Sensitivity of Different Human Lung Cancer Histologies to Photodynamic Therapy

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ABSTRACT

The relative sensitivities of different cancer histologies in a single site to photodynamic therapy (PDT) are unknown and methods to predict PDT sensitivity have not been described. The in vitro response to PDT of six established human lung cancer cell lines and one normal lung fibroblast cell line was studied using the clonogenic assay. Dose-response curves were determined for cells incubated in 25 μg/ml of Photofrin II for 2 h, followed by exposure to 630-nm light to total energies of 0–3150 J/m². None of the cell lines were sensitive to sensitizer alone or light alone. Differences in inherent PDT sensitivities as evaluated by survival curve parameters n, Dₐ, and light dose to yield 1% survival were observed among the cell lines. No clear correlation was found when inherent PDT sensitivity was compared with sensitizer uptake; however, a general association was noted between PDT sensitivity and the plating efficiency of the cell line. These data illustrate that differences in inherent PDT exist for in vitro systems. Such differences may explain some of the failures seen in clinical PDT.

INTRODUCTION

Thoracic malignancies generally have a poor prognosis. The overall 5-year survival rate for patients with carcinoma of the lung is approximately 10%, with or without multimodality therapy (1). The standard therapies for mesothelioma including pleureperoneumectomy, pleurectomy with radiation therapy, and radiation therapy combined with chemotherapy are generally ineffective (2). An innovative therapy which shows promise for the treatment of certain malignancies is PDT. PDT has been used to treat both early and advanced endobronchial disease (3), for the palliation of advanced carcinoma of the esophagus (4), and for the treatment of malignancies at other sites, such as the bladder (5), skin (6), and brain (7). The major side effect of this treatment is skin photosensitivity.

In spite of increasing clinical use, little is known about the relative PDT sensitivities of different malignancies or about the relative PDT sensitivities of different malignant histologies in the same site. Different transitional cell carcinoma of the bladder cell lines take up different amounts of sensitizer, and the PDT sensitivity of one of the cell lines correlates with cell sensitizer levels (8). Conditions which effect sensitizer uptake also impact on PDT sensitivity (9). Different concentrations of sensitizer are required to achieve 90% cytotoxicity of explanted brain cancer cells from different tumors (10). These studies suggest that the degree of PDT sensitivity may be related to cell sensitizer levels. However, the precise cell parameter which correlates with PDT sensitivity remains poorly defined.

In order to clarify the issue of the relative PDT sensitivities of different cancer histologies, the major human lung cancer histologies (adenocarcinoma, small cell carcinoma, large cell carcinoma, squamous cell carcinoma, and mesothelioma) were studied in vitro under identical conditions and compared to the PDT sensitivity of normal human lung fibroblasts. Different cell parameters, such as cellular sensitizer uptake, total cell protein, and cell size were examined to determine whether any of these parameters correlated with the degree of PDT sensitivity.

MATERIALS AND METHODS

Drugs. Photofrin II was obtained from Photofrin Medical Inc. (Quadra Logic, Vancouver, British Columbia, Canada) as a sterile solution of 2.5 mg/ml dissolved in 0.9% NaCl solution.

Light Source. Exposure to light was accomplished by placing the Petri dishes on top of an X-ray viewbox with daylight bulbs (General Electric, Milwaukee, WI) filtered with a thin sheet of ruby red acetate (Visual Systems Inc., Rockville, MD). The emission spectrum of the light box ranged from 595–700 nm (10% peak intensity) with the peak at 630 nm. This was measured using a set of calibrated interference filters (Oriel Corporation, Stratford, CT; typically 50% transmission, 10 nm band width) and a radiometer (Model 550; EG&G, Salem, MA). The fluence rate as measured by a photometer (Model 1140; International Light, Newbury, MA) was 3.5 W/m². Cells were exposed to light for periods of time ranging from 1–15 min, resulting in delivered energies of 210–3150 J/m².

Cell Lines. The characteristics of the six human lung cancer cell lines and the normal human lung fibroblast cell line are shown in Table 1. NCI-H23, NCI-H841, NCI-H460, NCI-H520, and JMN were supplied by Dr. John Minna. A549 and CCL-210 were obtained from American Type Culture Collection (Rockville, MD). The malignant cell lines were grown in RPMI 1640 medium (Biofluids, Inc., Rockville, MD) supplemented with penicillin, streptomycin, glutamine, and 10% fetal calf serum. Cells were maintained in stock monolayer cultures at 37°C in a 5% carbon dioxide/95% air environment. The lines were passaged once per week except for NCI-H520 (squamous cell) which was passaged every 2 weeks. The media were changed between passes every 3–4 days. The normal human lung fibroblast line (CCL-210, American Type Culture Collection) was grown in Ham’s F-12 medium (Biofluids, Inc.) supplemented with penicillin, streptomycin, glutamine, and 15% non-heat-inactivated fetal calf serum.

