**In Vitro Photodynamic Therapy of Human Lung Cancer: Investigation of Dose-Rate Effects**

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**ABSTRACT**

The influence of light dose-rate delivery was studied in human lung adenocarcinoma A549 cells treated with hematoporphyrin derivative (Photofrin II)-based photodynamic therapy. Clonogenic cell survival curves were generated for cells treated for 2 h with 25 μg/ml of Photofrin II followed by exposure to light delivered at 0.3, 0.15, 0.075, or 0.0375 milliwatts/cm². Cellular sensitizer levels, as determined by fluorescence measurements, remained constant over the entire time course of all light exposures. As the dose rate of light delivery was decreased, a significant increase in cell survival was observed at equal light energies (225 mJ/cm²). The enhancement in survival from the highest to the lowest dose rate used was 1.6-fold (at the 50% survival level). These findings may have important clinical implications relating to photodynamic therapy of tumors and may provide a partial explanation for treatment failure.

**INTRODUCTION**

The effect of PDT² or ionizing radiation delivered at high dose rates is similar in that both modalities produce cell survival curves characterized by an initial “shoulder” for low radiation doses followed by an exponential decrease in survival as the dose increases. For high dose rate X-rays, the shoulder portion of the survival curve is thought to result from the cellular capacity to accumulate and repair sublethal damage. Indeed, if a total dose of X-rays is delivered in two fractions with a time interval between fractions, significant enhancement in survival is observed compared to administering the dose in a single fraction (1). This repair process is also operative if X-rays are delivered at low dose rates over protracted periods of time, since cells can repair radiation damage during the exposure (2). This phenomenon, referred to as the “dose-rate effect,” results in increased cell survival as the dose rate is decreased.

There has been no general acceptance of a dose-rate effect for PDT. Ben-Hur et al. reported the presence of a visible light dose-rate effect when chloroaluminum phthalocyanine tetrasulfonate was used as the photosensitizer (3), and Bellnier and Lin reported that mammalian cells are able to repair HPD-sensitized sublethal photodamage (4). However, Gomer et al. using a wide range of dose rates were unable to demonstrate a dose rate effect for HPD-based PDT (5). In addition to the apparent conflicting aspects of dose rate effect for PDT, it is surprising that, although the primary use of PDT is the treatment of tumors, no investigation of light dose-rate effects with any photosensitizer has been conducted on human malignant cells.

As part of our general interest in the use of PDT for management of lung cancer, we have previously documented PDT sensitivity of a human lung adenocarcinoma line, A549 (6). Since documentation of a possible dose-rate effect in PDT has important clinical implications and since hematoporphyrin derivative (Photofrin II) is the only photosensitizer approved for human use, we investigated the in vitro survival of A549 cells subjected to porphyrin-PDT at various light dose rates encompassing dose rates lower than those previously used by Gomer (5). Because of the long time periods required to deliver the light, cellular fluorescence determinations were made to ascertain if the presence or absence of a dose-rate effect could be explained by loss of sensitizer from cells during treatment. In the present study a porphyrin-based PDT dose-rate effect was observed which was not dependent upon loss of sensitizer during the exposure.

**MATERIALS AND METHODS**

Drugs. Photofrin II was obtained from Photofrin Medical, Inc. (Cheektowaga, NY) as a sterile solution, 2.5 mg/ml, dissolved in 0.9% NaCl solution.

Light Source. Light exposure was accomplished by placing Petri dishes on top of an X-ray view box (General Electric, Milwaukee, WI; daylight bulbs) filtered with a thin sheet of ruby red acetate and appropriate neutral density filters. The emission spectrum of the light box ranged from 595 to 700 nm (10% peak intensity) with a peak at 630 nm. This was measured using a set of calibrated interference filters (Oriel Corp., Stratford, CT; typically 60% transmission, 10-nm bandwidth) and a radiometer (Model 550; EG and G, Salem, MA). The dishes were elevated above the light source on a transparent plexiglas platform to continually dissipate heat effects by fan-convected air currents. Temperature measurements of light-treated media were taken in duplicate plates exposed to the highest energies using a thermister probe (Model BAT-12; Baily Instruments, Inc., Saddlebrook, NJ). Temperature measurements prior to light exposure were 22.1°C and after a 100-min light exposure were 24.1°C. Therefore, this particular light exposure arrangement did not impose hyperthermic conditions.

