Effects of Various Photoradiation Regimens on the Antitumor Efficacy of Photodynamic Therapy for R3230AC Mammary Carcinomas

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ABSTRACT

Clinical photodynamic therapy consists of the systemic administration of a derivative of hematoporphyrin (Photofrin II) followed by exposure of malignant lesions to continuous visible laser irradiation. We investigated the effects of various modifications of laser light delivery on the efficacy of photodynamic therapy in controlling R3230AC mammary tumor growth. We observed a significant delay in growth (from initial to 2 times initial volume) of tumors exposed to periodic irradiation (100 mW/cm²/0.25 h, 1-h dark interval, total fluence, 180 J/cm²), compared to untreated controls or to tumors receiving continuous irradiation at the same total fluence. Other periodic light treatment regimens, consisting of 3-, 6-, or 24-hr dark intervals, delayed tumor growth but not significantly more than continuous irradiation at the same fluence. A biochemical basis was sought by comparing continuous versus periodic irradiation for effects on mitochondrial or cytosolic enzymes in vivo. Although both cytochrome c oxidase and pyruvate kinase activities were reduced dramatically during the first 24 h by continuous or periodic irradiation schemes, recovery of enzyme activity to initial levels took longer after the periodic irradiation protocol (168 h), compared to the continuous irradiation regimen (72 h). We observed a significantly greater delay in the growth of tumors exposed to 50 mW/cm²/2 h continuously, compared to controls or to tumors exposed to the same total fluence but with light delivered at 100 or 200 mW/cm². The data presented here indicate that the efficacy of photodynamic therapy could be significantly increased by modifications in the delivery of photoradiation.

INTRODUCTION

PDT, which shows considerable promise as a new treatment modality for the clinical management of a variety of cancers, has been under intensive investigation in the laboratory. The clinical treatment regimen usually consists of the systemic i.v. administration of Photofrin II, a porphyrin photosensitizer currently undergoing clinical trials, followed by an equilibration period of 24–72 h, during which time the "biologically active" porphyrin components of Photofrin II are apparently retained at greater concentrations in tumor tissue than in some, but not all, normal tissues, according to a majority of reports in the literature (1–6). The malignancies, subsequent to the equilibration period, are then exposed to visible radiation, usually 630-nm laser light. Excitation of the tumor-retained porphyrins by visible light leads to production of singlet oxygen, which is reported to be the toxic agent responsible for induction of necrosis and regression of malignancies (7–10).

There are at least three variable factors that can have an impact on the effectiveness of PDT in controlling malignancies: (a) the concentration of "active" porphyrin components in the tumor (photosensitizer delivery), (b) the exposure of malignant tissues to sufficient photon flux to elicit a response (light delivery), and (c) the presence of sufficient levels of dioxygen for formation of effective amounts of singlet oxygen throughout the light exposure period (oxygen availability). Some efforts have been made to modulate these factors in attempts to attain greater antitumor efficacy. To achieve higher intratumor levels of photosensitizer, Photofrin II has been administered by direct injection into tumors by Lin and his colleagues (2, 3) and by us (4), and Zhou et al. (11) incorporated hematoporphyrin into liposomes prior to systemic administration. Each approach appears to increase the concentration of photosensitizing porphyrins in tumors and the resulting efficacy of PDT. Maintenance of sufficient concentrations of molecular oxygen for continuous production of adequate amounts of singlet oxygen during irradiation has been explored. Hyperbaric oxygen and administration of fluorinated blood substitutes to increase concentrations of oxygen in tumors were investigated (12, 13). The authors concluded that these disappointing results suggest that maintenance of the O₂ level was not as critical as the amount of oxygen present in the tissue at the onset of PDT. Also, other processes may be responsible for the reduction in molecular O₂ levels, such as the photochemical reaction and/or metabolic utilization. On the other hand, delivery of laser light to the malignancies has been extensively investigated for improvement of the efficacy of PDT. Fiber optics, diffusing lenses, and interstitial devices have all been designed and developed for use in PDT and, in many cases, have dramatically increased the effectiveness of this emerging treatment (14–17).