For the clonogenic cell survival assay and sensitizer uptake experiments, 6 × 10⁵ to 1 × 10⁶ cells were inoculated into 100-mm Petri dishes with 15 ml of growth media for all cell lines except NCI-H520, for which 1.5–2 × 10⁵ cells were plated. After 48 h the media were removed and replaced with 10 ml of medium containing 25 μg/ml of Photofrin II and 1% fetal calf serum adjusted to pH 7.3. The cultures were handled in a dimly lighted room. The cells were incubated at 37°C for 2 h and then used for clonogenic cell survival assay and sensitizer uptake measurements. Controls for each experiment were cells exposed to light without sensitizer and cells exposed to sensitizer without light exposure.

Clonogenic Cell Survival Assay. Following a 2-h incubation in sensitizer, the media were removed, the plates were washed twice with PBS containing calcium and magnesium, and 10 ml of PBS was added to the plates. After light treatment, the PBS was removed and the cells were washed with PBS containing calcium and magnesium. The cells were trypsinized from the dishes, counted, diluted, and plated. Cells were plated in triplicate for each experimental point and each experiment was repeated a minimum of 3 times. The dilutions plated included 250 cells/dish, 5000 cells/dish, and 100,000 cells/dish. The dishes were incubated 7–21 days for macroscopic colony formation with a colony being defined as having 50 or more cells (11). The colonies were fixed

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2 The abbreviations used are: PDT, photodynamic therapy; Dₐ, dose required to decrease surviving fraction to 37% along the exponential portion of the survival curve; Spearman correlation coefficient; n, extrapolation number; PBS, phosphate-buffered saline.

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of the dose for 1% survival confirmed a significant difference among the cell lines ($P_2 = 0.003$). The relative resistance of A549 is not a general property of adenocarcinoma as NCI-H23, also an adenocarcinoma, was more sensitive to PDT than A549. NCI-H23 had a sensitivity comparable to several of the other cell lines (NCI-H841, NCI-H460, NCI-H520).

Sensitizer Uptake. The amount of sensitizer taken up by the cells after a 2-h sensitizer incubation is shown in Table 2. Sensitizer uptake was expressed in 3 ways: amount of sensitizer/10^6 cells, amount of sensitizer/mg cell protein, and amount of sensitizer/μm^3 cell volume. Kruskal-Wallis test of the sensitizer uptakes confirmed that there was a significant difference among the cell lines. CCL-210, which was the most sensitive cell line, took up the most sensitizer. However, there was no significant difference in sensitizer uptake between A549 and NCI-H460 ($P = 0.16$), yet H460 was much more sensitive to PDT. Therefore, PDT sensitivity did not correlate well with sensitizer uptake.

To statistically verify the lack of correlation between PDT sensitivity and sensitizer uptake, the $r$ was calculated comparing the dose at 1% survival for each cell line versus sensitizer uptake per million cells, per mg protein, or per μm^3 cell volume. The values of $r$ ranged from 0.43–0.57, none of which were statistically significant. Hence, no good correlation was seen between these parameters and sensitivity to PDT. Also, no correlation was seen between sensitizer uptake at 2 h and cell protein content ($r = 0.32$) or between sensitizer uptake and cell volume ($r = 0$).

Finally, the colony-forming ability of each cell line was evaluated against three measures of PDT sensitivity, the light dose at 1% surviving fraction, the extrapolation number $n$, and the survival curve $D_0$ (the inverse of the terminal slope of the PDT survival curve). The results of these comparisons are shown in Fig. 2. There was a much better correlation between the plating efficiency and light dose at 1% survival ($R = 0.81$) when compared to the extrapolation $n$ ($R = 0.46$) or the $D_0$ ($R = 0.37$). Despite the disparity among these comparisons, the general tendency was that the cell lines with the lowest plating efficiency were the most sensitive to PDT treatment.

**DISCUSSION**

Our results demonstrate differences among human lung cancer lines and their sensitivity to PDT under controlled conditions. Although the finding that such differences exist is not surprising, little work has been done to examine the relative PDT sensitivities of human cell lines. Cohen *et al.* (10) examined the PDT sensitivity of 12 explanted human central nervous system tumors by measuring the sensitizer concentration required for 90% cytotoxicity at a constant light dose. After a 1-h incubation with hematoporphyrin derivative, a 5-fold variation among the cell lines in the amount of sensitizer required for 90% cytotoxicity was observed. Melloni *et al.* (15), using rhodamine 123 as a sensitizer in a 24-h incubation, showed significant differences in sensitivity among human colon, breast and melanoma cell lines to the sensitizer alone and in combination with light. None of these studies, however, have attempted to correlate inherent characteristics of the cells with PDT sensitivity.

