Characterization of Variant Subclasses of Cell Lines Derived from Small Cell Lung Cancer Having Distinctive Biochemical, Morphological, and Growth Properties

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ABSTRACT

We have described the establishment and biochemical characterization of 50 small cell lung carcinoma (SCLC) cell lines. Further analysis of these data, combined with studies of morphology and growth characteristics, indicates that 35 (70%) of the lines retained typical morphology (SCLC, intermediate subtype), growth characteristics (growth as tightly packed floating cellular aggregates, long doubling times and low colony-forming efficiencies), and biochemical profile (presence of L-dopa decarboxylase, bombesin-like immunoreactivity, neuron-specific enolase, and high concentrations of brain isoenzyme of creatine kinase). They are referred to as classic SCLC lines. The remaining 15 (30%) lines had discordant expression of the biochemical markers; they retained high concentrations of brain isoenzyme of creatine kinase, but had significantly lower concentrations of neuron-specific enolase and lacked L-dopa decarboxylase and bombesin-like immunoreactivity. These cell lines are called variants. SCLC variant lines could further be divided into (a) biochemical variant lines having variant biochemical profile but retaining typical SCLC morphology and growth characteristics; and (b) morphological variant (SCLC-MV) lines having variant biochemical profile, altered morphology (features of large cell undifferentiated carcinoma) and altered growth characteristics (growth as loosely attached floating aggregates, relatively short doubling times and cloning efficiencies). Fifty-five clones derived from the three SCLC subclasses retained their parental phenotypes. In SCLC-MV lines there was a near constant relationship between variant morphology, altered growth characteristics and amplification of the c-myc oncogene; classic SCLC and biochemical variant SCLC lines were not amplified. Variant morphologies, especially large cell carcinoma, and at autopsy further, SCLC tumors may be admixed with or convert to non-SCLC morphologies (18, 19). At presentation, approximately 8% of SCLC tumors contain a subpopulation of cells with non-SCLC morphologies, especially large cell carcinoma, and at autopsy the incidence is much higher, and over one-third of previously "pure" SCLC tumors contain (or have been completely replaced by) non-SCLC morphologies. Similar conversions of SCLC to non-SCLC have been reported in vitro and in xenografts (13, 17, 20, 21). These changes, in vivo and ex vivo, may be accompanied by loss of some of the SCLC biochemical markers. Recently we have found that lines derived from SCLC tumors having variant morphologies have considerable amplification and increased expression of the c-myc oncogene (22). These findings may be of major clinical significance, as SCLC patients presenting with SCLC tumors having variant morphologies have poorer responses to therapy and shorter survival times than do those presenting with pure or SCLC-C morphologies (23).

INTRODUCTION

SCLC accounts for approximately 25% of bronchogenic carcinomas, and has distinctive clinical, pathological, and biological features (1). We have described the establishment and biochemical characterization of 50 SCLC cells lines (2). Initially considered to be an "undifferentiated" tumor on morphological grounds, SCLC exhibits many differentiated properties of neuroendocrine cells of the amine precursor uptake and decarboxylation series (3). These properties include: the presence of cytoplasmic dense core ("neurosecretory") granules, high concentrations of the key amine precursor uptake and decarboxylation cell enzymes DDC and NSE, and production of several peptides (4-9). The peptide most frequently associated with SCLC is the mammalian homologue of the amphibian neuropeptide bombesin, and will be referred to as BLI (10-12). In addition, SCLC tumors and cell lines have very high specific activities of the enzyme creatine kinase, expressed predominantly in the form of its brain isoenzyme (CK-BB) (13). Thus, most cases of SCLC can be distinguished from non-SCLC lung cancers (squamous cell, large cell, and adenocarcinoma) by morphology, ultrastructure, and a panel of biochemical markers (DDC, NSE, BLI, and CK-BB). The WHO classification (14) divides SCLC into oat cell and intermediate subtypes. However, we, and others, have found no significant differences in the clinical behavior, response to therapy, or survival of these subtypes (15). SCLC cell lines and xenografts almost always express intermediate subtype morphology (16), and we have postulated that this subtype is the true morphological expression of SCLC, while the oat cell subtype probably represents a degenerative form or artifact (17, 18). Furthermore, SCLC tumors may be admixed with or convert to non-SCLC morphologies (18, 19). At presentation, approximately 8% of SCLC tumors contain a subpopulation of cells with non-SCLC morphologies, especially large cell carcinoma, and at autopsy the incidence is much higher, and over one-third of previously "pure" SCLC tumors contain (or have been completely replaced by) non-SCLC morphologies. Similar conversions of SCLC to non-SCLC have been reported in vitro and in xenografts (13, 17, 20, 21). These changes, in vivo and ex vivo, may be accompanies by loss of some of the SCLC biochemical markers. Recently we have found that lines derived from SCLC tumors having variant morphologies have considerable amplification and increased expression of the c-myc oncogene (22). These findings may be of major clinical significance, as SCLC patients presenting with SCLC tumors having variant morphologies have poorer responses to therapy and shorter survival times than do those presenting with pure or SCLC-C morphologies (23).

