Addition of Ionizing Radiation or Hyperthermia Enhances PDT Efficacy in Glioma Spheroids

Steen J. Madsen[*a,b], Chung-Ho Sun[c], Bruce J. Tromberg[c], Josephine Ni[c] and Henry Hirschberg[c,d]

[a]Dept. of Health Physics and [b]UNLV Cancer Research Center, University of Nevada, Las Vegas, 4505 Maryland Pkwy., Box 453037, Las Vegas, NV 89154

[c]Beckman Laser Institute, University of California, Irvine, 1002 Health Sciences Rd. E, Irvine, CA 92612

[d]Dept. of Neurosurgery, Rikshospitalet, 0027 Oslo, Norway

ABSTRACT

The effects of 5-aminolevulinic acid (ALA)-mediated photodynamic therapy (PDT) and gamma radiation, and PDT and hyperthermia (HT) are investigated in human glioma spheroids. In the case of ionizing radiation and PDT, the degree of interaction between the two modalities depends in a complex manner on a number of factors, including light fluence, fluence rate and radiation dose. It is shown that, under certain conditions, PDT and gamma radiation interact synergistically to produce enhanced cell kill. The degree of interaction appears to be independent of both sequence and time intervals investigated. TUNEL assays show that low fluence-rate ALA-PDT is a very efficient inducer of apoptosis, whereas neither high-fluence rate PDT nor ionizing radiation results in significant apoptosis in this system.

Synergistic interactions between HT and PDT are observed when the two modalities are given concurrently. The degree of synergism increases with increasing temperature and light fluence. It is shown that apoptosis is the primary mode of cell death following combined HT and low-fluence rate ALA-PDT.

Keywords: glioma, photodynamic therapy, 5-aminolevulinic acid, gamma radiation, hyperthermia, synergism, spheroids.

*steenm@ccmail.nevada.edu; phone: 702.895.1805; Fax: 702.895.4819

1. INTRODUCTION

The prognosis for patients with high-grade gliomas has not improved significantly during the past four decades. Even with the best available treatments, median survival is less than 1 year[1]. In most cases, treatment failure is due to local recurrence - approximately 80% of tumors recur within 2 cm of the resection cavity[2]. This would indicate that a more aggressive focal treatment may be of benefit in prolonging survival and/or improving the quality-of-life in patients with high-grade gliomas. PDT is a local form of treatment that may prove useful as an adjuvant to radiation therapy in the treatment of resected margins following surgery.

The effects of combined ionizing radiation and PDT have been studied primarily in simple *in vitro* systems consisting of monolayer cell cultures and using the first generation photosensitizer, hematoporphyrin derivative[3]. The results are to some extent ambiguous: the degree of interaction appears to depend on numerous parameters, including the type of cell line, the dose and dose rate of both ionizing radiation and light, and the sequence and timing of treatments. The degree of potentiation between PDT and ionizing radiation is clinically relevant because patients undergoing investigative PDT treatments are likely to receive ionizing radiation concurrently.
Since PDT efficacy is critically dependent on the ability to deliver adequate light doses to malignant cells in the resection margin, commonly used intraoperative treatments are unlikely to be effective due to the inability to deliver threshold

Photonic Therapeutics and Diagnostics, Bartels, Bass, de Riese, Gregory, Hirschberg, Katzir, Kollias, Madsen, Malek, McNally-Heintzelman, Tate, Trowers, Wong, eds., Proc. of SPIE Vol. 5686 (SPIE, Bellingham, WA, 2005) · 1605-7422/05/$15 · doi: 10.1117/12.589669

light doses in a reasonable time period[4,5]. To overcome this limitation, an indwelling balloon applicator has been developed which allows for extended, long-term and fractionated PDT[5]. This applicator can also be use to deliver other types of treatments such as brachytherapy and thermal therapy. Although the balloon applicator facilitates novel light delivery schemes, high laser powers are still required to achieve threshold light fluences at cm depths in the resection margin[4]. Since most of the optical energy is converted to heat, tissues close to the light source are likely to undergo significant heating.

The effects of HT and PDT have been investigated in a number of *in vitro*[6-11] and *in vivo*[12-20] systems. Variable effects have been reported following combined HT and PDT, with the degree and type of interaction dependent on a number of factors including tumor type, treatment sequence, time interval between treatments and photosensitizer type. Since HT is an unavoidable effect of high-fluence rate PDT, knowledge of potentiation effects between the two modalities may help optimize PDT efficacy.

In this study, the combined efficacy of: (1) ionizing radiation and ALA-PDT and (2) HT and ALA-PDT are investigated in a human glioma spheroid model. Since three-dimensional multicellular spheroids have many characteristics in common with tumors *in vivo,* they are ideally suited to basic therapeutic studies in which the effects of numerous parameters are investigated. This can be accomplished in the absence of a tumor vasculature and complex immunological reactions characteristic of animal models.

2. MATERIALS AND METHODS

2.1 Cell cultures

The grade IV glioblastoma multiforme (GBM) cell line (ACBT) used in this study was a generous gift of G. Granger (University of California, Irvine, USA). The cells were cultured in DMEM (Invitrogen, Carlsbad, CA) 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 (Invitrogen, Carlsbad, CA). Cells were maintained at 37 ^{0}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 using a liquid-overlay technique[21]. Spheroids of 500 μm diameter were selected by passage through a screen mesh (Sigma, St. Louis, MO). It took approximately 21 days for spheroids to reach a size of 500 μm. In all cases investigated, the spheroid culture medium was changed three times weekly.

