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Effects of Combined Photodynamic Therapy and Ionizing Radiation on Human Glioma Spheroids¶

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ABSTRACT

The effects of combined photodynamic therapy (PDT) and ionizing radiation are studied in a human glioma spheroid model. The degree of interaction between the two modalities depends in a complex manner on factors such as PDT irradiation fluence, fluence rate and dose of ionizing radiation. It is shown that gamma radiation and PDT interact in a synergistic manner only if both light fluence and gamma radiation dose exceed approximately 25 J cm^{-2} and 8 Gy, respectively. Synergistic interactions are observed only for the lower fluence rate (25 mW cm^{-2}) investigated. The degree of interaction appears to be independent of both sequence and the PDT or ionizing radiation time intervals investigated (1 and 24 h). Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assays show that low-fluence rate PDT is very efficient at inducing apoptotic cell death, whereas neither high-fluence rate PDT nor ionizing radiation produces significant apoptosis. Although the mechanisms remain to be elucidated, the data imply that the observed synergism is likely not due to gamma-induced cell cycle arrest or to PDT-induced inhibition of DNA repair.

INTRODUCTION

The prognosis for patients with high-grade gliomas has not improved significantly during the past four decades. Even with the best available treatments, using surgery, ionizing radiation and chemotherapy, median survival is less than 1 year (1). In most cases, treatment failure is due to local recurrence, indicating that a more aggressive local treatment may be beneficial; approximately 80% of tumors recur within 2 cm of the resection cavity (2).

Photodynamic therapy (PDT) has been used successfully in the treatment of a wide variety of localized malignancies (3) and may prove useful as an adjuvant therapy in the treatment of resected margins after surgery. The tumoricidal mechanism of this form of treatment is based on the cytotoxic activation, by light, of a photosensitizing drug that is localized to the tumor tissue. Photosensitizers in current clinical use are typically activated by red light through optical fibers. Because models of light and thermal distributions in brain tissue suggest that it may be possible to eradicate tumor cells at depths of 1–1.5 cm (4), PDT has the potential of playing a significant role in the management of brain tumors.

Although PDT has been used in the treatment of brain tumors since 1980 (5–8), the results of clinical trials have been ambiguous, partly because of their limited scope. In almost all cases, PDT has been given in a single treatment immediately after surgery. Because of the complicated nature of PDT dosimetry, there have been relatively few attempts to optimize the PDT dose because it depends on a number of parameters, including light fluence and fluence rate, photosensitizer concentration and tissue oxygenation status. Furthermore, interactions with standard treatment modalities such as ionizing radiation are poorly understood.

The effects of combined ionizing radiation and PDT have been studied primarily in simple *in vitro* systems consisting of monolayer cell cultures. 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. In this study a simple human glioma spheroid model is used to investigate systematically the degree of interaction between the two treatment modalities as a function of various parameters. Because 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. Of particular interest is the observation that spheroids mimic the oxygen gradients found in solid tumors (9).

The primary aim of this study is to investigate the effects of combined PDT and ionizing radiation in human glioma spheroids incubated in 5-aminolevulinic acid (ALA)—a prodrug that is

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Abbreviations: AF, apoptotic fraction; ALA, 5-aminolevulinic acid; DAPI, 4,6-diamidino-2-phenylindole, dihydrochloride; GBM, glioblastoma multiforme; NA, numerical aperture; PDT, photodynamic therapy; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated deoxyuridine triphosphate nick-end labeling.

converted in cells to a potent photosensitizer, protoporphyrin IX. ALA is a commonly used drug that appears promising in the treatment of brain tumors because of its favorable localization characteristics (10) and short-lived cutaneous photosensitivity (11). Previous studies using hematoporphyrin derivative have been inconclusive; some indicate a potentiation effect between PDT and ionizing radiation, whereas others show no such effect (12). To our knowledge the present study is the first to examine potentiative effects in human glioma spheroids incubated in ALA. Knowledge of such effects is clinically relevant because patients undergoing investigative PDT treatments are likely to receive ionizing radiation concurrently.

MATERIALS AND METHODS

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, CA). The cells were cultured in Dulbecco modified Eagle medium (GIBCO, Grand Island, NY) with high glucose and supplemented with 2 m*M* L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% heat-inactivated fetal bovine serum (GIBCO). The cells were maintained at 37°C in a 7.5% CO_2 incubator. At a density of 70% confluence, the cells were removed from the incubator and left at room temperature for approximately 20 min. The resultant cell clusters (consisting of approximately 10 cells) were transferred to a petri dish and grown to tumor spheroids using a liquid-overlay technique (13). 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.