The neutral density filters were interfaceted between the dishes containing the cells being illuminated on the elevated transparent plexiglas platform. The light intensities available to the cell monolayer were measured by a photometer (Model 1140; International Light, Newbury, MA). The light intensities using neutral density filters of 0, ½, ¼, and ⅛ resulted in respective dose rates of 0.3, 0.15, 0.075, and 0.0375 milliwatts/cm². Light delivery studies at various dose rates were performed such that the total energy imparted was equal to 45, 135, 180, and 225 mJ/cm².

Cell Culture Procedure. Human lung carcinoma (A549) cells (7) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine. Cells were maintained in stock monolayer cultures at 37°C and in an environment of 5% carbon dioxide/95% air and were passaged once per week. Under these growth conditions, the population doubling time was 26 h, and the average cell volume was 1.5 × 10⁻⁴ ml.

For light dose experiments, 500,000 A549 cells were inoculated in 100-mm Petri dishes with 15 ml of growth medium. After 16 to 24 h, growth medium was removed and replaced with 10 ml of standard sensitizer medium containing 25 μg/ml of Photofrin II with 1% fetal calf serum adjusted at pH 7.3. Cultures were then handled in a dimly lighted room. Cells were then incubated at 37°C for 2 h. At the conclusion of the incubation period, medium was removed, the cells were washed twice with calcium- and magnesium-containing PBS, and then a final 10 ml of PBS were added to the cells for subsequent light exposure.
Following light exposure, cells were trypsinized from the dishes, counted, diluted, and plated. Each investigation point was plated in triplicate. Controls from each experiment were cells exposed to light without sensitizer and cells exposed to sensitizer but no light exposure (8). After incubation, the colonies were fixed in methanol:acetic acid and counterstained with crystal violet. A macroscopic colony was defined as being ≥50 cells. Plating efficiency was defined as the ratio of the number of cells plated compared to the number of colonies counted. Surviving fraction was defined as the plating efficiency of treated cells divided by the plating efficiency of the control cells.

Cellular Fluorescence. After light treatment cells were trypsinized and resuspended in 1 ml of PBS to obtain a single cell suspension. Cellular DHE was measured by excitation with the 515-nm line of an argon ion laser, and the fluorescence was measured on a cell-by-cell basis using an EPICS V cell sorter (Coulter Corp., Hialeah, FL). The fluorescence signal was filtered with a 515-nm interference filter and a 530 nm long pass absorption filter. The filtered light signal was converted into an electrical signal with a Mamamatsu R1923 photomultiplier tube. The resultant analogue signal was digitized and stored in the computer memory for subsequent display and analysis. Post-PDT fluorescence of cells exposed to the lowest (45 mJ/cm²) and highest (225 mJ/cm²) energies at the four different dose rates was measured after light exposure (2.5 to 100 min). Cellular fluorescence after prolonged PBS incubation of Photofrin II-loaded cells, but not exposed to light, was also performed for cells incubated 2.5 to 100 min in the PBS.

Statistical Analysis. Statistical analysis for comparisons of cellular fluorescences and surviving fraction was performed via Student’s t test for paired data with significance (two-tailed) at the P<0.05 level.

RESULTS

Control Plating Efficiencies. Table 1 illustrates the plating efficiencies of the controls used for the various survival curves. Plating efficiency varied from 46 to 51%, and there were no differences in the plating efficiencies of the control plates. Furthermore, plating efficiency was not significantly different for the plates that were not exposed to Photofrin II but were exposed to light for maximum times dictated by energy desired for the individual dose-rates.