2.2 Treatments

2.2.1 PDT

Spheroids were incubated in 1000 μg mL^{-1} ALA (Sigma, St. Louis, MO) for approximately 4 h. In all cases, spheroids were irradiated with 635 nm light from an argon ion-pumped dye laser (Coherent, Inc., Santa Clara, CA). Light was coupled into a 200 μm diameter optical fiber containing a microlens at the ouput end. Spheroids were irradiated in a petri dish. A 2 cm diameter gasket was placed in the dish to confine the spheroids (ca. 30) to the central portion of the dish (medium volume = 1 mL) and thus limit the extent of the irradiated field. Spheroids were subjected to fluence rates of either 25 or 150 mW cm^{-2}. In the case of the high fluence rate studies, spheroids were irradiated to a total fluence of 50 J cm^{-2} while, at the lower fluence rate, spheroids were irradiated to fluences of 12 and 25 J cm^{-2}. These light fluence and fluence rates were found to be sub-optimal in a previous investigation[22].

2.2.2 Ionizing radiation

In all cases, spheroids were irradiated in a petri dish at ambient conditions using a ^{137}Cs gamma source (0.66 MeV). Spheroids were irradiated to doses ranging from 4 to 16 Gy at a dose rate of approximately 1.6 Gy min^{-1}.

2.2.3 Combined PDT and gamma irradiation

Spheroids were incubated in ALA 3 to 4 hours prior to gamma irradiation. PDT and gamma irradiation were separated by 45 minutes (range: 30 to 60 minutes), or 24 hours (range: 22 to 26 hours). In each case, the effects of treatment sequence (PDT first vs. gamma first) were investigated. Following the combined treatment, individual spheroids were placed into separate wells of a 64-well culture plate and monitored for growth. This was accomplished by measuring two perpendicular diameters of each spheroid using a microscope with a calibrated eyepiece micrometer. Typically, 16 to 24 spheroids were followed for each treatment regimen. Since each trial was performed 3 or 4 times, a total of 48 to 96 spheroids were followed for a given set of parameters. Spheroids were followed for up to 35 days. Spheroids showing significant growth during the observation period were assumed to have survived treatment. The surviving fraction was evaluated by taking the ratio of surviving to total number of spheroids.

2.2.4 Combined PDT and HT

Spheroids were incubated in 100 μg ml^{-1} ALA (Sigma, St. Louis, MO) for approximately 4 h. The irradiation parameters and techniques are identical to those described in section 2.2.1, with the following exceptions. PDT was performed in a temperature-controlled incubator with the petri dishes placed on large metal blocks which had reached temperature equilibrium at 37, 40, 43, 46, or 49 ^{o}C. Temperatures chosen for the HT experiments were based on diffusion theory calculations of thermal distributions in brain tissues during PDT with spherical light applicators[4].

Temperatures were measured with thermocouples placed in parallel dishes containing no spheroids but an equal amount of culture medium. Temperature equilibrium was attained 15 – 20 minutes following placement of the culture medium in the incubator. PDT-only spheroids were irradiated at 37 ^{o}C, while HT dark controls were incubated in ALA but received no light. PDT-treated spheroids were irradiated to various fluences ranging from 12 to 100 J cm^{-2} using a fluence rate of 25 mW cm^{-2}. Total irradiation times were between 8 and 64 minutes. In all cases, HT dark controls and PDT-treated spheroids were incubated at each temperature for a minimum of 40 – 45 minutes.

Following treatment (single or combined), individual spheroids were placed into separate wells of a 48-well culture plate and monitored for growth. Determination of spheroid size was carried out by measuring two perpendicular diameters of each spheroid using a microscope with a calibrated eyepiece micrometer. Typically, 24 spheroids were followed in each trial. Since each trial was performed 2 or 3 times, a total of 48 to 72 spheroids were followed for a given set of parameters. Spheroids were observed for up to 4 weeks.

2.3 Analysis

2.3.1 Potentiation effects

The degree of interaction between the two treatment modalities was evaluated by a technique proposed by Drewinko *et al*.[23] In this scheme, the degree of interaction is given by:

$$\alpha = \frac{SF^{\gamma} x SF^{\psi}}{SF^{\gamma\psi}}, \qquad (1)$$

where SF^{γ} and SF^{ψ} represent surviving fractions with gamma and PDT (or HT and PDT) respectively, and $SF^{\gamma\psi}$ is the surviving fraction following combined treatments. In this analysis, $\alpha = 1$ indicates an additive effect (or absence of any effect), $\alpha > 1$ indicates a synergistic effect, and $\alpha < 1$ indicates an antagonistic effect.

2.3.2 TUNEL assay

The presence of apoptosis was evaluated using a TUNEL assay. Approximately 24 h after treatment, the spheroids were removed from the well plates and fixed in 2% formaldehyde for 24 h. The spheroids were washed three times in phosphate-buffered saline and subjected to the DeadEnd TM flourometric terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate nick-end labeling system (Promega Corp., Madison, WI)—a classic TUNEL assay that measures nuclear DNA fragmentation in apoptotic cells by incorporating fluorescein-12-deoxyuridine triphosphate at 3′ -OH DNA ends using the enzyme TdT. In comparison to nuclear morphology techniques, this assay has been shown to have a higher degree of accuracy for the detection of apoptosis[24] and, as such, has been used to quantify programmed

cell death in a number of brain tumor cell lines[25,26]. The fluorescein label was detected by two-photon fluorescence microscopy[27]. The fluorescein was excited at a wavelength of 400 nm, and the resultant fluorescence images were collected using a long-pass (530 nm cut-off) filter (CVI, Albuquerque, NM). Images were acquired over spheroid depths ranging from 30 to 100 μm. Depth discrimination was accomplished by adjusting the Z position of the 10X (0.3 numerical aperture (N.A.)) objective (Zeiss, Thornwood, NY). Image acquisition times were of the order of 15 s (10 frames at 1.5 frames s^{-1}).

