**In Vitro** Radiation and Chemotherapy Sensitivity of Established Cell Lines of Human Small Cell Lung Cancer and Its Large Cell Morphological Variants

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**ABSTRACT**

The in vitro response to radiation and chemotherapeutic drugs of cell lines established from 7 patients with small cell (SC) lung cancer were tested using a soft agarose clonogenic assay. Five cell lines retained the typical morphological and biochemical amine precursor uptake decarboxylation characteristics of SC, while two cell lines had undergone "transformation" to large cell (LC) morphological variants with loss of amine precursor uptake decarboxylation cell characteristics of SC. The radiation survival curves for the SC lines were characterized by D0 values ranging from 51 to 140 rads and extrapolation values (f) ranging from 1.0 to 3.3. While the D0 values of the radiation survival curves of the LC variants were similar (81 and 80 rads), the extrapolation values were 5.8 and 11.1. In vitro chemosensitivity testing of the cell lines revealed an excellent correlation between prior treatment status of the patient and in vitro sensitivity or resistance. No correlation was observed between in vitro chemosensitivity and radiation response. These data suggest that transformation of SC to LC with loss of amine precursor uptake and decarboxylation characteristics is associated with a marked increase in radiation resistance (f) in vitro. The observation of a 2- to 5-fold increase in survival of the LC compared to the SC lines following 200 rads suggests that the use of larger daily radiation fractions and/or radiation-sensitizing drugs might lead to a significantly greater clinical response in patients with LC morphology. This clinical approach may have a major impact on patient response and survival.

**INTRODUCTION**

SC² accounts for 20 to 25% of all cases of primary lung cancer. Unlike the other forms of primary lung cancer, it is initially highly responsive to both radiation therapy and chemotherapy, and the majority of patients will demonstrate a clinical complete response to combination chemotherapy, with or without radiation therapy (20). Unfortunately, most patients relapse and die, with a median survival for all patients of 10 to 14 months. With present-day treatment it appears that only 5 to 10% of all patients presenting with SC can be potentially cured of their disease (20). In addition, in patients who fail in the lung and/or the mediastinum following combination chemotherapy alone, the subsequent response rate to radiation therapy is quite low (16). This resistance to further chemotherapy or radiation therapy presumably represents the emergence of resistant "clones" of tumor cells. In autopsy studies of patients who initially presented with histologically "pure" SC, another histological type of lung cancer is present in 20 to 35% of cases (1, 4, 19). In some studies of relapsed patients, biochemical markers usually elevated in SC were markedly lower than those in "pure" SC (1). Thus, it is possible that resistance to therapy may be correlated with these morphological and biochemical transformations. It has also been shown that those patients who have a mixture of SC and LC histological types in their initial diagnostic biopsy have a lower response rate and survival compared to patients with pure SC (22).

Over the past several years, we and other laboratories have established cell lines of human SC lung cancer in culture (12, 21). These cell lines grow as floating cell aggregates, express human isozymes, form colonies in agarose, and form typical SC tumors in athymic nude mice. In addition, the cells express many of the properties associated with cells of the APUD series, including neurosecretory granules, high levels of the key APUD enzyme L-dopa decarboxylase, formaldehyde-induced fluorescence, and polypeptide hormone secretion (3, 12, 21, 25). More recently, we have demonstrated that these cell lines of SC and fresh specimens of SC have a specific cytogenetic abnormality, a deletion in the short arm of chromosome 3 [3p−(14-23)] (29). This cytogenetic abnormality has been identified only in cells of SC origin but not in lymphocytes from patients with SC or in cell lines of other histological types of lung cancer.

While the majority of established cell lines of SC remain stable in tissue culture, we have observed a gradual change in morphology from SC to LC in some cell lines (11). This "transformation" to LC is accompanied by a loss of APUD properties, an increase in colony-forming efficiency in agarose, and an increase in doubling time in liquid culture. Although these cells lose their biochemical markers associated with SC, the cytogenetic abnormality of SC is retained.

Since this in vitro transformation mimics the clinical observation of a histological change in up to 35% of relapsing SC patients, we studied the in vitro response to both chemotherapy and radiation therapy of SC cell lines and their transformed cell lines. In this paper, we demonstrate 2 novel findings: (a) SC has little or no shoulder on the radiation survival curve, while the large cell variants exhibit a significant shoulder; and (b) this shoulder is independent of in vitro response to chemotherapy.

