Small Cell Lung Cancer Cell Line Derived from a Primary Tumor with a Characteristic Deletion of 3p


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ABSTRACT

Chemotherapy plus surgery is feasible and potentially effective in selected patients with small cell lung cancer (SCLC) and provides a unique opportunity to study SCLC early in its biological history. The in vitro characteristics of a SCLC cell line derived from a resected lung primary tumor after treatment with 3 courses of chemotherapy is described. The original SCLC cell line UMC-SCLC-1 exhibited features of classic SCLC with typical morphology and growth characteristics, high levels of dopa decarboxylase, bombesin-like peptides, neuron-specific enolase and calcitonin, and the presence of neurosecretory granules and all demonstrated the deletion of the short arm of chromosome 3. After multiple passages, UMC-SCLC-1 gradually changed its characteristics to a cell line, UMC-SCLC-1A, with morphological features of large cell anaplastic carcinoma, an altered growth pattern, decreased in calcitonin, and increase in radiosensitivity but retained the other biochemical markers of classic SCLC (bombesin and dopa decarboxylase production). Serial DNA content analyses showed that increased aneuploidy during continuous culture in vitro was associated with the morphological changes. Both UMC-SCLC-1 and UMC-SCLC-1A demonstrated the deletion of chromosome 3p, amplification and abundant expression of N-myc, and increased expression of c-ras. Chemotherapy sensitivities were stable throughout multiple passages and correlated with in vitro response. UMC-SCLC-1A represents a unique SCLC cell line with heterogeneous properties of both classic and morphological variant SCLC cell lines. In addition, the characteristic deletion of 3p, previously described in cultures derived from metastatic lesions and heavily pretreated patients, is seen in a primary lesion early in the natural history of SCLC.

INTRODUCTION

Lung cancer is the leading cause of death from malignancy in both men and women in the United States (1). SCLC accounts for 20–25% of all lung cancer and is a distinct clinicopathological entity with its unique paraneoplastic syndromes, propensity to metastasize, aggressive clinical course, and sensitivity to chemotherapy and radiation (2). Although combination chemotherapy can achieve response rates of 80 to 90% in all SCLC patients and complete responses in 50 to 60% of patients with limited disease, the majority of patients ultimately relapse and die of their disease (3, 4). The addition of chest irradiation, prophylactic cranial irradiation, and surgery for selected early stage patients has further improved results, but in general a plateau has been reached in therapeutic advances (2, 5, 6).

While clinical improvements have slowed, the understanding of the biology of SCLC is rapidly expanding. Continuous tumor cell lines have been successfully cultured from SCLC patients in both serum-supplemented and serum-free defined media, providing a model for the in vitro study of SCLC (7–10). These cell lines have demonstrated small cell histology in nude mice xenografts, neurosecretory granules on electron microscopy, dopa decarboxylase activity (11, 12), the presence of neuron-specific enolase (13), bombesin-like peptides (14, 15), and a characteristic deletion of chromosome 3p (16, 17). In addition, morphological variants resembling large cell anaplastic carcinoma have been described with loss of amine precursor uptake and decarboxylation properties, higher cloning efficiency, relative radioresistance and amplification and increased expression of c-myc (18–21).

The great majority of the above studies was performed on tumor cell lines and tissues obtained from metastatic sites, autopsies, or heavily pretreated patients (17, 18). We report the in vitro characteristics of a continuous SCLC cell line established from a primary tumor resected after minimal chemotherapy.

MATERIALS AND METHODS

Clinical Information

The patient, T. A., a 56-year-old female, presented with a right upper lobe mass. Bronchoscopy revealed small cell lung cancer and evaluation showed limited disease. She was treated according to the combined chemotherapy-surgery protocol for selected patients with limited disease (6, 22). After achieving a partial response to 3 cycles of CAVE chemotherapy, she underwent a right pneumonectomy which revealed extensive necrosis but residual viable tumor in the mass and hilar nodes. She continued CAVE chemotherapy for approximately 11 months, refused further chemotherapy, and developed jaundice and an abnormal liver-spleen scan compatible with metastatic disease 13 months after diagnosis. She refused any further diagnostic procedures and died 18 months after diagnosis. An autopsy was not granted.

