Investigation of different light delivery schemes in photodynamic therapy of human glioma spheroids

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INVESTIGATION OF DIFFERENT LIGHT DELIVERY SCHEMES IN
PHOTODYNAMIC THERAPY OF HUMAN
GLIOMA SPHEROIDS

by

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ABSTRACT

Investigation Of Different Light Delivery Schemes
In Photodynamic Therapy Of Human
Glioma Spheroids

by

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The response of human glioma spheroids to ALA-mediated photodynamic therapy subjected to various optical dose delivery schemes is investigated. In particular, the effects of fluence, fluence rate, light dose fractionation and long-term repeat PDT is considered. Improved PDT response was found for low fluence rate photodynamic treatments i.e. those at or below 25 mW cm$^{-2}$. Results are consistent with the self-sensitized singlet oxygen mediated photobleaching model of Georgakoudi et al (Georgakoudi, 1998), in which the response is attributed to the deposition of singlet oxygen to a larger volume, i.e., deeper into the spheroid. The efficacy of high-fluence rate PDT (150 mW cm$^{-2}$) can be improved by the introduction of dark intervals – an approximately 35 % enhanced spheroid response was observed with cyclic intervals of between 15 and 45 s. Shorter on/off cycles appear more effective than longer cycles. Longer dark periods likely result in decreased singlet
molecular oxygen production per unit time and, hence reduced cell killing efficiency. The optimum fractionation schedule may well depend only on the oxygen diffusion distance, insensitive to either light fluence rate or photosensitizer properties and can be obtained by a simple mathematical model of oxygen diffusion (Pogue, 1997). Furthermore, results presented in this thesis suggest that the production of singlet molecular oxygen is maximized by the use of lower fluence rates as opposed to any fractionation schedule.

Repetitive PDT consisting of multiple weekly treatments at sub-optimal fluences (12.5 or 25 J cm\(^{-2}\)) revealed marked improvements in efficacy - as much as 90% spheroid kill was achieved for treatments employing low fluence rates (2.5 mW cm\(^{-2}\)). Additionally, cellular proliferative capacity was inhibited as evidenced by significant growth delays. Two-photon fluorescence microscopy imaging was used to evaluate morphological changes induced by low and high fluence rates. The images indicate preferential apoptotic induction at low fluence rates, while high fluence rates appear to cause necrosis. The general lack of cellular response to high fluence rate PDT can be improved through light modulation, as is shown in this study.
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CHAPTER 1

INTRODUCTION

Section 1.1 Glioblastoma Multiforme, Disease and Prognosis

Despite an arsenal of currently employed treatment methods for malignant gliomas including surgical tumor resection, radio- and/or chemotherapy, patient prognosis remains poor. Nearly half of these intracranial neoplasms present as the most aggressive malignant glioma variety known as Glioblastoma Multiforme (GBM). Although GBM accounts for only one percent of all cancer mortality in the United States each year, these patients suffer an astoundingly rapid deterioration in their quality of life. It is possible to extend median survival from three to ten months using the aforementioned modalities, though long-term tumor control is rarely accomplished. Fewer than five percent of GBM patients survive longer than five years. It is no wonder adjuvant therapies such as brachytherapy, hyperthermia and photodynamic therapy are being investigated for their efficacy in prolonging and improving life for brain tumor patients.

GBM originates in proliferative glial cells or their precursors within the central nervous system (CNS). The nature and location of this disease manifests with great complexity, which renders it quite resistant
to therapeutic intervention. Malignant cells of these high-grade gliomas (classified by the American Cancer Society as a grade IV brain tumor) exhibit migratory behavior, possibly owing to their developmental character within the CNS. These infiltrative cells, referred to as the secondary structures of Scherer (Holland, 2000), detach from the focal tumor site and invade normal brain parenchyma. Radical tumor resection becomes effectively unrealistic as it counters the preservation of brain regions vital to patient survival. As a consequence, patients relapse in eighty percent of all cases with recurrent tumor growth within a 2-3 cm margin of the surgical resection cavity (Wallner, 1989; Nieder, 2000). This attribute allows for the possibility that a more aggressive form of local therapy could potentially quell the frequency of these recurrences, thereby extending patient survival.

Section 1.2 Photodynamic Therapy

Photodynamic therapy is a local antineoplastic treatment with the potential for tumor cell specificity. The treatment involves the administration of a tumor localizing photosensitizing agent followed by photo-activation within the malignant tissue. Lasers having wavelengths within the visible spectrum are commonly employed and are chosen in accordance with the region of absorption maxima for the given photosensitizing drug. Red light is almost always used due to its deeper penetration in tissues. In response to laser irradiation, the
photosensitizer is raised to an excited triplet state. One mode of de-excitation occurs via collision with ground state triplet molecular oxygen yielding a highly reactive cytotoxic singlet oxygen species. Oxygen radical species have a lifetime in biological systems ranging from 100 nanoseconds within the lipid regions of membranes (Kanofsky, 1991) to 250 nanoseconds within the cytoplasm (Baker, 1992). Thus, diffusion is limited to approximately 20 nm before reacting with critical cellular structures and/or membranes to potentially bring about cellular death.