**MATERIALS AND METHODS**

**Cell Lines.** The methods for the initiation, maintenance, and characterization of the cell lines used in this study have been previously

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²The abbreviations used are: SC, small cell lung cancer; LC, large cell lung cancer; APUD, amine precursor uptake and decarboxylation.

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Two cell lines, NCI H82 and NCI N417, were transformed variants of SC. NCI H82, a gift from Dr. A. F. Gazdar, was derived from a patient who had relapsed from prior chemotherapy and whose initial tumor morphology to LC carcinoma (Fig. 1B). Thus, the NCI N417 large cell variant arose ex vivo.

Morphologically, the SC cultures grow as tight aggregates of cells while the LC converters grow in much looser aggregates. All of the cultures are continuous, clonable, aneuploid, and tumorigenic in athymic mice, and most had been in culture for more than 1 year when tested (Table 1). All cultures expressed human isozymes and were free of fibroblast contamination. Tests for Mycoplasma contamination were negative (performed by Microbiological Associates, Bethesda, Md.).

Radiation. Cells were plated 2 days prior to irradiation in 25-cm plastic flasks. At the time of irradiation, the cultures were in log-phase growth. Following irradiation, the cells were pipetted into single-cell suspensions, assayed for viability by trypan blue exclusion, counted, and plated at varying densities in soft agarose as described previously. Briefly, 1 to 1000 x 10^3 viable single cells were suspended in culture medium and 0.3% agarose (Sea Kem, Rockland, Mass.) at 40° (7). Two ml of the mixture were plated in triplicate in 60-mm plastic Petri dishes containing a base layer of 0.5% agarose in medium. Cultures were incubated in a well-humidified atmosphere of 5% CO2-95% air. Plates were then examined with an inverted-phase microscope biweekly for growth, and colonies (cell aggregates of more than 50 cells) were counted 21 days after plating when colony size and viability were optimal. Radiation survival curves were determined a minimum of 2 times for each cell line. Curves were plotted using least-squares regression analysis of data points below the first decade of survival.

Cell samples were irradiated at room temperature using a 6-MeV photon beam from a Mevatron VI linear accelerator. The dose rate was 200 rads/min. Dosimetry was carried out using a Baldwin Farmer ion chamber connected to a Keithly electrometer system having a direct National Bureau of Standards calibration. Full electron equilibrium was insured for all radiation.

In Vitro Chemosensitivity Studies. Detailed methods for cell culture and measurement of drug sensitivity of human tumor cells in soft agarose have been described previously (23, 26). In brief, a single-cell suspension of cells (3 x 10^6 to 3 x 10^5) in a volume of 1 ml were exposed in tubes for 1 hr at 37° to increasing concentrations of the drugs being tested. The concentrations tested included the peak achievable human plasma level of the drug and 1- and 2-log dilutions of that level. In this study, the drugs tested included those compounds with known clinical activity in the treatment of SC, including doxorubicin, vincristine, methotrexate, and 1,3-bis(2-chloroethyl)-1-nitrosourea. After incubation, the cells were washed twice by centrifugation in serum-free medium and then plated in 0.3% soft agarose in culture medium at a concentration of 1 x 10^4 to 1 x 10^5 cells per plate. The number of plated viable cells was chosen so as to give approximately 100 to 200 colonies in the control plate. All drug concentrations were tested in triplicate. After 3 weeks of incubation, colonies were scored for sensitivity or resistance. In drug-treated plates, the number of clonogenic cells surviving treatment was expressed as a fraction of untreated controls.

In Vitro Biological Characteristics of Cell Lines. Detailed methods for cell culture and measurement of drug sensitivity of human tumor cells in soft agarose have been described previously (23, 26). In brief, a single-cell suspension of cells in log-phase growth was plated in triplicate in 60-mm plastic Petri dishes containing a base layer of 0.5% agarose in medium. Cultures were incubated in a well-humidified atmosphere of 5% CO2-95% air. Plates were then examined with an inverted-phase microscope biweekly for growth, and colonies (cell aggregates of more than 50 cells) were counted 21 days after plating when colony size and viability were optimal. Radiation survival curves were determined a minimum of 2 times for each cell line. Curves were plotted using least-squares regression analysis of data points below the first decade of survival.