Cell Culture

Single cell suspensions were prepared from the primary tumor obtained at pneumonectomy by mincing, passing through a stainless steel mesh, and gently pipeting with culture media. For culture, single cell suspensions were placed into 25-cm² flasks at a density of approximately 1 x 10⁶ cells/ml in HITES media as described previously (9, 18). The cells were then incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air. Cells grew as floating cell aggregates and were passaged when cell concentration was greater than 1 x 10⁶ cells/ml. After initial establishment of the cell line, improved growth rate was found to be dependent on the addition of 5–10% heat-inactivated fetal calf serum (GIBCO). Once established, cultures were serially passed in RPMI 1640 with 10% fetal calf serum.

Biochemical Characterization

1-Dopa decarboxylase was determined on SCLC cell homogenates using a modification of the method of Beaven et al. (23, 24). Activity...
was expressed as nmol of $^{14}$CO$_2$ released per h incubation per mg of cell protein. Neuron-specific enolase activity was assayed by a triplicate radioimmunoassay procedure using rabbit antisera to human neuron-specific enolase (25). Calcitonin was assayed by immunoperoxidase reaction with rabbit antisera to unconjugated synthetic human calcitonin and expressed as pg per ml (12, 26). Bombesin was assayed after acetic acid extraction by radioimmunoassay using rabbit antisera to bombesin with results expressed as pmol per mg of cellular protein (14, 27).

Cytogenetics

The continuous cell lines were harvested for cytogenetic analysis after 1.5 h exposure to Colcemid (28). Cells with well spread metaphases were Giemsa-trypsin G banded using a procedure modified after the method of Wang and Fedoroff (29) and were analyzed as per ISCN (30).

DNA Index

DNA content was determined by flow cytometric analysis of ethidium bromide-stained nuclei according to the method of Vindelov (31). The ratio of the channel number of the aneuploid peak to the channel number of the diploid standard is the DNA index.

Chemotherapy Sensitivities

Drug sensitivities of SCLC cell lines were assayed using methods described previously (20). After 1-h incubations with single or combination chemotherapeutic agents (kindly provided by Bristol Laboratories, Syracuse, NY), 1–2 × 10$^5$ viable cells in 1 ml of HITES plus 10% fetal calf serum plus 0.3% agarose were placed over an underlayer of HITES plus 10% fetal calf serum plus 0.5% agarose in 35-mm Petri dishes. Semisolid cultures were plated in triplicate and incubated at 37°C in a humidified atmosphere of 5% CO$_2$/95% air for 10 to 14 days until viability and colony size were optimal. Colonies (>50 cells) were then counted. The drug concentrations and incubation time chosen represent approximately 10% of the expected in vivo concentration × time exposure (32). Hydroxypropoxycyclophosphamide, the synthetic active metabolite, was used rather than cyclophosphamide. In the combination chemotherapy experiments, drug concentrations were adjusted to be equivalent to those used in the single agent experiments.

Radiation Sensitivity. Cell cultures were split 48 h prior to the time of irradiation to obtain log phase growth. Cell samples were irradiated at room temperature using 10-MV photons at 1 m target-surface distance with full electronic equilibrium at a dose rate of 3 Gy (300 rads)/min. In each radiation experiment single cell suspensions were placed in 15-ml polystyrene test tubes (Corning Glass Works) and immersed in water. At each dose (e.g., 50, 100, 200 rad...), one tube was removed from the beam until the highest dose (1000 rads) was reached. Following irradiation, cells were assayed for viability by trypan blue exclusion and plated in soft agarose as described for chemotherapy sensitivities.

Electron Microscopy

Cell blocks were prepared from cells growing in liquid culture according to methods described previously (33).

Nucleic Acid Analysis

myc. Small cell lung cancer cell lines were harvested during log phase growth. DNA was prepared by the method of Hieter et al. (34) and RNA was prepared by the method of Chirgwin et al. (35). Ten µg of DNA from different SCLC cell lines were digested with EcoRI and Southern blots were prepared (36). Ten µg of total RNA were electrophoresed in 0.8% agarose and Northern blots were prepared. Three control SCLC cell lines were used: NCI H526, a cell line highly amplified for and expressing N-myc (37); NCI N417, a cell line highly amplified for and expressing c-myc (21); and NCI H378, a cell line highly amplified for and expressing L-myc (38). The probes used for evaluation of the myc family of genes were a second exon Nb-1 fragment (39) for N-myc, a third exon ClaI-EcoRI fragment (40) for c-myc, and a Smal-EcoRI fragment (38) for L-myc. A diploid copy gene control to demonstrate that similar amounts of DNA were loaded in each lane was a gastrin-releasing peptide probe, a PvuII-PvuII fragment (41). The blots were hybridized to c-myc, N-myc, and L-myc probes and washed as reported by Nau et al. (21, 37, 38). The blots were exposed to X-ray film and autoradiograms were prepared.