PDT-induced cell death may manifest as either a necrotic or apoptotic process that will depend on the parameters particular to treatment as well as on the subcellular localizing characteristics of the employed photosensitizer. Necrosis is the common mode of death for cells treated with ionizing radiation and ensues as a result of chromosomal damage sufficient to inhibit cellular mitosis. Conversely, apoptosis is a natural homeostatic mechanism responsible for maintaining certain bodily tissues in a healthy cellular balance. It is for this reason that it is commonly referred to as programmed cell death. The apoptotic response is initiated by the release of cytochrome C through a compromised mitochondrial membrane damaged by, for example, PDT. Apoptosis offers a somewhat more favorable biological response, the details of which will be discussed in the upcoming text. Understanding the causation of an apoptotic response is particularly relevant when one considers the exceedingly fragile organ under siege, the brain.
Since the onset of PDT research in the 1960's, countless preclinical and clinical studies worldwide have established its usefulness as an adjuvant or, in the case of superficial lesions, as a stand-alone treatment for a variety of cancers. Inaccessibility of the malignancy to a light source may preclude PDT treatment. PDT has been applied to cutaneous and gastrointestinal lesions, cancers of the mouth, larynx, esophagus, lung, bladder and brain. PDT offers the prospect of lengthening and/or improving the quality of life of cancer sufferers.

The aim of PDT in the treatment of high-grade gliomas is to eliminate the nests of malignant cells residing post-operatively at cm depths in the resection margin in an effort to mitigate the likelihood of recurrent tumor growths. PDT efficacy depends on a number of factors as well as the dynamic interplay amongst these during the course of treatment. The most critical factors affecting treatment outcome are intratumoral oxygenation status, photosensitizer concentration and localization, total light dose or fluence and the rate of light delivery or fluence rate. The vital role of each will be discussed at some length.

Since singlet molecular oxygen has been implicated as the primary cytotoxic species, the efficacy of PDT depends sensitively on the amount of oxygen present during treatment. There is a fine balance between oxygen depletion and reoxygenation during PDT. At any given time, tissue oxygenation status depends primarily on capillary density and on the rate at which the light is delivered, i.e., the fluence rate. Thus, in
principle, it should be possible to affect PDT outcome through judicious choice of fluence rate.

Section 1.3 The Role and Influence of the Photosensitizer

Photosensitizing compounds preferentially accumulate to varying degrees in neoplastic cells. Tumor cell targeting depends on the ability of the particular photosensitizer to pass across cellular membranes. This will depend on the biochemical characteristics of the photosensitizer employed. The drug may be applied topically, injected intravenously or administered orally.

A wide variety of third generation photosensitizers are currently being evaluated for their particular localization properties in combination with other desirable characteristics such as low toxicity and favorable absorption spectra. Photofrin®, a commonly employed photosensitizer in PDT, is a partially purified commercially available haematoporphyrin derivative compound approved for clinical application in PDT of early and advanced stage cancers of the lung, digestive tract, and genitourinary tract in Canada, The Netherlands, France, Germany, Japan and the United States (Dougherty, 1998). The United States Food and Drug Administration (US FDA) initially approved its application in 1995 for the palliation of patients with complete obstructing esophageal cancer and in 1998 for use in PDT of early and late stage non-small cell lung cancer. The efficacy of Photofrin-mediated PDT in the treatment of the bladder,
mouth, larynx, chest wall, brain, cutaneous and GI lesions is under wide study, much of which is in the clinical trial phase.

Several drawbacks limit Photofrin’s application in PDT, the most severe of which is the uncommonly long period of cutaneous photosensitization experienced by patients lasting two to three weeks. This feature precludes the use of Photofrin in fractionated treatment regimens, an attractive treatment protocol evaluated in this research. Additionally, inadequate tumor-to-normal tissue ratios limit the allowable dose due to the increased potential for normal tissue complications. Pharmacological efforts geared towards improving the characteristics of the available sensitizers have brought a number of second-generation photosensitizers to the market. Toxicity, bioavailability, excitation band, retention and excretion rates, side effects as well as the resultant biochemical pathway of photodynamic action are among the important photosensitizer attributes currently being evaluated by researchers worldwide (Stummer, 1998). At the forefront of drug development is 5-aminolevulinic acid (ALA). This prodrug stimulates endogenous porphyrin production and causes the accumulation of protoporphyrin IX (PpIX), a potent sensitizer via the heme biosynthetic pathway (Kennedy, 1990; Kennedy, 1992). Heme is synthesized from glycine and succinyl CoA to ALA, whose production is under negative feedback control by heme (Peng, 1997). Through the exogenous introduction of ALA, PpIX bypasses regulatory control resulting in
sensitizer accumulation. Since PpIX-induced patient photosensitivity lasts only 24 to 48 hours, ALA is well suited for repeat PDT treatments. Another attractive advantage of ALA is that it can be administered orally. Finally, a number of animal studies (Lilge, 1998) have reported high tumor-to-normal brain tissue concentrations of PpIX. This would make ALA an excellent candidate for PDT of human gliomas.

The mode of cell death following PDT depends on a number of parameters including photosensitizer type, incubation time, light fluence and fluence rate. Due to mitochondrial localization of PpIX, ALA-mediated PDT has been shown to preferentially induce apoptotic cell death. Following PDT, the integrity of the mitochondrial membrane is compromised resulting in the release of cytochrome C which, in turn, initiates a caspase-3 cascade event ending in apoptosis. This mode of destruction is morphologically characterized by cell condensation and budding to produce membrane-enclosed bodies that are subsequently phagocytized and digested by nearby cells. In contrast, a necrotic death induces inflammation, blebbing and edema- a particularly unfavorable response in the brain. Maximizing the non-inflammatory advantages of apoptosis may be particularly useful in controlling post photodynamic treatment edema of the brain. Morphologic evaluation of cell death is possible with a number of techniques, including two-photon fluorescence microscopy.
Section 1.4 Fluence Effects

In all likelihood, the limiting factor for successful PDT is the inability to deliver threshold light fluences in the brain-adjacent-to-tumor (BAT). This is due to the rapid attenuation of light in brain tissue. Assuming sufficient photosensitizer concentration, PDT response has been shown to depend critically on the amount of light delivered (Lilge, 1996). Photodynamic threshold models have been developed based on the observation of a distinct demarcation between tissue necrosis and undamaged tissue. In one such model (Patterson, 1990), under well oxygenated conditions, tissue necrosis occurs for a given photosensitizer if a certain number of photons are absorbed within a given volume.