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Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Historical type</th>
<th>Time in culture (mos.)</th>
<th>Colony-forming efficiency (%)</th>
<th>Dopa decarboxylase (units/mg)</th>
<th>EM (NSG)</th>
<th>3p-</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI H69</td>
<td>SC</td>
<td>44</td>
<td>2.6</td>
<td>279</td>
<td>++</td>
<td>Yes</td>
</tr>
<tr>
<td>NCI H146</td>
<td>SC</td>
<td>26</td>
<td>5.0</td>
<td>341</td>
<td>++</td>
<td>Yes</td>
</tr>
<tr>
<td>NCI H187</td>
<td>SC</td>
<td>19</td>
<td>3.1</td>
<td>22</td>
<td>++</td>
<td>Yes</td>
</tr>
<tr>
<td>NCI H209</td>
<td>SC</td>
<td>18</td>
<td>2.5</td>
<td>233</td>
<td>++</td>
<td>Yes</td>
</tr>
<tr>
<td>NCI H249</td>
<td>SC</td>
<td>10</td>
<td>2.3</td>
<td>320</td>
<td>++</td>
<td>Yes</td>
</tr>
<tr>
<td>NCI H82</td>
<td>LC</td>
<td>16</td>
<td>18</td>
<td>0.01</td>
<td>Absent</td>
<td>Yes</td>
</tr>
<tr>
<td>NCI N417</td>
<td>LC</td>
<td>72</td>
<td>20</td>
<td>0.01</td>
<td>Absent</td>
<td>Yes</td>
</tr>
</tbody>
</table>

a | Cytophilia exam in cell line; histology in athymic nude mice.

b | EM (NSG), neurosecretory granules present by electron microscopy.
percentage of control. In vitro sensitivity was defined as a 70% or more reduction in the survival of tumor cell colonies at one-tenth the peak achievable plasma level (23, 26). Anything less than a 70% reduction was considered to indicate resistance. These figures were chosen on the basis of prior correlations of in vitro chemosensitivity with in vitro clinical responses using fresh clinical tumor specimens (23, 26).

RESULTS

Radiation Survival. The in vitro radiation survival curves for the 7 human lung cancer cell lines evaluated are shown in Charts 1 to 3. The survival curve parameters, derived by linear regression analysis of the exponential region of the curves, are listed in Table 2. Radiation survival curves for the classic SC (Charts 1 and 2) are characterized by $D_0$ values ranging from 51 to 140 rads. With the exception of cell line NCI H146, the extrapolation numbers ($n$) are small, ranging between 1.0 and 1.49. In contrast, the radiation survival curves for the “convertor” LC lines (Chart 3) are considerably different. While the $D_0$ values of the convertors were similar to the $D_0$ values of the SC cell lines (80, 91), the extrapolation numbers were larger (5.6 and 11.1).

In Vitro Chemosensitivity. The in vitro chemosensitivity of the 7 cell lines tested are indicated in Table 3. Three cell lines (2 SC and 1 LC) were derived from newly diagnosed previously untreated patients, while the remaining 4 cell lines were established from patients who had relapsed from prior multiple-drug combi-
Radiation and Chemosensitivity of Human Lung Cancer

Chart 3. Radiation survival curves for the LC variants cell lines NCI H82 and NCI N417. Different symbols on each plot represent individual experiments conducted on different days.

