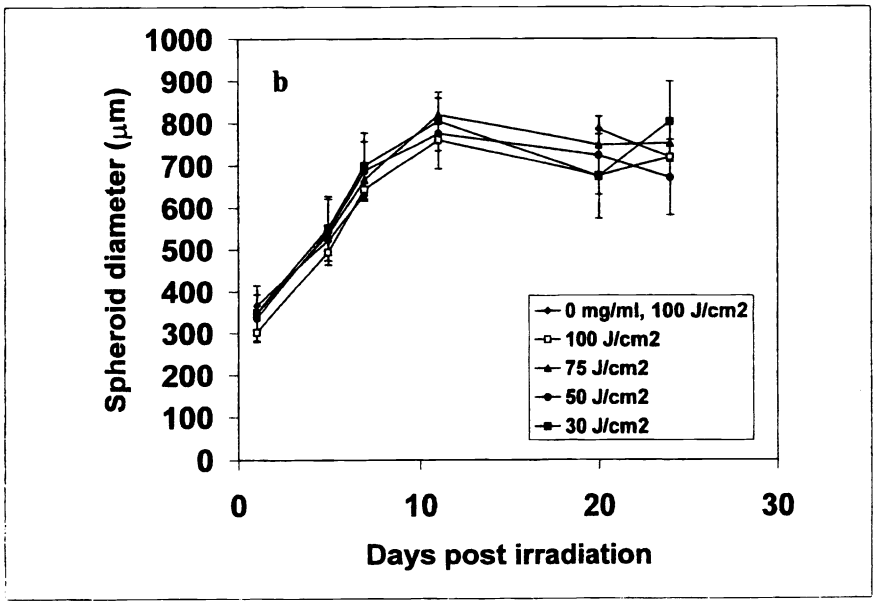


Figure 2. Spheroid growth delay as a function of light fluence. Each data point represents the mean of 25 spheroids. Spheroids (except controls) were incubated in ALA (100 μg ml^{-1}). (a) Fluence rate = 50 mW cm^{-2}. (b) Fluence rate = 100 mW cm^{-2}.

4. CONCLUSIONS

The results presented here for Photofrin-mediated PDT in human glioma spheroids show that spheroid growth is delayed at optical fluences of approximately 35 J cm^{-2}. Even though spheroid disintegration was observed at higher fluences, the resultant cells appeared to be viable and thus capable of forming new foci of tumor growth in the brain microenvironment. The Photofrin data presented here are limited by the small sample size; only ten spheroids were followed per treatment group. Additional experiments will be performed to confirm these results. The preliminary data is, however, in good agreement with similar studies performed in comparable spheroid cell lines by other investigators[1].

PDT with ALA had no effect on spheroid growth. This is most likely due to an inadequate concentration of ALA. Studies are under way to determine the dark toxicity, and hence the optimal ALA concentration for the human glioma spheroid cells used here.

5. FUTURE WORK

The clinical experience with PDT in the treatment of brain tumors is somewhat limited – only a few patients have been treated. However, preliminary results are encouraging as many patients have shown prolonged survival with few complications[19]. It has been suggested that further improvement to quality-of-life may be possible by combining PDT with ionizing radiation[20]. The search for additive and/or synergistic effects of PDT and ionizing radiation has been attempted previously in numerous cell lines[21]. These studies have been complicated by the fact that the interaction of ionizing radiation and PDT does not follow a general pattern at the cellular level. Due to different responses of different cell types to each treatment, the interaction may be additive in one and synergistic in another cell type and should thus be established for each cell type separately[21]. To our knowledge, the interaction of PDT and ionizing radiation has not been investigated in human glioma spheroids. A synergistic interaction between these modalities has been noted in a rat glioma model[21].

An indwelling balloon applicator designed for use in high dose rate (HDR) intercavity brachytherapy of brain tumors has recently been designed by one of the authors (Figure 3)[22]. The applicator is also ideally suited to PDT[23]. A combined HDR brachytherapy – PDT treatment has been proposed. The applicator is inserted into the resected tumor bed through a burr hole in the skull and left in place during the 5 to 6 day treatment period. During this time, high doses (60 – 70 Gy) of ionizing radiation are given in combination with PDT.

The clinical study described above provides the impetus for future studies with human glioma spheroids. A systematic examination of the effects of ionizing radiation and PDT on human glioma spheroids is proposed. Effects of ionizing radiation dose and dose rate, type of photosensitizer (Photofrin vs. ALA), and various fractionation schemes will be investigated.

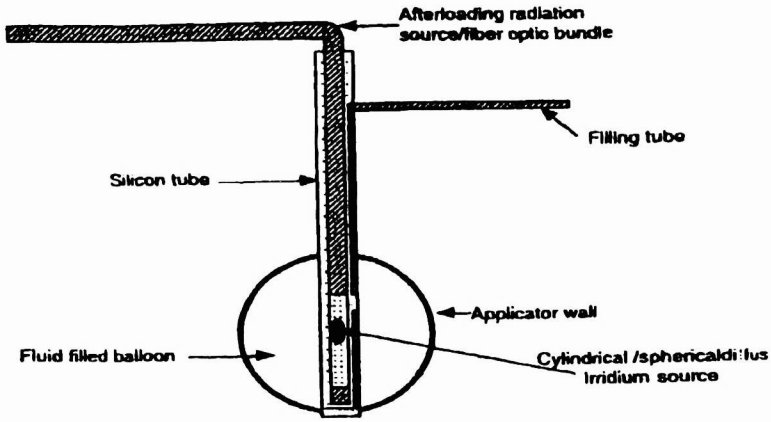


Figure 3. An indwelling balloon applicator for brachy and photodynamic therapy.

ACKNOWLEDGEMENTS

Steen Madsen is grateful for the support of the UNLV Office of Research. This work was made possible, in part, through access to the Laser Microbeam and Medical Program (LAMMP) and the Chao Cancer Center Optical Biology Shared Resource at the University of California, Irvine. These facilities are supported by the National Institutes of Health under grants RR-01192 and CA-62203, respectively. In addition, Beckman Laser Institute programmatic support was provided by the Department of Energy (DOE #DE-FG03-91ER61227), and the Office of Naval Research (ONR #N00014-91-C-0134).

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