Photodynamic therapy. Spheroids were incubated in 1000 μg mL^{-1} ALA (Sigma) 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 output 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}, whereas in the lower-fluence rate studies, spheroids were irradiated to fluences of 12 and 25 J cm^{-2}. These light fluence and fluence rates were found to be suboptimal in a previous investigation (14).

Ionizing radiation. In all cases, spheroids were irradiated in a petri dish under 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}.

Combined PDT and gamma irradiation. Spheroids were incubated in ALA 3–4 h before gamma irradiation. PDT and gamma irradiation were separated by 45 min (range: 30–60 min) or 24 h (range: 22–26 h). In each case the effects of treatment sequence (PDT first *vs* gamma first) were investigated. After 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, 16–24 spheroids were followed for each treatment regimen. Because each trial was performed 3 or 4 times, a total of 48–96 spheroids were followed for a given set of parameters. Spheroids were followed for up to 35 days. The spheroids showing significant growth during the observation period were assumed to have survived treatment. The surviving fraction was evaluated by taking the ratio of the surviving to the total number of spheroids.

The degree of interaction between the two treatment modalities was evaluated by a technique proposed by Drewinko *et al.* (15). In this scheme the degree of interaction is given by

$$\alpha = \frac{SF^{\gamma} \times SF^{\psi}}{SF^{\gamma\psi}} \quad (1)$$

where SF^{γ} and SF^{ψ} represent the surviving fractions with gamma and PDT, respectively, and $SF^{\gamma\psi}$ is the surviving fraction after 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.

Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling assay for apoptosis. 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™ flourometric terminal deoxynucleotidyl transferase (TdT)–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) 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. The fluorescein label was then detected by two-photon fluorescence microscopy (16). The fluorescein was excited at a wavelength of 800 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 20 to 120 μm. Depth discrimination was accomplished by adjusting the Z position of the 10× (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

$$AF = N_f / N_t \quad (2)$$

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 × 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. Nuclear morphology of representative DAPI-stained spheroids in each treatment group was studied from high-resolution (63×) two-photon fluorescence images.

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. In all cases, apoptosis was evaluated at a spheroid depth of 60 μm.

RESULTS

Effects of either gamma radiation or PDT on spheroid survival are summarized in Fig. 1. The control group represents true controls that were allowed to grow in the absence of light and ALA. Two other control groups (ALA only and light only) exhibited survival (100%) that was identical to that observed for the true controls (data not shown). 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 before ALA incubation, whereas the other was irradiated after incubation. Because 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 Fig. 1 are for the postincubation group only.

As illustrated in Fig. 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

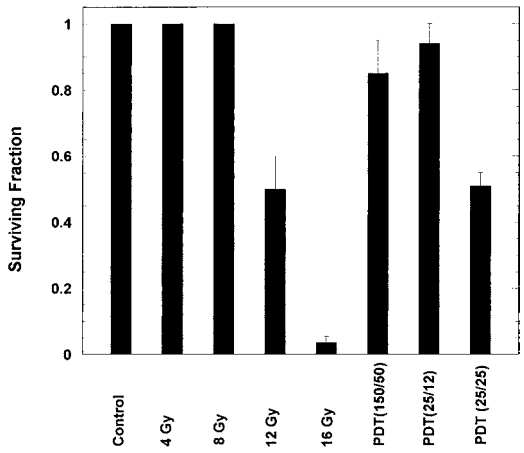

Figure 1. Spheroid survival after exposure to either gamma radiation or 635 nm light. Fluence rate (mW cm^{-2}) and fluence (J cm^{-2}) are indicated in parentheses (fluence rate/fluence). Each data point represents