raf. Dot-blot analysis of total RNA was performed using a "riboprobe" representing the 3' 1.5-kilobase c-raf-1 complementary DNA sequence as described elsewhere (42). The control SCLC cell lines for these analyses were NCI N417 and NCI H69, a classic SCLC cell line amplified for N-myc. NCI H157 is a large cell lung carcinoma cell line and MOLT 4 is a T-cell line (American Type Culture Collection). Genomic DNA (10 µg) was digested to completion with restriction endonucleases, electrophoresed in a 0.7% agarose gel, and transferred to nitrocellulose paper by the method of Southern (36). The blots were hybridized overnight to complementary DNA fragment probes (specific activity, 3 × 10$^6$ cpm/µg) at 65°C in 5 × standard saline citrate/5 × Denhardt's solution/10% (w/v) dextran sulfate/50 mM sodium phosphate buffer, pH 6.6/salmon sperm DNA (250 µg/ml)/0.1% (w/v) sodium dodecyl sulfate. After a washing under stringent conditions (0.1 × standard saline citrate/0.1% sodium dodecyl sulfate at 68°C), the blots were autoradiographed as above.

RESULTS

Establishment of a Continuous SCLC Cell Culture. Viable tumor was obtained from the primary tumor at thoracotomy and grew as a suspension of clustered cells in HITES medium. The cultured cells fulfilled the cytological criteria for classic SCLC on light and electron microscopic examination including a high nuclear/cytoplasmic ratio, a dense chromatin pattern, the presence of nuclear molding, the rarity of a distinct nucleolus, and the presence of neurosecretory granules (Fig. 1). The cells were negative for mucin and keratin production by the Kreyberg staining technique. The culture appearance of UMC-SCLC-1 as assessed by phase contrast microscopy showed irregular floating aggregates of cells lacking central necrosis, the so-called type 2 growth pattern described by Carney (Ref. 18; for definition see Table 1). During approximately 6 months of continuous culture (20 passages), UMC-SCLC-1 gradually changed its culture characteristics to a cell line, UMC-SCLC-1A, with cytological characteristics of large cell anaplastic lung cancer including an increase in amount of cytoplasm, an increased cell size, a large eosinophilic nucleolus, and a more dispersed chromatin pattern (Fig. 2). In conjunction with the morphological alteration, the growth pattern in culture changed to a pattern of more loosely associated clusters and cords of cells, typical of type 3 growth or variant morphology as described by Carney (Ref. 18; for definition see Table 1). While there was a decrease in neurosecretory granules on electron microscopy they were present in UMC-SCLC-1A. UMC-SCLC-1A has been in culture for more than 48 months. Both designations, UMC-SCLC-1 and UMC-SCLC-1A, have been retained because liquid nitrogen freezes of the early passages with classic characteristics have been maintained. Detailed biological studies were carried out in both populations and compared.

Biochemical Characteristics. Biochemical analysis was performed on both UMC-SCLC-1 and UMC-SCLC-1A. Neuron-specific enolase, dopa decarboxylase activity, and bombesin were present in equal amounts in both the original classic cell line, UMC-SCLC-1 and UMC-SCLC-1A (Table 1). Calcitonin production was markedly elevated in UMC-SCLC-1, whereas UMC-SCLC-1A exhibited decreased production consistent with passage in culture (Table 1).