In a companion paper (2), we describe the establishment and characteriztion of 50 continuous cell lines from SCLC tumor specimens. While most SCLC lines expressed the complete SCLC biochemical profile, a considerable number (30%) lacked one or more properties. In this manuscript we detail the biochem-
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MATERIALS AND METHODS

Establishment and Characterization of Cell Lines. Most methods for the establishment and characterization of SCLC cell lines are as described earlier and also in a companion manuscript (2, 5, 24). Cell lines were established from pathologically proven SCLC tumor materials, either directly or after heterotransplantation in athymic nude mice. Two basic growth media were used for establishment and maintenance of cell lines, RPMI 1640 supplemented with 10% fetal bovine serum, or with fully defined serum-free growth medium for SCLC culture (2, 24, 25). Methods for cell maintenance, xenografting into athymic nude mice, and assays for DDC are as described (2, 5, 16, 24).

Growth Characteristics. Population-doubling times were determined by seeding single-cell suspensions into replicate flasks (25 sq cm), and performing cell counts every 2 or 3 days for 14 days. Cultures were fed every 3 or 4 days, and 24 h prior to counting. Colony-forming efficiencies were determined by suspending single cells in a semisolid layer of 0.3% agarose in growth medium placed over a base layer of 0.5% agarose in RPMI 1640 supplemented with 10% fetal bovine serum that had hardened (26). Colonies, consisting of aggregates of more than 50 cells, were enumerated 14 to 21 days later, using an inverted-phase microscope. For clonal studies, colonies were picked using a Pasteur pipette and grown to mass culture.

Morphological Studies. The tumor materials used to initiate the cell lines were reviewed. For cytological examination of cell lines, cell suspensions were cytocolonized onto glass slides, fixed with either Saccochonno’s fixative or 95% ethanol, and stained with hematoxylin-eosin or the Papanicolaou technique. Formalin-fixed, paraffin-embedded, hematoxylin-eosin stained sections of nude mouse xenografts induced by inoculation of cell lines were also examined.

Copy Number of the c-myc Oncogene. Methodology and references for estimation of the copy number of the c-myc gene have been published previously (22). In brief, we analyzed EcoRI digests of genomic DNA extracts prepared from representative controls, classic and variant SCLC lines on agarose gels using the Southern nitrocellulose transfer method. The nitrocellulose filters were hybridized with the 1.6-kilobase SstII human c-myc probe and autoradiography was performed. Densitometer scans were used for the quantitative comparison of the c-myc 12.5-kilobase DNA signal between cell lines. The c-myc clone was kindly provided by Drs. Philip Leder and James Battey.

Statistical Methods. Values were compared by the nonparametric 2 rank test of Mann and Whitney (27).

RESULTS

Identification of Classic and Variant Subclasses. A total of 50 independent cell lines have been established in the laboratories of A. F. Gazdar or D. N. Carney from primary or metastatic SCLC tumors obtained from 45 patients (2). Analysis of data presented in Ref. 2 demonstrates that 35 of these lines expressed all 4 biochemical markers characteristic of SCLC, DDC, BLI, NSE, and high concentrations of CK-BB. Cytological examination of these lines and histological examination of xenografts induced by cell inoculation into athymic nude mice revealed that all of these lines had the characteristic morphological features of SCLC, intermediate subtype (Figs. 1 and 2). The cells were relatively small, with high nuclear:cytoplasmic ratios and narrow rims of cytoplasm. The chromatin was finely granular and distributed throughout the nucleus. Nucleoli were small and inconspicuous, and usually single. Cultures expressing all of the biochemical features and characteristic morphology are referred to as SCLC-C cell lines. The other 15 lines had discordant expression of SCLC markers (Table 1). These lines are referred to as SCLC-V cell lines.