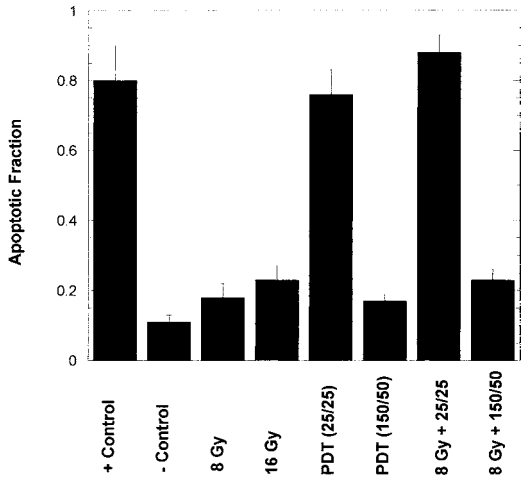

Figure 2. Fraction of cells in apoptosis as a function of treatment type. The AF was evaluated from two-photon fluorescence images (10×) acquired at spheroid depths of approximately 60 μm. Each data point represents the mean of six spheroids from two independent treatments. Positive and negative controls are denoted by +Control and −Control, respectively. Error bars denote standard deviations.

cm^{-2}), but the response is fluence dependent. For example, fluences of 12 and 25 J cm^{-2} result in surviving fractions of approximately 0.95 and 0.50, respectively.

The ability of various treatments to induce apoptosis is illustrated in Fig. 2. It is shown that low–fluence rate PDT is a very effective inducer of apoptosis—the AF (*ca* 0.76) is comparable with that observed in the positive controls. In contradistinction, both high–fluence rate PDT and ionizing radiation fail to produce apoptosis at levels significantly above the background levels (as denoted by the negative controls). The combined treatments yield slightly higher levels of apoptosis than does either of the single modalities.

The data in Fig. 3 illustrate that subthreshold gamma doses can have a significant effect on spheroid growth. The effect appears to be dose dependent, as illustrated by the greater growth delay induced by the higher dose. For example, the doubling time for the 8 Gy–exposed spheroids is approximately 25 days, compared with 8 days for both controls and the 4 Gy–exposed spheroids. As illustrated in Fig. 4, a similar dose-dependent effect is observed for light-exposed spheroids. Fluences of 10 and 25 J cm^{-2} yield doubling times of approximately 14 and 22 days, respectively.

The response of spheroids to combined gamma irradiation and high fluence rate (150 mW cm^{-2}) PDT is illustrated in Fig. 5. 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 Eq. 1, is listed above each data point. As shown in Fig. 5, there is no advantage in combining gamma radiation with high-fluence rate PDT—survival for spheroids treated with PDT only (Fig. 1) is similar to that observed for low gamma dose (4 or 8 Gy) combined treatments (Fig. 5). Furthermore, survival after high gamma dose (12 or 16 Gy) combined treatments is similar to that for gamma-only (12 or 16 Gy) treatment (Fig. 1). As shown in Fig. 5, response to treatment is independent of sequence (PDT first *vs* gamma first) and time interval (1 or 24 h).

As illustrated in Fig. 6, 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 in 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.

DISCUSSION

The management of patients with high-grade gliomas typically includes high doses of ionizing radiation. The aim of such treatments is to improve local control through eradication of tumor cells in the resection margin. Unfortunately, radiation therapy has proven relatively unsuccessful because of the radioresistance of glioma cells. This may be partly due to the inability of therapeutic doses of ionizing radiation to induce apoptosis in glioma cells; necrosis is the primary mode of cell death after gamma irradiation (17). Because of the inability of ionizing radiation to induce apoptosis in human glioma cells (Fig. 2), the observed cell death after high gamma doses ($\geq$12 Gy) was attributed to necrosis. This was confirmed by high-resolution (63×) two-photon fluorescence microscopy studies of DAPI-stained cells in gamma-exposed spheroids (data not shown). These cells showed morphologic changes consistent with a necrotic mode of cell death (*e.g.* cell swelling). In agreement with the data presented in Fig. 2, there was little evidence of apoptosis—cellular nuclei appeared normal with well-organized chromatin. In contradistinction, the mechanism of cell death after PDT is variable and depends on factors such as cell line, sensitizer and treatment conditions (*e.g.* light fluence, fluence rate and sensitizer concentration). PDT has been shown to cause apoptosis in a number of cell lines (18–24) including human glioma cells (25). This is confirmed by the present study, which shows that apoptosis is the primary mode of cell death after exposure to low-fluence rate PDT (Fig. 2).

A significant limitation of PDT is its inability to deliver adequate light doses to resection margins. This is due to the high attenuation of light in biological tissues—the penetration depth of 630 nm light in brain tissues is approximately 3 mm (26–28).