In a previous *in vitro* study using 500 μm diameter human glioma spheroids incubated in 5 mM ALA, Madsen et al (Madsen, 2000) observed a threshold fluence of approximately 50 J/cm² (delivered at a fluence rate of 25 mW cm⁻²). The delivery of threshold light fluences (50 J cm⁻²) to cm depths in the BAT require treatment times on the order of hours (Hirschberg, 1999). Such long treatment times are impractical with standard one-shot intraoperative procedures. Clearly, more sophisticated light delivery techniques are required for improved PDT outcome. It may be possible to improve outcome through multiple repeat PDT treatments given at weekly intervals.
Section 1.5 Fluence Rate Effects

Tissue oxygenation status during PDT is a critical factor in determining treatment outcome. Tissue oxygenation is a dynamic variable and, as has been shown in numerous in vitro (Ben-Hur, 1987; Matthews, 1989; Foster, 1993) and in vivo (Gibson, 1990; Van Geel, 1996; Sitnik, 1998a, b; Linuma, 1999) studies, is strongly dependent on the rate at which the light dose is given – low dose rates are more effective than high dose rates. The reduced effectiveness of high dose rate PDT is due to the fact that the rate of singlet oxygen production depends, in part, on the light dose rate. At high dose rates, PDT can photochemically deplete ambient tumor oxygen causing acute hypoxia thus limiting the overall treatment effectiveness.

The importance of adequate tissue oxygenation during PDT has also been demonstrated in a number of theoretical studies (Foster, 1991; Foster, 1992; Nichols, 1994; Henning, 1995; Pogue, 1997). These models indicate that, in addition to dose rate, oxygenation status can also be affected by microvasculature patency. The degree to which the microvasculature is affected by PDT is strongly dependent on the particular type of photosensitizer used, for example, some drugs exert their effects indirectly via vascular destruction (Linuma, 1999), while others, such as ALA, are generally considered to be cellular photosensitizers. It has been suggested that the effect of oxygen diffusion into hypoxic tumor cells is less important if the mechanism of
tumor destruction is due to PDT-induced microvasculature damage and that oxygen replenishment is crucial if the destructive photochemistry occurs via singlet oxygen-mediated destruction of tumor cells, as for example, in ALA-mediated PDT (Linuma, 1999). This suggests that the dose rate effect is strongly dependent on photosensitizer type. Since ALA has a negligible vascular component, the response to ALA-mediated PDT is expected to be critically dependent on the balance between oxygen consumption and replenishment. Since this balance is affected by the rate at which the light dose is given, ALA-mediated PDT is expected to exhibit significant dose rate dependence. This has been confirmed in a number of studies. For example, enhanced tumor destruction has been observed at low dose rates in a rat tumor model using ALA-mediated PDT (Linuma, 1999). A systematic study of dose rate effects in a human glioma spheroid model using ALA has significant clinical implications in that the results will guide us toward the development of optimum light delivery protocols.

Section 1.6 Light Dose Fractionation

Due to the rapid attenuation of light in brain tissue, light fluence and, hence fluence rate, decrease rapidly with distance from the light source. Thus, high laser powers are required in order to achieve significant cell damage at 1-2 cm depths in the BAT. Not surprisingly, the tissue in close proximity to the applicator will be exposed to very high fluence
rates. This is problematic since high fluence rates result in significant oxygen depletion and resultant poor PDT outcome. It has been postulated that modulating the output of the laser light source (e.g. by introducing brief dark intervals during irradiation) will allow for reoxygenation, thus producing a higher cytotoxic singlet oxygen yield and ultimately resulting in higher cell kill. The optimum dark interval depends on the rate of reoxygenation, i.e., on the distance of the cells from the oxygen source (d) and on the rate of oxygen diffusion (D). The characteristic diffusion time of oxygen in tissue is given by:

\[ t = \frac{d^2}{D}. \]  

Assuming a diffusion constant of 1460 \( \mu \text{m}^2 \text{s}^{-1} \) [Nichols, 1994] and using distances ranging from 150-200 \( \mu \text{m} \), characteristic diffusion times range from 15 to 27 s. Knowledge of the oxygen diffusion time allows optimization of the dark interval. The effectiveness of various dark intervals during high-fluence rate PDT will be investigated in an \textit{in vitro} human glioma spheroid system. The results will be compared to a simple theoretical model of oxygen diffusion.