Cytogenetics and DNA Content. Karyology and DNA content
were performed on both UMC-SCLC-1 and the variant UMC-SCLC-1A. Over 100 metaphases were examined and 17 cells were karyotyped. A deletion of chromosome 3p, with breakpoints at p14 and p25, was present in both the parent cell line and its variant (Figs. 3 and 4). Other chromosomal abnormalities that were clonal in both cell lines and present in at least 50% of the cells examined included the absence of a normal chromosome 4 (in UMC-SCLC-1A, an almost complete long arm of chromosome 4 was translocated to the short arm of chromosome 10), extra chromosomes 9 with deletions of the long arm, absence of one chromosome 13, extra derivative chromosome 19 with translocated segments of chromosome 1, extra derivative chromosome 19 with translocated segments of chromosome 9, and extra derivative chromosome 20 with translocated segments of chromosome 1 (for ISCN designations, see Table 2). Karyology on metaphases of early passages of UMC-SCLC-1 showed that there was a broad spectrum of karyotypic abnormalities with aneuploidy ranging from 35 to 85 chromosomes. This was reflected in the DNA content analysis with the dominant cells being pseudodiploid with a DNA index of 1.0 (Fig. 5). With the transition to UMC-SCLC-1A, two distinct aneuploid peaks were present after multiple passages in culture. Additional clonal structural chromosomal abnormalities specific to UMC-SCLC-1A included a long arm deletion of chromosome 1, presence of an abnormally staining region in chromosome 5 with the deletion of the short arm, extra unidentified chromatin material on chromosome 12, derivative chromosome 21 with a translocated short arm of chromosome 8, and derivative chromosome 22 with a translocated long arm of chromosome 12 (Fig. 4; Table 2). With continued passage in culture, the hyperdiploid population has predominated and the hypodiploid population is no longer evident (Fig. 5).
Chemotherapy Sensitivity. *In vitro* drug sensitivity assays performed in UMC-SCLC-1 revealed a 79% decrease in colony formation with CAVE chemotherapy over the control. This combination of drugs was superior to other single agents (doxorubicin 64%, cisplatin 64%, vincristine 44%, VP-16 41%, and the combination of VP-16 and cisplatin 49%). The pattern of drug sensitivity of UMC-SCLC-1A was similar and has remained stable despite its change in morphology and altered DNA content (Table 3).

Radiation Sensitivity. *In vitro* radiation survival curves for UMC-SCLC-1 and UMC-SCLC-1A are shown in Fig. 6. The survival curve parameters show comparable $D_0$ values of 180 and 110 rads for UMC-SCLC-1 and UMC-SCLC-1A, respectively. Extrapolation number ($n$) was low for UMC-SCLC-1 (1.2) and higher for the variant cell line, UMC-SCLC-1A at 2.8.

Nucleic Acid Analysis. UMC-SCLC-1, UMC-SCLC-1A, and appropriate SCLC controls were analyzed for amplification and expression of c-myc, N-myc, and L-myc. The control cell line NCI-H526 has been shown previously to be highly amplified for N-myc DNA and to express abundant N-myc mRNA (37) (Fig. 7). The intense band corresponding to a 2.0-kilobase EcoRI fragment demonstrates DNA amplification of N-myc in UMC-SCLC-1 and UMC-SCLC-1A. The 5.6-kilobase EcoRI fragment seen in NCI H526 is not present in UMC-SCLC-1 or UMC-SCLC-1A. Both cell lines express abundant N-myc mRNA. Neither UMC-SCLC-1 nor UMC-SCLC-1A had DNA amplification or detectable mRNA expression of c-myc or L-myc.

RNA dot-blot analysis of c-raf-1 showed increased expression in both UMC-SCLC-1 and UMC-SCLC-1A comparable to the c-myc-amplified variant SCLC line NCI N417 and the N-myc-amplified classic SCLC line NCI H69 (Fig. 8). No expression of c-raf was seen in NCI H157 or MOLT-4. Probes representing the 5' end of c-raf-1 gene were unable to detect genomic rearrangements or amplification in UMC-SCLC-1 or UMC-SCLC-1A (data not shown).

DISCUSSION

Since chemotherapy with or without radiotherapy forms the cornerstone of treatment for SCLC, resection of a primary tumor offers a rare opportunity to study SCLC early in its biological history. The *in vitro* chemotherapy sensitivity studies performed demonstrated superiority of combination che-
therapy with CAVE over other single agents or combinations [VP-16/cis-diammine-dichloroplatinum(II)] and showed excellent correlation with clinical response. In addition, chemotherapy sensitivities remained remarkably stable over multiple passages, in spite of changes in morphology and increased radioresistance. The lack of correlation between sensitivity to chemotherapy and ionizing radiation in SCLC cell lines has been described previously (20).