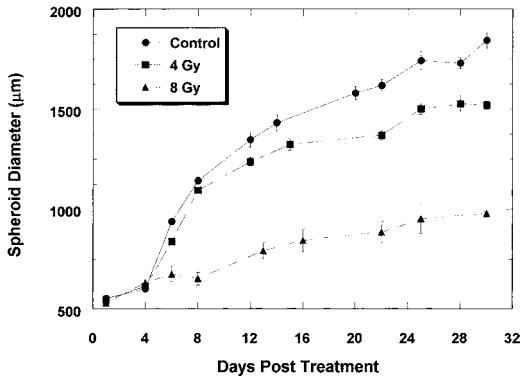

Figure 3. Growth kinetics of spheroids exposed to low doses of gamma radiation. Each data point represents the mean of approximately 80 spheroids. Error bars denote standard errors.

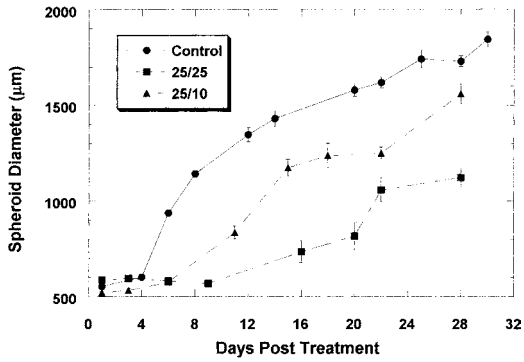

Figure 4. Growth kinetics of spheroids incubated in 1000 μg mL^{-1} ALA and exposed to fluences of 12 and 25 J cm^{-2} (fluence rate = 25 mW cm^{-2}). Each data point represents the mean of approximately 50

Through careful consideration of light-delivery technique, it may be possible to achieve fluence rates approaching 25 mW cm^{-2} at depths of 1 cm in the resection margin (4). This fluence rate has been found to be very effective in human glioma spheroids, provided that the total fluence is approximately 50 J cm^{-2} (14).

The clinical relevance of the gamma doses used in the present *in vitro* study is unknown, but it should be noted that in a current high–dose rate brachytherapy protocol, patients received doses of 72 Gy in 12 fractions (6 Gy $fraction^{-1}$, 2 fractions day^{-1}, 6 days) (29). Although the rationale for dose escalation is to improve local control, it is clear that even higher doses are required to achieve significant prolongation of life. Such high doses are problematic, however, because they are likely to result in unacceptable normal tissue complications. Thus, a fundamental problem associated with both radiation therapy and PDT is that malignant cells deep in the resection margin receive inadequate doses of ionizing and nonionizing radiation. The central question addressed in this study is to what extent, if any, suboptimal light and gamma doses interact in a human glioma spheroid model.

The results 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. Cell cycle effects have been investigated in a number of studies (30,31); in fact, the possibility of such effects provided the rationale for choosing time intervals of 24 h. Gamma irradiation is known to induce cell cycle arrest in G2 (32). It has also been shown that tumor cell sensitivity to ALA-PDT varies during the cell cycle—the cells in S and G2 being more sensitive (33). A 24 h delay after gamma irradiation allows maximum accumulation of cells in G2, when cells are most sensitive to PDT. However, the results of the present study, as well as those of others (30,31), do not support the hypothesis that PDT effectiveness is enhanced for time intervals of 24 h. As illustrated in Figs. 5 and 6, there is no difference in survival for time intervals of 1 and 24 h. Furthermore, there is no significant difference in survival as a function of treatment sequence—identical survival is observed if PDT is given first.

It has been suggested that synergistic interactions can be explained by PDT-induced inhibition of DNA repair (34). This would explain the variable response of different cell lines to combined therapies—in some cell lines PDT results in inhibition of DNA repair, whereas in others DNA repair is insensitive to PDT. Presumably, synergism can only occur if the two modalities are given within the time window of repair. In the case of human glioma cells, this implies that treatments must be given within minutes of each other. This is because of the finding that approximately half of the radiation-induced damage is repaired in the first 5 min, whereas the remainder is repaired within 30 min (35). Because the shortest time interval examined in this study is 1 h, the observed synergism is likely not due to inhibition of DNA repair.