Section 1.7 Repetitive PDT

To date all PDT trials involving brain tumors have employed short-term intraoperative or stereotactic light delivery techniques [Krishnamurthy, 2000; Papovic, 1996]. This is unlikely to eliminate cells
deep in the BAT due to the inability to deliver toxic threshold light fluences in a reasonable time period. For example, the fluence at a depth of 1 cm in brain tissue is approximately 2% of that in tissues immediately adjacent to the light source (Madsen, 2003). Due to the difficulties associated with rapid light attenuation in brain tissue, alternative treatment strategies have been proposed. In a series of simple \textit{in vitro} studies, Madsen et al. (Madsen, 2003) have investigated the efficacy of repetitive weekly or bi-monthly PDT treatments. In each treatment, light fluences were purposely kept below toxic thresholds to simulate conditions typically found at 1-2 cm depths in the BAT. The results show that multiple PDT treatments can be very effective, at least when the light is delivered at fluence rates of 25 mW cm\textsuperscript{-2}. The efficacy of multiple low-fluence rate (2.5 mW cm\textsuperscript{-2}) PDT treatments is unknown.

\textbf{Section 1.8 \textit{In vitro} Tumor Model: The Spheroid}

Preclinical research is typically conducted either \textit{in vitro} using traditional monolayer cell cultures, or in animal models. Multicell spheroids have a complexity intermediate to these models and offer unique research advantages. For example, the 3-dimensional multicellular architecture better mimics the compact spatial organization and intercellular communication of a solid tumor's microenvironment, but in the absence of the dynamic influences of an \textit{in vivo} model. Specifically, the 3-dimensional geometry results in heterogeneous
subpopulations of cells differing in proliferation, nutritional, metabolic and, most importantly, oxygenation status (Sutherland, 1971). The local environment surrounding the various cells in the spheroid is dependent on their position thus mimicking the gradients found in solid tumors.

Section 1.9 Purpose and Significance of the Study: Research Questions

In this thesis, the response of human glioma spheroids to various light delivery schemes is investigated. In particular, the effects of fluence, fluence rate, light dose fractionation and long-term repeat PDT is considered. Specifically, under high fluence rate conditions i.e. 150 mW cm\(^2\), the effect of interrupted photo irradiation is studied. Cyclic on/off light delivery ranging from 15 to 60 s is investigated based on theoretical estimates of oxygen diffusion times. This time allocation provides for varying degrees of oxygen depletion and subsequent replenishment. Overall PDT efficacy is evaluated by monitoring spheroid growth and by scoring spheroid survival. The efficacy of fractionated high fluence rate delivery schemes is compared to continuous low fluence rate delivery. It is hypothesized that for a given fluence, high fluence rate PDT can be made as effective as low fluence rate PDT by the introduction of an appropriate dark interval. The clinical significance is that treatment failure resulting directly from high fluence rate-induced oxygen depletion may be overcome through proper modulation of the light source. Tumor regions immediately adjacent to the light source where high fluence rates
are present would benefit most from this type of treatment protocol. Improved local tumor control is likely.

Low fluence rate light delivery is also investigated. The effect of fluence rate on outcome is evaluated by scoring spheroid survival. The effect of repeated low fluence rate delivery of sub-threshold fluences is also investigated. Evaluation will be in terms of both spheroid growth kinetics and overall survival. The results will be compared to various one-shot fluence treatments. It is hypothesized that at low fluence rates i.e. 25 mW cm\(^{-2}\) and below, the efficacy of repeated PDT treatments is independent of fluence rate. This implies that under conditions of ample reoxygenation, accumulation of PDT insult is possible by employing repetitive treatment protocols. Clinically, single intra-operative PDT of the brain has been ineffective. Failure is due in part to insufficient fluence delivery to cm depths in the resection margin. It may be possible to take advantage of low fluence rate efficacy without impractically long treatments times by repeating treatments at weekly intervals.

The mode of cell death as a function of fluence rate is examined using a TUNEL assay. The clinical significance of this study directly correlates to patient’s quality of life. Elucidation of the link between fluence rate and mode of cell death could potentially minimize post photodynamic treatment edema of the brain.
CHAPTER 2

METHODOLOGY

Section 2.1 Cell Cultures

This study was conducted on a grade IV human GBM cell line (ACBT), which was a generous gift from G. Granger, University of California, Irvine, USA. Cells were cultured in monolayer using Dulbecco's Modified Medium (DMEM) (Gibco, Grand Island, NY) with high glucose and supplemented with penicillin (100 U/ml), 2 mM L-glutamine, streptomycin (100 µg/ml) and 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). Cells were incubated at 37°C in a 7.5% CO₂ humidified atmosphere. Medium was changed three times weekly. At a 70% confluent density, cell aggregates, consisting of approximately 10 cells, were transferred to petri dishes. Resultant spheroids, consisting of approximately 40-50 spheroids per dish, were maintained in DMEM supplemented as described above and grown to sizes of 400-500 µm in diameter. Approximately twenty-one days were required to reach this size. Individual spheroids were selected by passage through a sized screen mesh (Sigma, St. Louis, MO) yielding the desired experimental size of 450 µm in diameter. Spheroid size was verified immediately.
following irradiation via measurement of two orthogonal axes using a microscope with a calibrated eyepiece micrometer.

Section 2.2 Fluence/Fluence Rate Studies

Spheroids were incubated in 1000 μg/ml concentration of 5-aminolevulic acid hydrochloride (Sigma, St. Louis, MO) for a period of 4 hours prior to irradiation. Light from an argon ion-pumped dye laser at 635 nm (Coherent, Inc., Santa Clara, CA) was coupled into a 200 μm-diameter optical fiber containing a microlens at the output. Spheroids were irradiated in a 35-mm petri dish container. In the fluence studies, spheroids were exposed to a constant fluence rate of 25 mW cm⁻² for fluences of 12.5, 25, 50 and 100 J cm⁻². In the fluence rate studies, fluence was held constant at 50 J cm⁻², delivered at fluence rates of 5, 25, 50, 75 and 150 mW cm⁻².