Bohmer and Morstyn (9) have shown that different amounts of sensitizer were taken up by different cell lines under identical conditions. These differences were dependent upon the cell size, with larger cells taking up more sensitizer than small cells. Williams and Runge (8) have shown that different cell lines will
take up different amounts of sensitizer. Our study showed differences between the PDT sensitivities of different human lung cancer lines. We then attempted to correlate these differences in sensitivity with measurable cell parameters such as sensitizer uptake, expressed per cell, per mg protein, and per cell volume. Although significant differences were noted among the cell lines and sensitizer uptake per million cells, per mg protein, and per cell volume, these differences did not correlate with differences in PDT sensitivity. It should be noted that such comparisons should be made with considerable caution, in that sensitizer uptake measurements were made using the entire population of cells, while the PDT sensitivity assessments represent only the proportion of cells that actually form colonies. The greatest concern lies with the cell lines that have low plating efficiencies. For these cell lines it is conceivable that the sensitizer uptake in the proportion of cells capable of forming colonies (those cells used to determine PDT sensitivity) differs from "average" sensitizer uptake of the entire population. Ideally, the appropriate way to correlate sensitizer uptake with PDT sensitivity would be to use only cell lines with 100% plating efficiencies, a rare characteristic of most human tumor cell lines in general. Another way to potentially resolve this issue would be to evaluate sensitizer uptake and PDT sensitivity in the same cell line under different conditions that yield a low and high plating efficiency. To our knowledge, at present there are few, if any, procedures to accomplish this objective.

The low plating efficiencies of some cell lines evaluated prompted yet another comparison. When the plating efficiency of the various cell lines was compared against PDT sensitivity, a general trend was observed. Cell lines with low plating efficiencies were found to be the most sensitive to PDT; however, this relationship was best correlated with the drug dose to yield 1% survival as opposed to the survival curve A, or n values. The reason for this discrepancy is unclear. Whether this observation is a uniform finding among lung cancer cell lines or other human tumor cell lines will require further study. There is

### Table 2: Cell survival and fluorescence versus cell characteristics

<table>
<thead>
<tr>
<th>Cell line</th>
<th>n*</th>
<th>D_{50} (J/m^2)</th>
<th>Dose for 1% survival</th>
<th>μg Sensitizer/10^6 cells*</th>
<th>ng Sensitizer/mg protein*</th>
<th>ng Sensitizer/μm^3 volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>51.13 ± 37.00</td>
<td>341 ± 31</td>
<td>2602 ± 147</td>
<td>0.98 ± 0.13</td>
<td>3.83 ± 0.52</td>
<td>0.72 ± 0.10</td>
</tr>
<tr>
<td>NCI-H23</td>
<td>11.96 ± 8.30</td>
<td>194 ± 19</td>
<td>1192 ± 109</td>
<td>1.68 ± 0.20</td>
<td>5.75 ± 0.68</td>
<td>1.03 ± 0.12</td>
</tr>
<tr>
<td>NCI-H841</td>
<td>2.64 ± 1.10</td>
<td>259 ± 16</td>
<td>1036 ± 41</td>
<td>1.80 ± 0.20</td>
<td>6.91 ± 0.77</td>
<td>1.26 ± 0.14</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>4.45 ± 2.20</td>
<td>224 ± 15</td>
<td>1078 ± 78</td>
<td>0.69 ± 0.06</td>
<td>2.76 ± 0.24</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>JMN</td>
<td>1.24 ± 0.17</td>
<td>378 ± 11</td>
<td>1503 ± 17</td>
<td>1.45 ± 0.13</td>
<td>4.41 ± 0.40</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>NCI-H520</td>
<td>5.65 ± 2.30</td>
<td>353 ± 20</td>
<td>1121 ± 194</td>
<td>1.80 ± 0.24</td>
<td>8.67 ± 0.11</td>
<td>1.66 ± 0.22</td>
</tr>
<tr>
<td>CCL-210</td>
<td>2.38 ± 1.10</td>
<td>175 ± 11</td>
<td>473 ± 26</td>
<td>3.16 ± 0.17</td>
<td>9.32 ± 0.51</td>
<td>1.66 ± 0.09</td>
</tr>
</tbody>
</table>