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

$$AF = \frac{N_f}{N_t} \qquad (2)$$

where N_f is the number of fluorescing nuclei, and N_t is the total number of cells in the field of view (200 μm x 200 μm). To determine the total number of cells, control spheroids were stained with 100 μg ml^{-1} 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI; Molecular Probes, Inc., Eugene, OR) - a nucleic acid stain that associates with the minor groove of double-strand DNA, preferentially binding to AT base pairs. Excitation of bound DAPI (λ_{peak} = 370 nm) results in blue fluorescence (λ_{peak} = 465 nm). DAPI fluorescence was imaged using the two-photon fluorescence microscope system.

Selected spheroids (positive controls) were exposed to deoxyribonuclease I which mimics apoptosis by inducing fragmentation of chromosomal DNA. The resultant exposed 3′ -OH DNA ends were labeled with fluorescein and imaged as described previously. Negative controls denote spheroids that were not subjected to any treatment. They represent the ambient level of apoptosis in this *in vitro* system. The AF was determined for three spheroids in each control or treatment group. Because each treatment was repeated, the AF was averaged over six spheroids.

3. RESULTS

3.1 Single treatments

Effects of PDT, gamma radiation or HT are illustrated in Figs. 1 and 2. The control group represents true controls that were allowed to grow in the absence of light and ALA. Figure 1 shows that relatively high gamma doses are required for significant response - the dose required for 50 % survival is approximately 12 Gy. Significant spheroid kill (3.5 % survival) is observed at doses of 16 Gy. For each dose, two groups of spheroids were irradiated; one group was irradiated prior to ALA incubation while the other was irradiated post incubation. Since there was no difference in survival between the two groups, it can be concluded that ALA does not act as a radio-sensitizer. The data shown in Figure 1 is for the post incubation group only.

As illustrated in Figure 1, spheroid survival is dependent on both light fluence and fluence rate. A fluence of 50 J cm^{-2} has a limited effect if delivered at high fluence rates (150 mW cm^{-2}). Improved response is observed at the lower fluence rate (25 mW cm^{-2}), but the response is fluence dependent. For example, fluences of 12 and 100 J cm^{-2} result in surviving fractions of approximately 0.95 and 0.04, respectively.

Figure 2 shows that relatively high temperatures are required for a significant cytotoxic response - the temperature required for 5% survival is approximately 49 ^{o}C. No significant spheroid kill was observed at temperatures up to 46 ^{o}C. Since the accuracy of the temperature measurements was of the order of 1 degree, no attempt was made to determine survival rates in the interval between 46 and 49 ^{o}C.

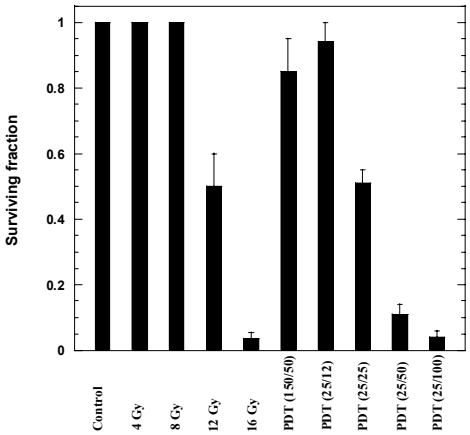

Figure 1. Spheroid survival after exposure to either gamma radiation or PDT. Fluence rate (mW cm^{-2}) and fluence (J cm^{-2}) are indicated in parentheses (fluence rate/fluence). Each data point represents the mean (± SE) of approximately 80 spheroids.

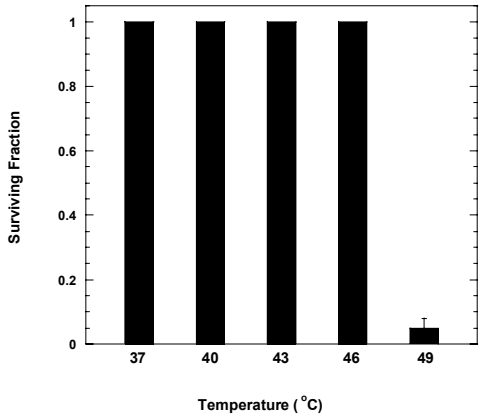

Figure 2. Effects of HT on spheroid survival. Each data point represents the mean (± SE) of 48-56 spheroids.

3.2 PDT + ionizing radiation

The response of spheroids to combined gamma irradiation and high fluence rate (150 mW cm^{-2}) PDT is illustrated in Fig. 3a. In all cases, spheroids were subjected to fluences of 50 J cm^{-2}. Treatments were separated by approximately 24 h unless otherwise indicated. The degree of interaction between the two modalities, as indicated by the α coefficient from equation (1), is listed above each data point. As shown in Fig. 3a, there is no advantage to combining gamma radiation with high-fluence rate PDT – survival for PDT-only treated spheroids (Fig. 1) is similar to that observed for low gamma dose (4 or 8 Gy) combined treatments (Fig. 3a). Furthermore, survival following high gamma dose (12 or 16 Gy) combined treatments is similar to gamma-only (12 or 16 Gy) treatment (Figure 1). As shown in Fig. 3a, response to treatment is independent of sequence (PDT first vs. gamma first) and time interval (1 or 24 h).