A continuous clonable culture (UMC-SCLC-1) was obtained which after 6 months in culture yielded a variant hyperdiploid culture UMC-SCLC-1A with characteristics of large cell anaplastic carcinoma. The changes in DNA content were associated with the continuum of pure classic morphology in the early passages (up to P15), both classic and variant morphology in midpassages (P15–P30), and only the large cell component present in the later passages (after P30). Studies confirmed the amine precursor uptake and decarboxylation features of UMC-SCLC-1 with characteristic elevations of dopa decarboxylase, bombesin-like peptides, calcitonin, neuron-specific enolase, and the presence of neurosecretory granules on electron microscopy. UMC-SCLC-1A, with morphological characteristics of large cell carcinoma, showed a marked decrease in calcitonin. These data could be explained by the presence of both populations in the original tumor with the emergence of a dominant clone or by an in vitro phenomenon. The presence at passage 11 of a very small hyperdiploid peak and metaphases with hyperdiploid karyotypes support the former mechanism. Also, it is known that 10–20% of SCLC specimens will have multiple stem lines present by DNA content analysis (43, 44), and clinical observations including autopsy studies corroborate that SCLC can coexist with or perhaps transform to non-small cell histological types (45–50).

The variant cell line UMC-SCLC-1A described here differs somewhat from those previously described. Gazdar et al. (19) recently defined variant SCLC cell lines on the basis of biochemical and morphological characteristics. In the presently described SCLC cell lines, UMC-SCLC-1 demonstrates the features of classic SCLC by light and electron microscopy, growth pattern, and biochemical properties. UMC-SCLC-1A has the cytological and growth characteristics typical of the morphological variants and relative radioresistance. However, with elevated dopa decarboxylase, bombesin-like peptides, and dense core granules, UMC-SCLC-1A has features of both classic and morphological variant SCLC. Thus, the clinical and in vitro data support the concept of a continuum of cell types in lung cancer and challenge the notion that different tumors arise from different stem cells (51).

While 7 of 9 morphological variants described by Gazdar et al. (19) had amplification of c-myc, UMC-SCLC-1A demonstrated N-myc amplification and expression. While c-myc is strongly associated with morphological variants and transfection studies suggest an etiological role (52), the significance of N-myc is less clear. SCLC cell lines with amplification and increased expression of N-myc have been described with both classic and variant features morphologically and biochemically (37). While the mechanism of increased N-myc expression in UMC-SCLC-1 and -1A appears to be amplification, the role of the chromosomal abnormalities observed (Table 2) is unclear. It is possible that the abnormal staining region noted on chromosome 5 (Fig. 4) could represent an area of gene amplification.

Table 2 Chromosomal abnormalities present in at least 50% of metaphases examined

<table>
<thead>
<tr>
<th>A. Both UMC-SCLC-1 and UMC-SCLC-1A</th>
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<tbody>
<tr>
<td>del(3)(p14;p25)</td>
</tr>
<tr>
<td>del(9)(q13)</td>
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<tr>
<td>del(19)(p19;q21;q13.1)</td>
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<tr>
<td>del(19)(p19;q13;p13.3)</td>
</tr>
<tr>
<td>del(20)(p12;q12p13)</td>
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<tr>
<td>B. UMC-SCLC-1A only</td>
</tr>
<tr>
<td>del(1)(q25)</td>
</tr>
<tr>
<td>del(5)(p13)</td>
</tr>
<tr>
<td>12p+</td>
</tr>
<tr>
<td>del(21)(p11.2;p11.1)</td>
</tr>
<tr>
<td>del(22)(q13;p11.2)</td>
</tr>
<tr>
<td>del(10)(q12p13)</td>
</tr>
</tbody>
</table>

* Designations according to ISCN, 1985 (30).
Double minutes, homogeneously staining regions, and abnormalities of chromosome 2, the site of N-myc, were not seen. Other factors including additional oncogenes may have a role in the observed changes (53).

The nonrandom chromosomal abnormality noted by Whang-Peng et al. (17), the deletion of chromosome 3p, was noted in both UMC-SCLC-1 and UMC-SCLC-1A. Whereas in previous reports this abnormality was noted only in metastatic lesions, in heavily pretreated patients, and in one instance from a primary tumor after long-term culture, we were able to confirm this finding in a primary tumor relatively early in the clinical course of SCLC. The significance of this finding is unclear, particularly in view of the findings of Wurster-hill et al. (54) who found abnormalities in chromosome 3 in a minority of SCLC cell lines. It is possible that the different culture techniques used by the Dartmouth and NCI-Navy groups may be responsible for the discrepancy, the former using serum-supplemented media and the latter using serum-free defined media (7, 9). UMC-SCLC-1 was initiated in serum-free defined media (HITES).

The finding of the deletion of 3p early in cultured SCLC...


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