Morphological and Biochemical Features of Variant Subclasses. Cytological and histopathological examination of SCLC-V lines and xenografts induced by inoculating them into nude mice indicated that 5 had characteristic SCLC morphology. These lines are referred to as SCLC-BV. In contrast, 10 SCLC-V lines lacked characteristic SCLC morphology. The cells of these lines were somewhat larger, with moderate amounts of palely staining cytoplasm. Their characteristic feature was the presence of one or multiple prominent nucleoli. The chromatin pattern varied; in some lines it resembled that of SCLC-C lines (i.e., finely granular and evenly distributed); in others it was more variable in size and distribution, with perinucleolar clearing. Lines having the latter morphologies closely resembled the appearance of large cell undifferentiated carcinomas (Figs. 3 and 4). SCLC cultures having noncharacteristic morphologies are referred to as SCLC-MV.

The biochemical characteristics of SCLC-C, SCLC-BV, and SCLC-MV lines, as extracted from Table 4 of Ref. 2, are summarized in Table 1. There were no significant differences between the biochemical properties of the 2 variant subtypes. SCLC-V lines lacked DDC and BLI expression (except for one SCLC-BV line which had low levels of DDC), they expressed NSE, but at significantly lower concentrations than did SCLC-C lines, and had high concentrations of CK-BB, similar to those of SCLC-C lines.

Origin of Variant Lines. Because of major differences in biochemical and morphological characteristics between SCLC-C and SCLC-V lines, we examined the latter for possible differences in patient characteristics or growth conditions. As presented in Table 2, there were no major differences in the patients’ sex or prior therapy status, or in the growth conditions. However, there were important differences in the pathology of the tumor material from which some of the SCLC-MV lines originated. The original tumor morphologies of 34 of 35 SCLC-C and all 5 SCLC-BV lines were characteristic of SCLC. However, 5 of 10 SCLC-MV lines originated from tumors having variant morphologies (NCl lines H82, N177, H360, H437, and H446).

Another important question we addressed is whether SCLC-

Table 1

<table>
<thead>
<tr>
<th>Cell line subclass</th>
<th>DDC (units/mg)</th>
<th>BLI (pmol/mg)</th>
<th>NSE (ng/mg)</th>
<th>CK-BB (ng/mg)</th>
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</thead>
<tbody>
<tr>
<td>SCLC-C</td>
<td>199 ± 32.9*</td>
<td>3.97 ± 0.90</td>
<td>1495 ± 247</td>
<td>6486 ± 829</td>
</tr>
<tr>
<td>(n = 35)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SCLC-MV</td>
<td>&lt;1</td>
<td>&lt;0.01</td>
<td>467 ± 112</td>
<td>5055 ± 1046</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SCLC-BV</td>
<td>&lt;1</td>
<td>&lt;0.01</td>
<td>522 ± 130</td>
<td>7699 ± 2050</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SCLC-MV</td>
<td>&lt;1</td>
<td>&lt;0.01</td>
<td>481 ± 87</td>
<td>5936 ± 990</td>
</tr>
<tr>
<td>(n = 15)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>P&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.94</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SE.

Because there were no significant differences between the values of the 2 variant subclasses, their data were pooled and compared statistically to the values of the SCLC-C group.
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Table 2

<table>
<thead>
<tr>
<th>Property</th>
<th>SCLC-C (n = 35)</th>
<th>SCLC-MV (n = 10)</th>
<th>SCLC-BV (n = 5)</th>
<th>SCLC-V (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>26</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Prior therapy</td>
<td>Yes</td>
<td>21</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>14</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Growth medium&lt;sup&gt;b&lt;/sup&gt;</td>
<td>SSM</td>
<td>11</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Fully defined for SCLC culture (2)</td>
<td>20</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Combined data of the 2 variant subclasses.

<sup>b</sup> Cell lines were established in 10% fetal bovine serum-supplemented medium, serum-free fully defined growth medium for SCLC culture (2), or in both (9).

Table 3

Growth patterns of SCLC cell lines

As detailed in Ref. 2, SCLC lines exhibit 4 growth patterns. These patterns are: type 1, cell growth in tightly packed floating spheroids; type 2, cell growth in amorphous floating aggregates; type 3, large floating cells growing in intertwined cords or loose aggregates; type 4, adherent cells lacking the epithelioid features of non-SCLC lung cancer lines.

<table>
<thead>
<tr>
<th>Growth pattern type</th>
<th>Cell line subclass</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>SCLC-C (n = 35)</td>
</tr>
<tr>
<td></td>
<td>SCLC-MV (n = 10)</td>
</tr>
<tr>
<td></td>
<td>SCLC-BV (n = 5)</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
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<tr>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

<sup>a</sup> One SCLC-MV cell line, NCI-H446, exhibited dual type 2 and 4 patterns.