Table 2
In vitro radiation response of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Prior X-ray therapy</th>
<th>D0 (rads)</th>
<th>n</th>
<th>Surviving fraction for 200 rads</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI H187</td>
<td>No</td>
<td>110</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td>NCI H209</td>
<td>No</td>
<td>137</td>
<td>1.43</td>
<td>0.32</td>
</tr>
<tr>
<td>NCI H249</td>
<td>Yes</td>
<td>80</td>
<td>1.49</td>
<td>0.12</td>
</tr>
<tr>
<td>NCI H89</td>
<td>No</td>
<td>140</td>
<td>1.08</td>
<td>0.25</td>
</tr>
<tr>
<td>NCI H146</td>
<td>No</td>
<td>51</td>
<td>3.3</td>
<td>0.066</td>
</tr>
<tr>
<td>LC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI N417</td>
<td>No</td>
<td>91</td>
<td>5.6</td>
<td>0.56</td>
</tr>
<tr>
<td>NCI H82</td>
<td>No</td>
<td>80</td>
<td>11.1</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Table 3
In vitro chemosensitivity of cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Prior chemotherapy</th>
<th>Responses to agents tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC</td>
<td></td>
<td>V, D, N, MTX R R R R</td>
</tr>
<tr>
<td>NCI H69</td>
<td>V, D, N, MTX</td>
<td>S R R R R</td>
</tr>
<tr>
<td>NCI H146</td>
<td>V, D, N, MTX</td>
<td>R R R R R</td>
</tr>
<tr>
<td>NCI H187</td>
<td>None</td>
<td>S S S S S</td>
</tr>
<tr>
<td>NCI H209</td>
<td>None</td>
<td>S S S S S</td>
</tr>
<tr>
<td>NCI H249</td>
<td>V, D, N, MTX</td>
<td>R R R R R</td>
</tr>
<tr>
<td>LC</td>
<td></td>
<td>S S S S S</td>
</tr>
<tr>
<td>NCI H82</td>
<td>V, D, N, MTX</td>
<td>R R R R R</td>
</tr>
<tr>
<td>NCI N417</td>
<td>None</td>
<td>S S S S S</td>
</tr>
</tbody>
</table>

a Only one patient had received chest irradiation prior to culture of these cell lines. A partial response was achieved with chest irradiation following local failure to combination chemotherapy (Table 3).

DISCUSSION

Histological variants of SC have long been recognized, including the lymphocyte-like type, the fusiform, the polygonal, and other subtypes. However, no significant difference has been observed in the clinical presentation, extent of disease, sites of metastases, nor response to therapy among these histological subtypes of SC (8). In contrast, in those patients in whom a mixture of SC and LC carcinoma are detected at diagnosis, the prognosis for these patients is significantly worse than for those patients with SC (22). These mixed-histology patients have a significantly lower complete response rate to cytotoxic therapy and poorer survival.

We and others have shown that mixed cell types of SC with LC or other histological types of lung cancer are frequently recognized at autopsy in patients who initially presented with pure SC in their initial biopsy specimen (1, 4, 19). It is unclear whether this observation represents the evolution of 2 separate lung cancers or the transformation of the initial “pure” SC to another cell type. Similar to patients who initially present with a mixed cell type, patients with SC who relapse from initial cytotoxic therapy are frequently resistant to the effects of further cytotoxic therapy (16, 20). In established cell lines of SC and in nude mouse heterotransplants, transformation to LC carcinoma has been recognized in long-term cultures (11). Interestingly, this
morphological change is usually associated with a loss of APUD properties, a loss of surface receptors to SC specific monoclonal antibodies, and an increase in both the doubling time and plating efficiency in soft agarose. However, these cell lines retain the 3p chromosomal deletion of SC (29).

The major question addressed in this study is whether there was a difference in the survival response of these well-characterized SC and LC variant tumor lines to chemotherapy or radiation, which might explain the observed poor clinical response to the large cell variants of SC. One might predict that these cell lines would demonstrate in vitro resistance (<70% reduction in survival) to chemotherapy drugs which they had been previously exposed to in vivo. Indeed, the in vitro chemosensitivity results on both the classic SC and large cell variant cell lines underscores the correlation of in vitro and clinical response to chemotherapy (Table 3).

The clinical experience with conventional fractionated radiation therapy (180 to 200 rads given daily to a total dose of 4000 to 7000 rads) reflects a difference in the radioresponsiveness between SC at presentation and at relapse (5, 16). Indeed, a recent analysis of a prospective randomized trial at our institution on SC reveals that patients initially treated with combination chemotherapy and later treated with radiation therapy for a central failure (mediastinum and lung) have a low complete response rate (16). A marked difference in the clinical radiation response is also evident between SC and non-SC lung cancer at presentation (i.e., without prior treatment) (20). The reason(s) for this is unknown but is of paramount importance to clinical fractionated radiation therapy.