Following irradiations, spheroids from each group were transferred to individual wells of a 48-well culture plate where they were maintained as described above. A microscope with a calibrated eyepiece micrometer was used to measure orthogonal axes of each spheroid diameter twice weekly. Typically, 10 to 12 spheroids were followed for each irradiation condition. Since each trial was performed 3 or 4 times, a total of 30 to 50 spheroids were followed for a given set of parameters. Spheroids were followed for up to 35 days.
Section 2.3 Mode of Cell Death

Approximately 24 hours following treatment, spheroids were removed from the well plates and fixed in 2% formaldehyde for 24 hours. Spheroids were washed three times in PBS and subjected to the DeadEnd™ Fluorometric TUNEL system (Promega Corp., Madison, WI, USA) – a classic TUNEL assay that measures nuclear DNA fragmentation in apoptotic cells by incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the enzyme TdT. The fluorescein label was then detected by two-photon scanning fluorescence microscopy (Coleno, 1999). Fluorescein was excited at a wavelength of 800 nm from 100 fs light pulses produced by a mode locked titanium sapphire laser (Coherent, Inc). The resultant fluorescence images were collected using a long-pass (530 nm cut-off) filter (CVI, Albuquerque, NM, USA). Images were acquired over spheroid depths ranging from 20 to 120 μm. Depth discrimination was accomplished by adjusting the Z position of the 10X (0.3 N.A.) objective, (Zeiss, Thornwood, NY, USA). Image acquisition times were of the order of 15 s (10 frames at 1.5 frames/s). The total number of apoptotic cells in each image was determined by counting the number of fluorescing nuclei.

The apoptotic fraction (AF) was determined from the following equation:

\[ AF = \frac{N_f}{N_t} \]  

(2)
where $N_f$ is the number of fluorescing nuclei and $N_t$ is the total number of cells in the field of view (200 $\mu$m x 200 $\mu$m). In order to determine the total number of cells, control spheroids were stained with 100 $\mu$g ml$^{-1}$ DAPI (Molecular Probes, Inc., Eugene, OR, USA) – a nucleic acid stain that associates with the minor groove of double-strand DNA, preferentially binding to AT base pairs. Excitation of bound DAPI ($\lambda_{\text{peak}} = 370$ nm) results in blue fluorescence ($\lambda_{\text{peak}} = 465$ nm). DAPI fluorescence was imaged using the two-photon fluorescence microscope system. Nuclear morphology of representative DAPI-stained spheroids in each treatment group was studied from high resolution (63X) two-photon fluorescence images.

Selected spheroids (positive controls) were exposed to DNase I, which mimics apoptosis by inducing fragmentation of chromosomal DNA. The resultant exposed 3'-OH DNA ends were labeled with fluorescein and imaged as previously described. Negative controls denote spheroids that were not subjected to any treatment. They represent the ambient level of apoptosis in this in vitro system.

The apoptotic fraction was determined for 3 spheroids in each control or treatment group. Since each treatment was repeated, the apoptotic fraction was averaged over 6 spheroids. In all cases, apoptosis was evaluated at a spheroid depth of 60 $\mu$m.
Section 2.4 Short-Term Fractionation Studies

Spheroids were incubated in 1000 µg/ml concentration of 5-aminolevulic acid hydrochloride (Sigma, St. Louis, MO) for a period of 4 hours prior to irradiation. Light from an argon ion-pumped dye laser at 635 nm (Coherent, Inc., Santa Clara, CA) was coupled into a 200 µm-diameter optical fiber containing a microlens at the output. Light was projected onto the spheroids in an open 35-mm petri dish container for various treatment times, i.e. the dark compensated time necessary to achieve total light fluences of 50 J. Light was delivered and interrupted for equal periods of 5, 15, 30, 45 and 60 seconds. Control groups of continuous light delivery (50J + ALA) and light-only controls using the 30 s on/off fractionation regimen were used for comparative purposes. All treatments were carried out at a fluence rate of 150 mW cm⁻².

Following irradiation, between 16 and 24 spheroids from each group were transferred to individual wells of a 48-well culture plate where they were maintained as described above. Spheroid growth was monitored for up to 28 days using a microscope with calibrated eyepiece micrometer to measure orthogonal axes of each spheroid diameter twice weekly. The experiment was repeated, totaling between 32 to 48 spheroids evaluated for each set of experimental parameters.
Section 2.5 Repetitive PDT Studies

Spheroids were incubated in 100 μg ml⁻¹ ALA (Sigma, St. Louis, MO) for approximately 4 hours. 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 output 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 to 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 mW cm⁻² or 25 mW cm⁻². Some of the cultures were treated only once, while the others were treated 4 times at weekly intervals. Spheroids were incubated in ALA prior to each treatment. In the case of the low fluence rate study (2.5 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. Spheroid sizing was accomplished by measuring two perpendicular diameters of each spheroid using a microscope with a calibrated eyepiece micrometer. Spheroids were followed for up to 8 weeks.
CHAPTER 3

RESULTS

Section 3.1 Data Analyses

Section 3.1.1 Fluence/Fluence Rate Studies

The effect of light fluence on spheroid survival was evaluated by measuring spheroid growth following PDT. A spheroid was considered to have survived treatment if growth was observed within the 30 days. It is shown that treatment outcome depends critically on light fluence. At the investigated fluence rate of 25 mW cm\(^{-2}\), a threshold fluence value of approximately 50 J cm\(^{-2}\) is observed below which the treatment has no effect on overall spheroid survival. In 95\% of the spheroids treated at or above 50 J cm\(^{-2}\) proliferative capacity was not regained during the 30-day observation period. The data are consistent with the hypothesis that complete response requires the absorption of a critical amount of photon energy by the photosensitizer in order to exact damage.

