Characterization of Three Small Cell Lung Cancer Cell Lines Established from One Patient during Longitudinal Follow-up


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ABSTRACT

Three classic-type, small cell lung cancer cell lines (GLC-14, GLC-16, and GLC-19) have been established from one patient during longitudinal follow-up. During this period the tumor changed from sensitive to completely resistant to (chemo)therapy. A phenotypical and functional characterization of the different cell lines is given in combination with the matching clinical data.

(a) The cell lines have been compared with the biopsies from which they were derived. There was a good match between the morphological, biochemical, and immunohistological findings in the cell lines as compared to those obtained in the biopsies. When the biopsy and cell line (GLC-14) obtained before the start of therapy were compared to the biopsies and cell lines (GLC-16 and GLC-19) acquired after the first and second reinduction therapy, respectively, no major changes could be observed. The only clear alteration was the loss of a neuroendocrine antigen (defined by monoclonal antibody MOC-51) in the posttherapy specimens.

(b) The doxorubicin, melphalan, and etoposide sensitivity in vitro reflected the clinically observed development of resistance to treatment. The cell line (GLC-14) established before the start of therapy was more sensitive than the lines (GLC-16 and GLC-19) obtained after treatment.

It is concluded that the cell lines described in this paper represent a well-characterized in vitro model in which the development of drug resistance in small cell lung cancer can be studied.

INTRODUCTION

SCLC accounts for 20 to 25% of newly diagnosed cases of lung cancer and is characterized by early and widespread metastases. With no treatment the patient's life expectation after diagnosis rarely exceeds 35 wk. Therefore it is the most aggressive form of lung cancer. Combination chemotherapy may induce remission in up to 80% of the cases, but most patients relapse within 6 mo and, as reinduction therapy is generally less successful, long-term survival is poor (1–6). Apparently the main obstacle in the management of these patients is the development of tumor resistance to cytotoxics during treatment.

The recognition of changes in the tumor cell, coinciding with the development of clinical resistance in the tumor, might contribute to an understanding of the mechanisms involved. A subpopulation of established SCLC cell lines, the so-called "variant" lines, has been proposed to reflect the tumor cells showing in vivo refractoriness to treatment. The main characteristics of "variant" SCLC cell lines are a changed, "large cell"-like phenotype and a reduced content of the neuroendocrine markers DDC and gastrin releasing peptide (7–11). There is, however, a paucity of clinical data to decide whether it is justified to extend these in vitro findings to the clinical situation. In addition, it is only rarely possible to evaluate such characteristics in one patient. In this paper we describe the isolation and characterization of a series of three cell lines sequentially obtained from one patient. The laboratory parameters measured in these lines were compared to the clinical data.

MATERIALS AND METHODS

Patient History and Establishment of Cell Lines. A 55-year-old woman was seen in our clinic in December 1984. Her chest X-ray showed enlarged lymph nodes in the left hilar and right paratracheal region. Rigid bronchoscopy revealed granulomatous, easily bleeding lesions at the carina. The lower lobe bronchus was completely obstructed by compression. Histology from the biopsies of the lesions at the main carina and from a right supravacular node revealed SCLC. Ultrasound investigation of liver and adrenals suggested metastases. Immunohistology was performed on a part of the supravacular node biopsy (Table 1), and a cell line (GLC-14) was derived from another part.

Treatment was started with cyclophosphamide (1 g/m2/day, every 3 wk), doxorubicin (45 mg/m2/day, every 3 wk, and etoposide (100 mg/m2/days 1, 3, and 5, every 3 wk) (CDE). In April 1985, after 5 cycles, restaging showed a complete response. No signs of tumor were found with clinical evaluation including bronchoscopy. Prophylactic cranial irradiation (10 times, 30 Gy) was given. In July 1985 the chest X-ray showed tumor recurrence. Rigid bronchoscopy showed an easily bleeding tumor in the left upper lobe bronchus. Histopathological and immunohistological (Table 1) evaluation of the biopsy revealed SCLC. No cell line was established from this specimen. Reinduction chemotherapy with CDE was started.

According to the patient's wishes, after 4 cycles of CDE, the therapy was discontinued in October 1985. The chest X-ray showed a partial response. On bronchoscopy the upper division of the left upper lobe appeared normal. However, a tumor was visible in the orifice of the lingula segment. Histopathological and immunohistological (Table 1) examination of a biopsy revealed SCLC, and a cell line (GLC-16) could be derived from the same biopsy specimen. Radiotherapy (10 times, 30 Gy) to the left hilar region and mediastinum was given. In November 1985 the chest X-ray was normal.