In this report, we describe studies undertaken to address the question of light delivery by modifying the usual continuous irradiation protocols in two ways. First, we introduced a periodic photoradiation protocol, a scheme based on previous results obtained from experiments with in vivo nuclear magnetic resonance spectroscopy (18). In those studies, we proposed that, during the time period when the tumor β-nucleotide triphosphate/P₃ ratios were at their lowest levels, delivery of a second dose of irradiation might increase the efficacy of PDT (18–20). The second modification investigated here was to decrease the power density delivered to the tumors, from 200 to 50 mW/cm², and to extend the length of time of the exposure period. Evaluation of these modifications of light delivery was assessed by tumor growth behavior in vivo. Additionally, the effects of periodic photoradiation on the activity of a selected mitochondrial and cytosolic enzyme in tumors were measured in vivo.

MATERIALS AND METHODS

Materials. All chemicals and reagents used for experiments were purchased from Sigma Chemical Co. (St. Louis, MO), unless indicated otherwise. Photofrin II, provided by Quadra Logic Technologies Inc. (Vancouver, British Columbia, Canada), was received frozen, thawed in the dark at room temperature, divided into 1-ml aliquots, and stored at −70°C until used.

Animals and Tumors. The R3230AC mammary adenocarcinoma was...
maintained by transplantation in the axillary region of 80–100-g Fischer female rats, using the sterile trochar procedure described earlier (21). All animals were cared for under the guidelines of the University Committee on Animal Resources at the University of Rochester.

Measurement of Tumor Growth and Determination of Treatment Efficacy. Once palpable, tumors were measured at regular intervals, usually every other day, using calipers to obtain two perpendicular dimensions. Tumor volume was calculated as $V = \pi r^2 H$, where $r$ is the width and $H$ the length. The actual tumor volume was assessed in a sample of tumors by surgically removing them and determining their wet weight. A comparison of wet displacement data with values calculated from the equation resulted in a consistent overestimate of ~20% by the latter.

Tumor growth was monitored by the calculated volumes, and the rate of growth is presented as the number of days required for an individual tumor to reach 2, 5, or 10 times initial volume. Linear interpolation was used to estimate tumor volume on days when measurements were not taken. This method of analysis of tumor growth accounts for variations due to differing initial tumor volume.

Administration of Photofrin II and Photoradiation of Tumors in Vivo. Photofrin II was administered at 5 or 10 mg/kg, i.p., 24 h prior to photoradiation. Animals were anesthetized by i.m. administration of 75 mg/kg ketamine and 6 mg/kg Rompun and the skin overlying the tumor was shaved with electric clippers to remove the hair. Tumors were photoradiated with an argon-pumped tunable dye laser (Inova 90; Coherent Inc., Palo Alto, CA) coupled to 400-μm optical fibers fitted with cylindrical lenses (Laserguide, Santa Barbara, CA). Intensity of the 630-nm beam emitted from the fibers was monitored using a power radiometer (RK 5200; Laser Precision, Utica, NY) connected to an RK 545 radiometer probe. The fibers, placed at 1.5 cm from the surface of the tumor, provided a 1-cm-diameter light spot. Tumors were irradiated transcutaneously either by selected continuous wave schedules or by selected periodic photoradiation schedules, resulting in a total fluence exposure of 90, 180, or 360 J/cm². Tumor temperature was monitored during photoradiation using a YSI Telethermometer (model 411TD; Yellow Springs Instruments, Yellow Springs, Ohio) connected to a needle probe. The thermometer probe was inserted into various treated tumors, at different distances from the incident source (depths in the tumors), and into tumors exposed to different power densities and fluences. Tumor temperature did not rise above 39°C at any site during exposure to 200-mW/cm² fluence rate and 360-J/cm² total fluence. Tumors of animals entered into treatment regimens had initial dimensions ranging from 0.7 to 1.0 cm in their longest diameter, corresponding to a volume ranging from 0.18 to 0.54 cm³. Animals were entered into control and treatment protocols randomly, so that one group would not contain a disproportional number of tumors with the same initial volume. Tumor growth was followed until each tumor reached a size calculated to be 10 times its initial volume, at which time animals were euthanized.