As illustrated in Fig. 3b, a significant synergistic response ($\alpha > 1$) is observed in spheroids treated with 8 Gy and 25 J cm^{-2} (fluence rate = 25 mW cm^{-2}). The degree of synergism is independent of both sequencing and time interval. In all other cases, there appears to be no significant advantage of combining the two modalities. The data support the existence of gamma and fluence thresholds, both of which must be exceeded for synergistic interactions to occur.

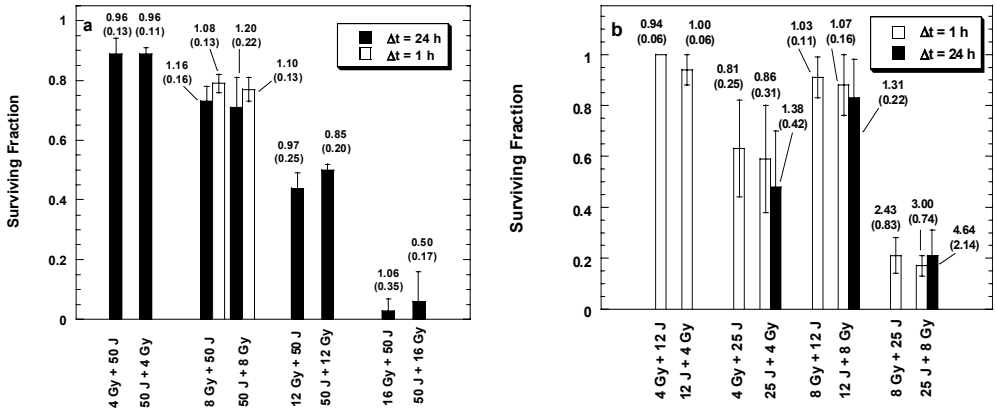

Figure 3. Spheroid survival after combined gamma radiation and PDT. (a) High-fluence rate (150 mW cm^{-2}) PDT (fluence = 50 J cm^{-2} – denoted by 50 J). (b) Low-fluence rate (25 mW cm^{-2}) PDT. The interaction coefficient (α in equation (1)) and its uncertainty (in parenthesis) are shown above each data point. Time intervals of 1 and 24 h were investigated. Each data point represents the mean (± SE) of approximately 70 spheroids.

The ability of various treatments to induce apoptosis is illustrated in Figure 4. It is shown that low-fluence rate PDT is a very effective inducer of apoptosis – the apoptotic fraction (ca. 0.76) is comparable to that observed in the positive controls. In contradistinction, both high-fluence rate PDT and ionizing radiation fail to produce apoptosis at levels significantly above background (as denoted by the negative controls). The combined treatments yield slightly higher levels of apoptosis than either of the single modalities.

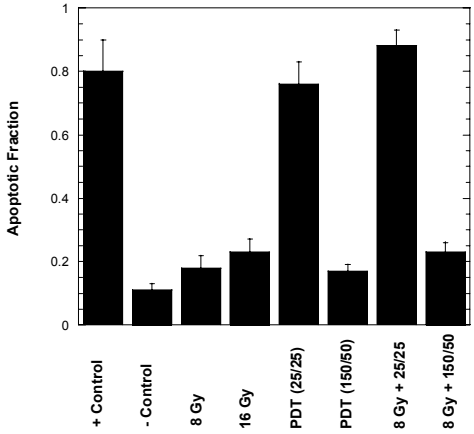

Figure 4. Fraction of cells in apoptosis as a function of treatment type. The apoptotic fraction was evaluated from two-photon fluorescence images (10X) acquired at spheroid depths of approximately 60 μm. Each data point represents the mean of six spheroids from two independent treatments. Positive and negative controls are denoted by +Control and –Control, respectively. Error bars denote standard deviations.

3.3 PDT + HT

A significant inhibitory response was observed in spheroids subjected to concurrent HT and PDT (Fig. 5). The degree of response is clearly temperature and fluence dependent below 49 ^{o}C – treatment efficacy improves with increasing temperature and light fluence.

The degree of interaction between the two modalities, as indicated by the α coefficient, is summarized in Figure 6. At temperatures below 49 °C, HT and PDT interact in a synergistic manner ($\alpha > 1$) if the two treatments are given concurrently. The highest degree of potentiation ($\alpha \approx 5$) is obtained at a temperature and fluence of 46 °C and 25 J cm^{-2}, respectively. In all cases, the introduction of a 24 h delay between the two treatments results in a decreased PDT effect as indicated by α coefficients below unity.

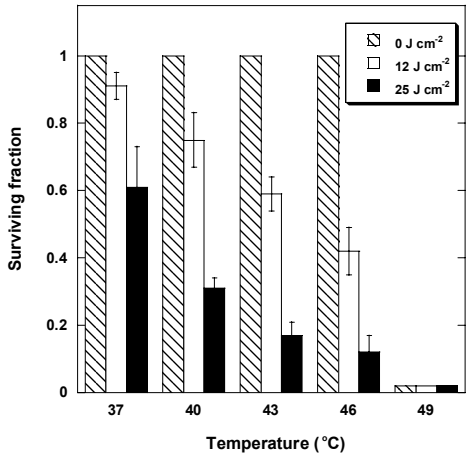

Figure 5. Effects of concurrent PDT and HT on human spheroid survival. The HT-only spheroids (0 J cm^{-2}) were incubated in 100 μg ml^{-1} ALA 4 h prior to treatment. In all PDT treatments, a fluence rate of 25 mW cm^{-2} was used. Each data point represents the mean (± SE) of 56-72 spheroids.