In February 1986 a chest X-ray indicated recurrence of disease. Bronchoscopy showed a tumor obstructing the left upper lobe. This is in the area which had been irradiated. Histopathological and immunohistological (Table 1) examination of a biopsy revealed SCLC. A cell line (GLC-19) was established from the same biopsy specimen. Two additional cycles of CDE were given. Because of tumor progression, treatment was discontinued. The patient died in May 1986. Autopsy was not performed.

Biopsies. Each biopsy was split. One part was used for tissue culturing, and the other was immediately placed in Nakane fixative (12). After fixation the specimen was further divided into two parts (13). One part was rinsed and kept in PBS (Dulbecco "A"; Oxoid Limited, London, England) for at least 12 h. This tissue part was subsequently
Table 1: A series of parameters developed on biopsy specimens and matching cell lines obtained during longitudinal follow-up in one patient

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Biopsy 1</th>
<th>GLC-14</th>
<th>Biopsy 2</th>
<th>Biopsy 3</th>
<th>GLC-16</th>
<th>Biopsy 4</th>
<th>GLC-19</th>
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<tr>
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<td>DDC</td>
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<td>72.5</td>
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<tr>
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<td>RGE-53</td>
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<td>Vimentin</td>
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**RESULTS**

Histology of Biopsies. Biopsies were procured during the course of disease at different time points (see patient history and establishment of cell lines data in “Materials and Methods”). All tumor specimens (Fig. 1) were classified as SCLC of the intermediate type by histopathological criteria (revised WHO classification [29]). Apparent differences in histological appearance between the extirpated lymph node (Fig. 1A) and the bronchoscopically procured specimens (Fig. 1, B to D) are probably crushing artifacts due to differences in sampling.

Immunohistochemistry of Biopsies. An immunohistological analysis of the tumor cells in the biopsy specimens (Table 1) revealed that the neuroendocrine-related antigens recognized by Moab MOC-1, MOC-21, MOC-32, and MOC-52 as well as the epithelial antigen detected by MOC-31 were present in all tumor cells in both pretreatment and recurrent disease biopsies, whereas the neuroendocrine-related antigen recognized by MOC-51 was expressed in the pretreatment specimen only. Immunostaining with Moab RGE 53, which recognizes cytokeratin 18, showed a heterogeneous reaction pattern in the

Biochemical Analysis. DDC determinations were carried out according to Baylin (25). Creatine kinase determinations and isoenzyme analysis were done with the CK Merckotest (Merck, Federal Republic of Germany).

Drug Sensitivity Assay. The Fast green assay as described by Weisenthal et al. was used with slight modifications (26–28). The cells were suspended in fresh Dulbecco’s modified Eagle’s medium (Flow Lab, Irvine, Scotland); F12 nutrient mixture (Flow Lab) (1:1) and 20% FCS with a minimum concentration of 10^6 cells/ml with 10^6 fixed chicken RBC as internal standard and incubated for 1 h at 37°C with doxorubicin, etoposide, melphalan, vinblastine, vincristine, actinomycin, and PBS as a control.

After the incubation, the cells were washed twice and cultured in fresh medium for a period of 4 days. At Day 4, cells were stained for 10 min with 1% (w/v) Fast green (Sigma, St. Louis, MO) in PBS. Living tumor cells excluded the dye and were identified after hematoxylin-eosin staining. The dead tumor cells as well as the fixed chicken RBC, which are oval and nucleated, stained green. The ratio of living tumor cells was determined for each triplicate of cytospin preparations at each drug concentration. The results are expressed as a percentage of control. Each indicated point, in Fig. 5, is the mean of triplicate determinations in two independent experiments. The areas under the curve (Fig. 6) are expressed as a percentage of the area obtained after a presumed 100% survival at all drug concentrations.

Statistical Analysis. The results of the Fast green assay were analyzed for statistical significance by the Student t test. The test was considered positive for P < 0.05.
biopsy specimens. A majority of tumor cells stained positively in the pretreatment and the second recurrence biopsy (Biopsies 1 and 4). The biopsies obtained before and after the first reinduction chemotherapy (Biopsies 2 and 3) contained a minority of tumor cells positively reacting with RGE 53. The M, 210,000 and 68,000 components of neurofilaments, as detected by Moab MNF, were not encountered in the tumor cells present in any of the assessed biopsy specimens (Table 1).

Growth Characteristics of Cell Lines. All cell lines (GLC-14, GLC-16, and GLC-19, established from Biopsies 1, 3, and 4, respectively; see also patient history and establishment of cell lines in "Materials and Methods") grew as floating aggregates, in which no central necrosis was apparent (Fig. 2). The aggregates present in GLC-16 and GLC-19 had a more loose appearance than those in GLC-14. The culture-doubling time was 65, 40, and 40 h for GLC-14, GLC-16, and GLC-19, respectively. The cloning efficiency in semisolid medium as defined earlier (23) was 10% for all three cell lines.