Effects of Different Light Exposure Schedules on Cytosolic or Mitochondrial Enzyme Activities. Tumor-bearing rats were administered 5 mg/kg Photofrin II, i.p., 24 h prior to exposure, to tumors without Photofrin II (light control), tumors grown in animals that received anesthetic only (anesthetic control), or tumors in animals that received neither light nor drug (tumor growth control), photoradiation of tumors without Photofrin II (light control), tumors grown in animals that received anesthetic only (anesthetic control), or tumors in animals that received neither light nor drug (tumor growth control), as well as among the various treatment groups. The statistical analyses were conducted on logarithmic transformations of the number of days for each tumor to double its initial volume ($T_2$) and the number of days for tumor volume to progress from 2 times initial volume to 10 times initial volume ($T_{10} - T_2$); a value of $P < 0.05$ was considered to be significant. Treatment regimens were tested on 4–21 individual tumors. No significant differences were observed among animals in the control regimens; these groups were combined and used as a pooled control for comparisons with treatment regimens.

RESULTS

Effects of PDT on Tumor Growth. All PDT protocols investigated were effective in delaying tumor growth from initial to 2 times initial volume ($T_2$) compared to controls, except for the protocol with the lowest total fluence (90 J/cm²; 100 mW/cm²/15 min). The typical pattern observed in responsive tumors after PDT was a decrease in tumor volume, i.e., reduction in tumor dimensions by caliper measurement, and in some cases tumors became undetectable by palpation. However, none of the protocols employed under the experimental conditions used here produced a "cure" of the R3230AC tumor. It should be noted that two groups also showed significantly slower tumor growth from 2 times to 10 times initial volume ($T_{10} - T_2$), compared to controls. Both involved longer irradiation periods: 50 mW/cm² for 1 h (fluence, 180 J/cm²) or 2 h (360 J/cm²).

Effects of Photofrin II Dose on Tumor Growth. Groups of tumor-bearing animals were tested at each combination of two doses of Photofrin II (5 or 10 mg/kg) and two power densities (100 or 200 mW/cm² for 0.5 h) (data not shown). A two-way analysis of variance indicated no significant differences between the two doses or the two power densities in their effect on tumor doubling time ($T_2$) or on time from doubling to 10 times initial volume ($T_{10} - T_2$). Additionally, no significant difference in tumor growth behavior was found between the 5 mg/kg Photofrin II, 200 mW/cm² and the 10 mg/kg Photofrin II, 100 mW/cm² groups, consistent with reciprocity.

Comparison of Periodic versus Continuous Irradiation on Tumor Growth. Tumors were exposed to 180 J/cm² total fluence at a delivered power of 100 mW/cm², following administration of 5 mg/kg Photofrin II 24 h earlier. One group of animals received the light treatment continuously for 30 min. Other treatment groups received light for a 15-min period and then a dark period of 1, 3, 6, or 24 h, followed by another 15-min period of light. Fig. 1 shows tumor growth data for these treatment groups. Groups were compared using Tukey's multiple comparison procedure. There were no significant differences in $T_{10} - T_2$ among the groups. However, initial doubling time was significantly longer in the group that received a 1-h interruption of light than in the continuous irradiation group. The other treatment groups exposed to periodic light had $T_2$ values intermediate between the 1-h periodic regimen and the continuous light groups but were not significantly different from either.
60% at 24 h after irradiation (Fig. 3A). By 168 h after either continuous and 360 J/cm² (P = 0.52).

A similar trend for Tu−7% is nearly statistically significant (P = 0.053; data not shown). However, there is no significant difference in Tu−7% between the two fluence levels, 180 J/cm² and 2 x 200 mW/cm²/0.25 h separated by a 1-h dark interval, 8 tumors (– – – – – –). Data are presented as the number of days required for tumors in each group to reach 2.5 or 10 times their initial volume. Each data point represents the mean; bars are the SE.