Figure 3.1 illustrates treatment outcome as a function of fluence rate. In all cases, a threshold fluence of 50 J cm\(^{-2}\) was delivered. Results indicate that survival is critically dependent on the rate of light delivery. As fluence rate is increased from 25 to 150 mW cm\(^{-2}\), the maximum fluence rate investigated in this study, spheroid survival increases.
There appears to be a threshold at ca. 25 mW cm\(^{-2}\) below which outcomes are similar. The upper limit on fluence rate was chosen to avoid confounding hyperthermic effects which are possible at

![Graph showing survival as a function of fluence rate for spheroids exposed to fluences of 50 J cm\(^{-2}\). Each data point represents the mean of 30-50 spheroids. Error bars denote standard errors.]

approximately 200 mW cm\(^{-2}\). The data strongly suggest enhanced PDT outcome at low fluence rates. The possibility of significant PDT effect at fluence rates lower than 5 mW cm\(^{-2}\) cannot be ruled out. Such
experiments are difficult to perform due to the long irradiation times required to deliver threshold fluences.

Section 3.1.2 Mode of Cell Death

The fraction of apoptotic cells in spheroids subjected to various treatment regimens are summarized in Figure 3.2. Low fluence rates appear to be effective at inducing apoptosis, as evidenced by a high apoptotic fraction of 0.76. This value is statistically equivalent to DNase I exposed spheroids (positive control) which is an artificially induced DNA fragmentation process used for control purposes. In contrast, high fluence rates appear to be rather ineffective at inducing apoptosis - only

<table>
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<tr>
<th>Apoptotic Fraction</th>
<th>0.8</th>
<th>0.11</th>
<th>0.76</th>
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<tr>
<td>Pos. Control</td>
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<tr>
<td>Neg. Control</td>
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<tr>
<td>PDT (25/25)</td>
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<tr>
<td>PDT (150/50)</td>
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Figure 3.2 TUNEL assay - derived apoptotic fractions of representative human glioma spheroids subjected to specified PDT fluence rate / fluence, positive control (DNase I exposed) and negative control (untreated). Error bars denote standard errors.
17% of the imaged cells died by apoptosis. The images acquired via two-photon fluorescence microscopy are displayed in figure 3.3. Cells in untreated spheroids (Figure 3.3a) are characterized by well-defined, oval-shaped nuclei.

![Figure 3.3](image)

Figure 3.3 Two-photon fluorescence images (X-Y plane, Z = 30 - 50 μm) of DAPI-stained nuclei from (a) Cells in untreated spheroids (b) Cells in low fluence rate PDT treated spheroids (25 mW/25 J) (c) Cells in high fluence rate PDT treated spheroids (150 mW/50 J). The scan region is approximately 35 x 35 μm. In each image, the nucleus is denoted by n.

Low fluence rate PDT (Figure 3.3b) produces irregular shaped nuclei - most nuclei appear to be in the process of disintegrating. This appearance is consistent with the morphologic changes observed during apoptosis. There appears to be nuclear fragmentation and separation into phagocytotic bodies. In contradistinction, high-fluence rate PDT
(Figure 3.3c) appears to have little effect on nuclear morphology. The enlarged, swollen appearance of the cell may indicate that it is in the early stages of necrosis.

Section 3.1.3 Short-Term Fractionation Studies

Mean spheroid survival as a function of fractionated light delivery scheme is shown in Figure 3.4. As expected, all spheroids exposed to continuous high-fluence rate PDT survived treatment. This result is consistent with the data presented in Figure 3.1. There appears to be a

![Graph showing spheroid survival](image)

Figure 3.4 Spheroid survival as a function of fractionated light delivery scheme- (continuous), 5, 30, 45, 60s on/off irradiation (150 mW cm⁻² Fluence Rate; 50 J cm⁻² Fluence; 1000 μg/ml ALA). Control indicates light/no drug, 60 sec on/off cycle. Error bars denote standard errors.
slight decrease in spheroid survival with decreasing on/off times, however, this is likely not statistically significant. Each regimen results in approximately 65% survival—essentially a 35% improvement over continuously delivered light.

The data in Figure 3.4 show that a significant reduction in spheroid survival can be achieved by introducing dark intervals during high-fluence rate PDT. It should be noted that the reduction in spheroid survival is not as pronounced as that which can be achieved by lowering the fluence rate (Figure 3.1). The data in Figure 3.4 suggest an optimal on/off cycle of somewhere between 5 and 45 s for 475 μm diameter spheroids.

Section 3.1.4 Repeat PDT Studies

The effects of multiple versus a single treatment protocol are illustrated in Figure 3.5a and b. The results here are dramatic. A single treatment consisting of a sub-threshold fluence of 12 J cm\(^{-2}\) or 25 J cm\(^{-2}\) was inefectual at a 25 mW cm\(^{-2}\) fluence rate. Overall treatment efficacy was markedly improved for both fluence values when repeating the treatment weekly. The most favorable response was observed at the lower fluence rate (2.5 mW cm\(^{-2}\)), in which case a 90% spheroid kill was achieved.

