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

Steen J. Madsen*1–3, Chung-Ho Sun3, Bruce J. Tromberg3, Alvin T. Yeh3, Rogelio Sanchez3 and Henry Hirschberg3,4

1Department of Health Physics, University of Nevada–Las Vegas, Las Vegas, NV; 2Las Vegas Cancer Institute, University of Nevada, Las Vegas, NV; 3Beckman Laser Institute and Medical Clinic, University of California, Irvine, CA and 4Department of Neurosurgery, Rikshospitalet, Oslo, Norway

Received 17 June 2002; accepted 22 July 2002

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 various 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
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 grown in Dulbecco modified Eagle medium (GIBCO, Grand Island, NY) 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 (GIBCO). The cells were maintained at 37°C in a 7.5% CO2 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. All experiments were performed in triplicate. 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 rate experiments were found to be suboptimal in a previous investigation (14).

Irradiation. In all cases, spheroids were irradiated in a petri dish under ambient conditions, using a 137Cs 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 with a microscope with a calibrated eyepiece to measure the diameter of each spheroid using a microscope with a calibrated eyepiece. In all cases, spheroids were irradiated in a petri dish under ambient conditions, using a 137Cs 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.

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 (mean ± S.D.) of at least six independently treated spheroids. 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.

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.

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).
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
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 (<60 μm) was not significantly different from that found in the negative control (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.

Acknowledgements—The authors are grateful to Linda Lee for her experimental help and to J. L. Redpath for providing the gamma source and for many useful discussions. S.M. is grateful for the support of the UNLV Office of Research and the UNLV Cancer Institute. H.H. is grateful for the support of the Norwegian Cancer Society. A.T.Y. was supported, in part, by a NIH carcinogenesis training grant (CA-09054). 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, CA. 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