Dose-Rate Survival Differences. Fig. 1 represents the results of the colony formation assays for three experiments performed in triplicate comparing different dose rates at equal energy for A549 lung carcinoma cells. For each dose rate, as one increased energy delivery, there was increased cytotoxicity. At equal energy input at varying dose rates, there was no significant difference in surviving fraction at the lower energies. However, at the two highest energies (180 and 225 mJ/cm²), there were significant differences in surviving fraction comparing cells that were subjected to the highest dose rate to those subjected to the lower dose rates. For 225 mJ/cm², 52% of the cells treated at 0.0375 and 0.075 milliwatts/cm² survived as compared to 24% at 0.15 and 8% at 0.3 milliwatts/cm². At 50% survival, the dose-modifying factor is 1.6.

Fluorescence after PDT Treatment: Equal Energies-Varying Dose Rates. When cells were processed after PDT at 45 and 225 mJ/cm², there were no statistical differences seen in the fluorescences from the cells between the dose rates seen at the lowest or the highest energies (Fig. 2). The fluorescence of the cells that were treated with the lowest dose rate was not different at the end of treatment compared to the cells that were treated at higher dose rates at either energy input. A nonstatistically significant decrease in the fluorescence of cells treated at the higher energy compared to the lower energy at equal dose rates seemed to occur, and this decrease was most marked at the highest energy level and highest dose rate.

The fluorescence of the cells which were incubated in PBS for 2.5 to 100 min without light treatment did not change during this time period (Fig. 3). Moreover, there were no significant differences between the fluorescence of the cells which were exposed to light treatment compared to those which were not exposed to light treatment.
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Fig. 3. Relative fluorescence of A549 cells as a function of incubation time in PBS after Photofrin II exposure. Cells were incubated for 2 h in RPMI medium containing sensitizer (25 μg/ml) and 1% fetal calf serum. Cells were rinsed twice with PBS and then incubated in PBS for 2.5 to 10 min. For relative fluorescence measurements, 10,000 cells were collected for each fluorescence histogram and compared to the mean endogenous fluorescence from cells untreated with sensitizer.

DISCUSSION

Our results establish the presence of a dose-rate effect for porphyrin-based photodynamic therapy of a human lung carcinoma line. Although the effect with Photofrin II has not been documented before with either normal or malignant cells in vitro, it is not altogether surprising that a dose-rate effect should be present. Ben Hur, using chloroaluminum phthalocyanine tetrasulfonate, clearly showed differences in PDT cytotoxicity via colony formation of V79 cells when the cells were treated at dose rates of 0.165 to 3.3 kJ/m2/min. The role of sensitizer concentration as a function of time and treatment energy, however, was not addressed in that report (3). The only previous investigation of in vitro dose-rate effects for HPD-based PDT was reported by Gomer (5) who was unable to demonstrate such an effect. Moreover, examination of his survival curves for V79 cells at varying dose rates revealed greater apparent cytotoxicity at the low dose rate (0.5 milliwatts/cm²) compared to the highest dose rate (60 milliwatts/cm²), although not statistically greater. Since laser light via an optical fiber was used for illumination in Gomer's experiment, one must consider the possibility of “back-scatter” of light to the cells over a longer period of time with the lowest treatment dose rate. Such a possibility would thus underestimate the total PDT energy delivered. We have observed that, when plates containing cells as a bottom-dish monolayer are illuminated from above by an optical fiber, if the plates are not adequately filtered, photons may be redelivered to the cells after striking the surface supporting the plates. Moreover, if neutral density filters, just large enough to cover the tops of the plates, are used in such a system to alter dose rate without changing laser output, light of high dose rate will surround the plate and artifactually increase energy delivery. By using the “illumination from the bottom” system, we have avoided such artifactual differences in dose rate. Another explanation for Gomer’s observations, although unlikely, is that hyperthermia may have occurred with prolonged exposure during the low dose rate treatment. No in vitro temperature effects were reported by Gomer.