Effects of multiple treatments on growth kinetics are illustrated in Figures 3.6a and b. Compared to controls which show 100% of spheroids having discernable clonogenic capacity at 4 weeks, 100% of
the spheroids exposed to 25 J x 4 treatments were growth inhibited for an identical period. Approximately 70% of the spheroids having undergone repeated sub-threshold fluence (25 J cm\(^{-2}\)) treatments appeared to have lost proliferative capacity for the length of observation, i.e. eight weeks.

In the case of the lower fluence delivery (12.5 J cm\(^{-2}\)), a significant 55% of the spheroids remained suppressed for the full eight week observation period.

![Figure 3.5a Survival as a function of PDT treatment protocol. All spheroids were exposed to a fluence rate of 25 mW cm\(^{-2}\). Error bars denote standard errors.](image)
Figure 3.6b shows the lower fluence rate to be superior at tumor spheroid growth suppression. No growth was observed for the 25 J x 4 group for 6 weeks post irradiation as compared to a completely ineffectual single fraction. This is a marked improvement over the higher fluence rate (Figure 3.6a) where approximately 20% of the spheroids were viable 6 weeks post irradiation.

![Graph showing survival as a function of PDT treatment protocol](image)

**Figure 3.5b** Survival as a function of PDT treatment protocol. All spheroids were exposed to a fluence rate of 2.5 mW cm$^2$. Error bars denote standard errors.
Figure 3.6a Growth kinetics of spheroids exposed to either 12.5 or 25 J cm\(^{-2}\) by 4 repeated treatments compared to a single 25 J cm\(^{-2}\) fraction. In all cases, spheroids were exposed to a 25 mW cm\(^{-2}\) fluence rate. 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. Controls represent untreated spheroids. Each point represents the mean of between 80 and 150 spheroids. Error bars denote standard errors.
Figure 3.6b Growth kinetics of spheroids exposed to either 25 J cm$^{-2}$ in 4 repeated treatments compared to a single 25 J cm$^{-2}$ fraction. In all cases, spheroids were exposed to a 2.5 mW cm$^{-2}$ fluence rate. 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. Controls represent untreated spheroids. Each point represents the mean of between 80 and 150 spheroids. Error bars denote standard errors.
Section 3.2 Discussion and Statistical Analysis of Research Questions

Section 3.2.1 Fluence/Fluence Rate Effects

It has been shown in this study that the response of human glioma spheroids to ALA-mediated PDT depends not only on the total light fluence but also on the rate at which the light fluence is delivered. The fluence rate results, which are summarized in Figure 3.2, are in qualitative agreement with the findings of Foster et al. (Foster, 1993) who observed significant fluence rate effects in a murine mammary carcinoma spheroid model using Photofrin - a first generation photosensitizer. The results of the study presented in this thesis can be interpreted by invoking the self-sensitized singlet oxygen-mediated bleaching model of Georgakoudi et al. (Georgakoudi, 1998). In this model, the details of the spatial distribution of singlet oxygen and, therefore of bleaching, depend on the fluence rate. 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 response since singlet oxygen is delivered to a larger volume of tumor cells.

The results presented here are of significant clinical relevance since they suggest that it may be possible to effect significant damage to cells residing deep in the BAT which are exposed to relatively low fluence rates. Thus, it may be possible to achieve local control with ALA-
mediated PDT. A potential problem is the lack of cellular response to high-fluence rate PDT, such as would be expected in brain tissue close to the light source. Improved PDT response in high-fluence rate regions may be possible through the introduction of dark cycles during treatment. The clinical necessity of such short-term light fractionation is unknown, especially in view of recent studies (Madsen et al. 2000) showing only a weak fluence rate dependence on PDT outcome at higher fluences ($\geq 200 \text{ J cm}^{-2}$).

Section 3.2.2 Mode of Cell Death

As illustrated in Figure 3.2, low-fluence rate ALA-PDT results in significant apoptotic death. This is likely due to the fact that PpIX, the active photosensitizer, localizes to the mitochondrial membrane. The ineffectiveness of high-fluence rate PDT is likely due to the fact that the photodynamic dose is confined to the outer rim of the spheroid (Foster, 1993). Since the level of apoptosis in this superficial layer ($\leq 60 \text{ m m}$) was not significantly different from that found in the negative controls (Figure 3.2), the observed cell death was assumed to have occurred via necrosis. Indeed, the appearance of DAPI-stained cells in the outer rim of spheroids exposed to high-fluence rate PDT is consistent with necrosis. This is not unexpected since necrosis is often observed when cells are subjected to extreme treatment conditions such as those encountered in the high-fluence rate case - the fluence rate used (150 mW cm$^{-2}$) is just below the hyperthermic threshold (approximately 200 mW cm$^{-2}$ for most...
tissues). In addition, high fluences have been shown to kill, by a non-apoptotic mechanism, cells that undergo apoptosis with lower fluences (Luo, 1997; Separovic, 1998; Ball, 1998). This phenomenon has been attributed to the induction of extensive membrane photodamage following high light doses, and has been observed even for photosensitizers having significant mitochondrial localization (Agostinis, 2000).