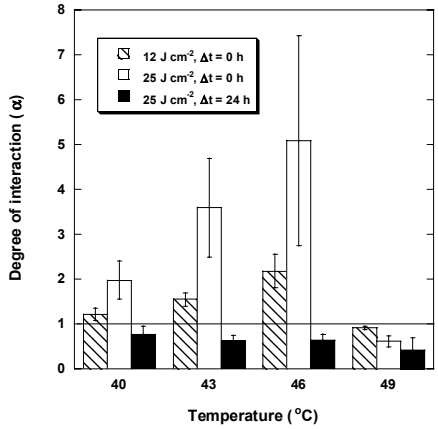

Figure 6. Degree of interaction between HT and PDT as a function of temperature. Three cases are considered: concurrent HT and PDT ($\Delta t = 0$) at two light fluences, and HT given 24 h prior to PDT ($\Delta t = 24$ h). In all PDT treatments, a fluence rate of 25 mW cm^{-2} was used. The horizontal line denotes the boundary between synergism ($\alpha > 1$) and antagonism ($\alpha < 1$). Each data point represents the mean (± SE) of 48-72 human spheroids.

The ability of various treatments to induce apoptosis in the cells comprising the human spheroids is illustrated in Figure 7. It is shown that low-fluence rate PDT is a very effective inducer of apoptosis - the AF (ca 0.9) for PDT at 37 °C is comparable to that observed in the positive controls (data not shown). In contrast, HT is a very poor inducer of apoptosis – the AF (ca. 5%) is equivalent to that observed in the negative controls (data not shown). Spheroids subjected to concurrent HT and PDT showed similar levels of apoptosis as those subjected to PDT only. Interestingly, PDT given at

49 °C, a degree of HT yielding 100% spheroid kill in the absence of light, also resulted in significant apoptosis. The absence of apoptosis in spheroids subjected to HT at 49 °C suggests a necrotic mode of cell death.

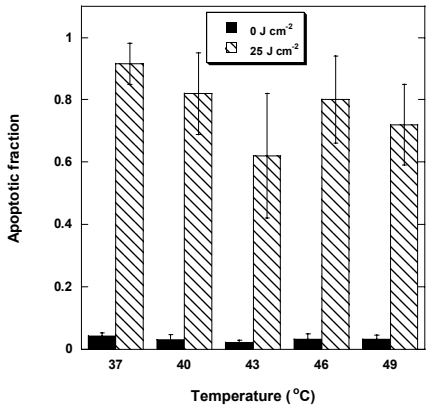

Figure 7. Fraction of cells in human spheroids showing apoptosis as a function of treatment type. The AF was evaluated from two-photon fluorescence images (10X) acquired at a spheroid depth of approximately 60 μm. Each data point represents the mean of 6 spheroids from two independent treatments. Error bars denote standard deviations.

4. DISCUSSION

Results of the combined PDT and gamma radiation studies can be summarized by stating that synergistic interactions are observed only under very specific irradiation conditions. The degree of interaction appears to be independent of both sequence and the time intervals (1 and 24 h) investigated in this study. The mechanisms have not been elucidated; they are the subject of ongoing investigations.

As illustrated in Fig. 4, low-fluence rate PDT and gamma radiation differ significantly in their ability to induce apoptosis in glioma cells. While low-fluence rate PDT is a very efficient apoptotic inducer, gamma radiation fails to induce apoptosis at levels significantly above background in exposed spheroids. Morphologic evidence suggests that gamma radiation results in necrotic cell death (data not shown). Since the main targets of gamma radiation and ALA-PDT are the cell nucleus and mitochondrion respectively, it is conceivable that changes at the mitochondrial level can interact with nuclear damage produced by gamma radiation, thus providing a possible explanation for the synergism observed for combined low- fluence rate PDT and gamma radiation. As illustrated in Fig. 3b, synergistic interactions occurred only for a very limited set of treatment conditions (8 Gy and 25 J cm^{-2}). The lack of synergism for the combinations of 4 Gy and 12 J cm^{-2} is likely due to inadequate gamma or light doses. This is consistent with the findings of Luksiene *et al*[28], who observed that neither apoptosis nor necrosis is triggered by sub-lethal doses of light or gamma radiation. A fluence of 12 J cm^{-2} can be considered sub-lethal as it has minimal effect on spheroid survival and does not result in significant growth delay compared to controls. In contradistinction, the higher fluence results in both decreased survival and increased growth delay. Although a gamma dose of 8 Gy results in 100 % survival, the spheroids are affected by this treatment as evidenced by significant growth delay compared to spheroids treated with 4 Gy (data not shown). Thus, the effect of 8 Gy on spheroid growth kinetics may be sufficient to cause synergistic effects when combined with 25 J cm^{-2}.

Although high-fluence rate PDT is relatively inefficient, resulting in approximately 85 % spheroid survival (Fig. 1), the lack of synergism may be due, in part, to similar modes of cell death for both treatments. It is shown in Fig. 4 that, unlike the low-fluence rate case, high-fluence rate PDT is a very inefficient inducer of apoptosis. In fact, there appears to be no significant difference in the levels of apoptosis produced by high- fluence rate PDT and ionizing radiation. 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[29]. Since the level of apoptosis in this superficial layer (≤ 60 μm) was not significantly different from that found in the negative controls (Fig. 4), 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 that 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[30-32]. 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[33].