V lines reflect unusual properties of the tumors from which they were derived, or whether they arose as a result of alterations occurring during in vitro culture. One SCLC-MV line (NCI-N417) and 2 SCLC-BV lines (NCI-N179 and NCI-H289) were typical SCLC-C lines when initiated, but altered during in vitro culture. The other 9 SCLC-MV and 3 SCLC-BV lines expressed their variant properties when first examined within a few weeks (Pases 2 to 4) of culture initiation.

Growth Patterns and Culture Characteristics. As previously described (2), most SCLC lines (47 of 50) lacked substrate adhesion and replicated as tightly packed floating cell aggregates, either as spheroids or as amorphous masses (type 1 and type 2 growth patterns, respectively). Some floating lines demonstrated much less cell-to-cell adherence, and grew as small, loosely attached aggregates or as intertwined cords (type 3 pattern). A small number of SCLC lines demonstrated substrate adherence similar to that of non-SCLC lung carcinoma cultures, but lacked the orderly epithelioid appearance of the latter (type 4 pattern). Table 3 presents the growth patterns of the SCLC subtypes. Lines retaining characteristic SCLC morphology (SCLC-C and SCLC-BV) usually exhibited tight adherence (type 1 and 2 patterns). Most SCLC-MV lines demonstrated much looser cell-to-cell attachment (type 3 pattern), and some adhered to the substrate (type 4 pattern).

SCLC-C and SCLC-BV lines had relatively long population doubling times (mean values of 79 and 97 h, respectively) (Table 4). In contrast, SCLC-MV lines had significantly shorter doubling times (mean value 32 h). Typical growth curves of a SCLC-C and a SCLC-MV line are illustrated in Chart 1.

The cloning efficiencies (in soft agarose) of SCLC-C and SCLC-MV lines were significantly different (Table 4); SCLC-C lines cloned inefficiently (mean value of 2.0%), while SCLC-MV lines had significantly higher cloning efficiencies (mean value of 14.0). SCLC-BV lines had low cloning efficiencies (mean value, 0.2%).

Clonal Expression of Parental Phenotypes. We examined
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55 clones derived from 3 SCLC-C lines (38 clones), one SCLC-MV line (12 clones), and one SCLC-BV line (5 clones) for cytological morphology and expression of DDC (Table 5). In all cases, the clones retained their respective parental phenotypes.

Relationship between c-myc Gene Amplification and Growth Characteristics. As illustrated in Charts 2 and 3 and summarized in Table 4, 7 of 9 SCLC-MV lines had amplification of the c-myc gene ranging from 5- to 76-fold (median, 26-fold). In contrast, only one of 23 SCLC-C lines and 0 of 3 SCLC-BV lines had amplification of the gene. As illustrated in Charts 2 and 3, all SCLC-MV lines tested had significantly shorter doubling times and higher colony-forming efficiencies ($P < 0.01$) than did SCLC-C or SCLC-BV cell lines, including a SCLC-MV line lacking c-myc amplification (NCI-H526, doubling time of 36 h and colony-forming efficiency of 12%). Examples of c-myc gene amplification in SCLC-MV cell lines are illustrated in Fig. 5.

DISCUSSION

With improved culture methods, especially the use of fully defined growth medium for SCLC culture (2), the vast majority of adequate SCLC tumor specimens can be established as permanently replicating cell lines (2, 24). In a companion paper (2) we describe 50 continuous cell lines established from SCLC tumors. Herein we analyze the biochemical, morphological, and growth properties of these 50 SCLC lines. Our studies indicate that SCLC cell lines can be divided into 3 distinctive subclasses. The majority of SCLC lines (70%) expressed all of the characteristic features of SCLC lines as detailed previously (5, 8, 10, 13, 16, 24), and are referred to as SCLC-C cell lines. SCLC-C lines had morphological features of the intermediate subtype of SCLC, expressed the entire range of SCLC biochemical features (DDC, BLI, NSE, and CK-BB), replicated slowly as tightly packed floating cellular aggregates, and cloned inefficiently in semisolid medium. The other 15 (30%) cell lines had a distinct biochemical profile; they lacked DDC and BLI, had significantly reduced concentrations of NSE, but retained high concentrations of CK-BB. These lines are referred to as SCLC-V cell lines.