Section 3.2.3 Short-Term Fractionation Effects

The results presented in Figure 3.5 suggest that shorter on/off cycles are more effective than longer cycles. This is in qualitative agreement with a simple mathematical model of oxygen diffusion developed by Pogue and Hasan (Pogue, 1997). In this model, the optimal fractionation time is given by:

$$t_{\text{frac}} = \frac{d^2}{8D},$$

(3)

where $t_{\text{frac}}$ is the time for the maximum oxygen diffusion rate during a single fraction, $d$ is the distance from the oxygen supply, and $D$ is the diffusion constant (1460 $\mu$m$^2$ s$^{-1}$ in human mammary carcinoma spheroids). Since a necrotic core typically develops in spheroids at distances of 150 – 200 $\mu$m from its surface, dark intervals should be chosen such that cells at these depths can be reoxygenated. From equation (3), this would suggest optimal on/off intervals of 1.9 – 3.4 s. The model predicts that longer on/off cycles result in a decrease in the
production of singlet molecular oxygen per unit time and, hence to a
decrease in cell killing efficiency, assuming the same total fluence is
delivered (Pogue and Hasan, 1997). It is interesting to note that in this
simple model, the optimum fractionation schedule depends only on the
oxygen diffusion distance – the optimum dark interval is insensitive to
either the light fluence rate or any of the properties of the
photosensitizer. Furthermore, the model predicts that the production of
singlet molecular oxygen is always maximized by the use of lower fluence
rates as opposed to any fractionation schedule. This is in agreement
with the experimental findings in this thesis which show that a reduction
in fluence rate is much more effective than high-fluence rate
fractionation schemes.

If it is assumed that a significant fraction of viable cells can be
sufficiently oxygenated during a 5 s dark interval, the observed data in
Figure 3.5 can be explained in terms of the number of on/off cycles.
Henning et al. (Henning, 1995) have shown that the total singlet oxygen
concentration in remote cells depends not only on the on/off time
interval, but also on the total number of cycles in a fractionated
treatment. Although it is true that longer off times result in better
reoxygenation of remote cells, the total singlet oxygen concentration
exhibits a greater dependence on the total number of cycles. The greater
the number of cycles, the higher the singlet oxygen concentration in
remote cells and, hence the greater the efficacy of the 5 s on/off fractionation scheme.

Section 3.2.4 Repeat Treatments

The primary finding of this study is that multiple PDT treatments at sub-threshold light fluences result in significant inhibition of spheroid growth. In all cases, suppression of growth is observed during the entire treatment period. Low fluence rates (Figures 3.5b and 3.6b) appear to be more effective than higher ones (Figures 3.5a and 3.6a) 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. Based on calculations of Madsen et al. (Madsen, 2003), it should be possible to treat to depths of 1.5 cm in the BAT. 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. 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 weekly treatment interval chosen in this study was 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 (Webber, 1997). Furthermore, due to logistical and quality-of-life issues, weekly or bi-monthly treatments are much more appealing than daily fractionation.

The increased efficacy of multiple PDT is probably due to a number of factors. The spheroids used in this study consist of three 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 (Bigelow, 2001). The outer layer of proliferating cells is therefore killed and sloughs 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 and will commence growth after treatment is curtailed when they form a new outer layer. 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 (Madsen, 2001).
CHAPTER 4

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

4.1 Fluence Rate/ Rapid Fractionation Studies

The primary finding of this thesis is that PDT efficacy depends sensitively on the light delivery scheme. For example, the effects of ALA-mediated PDT on human glioma spheroids depend on the rate at which the light is delivered – lower fluence rates are clearly more effective than higher ones. This finding is clinically significant since it suggests that the low fluence rates found at cm depths in the resection cavity are capable of destroying tumor cells. Unfortunately, due to the rapid attenuation of light in brain tissues, input laser powers on the order of several watts are required to achieve fluence rates of a few mW cm\(^{-2}\) at 1 – 2 cm depths. Obviously, tissues in close proximity to the light source will be subjected to very high fluence rates that have been shown to be ineffective in human glioma spheroids. A possible strategy to overcome the ineffectiveness of high fluence rates is to introduce a dark interval which allows for reoxygenation. It was shown in this thesis that the efficacy of high-fluence rate PDT could be improved by approximately 35% simply by the introduction of 5 – 45 s dark intervals during light irradiation.
The use of spheroids to study the effects of light fractionation on oxygenation status is greatly simplified by the assumption that the culture medium acts as an infinite source of oxygen. The situation \textit{in vivo} is somewhat more complex due to the effects of PDT on tumor vasculature patency. Thus, a full understanding of the efficacy of fractionated PDT schemes requires further investigation in experimental animal models.

4.2 Repeat PDT Studies

The commonly used one-shot intraoperative protocols are incapable of local control due to the prohibitively long treatment times required to deliver threshold light fluences to tumor cells residing deep in the resection margin. The development of permanent indwelling balloon applicators may provide a solution to this problem as they would allow ready access to the resection cavity (Madsen, 2001). This would allow for novel treatment protocols such as long-term repetitive PDT. Results presented in this thesis show that long-term repeat PDT, using sub-optimal light fluences, is much more effective than single treatments in a human glioma spheroid model. The data suggest that repeat PDT is effective at fluence rates as low as 2.5 mW cm\(^{-2}\). These results have significant clinical implications since they suggest the possibility of long-term management of patients with brain tumors through repetitive PDT treatments delivered at weekly intervals.
The feasibility of weekly PDT treatment regimens is uncertain due to the possibility of systemic toxicity from PpIX accumulation. Studies are currently underway in animals to investigate the potential of repetitive PDT. Finally, a fluence rate threshold was not found in these studies – it may be possible to elicit damage to glioma cells at fluence rates below 2.5 mW cm\(^2\) and thus extend the depth of treatment.
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