The results presented in this study suggest that the ability of PDT to interact synergistically with ionizing radiation depends strongly on the light fluence rate. Thus, it is possible that the mechanism of synergism is an oxygenation phenomenon. This is not unreasonable since the efficacy of both PDT and gamma radiation depends sensitively on the presence of oxygen during treatment. It has been shown that spheroid oxygenation status depends on fluence rate – high-fluence rate PDT results in rapid depletion of oxygen[29]. Consequently, only the well-oxygenated and rapidly proliferating cells in the outer rim of the spheroid will be damaged. In contrast, since low-fluence rate PDT does not result in significant oxygen depletion, the photodynamic dose is extended further into the central regions of the spheroid and may render the radioresistant quiescent cells more susceptible to ionizing radiation. Although this is a plausible explanation if PDT is given prior to ionizing radiation, it fails to account for the synergism observed when gamma radiation precedes PDT.

The possibility of a synergistic effect between ALA-mediated PDT and HT prompted the present study since tissue heating is an unavoidable consequence of high-fluence rate light irradiations such as those required for PDT treatments of GBM cells residing deep in resection margins. Theoretical calculations indicate that significant temperature increases are likely to occur in brain tissue immediately adjacent to a spherical light applicator following even modest input laser powers[4]. The higher input powers that are likely required for the elimination of GBM cells at 1 – 2 cm depths would result in even greater temperature increases, perhaps as high as 8 ^{o}C. It is imperative to keep temperatures below about 43 ^{o}C in order to avoid large-scale hyperthermic damage to normal brain tissues.

The human glioma spheroids used in this study appeared to be very thermoresistant – significant suppression was observed only at temperatures approaching 49 ^{o}C (Fig. 2). Furthermore, in contrast to sub-optimal fluence PDT, HT at sub-threshold temperatures (< 49 ^{o}C) had no observable effect on spheroid growth kinetics (data not shown). In comparison, GBM cells in monolayer are sensitive to much lower temperatures – exposures to 44 ^{o}C results in significant cell kill[34]. The relative ineffectiveness of HT on GBM cells in spheroids, is likely due to the close three-dimensional contact between cells (the contact effect) which has been shown to confer increased resistance of cells to various therapies, including HT[35,36]. Although neither PDT nor HT was particularly effective, a significant reduction in spheroid survival was nevertheless observed when the two treatments were given concurrently (Fig. 5). The efficacy of the combined treatment improved with increasing temperature and light fluence. As shown in Fig. 6, the two treatment modalities interacted synergistically at all temperatures below 49 ^{o}C. Not surprisingly, the degree of synergism was observed to increase with increasing temperature and light fluence.

In order to gain insight into the mechanism(s) of synergism, the mode of cell death was determined as a function of treatment modality. As illustrated in Fig. 7, HT is a poor inducer of apoptosis in GBM cells. The inability of HT to induce apoptosis in this cell line is likely due to the high constitutive expression of heat shock proteins (HSPs) in GBM cells[37]. Apoptosis and induction of HSPs have been found to be mutually exclusive due to the protective effect of HSPs[38]. Due to the inability of HT to induce apoptosis, the observed cell death at 49^{o} C was attributed to necrosis. This is in good agreement with the findings of others that necrosis is the predominant mode of cell death following exposures to high temperatures[39].

Apoptosis is the primary mode of cell death following exposure to low-fluence rate PDT at all temperatures investigated, including 49^{o} C, a temperature that resulted in pronounced cytotoxicity in the absence of light. Since the cytotoxic effects of HT at 49^{o} C are likely due to necrosis, the addition of PDT would appear to have the effect of switching the cells to an apoptotic mode of cell death. Although the details of this effect are unknown, the clinical implications are potentially significant since lower levels of brain edema are thought to be present following apoptosis-inducing therapies compared to those in which necrosis is the primary mode of cell death.

Although the mechanism of synergism between PDT and HT is not known, it likely has several components. For instance, it might be due, in part, to photodynamically-induced inhibition of cellular repair following sub-lethal thermal damage[6]. Another hypothesis for the observed synergism is the concerted action of both treatment modalities on cellular proteins[9]. For example, it has been shown that PDT can result in the photooxidation of intracellular enzymes such as glyceraldehyde-3-phosphate dehydrogenase and cytochrome c oxidase[40]. As a result, the enzymes undergo a conformational change which, in turn, affects their susceptibility for thermal inactivation. The net effect of PDT is thus to lower the activation energy of protein denaturation, thus making the proteins more susceptible to thermal damage. The observation of high levels of apoptotic cell death following combined HT and PDT (Fig. 7) is consistent with this hypothesis since these proteins can be found in the mitochondrial membrane and ALA-induced protoporphyrin IX has significant mitochondrial localization[41]. In other words, ALA-induced PDT may result in significant damage to proteins in the mitochondrial membrane thus making them more susceptible to thermal inactivation. Damage to these proteins would presumably result in damage to the mitochondrial membrane thus increasing the likelihood of apoptosis.

5. CONCLUSIONS

The response of human glioma spheroids to combined gamma radiation and PDT is highly dependent on irradiation parameters such as, gamma dose and light fluence and fluence rate. The results suggest that synergistic interactions occur only if both gamma and light dose thresholds are exceeded. The exact values of these thresholds are unknown, however, they must be greater than 4 Gy and 12 J cm^{-2} since synergism is only observed for combinations of 8 Gy and 25 J cm^{-2}. The degree of interaction between the two modalities is unaffected by treatment sequence and the time intervals studied. Although the precise mechanisms remain to be elucidated, combined activation of necrotic and apoptotic pathways is plausible, however, other phenomena, especially those involving cellular oxygenation status, cannot be ruled out.

The addition of concurrent HT appears to be an efficient strategy to increase the anti-tumor effect of ALA-mediated PDT in the therapy of gliomas. Although the mechanisms of synergism remain to be clarified, they may be due, in part, to a common mode of action on cellular proteins.