* Extrapolation number (extrapolation of terminal slope of the survival curve to the y axis).
* Cells were incubated in 25 μg/ml of Photofrin-II for 2 h and then treated with 630 nm light to total energies of 0–3150 J/m^2. Viability was assessed at 7–21 days using the Albright program (14). D_{50} dose to reduce survival to 37% along the terminal exponential portion of the survival curve.
* P = 0.003 for at least one difference among the means (Kruskal-Wallis test).
* Cells were incubated in 25 μg/ml of Photofrin-II for 2 h. The sensitizer was extracted using acetic acid/saturated sodium acetate and read using a spectrophotometer (P = 0.004, Kruskal-Wallis test).
* Protein measured using Bradford method (13) (P = 0.004, Kruskal-Wallis test).
* Mean ± SEM.
PHOTODYNAMIC THERAPY OF LUNG CANCER

always a concern when conducting cell survival studies, regardless of the modality, when plating efficiencies are low. Whether the sensitivity to a given modality of the few percent of cells that actually are able to form colonies is representative of the entire population of cells is a question most difficult to answer. As mentioned above, the best way to gain insight into this potential problem would be to compare the PDT sensitivity of the same cell line under conditions that yield both high and low plating efficiencies. Resolution of this issue will require more work and perhaps refinement and improvement of cell culture techniques for human tumor cell lines. Alternatives to this experimental design to investigate the role of sensitizer concentration and inherent PDT differences could be performed. Instead of using a constant incubation time for each cell line, one could incubate each cell line for an appropriate interval with sensitizer concentrations to ensure that the final sensitizer concentration is equal in all cell lines. By exposing the cells to equal light energy, any resulting differences in survival could be attributed to "inherent differences." This approach, however, would not address the important issue of multiple cellular PDT targets (i.e., nuclear membrane, plasma membrane, mitochondria, and lysosomes), and although total cellular fluorescence may be the same, differences in cellular organelle sensitizer uptake may account for survival differences. We have demonstrated this phenomenon of survival differences despite equal total cellular fluorescence in A549 cells when plateau levels of sensitizer uptake is reached (17). Thus, it would be necessary to define the organelle-sensitizer kinetics in such a study, possibly by using video fluorescence-imaging techniques with image analysis.

For the NCI-H460 cell line, it could be surmised that there may be a modest correlation between PDT sensitivity and sensitizer uptake. When examining the characteristics of this line, we noted that this cell line has a short doubling time compared with the other cell lines and it was a possibility that different distribution of cells in the cell cycle might influence the PDT sensitivity. Whether cell cycle variations influence the PDT response, however, remains an unanswered question. Moan et al. (18) showed that, for human NIH3K 3025 cells, S phase cells were more sensitive (100-fold) than cells in G1. However, Gomer and Smith (19) did not observe significant variation in PDT sensitivity with the cell cycle for Chinese hamster ovary cells. Hence, further work would be necessary to elucidate growth fraction differences with regard to these cell lines and the influence on PDT efficacy.

It is known that tumors in situ retain more hematoporphyrin derivative than normal tissue and for the most part all lung cancer types have been responsive to PDT (20); however, the degree of response (partial or complete) and duration of response are not well documented at present. These clinical findings are somewhat in conflict with the present in vitro findings. The normal lung fibroblast used as a control in this study exhibited the greatest dihematoporphyrin ether uptake and relative PDT sensitivity of all the cell lines studied. This cell line also had the lowest plating efficiency of the cell lines studied. While it may be desirable in comparative in vitro studies to include a "normal tissue" cell line, perhaps a lung fibroblast cell line is not representative of normal tissues evaluated thus far in clinical PDT. Unfortunately, there are no data evaluating normal thoracic organs for dihematoporphyrin ether retention and PDT sensitivity in vivo. Despite the qualitative clinical observation that all lung cancer cell types respond similarly to PDT, one cannot surmise that differential inherent tumor PDT sensitivities fail to exist in vivo. The fact that PDT treatment...
opens "tumor-obstructed" airways does not address the ultimate histologic tumor response and the duration of PDT response. As clinical trials continue and protocols become more uniform, differences in PDT response among lung cancer histologies as measured by conventional oncological parameters may be revealed.

The findings of the present study show inherent differences in PDT sensitivities among different lung tumor types; however, these differences do not take into account that in vitro PDT sensitivity among different cell lines may relate to different targets (mitochondria or membranes) or differences in sensitizer receptor number or binding affinity, specifically for low density lipoprotein receptors (21). Moreover, the importance of in vitro PDT sensitivity studies, with their inability to have consistent predictive indicators of PDT efficacy, are difficult to extrapolate to in vivo data since differences in tumor vasculature (22) or the tumor extracellular milieu (23) can markedly influence PDT tumor response. Resolution of these issues will hopefully come as more is understood about the basic mechanisms of PDT action for both in vitro and in vivo systems.

REFERENCES
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