Effect of Photoradiation Time and Power Density on Tumor Growth. Tumors were exposed to either 180 or 360 J/cm² total fluence at various power densities (50, 100, or 200 mW/cm²), commencing 24 h after i.p. administration of 5 mg/kg Photofrin II. The data in Fig. 2 indicate that animals receiving laser irradiation over a 2-h period at 50 mW/cm² (360 J/cm²) had the greatest delays in early tumor growth (Tu), compared with the other exposure time/power density combinations tested.

A compilation of the results, demonstrating the relationship between power density, fluence, and tumor doubling time, is presented in Table 1. At a given power density, higher fluence (i.e., longer exposure) is associated with longer doubling time (P = 0.02; two-way analysis of variance). At a constant fluence of 360 J/cm², there is a significant negative trend in Tu−7% as power increases and exposure time decreases (P = 0.004; linear regression). A similar trend for T10 − T2 is nearly statistically significant (P = 0.053; data not shown). However, there is no significant difference in T10 − T2 between the two fluence levels, 180 and 360 J/cm² (P = 0.52).

Comparison of Periodic versus Continuous Irradiation in Vivo on the Activities of Cytochrome c Oxidase and Pyruvate Kinase. We measured mitochondrial cytochrome c oxidase and cytosolic pyruvate kinase activities in tumors obtained at selected times subsequent to photoradiation of tumors in vivo, seeking to determine whether a biochemical response was correlatable to the observed differences in tumor growth arising from continuous versus periodic light exposure. The results of these experiments are depicted in Fig. 3. Both continuous and periodic photoradiation significantly lowered mitochondrial cytochrome c oxidase activity in tumors, with the reduction being 60% at 24 h after irradiation (Fig. 3A). By 168 h after either photoradiation regimen, cytochrome c oxidase activity returned to 75 to 80% of that found in controls. However, in tumors that were exposed to the periodic irradiation regimen (100 mW/cm² for 15 min, 1 h dark, 100 mW/cm² for 15 min), cytochrome c oxidase activity displayed a more gradual time course of recovery than in tumors treated by a continuous light regimen of the same total fluence.

An even more striking difference in time course was seen for pyruvate kinase activities (Fig. 3B). Although both photoradiation regimens produced a 60% reduction of pyruvate kinase activity by 4 h after irradiation, the extent of reduction and pattern of recovery of pyruvate kinase activity differed for the two light exposure regimens. Tumors subsequent to periodic irradiation displayed a maximum reduction of 75% in pyruvate kinase activity occurring at 24 h after treatment, and this reduced activity was followed by a gradual return of enzyme activity to control levels by 168 h. In contrast, pyruvate kinase activity had returned to control levels by 72 h after the continuous light exposure regimen. These findings indicate that the

![Graph](image-url)
periodic photoradiation schedule produced greater and/or more prolonged inhibition of the enzymes selected for study, a treatment regimen that was also more effective in reducing initial tumor growth.

DISCUSSION

Under the most commonly used clinical protocols, PDT has been reported to produce complete or partial response rates of 60 to 80% for lung, bronchial, skin, and head and neck tumors (26–30). The majority of the clinical treatment protocols utilize an argon-pumped tunable dye laser operated continuously for the selected exposure period. Modifications of light delivery systems have been studied, including bulb diffuser tips fitted to fiber optics to provide 360-degree illumination of the bladder, cylinder diffusers for exposure of the lumen of the bronchus or esophagus, and fibers modified for interstitial delivery of adequate light fluence to bulky solid tumors (14–17). However, much less attention has been given to modification of the light delivery schedule as one means to enhance the efficacy of PDT. One series of clinical trials used interstitial fiber implantation and laser irradiation fractionated by intervals of 2–3 days. Another study used intervals of 2–3 weeks; both of these regimens produced favorable responses of the lesions (31, 32). However, no direct comparisons were made with controls or with lesions receiving continuous illumination. In another report, Dougherty et al. (33) treated recurrent breast carcinomas with a fractionated light schedule containing a 24-h period between photoradiation times. The results of this study did not produce definite conclusions (33). Using the Lewis lung carcinoma transplanted in mice, Cowled et al. (34) demonstrated no significant difference in tumor response when either an argon-pumped dye laser, operated continuously, or a gold vapor laser, operated with 50-ns pulses at a repetition rate of 10–14 kHz, was employed as irradiation source for PDT. Results obtained in vitro, comparing light dose rate effects of PDT, demonstrated that surviving fractions of Chinese hamster ovary cells were identical at equal total fluence when light was delivered at power densities ranging from 0.5 to 60 mW/cm² (35). On the other hand, Ben Hur et al. (36) reported that Chinese hamster cells, exposed to chloroaluminum phthalocyanine tetrasulfonate and light delivered at various dose rates, displayed greater cell survival after receiving the lower light dose rates, a finding similar to that of Matthews et al. (37), who investigated effects on cultured A549 human lung adenocarcinoma cells using Photofrin II as the photosensitizer.