ACKNOWLEDGMENTS

The authors wish to acknowledge the help of Jessica Ganser, Brian Hsieh and Mickey Ni. Steen Madsen is grateful for the support of the UNLV Office of Research and the UNLV Cancer Research Center. Henry Hirschberg is grateful for the support of the Norwegian Cancer Society and the Norwegian Research Council. 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).

REFERENCES

1. M. Salcman "Epidemiology and factors affecting survival." In Malignant Cerebral Glioma. (Edited by M.L.J. Apuzzo), pp 95-109. American Association of Neurological Surgeons: Park Ridge, IL. 1990
2. K.E. Wallner, J.H. Galicich, G. Krol, E. Arbit, and M.G. Malkin, "Patterns of failure following treatment for glioblastoma multiforme and anaplastic astrocytoma," Int. J. Radiat. Oncol. Biol. Phys. **16**, pp. 1405-1409, 1989.
3. H. Kostron, A. Obwegesser and R. Jacober, "Photodynamic therapy in neurosurgery: a review," J. Photochem. Photobiol. B: Biol. **36**, pp. 157-168, 1996.
4. S.J. Madsen, L.O. Svaasand, B.J. Tromberg and H. Hirschberg, "Characterization of optical and thermal distributions from an intracranial balloon applicator for photodynamic therapy." In: Duncan DD, Jacques SL, Johnson PC (eds) Laser-Tissue Interaction XII: Photochemical, Photothermal and Photomechanical, Proceedings SPIE, Vol. 4257, January 2001, SPIE Publishers, Bellingham, WA, 2001, pp 41-49.
5. S.J. Madsen, C.-H. Sun, B.J Tromberg and H. Hirschberg, "Development of a novel balloon applicator for optimizing light delivery in photodynamic therapy," Lasers Surg. Med. **29**, pp. 406-412, 2001.

6. T. Christensen, A. Wahl and L. Smedshammer, "Effects of haematoporphyrin derivative and light in combination with hyperthermia on cells in culture," Br J Cancer **50**, pp. 85-89, 1984.
7. T.S. Mang and T.J. Dougherty, "Time and sequence dependent influence of *in vitro* photodynamic therapy survival by hyperthermia," Photochem. Photobiol. **42**, pp. 533-540, 1985.
8. N. Miyoshi, N. Matsumoto, H. Hisazumi and M. Fukuda, "The effect of hyperthermia on murine leukaemia cells in combination with photodynamic therapy," Int. J. Hyperthermia **4**, pp. 203-209, 1988.
9. C. Prinsze, L.C. Penning, T.M.A.R. Dubbelman and J. VanSteveninck, "Interaction of photodynamic treatment and either hyperthermia or ionizing radiation and of ionizing radiation and hyperthermia with respect to cell killing of L929 fibroblasts, Chinese hamster ovary cells and T24 human bladder carcinoma cells," Cancer Res. **52**, pp. 117-120, 1992.
10. M.H. Rasch, K. Tijssen, J. VanSteveninck and T.M.A.R. Dubbelman, "Synergistic interaction of photodynamic treatment with the sensitizer aluminum phthalocyanine and hyperthermia on loss of clonogenicity of CHO cells," Photochem. Photobiol. **64**, pp. 586-593, 1996.
11. B. Chen, Y. Xu, P. Agostinis and P.A.M. DeWitte, "Synergistic effect of photodynamic therapy with hypericin in combination with hyperthermia on loss of clonogenicity of RIF-1 cells." Int. J. Oncology **18**, pp. 1279-1285, 2001.
12. B.W. Henderson, S.M. Waldow, W.R. Potter and T.J. Dougherty, "Interaction of photodynamic therapy and hyperthermia: tumor response and cell survival studies after treatment of mice *in vivo*.," Cancer Res. **45**, pp. 6071-6077, 1985.
13. S.M. Waldow, B.W. Henderson and T.J. Dougherty, "Hyperthermic potentiation of photodynamic therapy employing photofrin I and II: comparison of results using three animal models," Lasers Surg. Med. **7**, pp. 12-22, 1987.
14. T.S. Mang, "Combination studies of hyperthermia induced by the Neodymium: Yttrium-Aluminum-Garnet (Nd:YAG) laser as an adjuvant to photodynamic therapy," Lasers Surg. Med. **10**, pp. 173-178, 1990.
15. Q. Jiang, M. Chopp and F.W. Hetzel, "*In vivo* ^{31}P NMR study of combined hyperthermia and photodynamic therapies of mammary cell carcinoma in the mouse, "Photochem. Photobiol. **54**, pp. 795-799, 1991.
16. S. Kimel, L.O. Svaasand, M. Hammer-Wilson, V. Gottfried, S. Cheng, E. Svaasand and M.W. Berns, "Demonstration of synergistic effects of hyperthermia and photodynamic therapy using the chick chorioallantoic membrane model," Lasers Surg. Med. **12**, pp. 432-440, 1992.
17. M.O. Dereski, L. Madigan and M. Chopp, "The effect of hypothermia and hyperthermia on photodynamic therapy of normal brain," Neurosurgery **36**, pp. 141-146, 1995.
18. Q. Chen, H. Chen, H. Shapiro and F.W. Hetzel, "Sequencing of combined hyperthermia and photodynamic therapy," Rad. Res. **146**, pp. 293-297, 1996.
19. D.L. Liu, S. Andersson-Engels, C. Sturesson, K. Svanberg, C.H. Haakansson and S. Svanberg, "Tumor vessel damage resulting from laser-induced hyperthermia alone and in combination with photodynamic therapy," Cancer Lett. **111**, pp. 157-165, 1997.
20. B. Chen, T. Roskams T and P.A.M. deWitte, "Enhancing the antitumoral effect of hypericin-mediated photodynamic therapy by hyperthermia," Lasers Surg. Med. **31**, pp. 158-163, 2002.
21. R.M. Sutherland, J. Carlsson, R.E. Durand and J. Yuhas, "Spheroids in cancer research," Cancer Res. **41**, pp. 2980-2994, 1981.
22. S.J. Madsen, C.-H. Sun, B.J. Tromberg, V.P. Wallace and H. Hirschberg, "Photodynamic therapy of human glioma spheroids using 5-aminolevulinic acid," Photochem. Photobiol. **72**, pp. 128-134, 2000.
23. B. Drewinko, T.L. Loo, B. Brown, J.A. Gottlieb and E.J. Freireich, "Combination chemotherapy in vitro with adriamycin. Observation of additive, antagonistic, and synergistic effects when used in two-drug combinations on cultured human lymphoma cells," Cancer Biochem. Biophys. **1**, pp. 187-195, 1976.
24. Y. Gavrieli, Y. Sherman and S.A. Ben-Sasson, "Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation," J. Cell Biol. **19**, pp. 493-501, 1992.
25. S.K. Ray, G.G. Wilford, C.V. Crosby, E.L. Hogan and N.L. Banik, "Diverse stimuli induce calpain overexpression and apoptosis in C6 glioma cells," Brain Res. **829**, pp. 18-27, 1999.
26. S.K. Ray, M. Fidan, M.W. Nowak, G.G. Wilford, E.L. Hogan and E.L. Banik, "Oxidative stress and Ca2+ influx upregulate calpain and induce apoptosis in PC12 cells," Brain Res. **852**, pp. 326-334, 2000.
27. V.P. Wallace, A.K. Dunn, M.L. Coleno and B.J. Tromberg, "Two photon microscopy in highly scattering tissue." In: Periasamy A (ed) Methods in Cellular Imaging. Oxford University Press, Cary, NC, pp 180-199, 2001.