Our data extend Gomer’s work, and we agree with his explanation that “the lowest dose rates (he) examined (0.4 milliwatts/cm²) were possibly too high for the documentation of repair during treatment.” By using lower dose rates in our studies, we were able to show a dose-rate effect. The design of our investigation, however, was of significant importance minimizing potential artifactual temperature rises during the period of light illumination. The rise in temperature was minimized (22.1–24.1°C) by fan-convected air currents, and temperature of the medium never approached the described 40–45°C necessary to produce the described PDT-hyperthermia synergism (9).

At present, we cannot comment whether A549 cells and the Chinese hamster ovary cells used by Gomer have similar repair responses to low dose-rate PDT. A549 and Chinese hamster ovary cells, however, do exhibit similar dose-rate effects to X-rays.

Since PDT efficacy with our A549 line is influenced in vitro by cellular sensitizer concentration (6), it was necessary to document that there was no difference in sensitizer concentration after PDT which could explain survival differences. The use of cellular sensitizer concentration as determined by fluorescence techniques, however, may only be a first order or partial approximation of a cell-line’s PDT sensitivity due to the inability to adequately identify the “active” component of the Photofrin II porphyrin mixture. These inadequacies have been demonstrated recently by Gomer (10) in normal cell lines. Nevertheless, we have previously shown that A549 PDT efficacy does correlate with cellular sensitizer levels (6) and thus investigated cellular fluorescence to try to explain the dose-rate effects. Sensitizer loss as a function of illumination of porphyrin-loaded cells in PBS without sensitizer during treatment could also negatively impact on PDT efficacy and artifactually produce a “dose-rate effect.” This would theoretically be most pronounced in the low dose-rate-treated group, for to deliver the desired energy, treatment time had to be extended to 100 min. We found no loss of sensitizer from A549 cells incubated in PBS from 2.5 to 100 min, thus eliminating the possibility of decreasing PDT efficacy by sensitizer efflux. Fluorescence-activated cell sorter analysis of cellular fluorescence after PDT at varying dose rates at the lowest and highest energy treatments did not reveal any significant differences in cellular sensitizer concentration. Samples were analyzed on a single cell basis, and due to the small path length (10 to 20 μm) of a single cell, an inner filter effect was not significant at the concentration used. This remains an advantage when using the cell sorter for these measurements. On the other hand, quantitation of Photofrin II is more difficult with cell sorters, and hence only relative measurements are reported. Single cell measurement also allows one to identify any subpopulations with low sensitizer fluorescence. All measurements in our study revealed a uniform labeling profile with no low fluorescence populations being noted. The apparent but insignificant decrease in sensitizer concentration at the highest energy and dose rate combination may be a reflection of photobleaching as described by Moan (11) and Mang (12). If photobleaching were of significance, however, one would expect a decreased PDT effect for this treatment group and, in fact, the greatest PDT effect was seen in the higher dose rate, high energy group.

The clinical implications of our findings should not be min-

3 J. B. Mitchell, unpublished observations.
imized. Our use of a short Photofrin II incubation time (2 h) may not totally reflect the clinical situation and may only represent membrane PDT effects. Future investigations with longer incubation times investigating dose-rate effects should be performed. Nevertheless, dosimetry guidelines for clinical PDT remain poorly defined because of the absence of on-line light monitoring of the treatment portal. Dose is arbitrarily defined as the amount of light at the end of a quartz fiber from a laser. The treatment portal, however, might be viewed as a series of neutral density filters, which by absorptive and scattering characteristics will alter the assumed dose rate. The light dose delivery to tissue will fall off exponentially as a function of distance from the point of light entry and degree of light absorption and scatter. If dose-rate effects are important, it is probably the edges as well as the depths of the treatment fields that will be most vulnerable to failure. Further, these dose-rate effects will be a function of the varying photosensitizer concentration, light absorption, and oxygen supply, and at the lower limits of concentrations of photons, sensitizer, and oxygen, dose rate effects would be most dramatic, possibly explaining treatment failures after presumed adequate PDT dosing.

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