In this study, considerable heterogeneity was observed in the 5 SC lung cancer lines with respect to the $D_0$ values of the radiation survival curves while the $D_0$ values for the 2 LC variants were quite similar. The range of $D_0$ values we report here are well within published reports of other human tumor lines (10). The in vitro radiation survival has been studied in 2 other SC lines in the literature with $D_0$ values of 45 rads (14) and 131 rads (24). The reason for the variation in $D_0$ values in our study and others is not understood. There was considerable variation in the plating efficiency between the SC and LC variants. A recent survey of radiation survival curve parameters of human tumor cell lines demonstrated that, to date, there is no correlation between plating efficiency and radiosensitivity (10). This survey encompassed plating efficiency ranges between 0.3 and 100%. Other investigators have reported similar observations (23).

Of interest is the difference in the $n$ value for 4 of the 5 SC lines (Lines 1 to 4) compared to the 2 LC variants. The larger $n$ observed for the LC variants implies that they have a greater capacity to accumulate sublethal damage. Since clinical radiation therapy typically involves daily fractions of 180 to 200 rads, we have indicated the relative survival after 200 rads for these cell lines (Table 2). The surviving fraction following a dose of 200 rads for the first 4 SC lines varies from 0.12 to 0.32 compared to 0.56 and 0.58 for the LC variants. This 2- to 5-fold difference in relative survival following a dose typically used in clinical fractionated external beam therapy may explain in part the difference in clinical response of SC and mixed cell lung cancer.

In vitro radiosensitivity is traditionally defined by the slope ($D_0$) of the linear portion of the survival curve as plotted on a semilogarithmic scale. However, the shoulder of the survival curve ($n$) may be the more important parameter to extrapolate information for clinical fractionated radiation therapy. The $n$ is postulated to reflect the ability of a cell population to accumulate sublethal damage and subsequently repair the damage (9). The mechanism of sublethal damage repair is poorly understood. If multiple fractions of radiation are given to a cell population with a defined shoulder, then the same shoulder is repeated following each radiation fraction, provided that the radiation dose is greater than the shoulder value and a time period of at least 6 hr is allowed between the radiation fractions. There is some controversy in the literature regarding the correlation of the magnitude of the in vitro extrapolation number ($n$) and clinical responsiveness (2, 28). Some studies suggest that there is good correlation both in clinically responsive tumors like leukemia (27) as well as in poorly responsive tumors like glioblastoma (13) and melanoma (10).

Our study and a recent report on the radiation response of a single SC and a morphological variant of the same cell line (14) also supports this correlation. These observations may be of considerable biological importance in exploring the underlying molecular mechanism of radiation damage and repair in human tumors. It is interesting to speculate on our results in SC line NCI-H146. This cell line appears at this point in culture to be in transition with respect to the $n$ and thus with respect to its ability to repair sublethal damage. While this cell line retains the morphological and biochemical profile of SC, its larger $n$ may be an early predictor of subsequent transformation to large cell morphology.

In summary, using an in vitro clonogenic assay, we have demonstrated that cell lines of classic SC and cell lines of SC origin that have undergone conversion to LC variants differ markedly in their capacity to accumulate sublethal radiation damage. By comparing the radiation survival curve extrapolation numbers, the LC variants, with lost APUD properties, are clearly more resistant to radiation than the classic SC lines. We have also shown that no correlation has been found between sensitivity of a given cell line, either SC or LC, to chemotherapeutic drugs or ionizing radiation. A similar lack of correlation has been shown for other human tumor cancer cell lines (18). While the extrapolation of the in vitro data to clinical situations should be tempered with caution, the observation of a 2- to 5-fold difference in survival between the SC and LC lines following 200 rads (the usual dose administered in fractionated schedules for lung cancer) (Table 2) suggests that a larger radiation fraction (e.g., >300 rads), radiation-sensitizing drugs, or both might lead to a significantly greater clinical response in relapsed SC patients suspected or documented to have undergone conversion or change to a large cell morphology. However, it is important to point out that many of these relapsed patients will have received Adriamycin and caution must be used in designing the radiation portals to minimize irradiation of the esophagus and heart. These clinical approaches have been used, with apparent improvement in local control in patients with melanoma and glioblastoma (15, 17).

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