28. Z. Luksiene, A. Kalvelyte and R. Supino, "On the combination of photodynamic therapy with ionizing radiation," J. Photochem. Photobiol. B **52**, pp. 35-42, 1999.
29. T.H. Foster, D.F. Hartley, M.G. Nichols and R. Hilf, " Fluence rate effects in photodynamic therapy of multicell tumor spheroids," Cancer Res. **53**, pp. 1249-1254, 1993.
30. Y. Luo and D. Kessel, "Initiation of apoptosis versus necrosis by photodynamic therapy with chloroaluminum phthalocyanine," Photochem. Photobiol. **66**, pp. 479-483, 1997.
31. D. Separovic, K.J. Mann and N.L. Oleinick, "Association of ceramide accumulation with photodynamic treatment-induced cell death," Photochem. Photobiol. **68**, pp. 101-109, 1998.
32. D.J. Ball, Y. Luo, D. Kessel, J. Griffiths and S.B. Brown, "The induction of apoptosis by a positively charged methylene blue derivative," J. Photochem. Photobiol. B **42**, pp. 159-163, 1998.
33. P. Agostinis, Z. Assefa, A. Vantieghem, J.R. Vandenheede, W. Merlevede and P. DeWitte, "Apoptotic and anti-apoptotic signalling pathways induced by photodynamic therapy with hypericin," Advan. Enzym. Regul. **40**, pp. 157-182, 2000.
34. F. Takahisa, K.-W. Yoon, T. Kato and Y. Kazuo, "Heat-induced apoptosis in human glioblastoma cell line A172," Neurosurgery **42**, pp. 843-849, 1998.
35. P.L. Olive and R.E. Durand, "Drug and radiation resistance in spheroids: cell contact and kinetics," Cancer Metastasis Rev. **13**, pp. 121-138, 1994.
36. J.C. Wigle and P.M. Sutherland, "Increased thermoresistance developed during growth of small multicellular spheroids," J. Cell Physiol. **122**, pp. 281-289, 1985.
37. M. Hermisson, H. Strik, J. Rieger, J. Dichgans, R. Meyermann and M. Weller, "Expression and functional activity of heat shock proteins in human glioblastoma multiforme," Neurology **54**, pp. 1357-1364, 2000.
38. A.M. Gorman, B. Heavey, E. Creagh, T.G. Cotter and A. Samali, "Antioxidant-mediated inhibition of the heat shock response leads to apoptosis," FEBS Lett. **445**, pp. 98-102, 1999.
39. B.V. Harmon, A.M. Corder, R.J. Collins, G.C. Gobe, J. Allen, D.J. Allan and J.F.R. Kerr, "Cell death induced in a murine mastocytoma by 42-47^{o}C heating *in vitro*: evidence that the form of death changes from apoptosis to necrosis above a critical heat load," Int. J. Radiat. Biol. **58**, pp. 845-858, 1990.
40. C. Prinsze, T.M.A.R. Dubbelman and J. VanSteveninck, "Potentiation of the thermal inactivation of glyceraldehydes-3-phosphate dehydrogenase by photodynamic treatment. A possible model for the synergistic interaction between photodynamic therapy and hyperthermia," Biochem. J. **276**, pp. 357-362, 1991.
41. A. Verma, S.L. Facchina, D.J. Hirsch, S.-Y. Song, L.F. Dillahey, J.R. Williams and S.H. Snyder, "Photodynamic tumor therapy: mitochondrial benzodiazepine receptors as a therapeutic target," Mol. Med. **4**, pp. 40-48, 1998.