SCLC-V lines could further be divided into 2 subclasses. SCLC-BV had the variant cell line biochemical profile as described above, but otherwise resembled SCLC-C lines, i.e., they had similar morphological and growth characteristics. SCLC-MV lines and their xenografts had some or all of the morphological features of large cell undifferentiated carcinoma. Because there appeared to be a continuous spectrum of changes between SCLC-MV cell lines, as well as within individual lines, we collectively refer to these lines as morphological variants, replacing older terminologies such as mixed small cell-large cell tumors,
and transition or conversion to large cell carcinoma (13, 18, 20, 21, 23, 26). SCLC-MV lines had growth properties very different from the other subclasses; they grew as loosely adherent floating cells (a few demonstrated substrate adherence), they had relatively short doubling times, and they cloned relatively efficiently. Fifty-five clones derived from 6 cell lines representing all 3 SCLC subclasses expressed their respective parental phenotypes, further evidence that SCLC lines do not consist of mixtures of different subclasses.

We investigated the possible origin of SCLC-V lines. There were no major differences in patient characteristics, therapy status, or growth conditions. With one exception, the 35 SCLC-C and 5 SCLC-BV lines were derived from tumor material having pure or characteristic SCLC morphology. However, 5 of 10 SCLC-MV lines originated from tumors having variant morphologies. In addition, 4 other SCLC-MV lines had variant morphology when first examined shortly after culture initiation. Initially we believed that SCLC-MV lines arose from changes occurring in long-passaged SCLC-C xenografts and cell lines (20, 21). While this mechanism may exist, analysis of the larger number of SCLC-V lines presented herein suggests that most SCLC-MV lines reflect alterations that had already occurred or commenced in the tumors from which they were derived.

Of major interest, 7 of 9 SCLC-MV lines tested had considerable amplification of the c-myc oncogene (median value, 26-fold). Only one of 23 SCLC-C lines and none of 3 SCLC-BV lines tested had amplified levels of this gene. Thus, amplification of c-myc is associated with alterations in growth and morphological characteristics of variant lines, but not with the variant biochemical profile. As we and others have found amplification and/or increased expression of other oncogenes in association with SCLC, including c-myb, and n-my c (28, 29), it is tempting to speculate that variant lines not associated with c-myc amplification are associated with amplification or rearrangements of other oncogenes. Of interest, the one SCLC-MV line lacking c-myc amplification (NCI-H526) had amplification and increased expression of n-myc.

Most non-SCLC lung cancer cell lines demonstrate substrate adherence, while SCLC lines usually lack this property (2, 5). These differences may be due to the markedly different cell surface protein phenotypes of these 2 major subdivisions of lung cancer (30). SCLC-MV lines usually lack substrate adherence, but their floating aggregates are much less cohesive than are SCLC-C lines, suggesting differences in their respective cell surface protein phenotypes. Preliminary data support this hypothesis (31).

We, and others, have reported the radiobiological properties of SCLC-C and SCLC-MV lines (26, 32). While SCLC-C lines were radiosensitive in vitro, with D0 values ranging from 51 to 140 rads and extrapolation values (n) ranging from 1 to 3.3, SCLC-MV lines, while having similar D0 values, had n values ranging from 5.6 to 11.1 (26). These studies indicate that SCLC-MV lines have a much greater capacity to recover from sublethal doses of radiation.

We have identified variant subclasses of SCLC cell lines having altered biochemical, morphological, and growth properties, and have related these features to amplification of the c-myc oncogene. Because SCLC patients presenting with variant morphologies respond less well to therapy and have shorter survival times, our findings may be of considerable clinical and biological importance.

REFERENCES


* Unpublished data.
* S. B. Baylin, A. F. Gazdar, and D. N. Carney, unpublished data.
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Fig. 1. Cytological appearance of SCL-C cell line NCH-128. The cytoplasm is scanty, the chromatin pattern granular, and nucleoli are inconspicuous. Ethanol fission. Papainzymed stain. x 300.  

Fig. 2. Histological appearance of xenograft induced by inoculation of SCL-C cell line NCH-128 into athymic nude mice. The neoplasms of the tumor are typical of the intermediate subtype of SCLC. H & E, x 375.  

Fig. 3. Cytological appearance of SCL-MV cell line NCH-182. The cells contain a moderate amount of cytoplasm, the chromatin is dispersed, and one or 2 prominent nucleoli are present. Ethanol fission.  

Fig. 4. Histological appearance of xenograft induced by inoculation of SCL-CMV cell line NCH-182 into athymic nude mice. The neoplasms of the tumor resemble that of large cell undifferentiated carcinoma. H & E. x 325.
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