Reports in the literature on the effects of light dose fractionation on cell toxicity in vitro are inconsistent in outcome. Moan and Christensen (38) demonstrated that NIHK 3025 carcinoma cells in culture were inactivated more readily using a split light dose regimen (two light doses separated by a 70-s dark period), compared to a continuous irradiation protocol of the same total fluence. In contrast, Bellnier and Lin (39) demonstrated that cultured EJ human urinary bladder carcinoma cells were less sensitive to damage when the light dose was fractionated, compared to damage after continuous illumination, and they suggested that the cells developed an increased tolerance to PDT subsequent to an earlier course of treatment. The lack of studies comparing efficacy of various light doses in vivo and the above conflicting results in vitro prompted us to undertake experiments to make a direct comparison of periodic versus continuous irradiation in vivo, using tumor growth behavior as an indicator of antitumor efficacy of PDT. As shown here, the antitumor effects of a periodic PDT protocol, consisting of 100 mW/cm²/0.25 h, a 1-h dark interval, and a second and equal photoradiation period, were significantly greater in prolonging the initial doubling time of R3230AC tumors (T2), compared to those obtained with continuous irradiation of equal total fluence.

The basis for this improved efficacy using periodic irradiation schedules may be due to any one or a combination of the following reasons. From our previous studies to define phototherapy mechanisms, e.g., experiments in vitro and in vivo-in vitro (40–42) and by use of ³¹P nuclear magnetic resonance
spectroscopy in situ (18–20), we observed a dramatic decrease in tumor ATP levels, along with a concomitant increase in P<sub>i</sub>, beginning by 30 min and continuing up to 6 h after continuous wave laser irradiation (20). From these findings, we speculated that delivery of the second dose of photoradiation, at a time when ATP levels were at or near their nadir, might enhance the efficacy of PDT by producing additional \(^{1}O_2\), which could interact with additional tumor cells that were sublethally damaged. The results reported here could support such a contention, although at this time we have not obtained sufficient data to prove the existence of a direct correlation between extent or duration of metabolite depletion and tumor growth control. PDT was reported to cause hypoxia in irradiated tumor tissue, due to damage to the vasculature (12, 43, 44), resulting in a reduction of tissue oxygen and, hence, diminished production of \(^{1}O_2\). Periodic delivery of light may cause only partial vascular damage and could provide sufficient time for repair and/or reoxygenation of tissues in the time between irradiation doses, enabling molecular oxygen concentrations to return to levels sufficient to permit a Type II photochemical reaction to occur and produce additional \(^{1}O_2\). A third possibility can be proposed from findings applicable to ionizing radiation therapy. It was suggested that, when layers of cells near the surface are irreversibly damaged, such cells no longer utilize oxygen, permitting oxygen to become available via diffusion to cells located at deeper sites. If this occurs, then the second dose of light in the periodic delivery schedule may arrive at a time when reoxygenation of deeper cells has occurred, providing an environment conducive to \(^{1}O_2\) formation and further cytotoxicity. If complete vascular occlusion had occurred prior to the second period of photoradiation and severe hypoxia resulted, no additional tumorstatic effects would be expected.