As illustrated in Fig. 2, low-fluence rate PDT and gamma radiation differ significantly in their ability to induce apoptosis in glioma cells. Although low-fluence rate PDT is a very efficient apoptotic inducer, gamma radiation fails to induce apoptosis at levels significantly above the background level in exposed spheroids. Morphologic evidence suggests that gamma radiation results in necrotic cell death. Because the main targets of gamma radiation and ALA-PDT are 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. 6, 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.* (36), who observed that neither apoptosis nor necrosis is triggered by sublethal doses of light or gamma radiation. A fluence of 12 J cm^{-2} can be considered sublethal because it has minimal effect on spheroid survival and does not result in significant growth delay compared with controls (Fig. 4). 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 with the spheroids treated with 4 Gy (Fig. 3). 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. 2 that, unlike the low-fluence

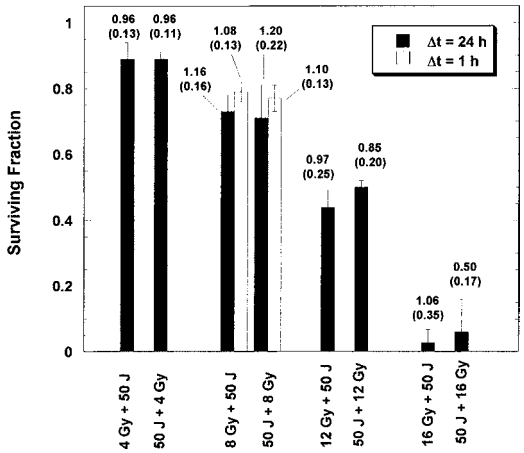

Figure 5. Spheroid survival after combined gamma radiation and high–fluence rate (150 mW cm^{-2}) PDT. In all cases spheroids were exposed to fluences of 50 J cm^{-2} (denoted by 50 J). The interaction coefficient and its uncertainty (in parentheses) are shown above each data point. Time intervals of 1 and 24 h were investigated. Each data point represents the mean of approximately 70 spheroids.

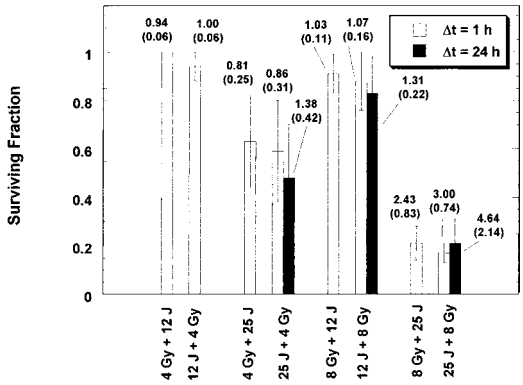

Figure 6. Spheroid survival after combined gamma radiation and low–fluence rate (25 mW cm^{-2}) PDT. Spheroids were exposed to fluences of 12 or 25 J cm^{-2} (denoted by 12 and 25 J). The interaction coefficient and its uncertainty (in parentheses) are shown above each data point. Time intervals of 1 and 24 h were investigated. Each data point represents the mean of approximately 70 spheroids.

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 (37). Because the level of apoptosis in this superficial layer ($\leqslant$60 μm) was not significantly different from that found in the negative controls (Fig. 2), the observed cell death was assumed to have occurred through 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 because 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 nonapoptotic mechanism, cells that undergo apoptosis with lower fluences (38–40). This phenomenon has been attributed to the induction of extensive membrane photodamage after high light doses and has been observed even for photosensitizers having significant mitochondrial localization (41).

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 because the efficacy of both PDT and gamma radiation depends 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 (37). Consequently, only the well-oxygenated and rapidly proliferating cells in the outer rim of the spheroid will be damaged. In contrast, because 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 radio-resistant quiescent cells more susceptible to ionizing radiation. Although this is a plausible explanation if PDT is given before ionizing radiation, it fails to account for the synergism observed when gamma radiation precedes PDT.

Although the simple spheroid model used in this study is not an accurate representation of the *in vivo* environment, it represents a more sophisticated system than do the monolayer suspensions used in most of the studies investigating combined therapies. The inability of the model to account for vascular effects is probably not critical because ALA is primarily a cellular photosensitizer (42).

In conclusion, the key finding of this study is that the response of human glioma spheroids to combined gamma radiation and PDT is highly dependent on irradiation parameters such as gamma dose, 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, but they must be greater than 4 Gy and 12 J cm^{-2} because 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; other phenomena, especially those involving cellular oxygenation status, cannot be ruled out. These are the subject of ongoing investigations.

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