The enhanced retardation of tumor growth observed after periodic photoradiation could be related to tumor enzyme activities from studies in vivo. This experimental protocol allows host metabolism and subsequent tissue and subcellular localization of the biologically active porphyrin components of Photofrin II to occur under conditions analogous to clinical PDT. Photoradiation of the lesion in situ at 24 h after Photofrin II administration, followed by excision of tumors at selected times and enzyme analysis, enables one to monitor the time course of selected biochemical parameters. Mitochondrial cytochrome c oxidase and cytosolic pyruvate kinase were previously found to be susceptible to inhibition by PDT, using an \(in \ vino-in \ vitr \) protocol (45) in which subcellular components prepared from tumors after injection of Photofrin II were exposed to light \(in \ vitr \). Each enzyme presented a different time course of inhibition following administration of Photofrin II. The data from the present study, using photoradiation regimens \(in \ vitr \), were similar to results obtained earlier, which employed continuous irradiation (45). The periodic light regimen consisting of 100 mW/cm\(^2\)/15 min (90 J/cm\(^2\)), a 1-h dark interval, and a second identical photoradiation period caused an inhibition of cytochrome c oxidase activity (50–60%) that remained for 120 h after PDT, whereas with continuous light exposure enzyme activity had returned to 75% of control levels by 72 h. This difference in response was even more dramatic for pyruvate kinase activity, in which periodic light treatment not only prolonged the duration of enzyme inhibition but also caused a greater inhibition, 75% versus 55% for periodic versus continuous treatment, respectively, at 24 h after PDT. These data indicate that, regardless of the mechanism responsible for the prolonged delay of tumor growth following PDT with periodic irradiation, the greater inhibition of enzymes involved in glycolysis and ATP synthesis appears to correlate with subsequent tumor growth behavior. Previous studies in our laboratory have demonstrated that inhibition of enzyme activity \(in \ vito\) apparently precedes loss of cell viability (40) but depletion of ATP can mimic the cytotoxicity profile (41). These data suggest that the inhibition of enzyme activity observed could precede loss of tumor cell viability in situ.

We investigated the effect of various power densities and fluence levels on growth of R3230AC tumors. We observed a significant delay in tumor growth for those lesions that were exposed to lower power densities over an extended period of time, e.g., 50 mW/cm\(^2\)/2 h versus 200 mW/cm\(^2\)/30 min (360 J/cm\(^2\)) (\(P = 0.002\) for T.<sub>2</sub>). Similar observations have been made for a human mesothelioma cell line grown in nude mice and treated by PDT (46). Several possible explanations can be offered for these results. Reports by Potter and others (47, 48) indicate that the active components of Photofrin II responsible for tumor cytotoxicity are photobleached when exposed to high power densities of light. At lower power densities, less bleaching due to complex photochemistry would be expected to occur than at higher power densities, thereby increasing the efficacy of PDT by continuous formation of \(^{1}O_2\). A second possibility, as noted above, is that vascular damage is less severe after exposure to lower power densities, enabling a continued reoxygenation of the tumor during photoradiation. On the other hand, impaired delivery of oxygen to the tumor may not be the only cause of hypoxia during PDT. Other considerations include increased utilization of O\(_2\) for ATP production to repair PDT-induced cell damage and/or reduction in dioxygen by its participation in Type II reactions for formation of \(^{1}O_2\), which we have determined may be the major factor in induction of hypoxia \(in \ vitr \) (49). Irradiation at lower power densities diminishes the rate of depletion of cellular O\(_2\), thus providing adequate levels for continued \(^{1}O_2\) production leading to greater cytotoxicity. Certainly, the lower power densities are less likely to elevate tumor temperatures, precluding adventitious hyperthermia.

In conclusion, the data presented here demonstrate that modification of photoradiation schedules can significantly enhance the effectiveness of PDT on \(in \ vitr \) tumor growth. Investigation into other light treatment schedules and various combinations of periodic light exposure at lower power densities is currently under way. Effects on tumor growth will be compared to effects on selected biochemical parameters as one approach to seeking out the basis for differences in tumor growth control.

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