Immunochemical Analysis of the Cell Lines. In agreement with the antigenic profile of the biopsies from which they were derived, all cell lines expressed the neuroendocrine-related SCLC differentiation antigens characterized by MOC-1, MOC-21, MOC-32, and MOC-52, whereas the neuroendocrine-related antigen recognized by MOC-51 was present in GLC-14 only (Table 1). Slight discrepancies were noted, however, in the expression of intermediate filament proteins. Although cytokeratin 18, as probed with RGE 53, was not equally well expressed in the assessed biopsies, all matching cell lines appeared to contain this intermediate filament protein in the vast majority of cells (Table 1). In addition, although no neurofila-
ments were present in any of the assessed biopsies, these intermediate filament proteins, as probed with MNF, proved to be present in <10% of the cells in both GLC-14 and GLC-16 (Table 1). Also vimentin was recognized in GLC-14 and GLC-16. None of the cell lines expressed gastrin-releasing peptide.

Enzyme Content of the Cell Lines. Creatine kinase proved to be present completely in the brain-type isoenzymatic form (CK-BB). The CK-BB and DDC content of the different cell lines are given in Table 1.

Chromosome Analysis. In all cell lines numerous karyotypic abnormalities were found. These can be summarized as follows.

From the cell line GLC-14, 5 metaphases have been analyzed. We found a 77,XX,+1,+4,+4,+5,+5,+6,+6,+8,+9,+9,+12,+14,+15,+17,+17,+18,+19,+20,+20,+21,+22,+der(2),+der3t(3;?), +der3t(3;?), +del(8), +del(8), +11q+, +12q+, +16q+, 19p+ chromosome pattern and related karyotypes. In three of the five metaphases double minutes were found (Fig. 3, top).

From GLC-16, 7 metaphases were analyzed. We found a 77,XX,+1,+2,+4,+4,+11,+12,+12,+14,+14,+15,+18,+20,+20,+20,+21,+21,+22,+Xp+, +der11(1;?), +der3t(3;?), +6q+, +der7t(7;?), +der9t(9;10), +11q+, +11q+, +16q+, +17p+, +19p+, +21p+, +m1,+m2,+m3 chromosome pattern and related karyotypes. In 3 of the 7 metaphases, double minutes were present (Fig. 3, middle).

From GLC-19, 6 metaphases were analyzed. The chromosomal pattern was 78,XX,+1,+2,+7,+8,+9,+12,+12,+14,+14,+14,+15,+15,+16,+21,+21,+21,+21,+der11(1;?), +1p+, +der3t(3;?), +der3t(3;?), +9q+, +11q+, +11q+, +16 markers, and related chromosomal patterns. Two metaphases had double minutes (Fig. 3, bottom).

Electron Microscopy of the Cell Lines. The electron microscopic appearance of all cell lines is compatible with SCLC of the "classic" type. Dense core vesicles are present in moderate amounts in all cell lines, whereas desmosomes with associated tonofilaments were identified in GLC-19 (Fig. 4).

In Vitro Drug Sensitivity Assay. The IC50 in the Fast green assay for doxorubicin increased 3-fold from 0.44 mM for GLC-14 to 1.4 mM for GLC-16 and to 0.75 mM for GLC-19. The IC50 for etoposide increased 12-fold from 4.5 μg/ml for GLC-14 to 53.4 μg/ml for GLC-16 and to 24.8 μg/ml for GLC-19. IC50 values in GLC-14, GLC-16, and GLC-19 for melphalan were, respectively, 0.33, 2.0, and 1.59 μg/ml. For vinblastine, 445, 1803, and 1936 ng/ml. For vincristine, 145.7, 156.0, and 179.9 ng/ml. For actinomycin, 0.337, 0.314, and 0.084 ng/ml (Fig. 5). Fig. 6 summarizes these results by indicating the integrated area present under the curves shown in Fig. 5. Using this parameter, an increase in drug resistance for GLC-16 and GLC-19 for doxorubicin, etoposide, melphalan, and vinblastine can be appreciated.

DISCUSSION

The case history described here is illustrative for the clinical course observed in the majority of patients suffering SCLC. After an initial good response to induction polychemotherapy, often resulting in apparently complete remission, the tumor relapses after 5 to 10 mo, and reinduction chemotherapy is far less successful or fails completely.

In this study biopsies were taken in the course of disease, and cell lines could be established from three of these. These cell
Fig. 3. Chromosomes from GLC-14 (top), GLC-16 (middle), and GLC-19 (bottom). In all three cell lines, both normal and abnormal chromosomes 3 are present. The three cell lines have unique as well as common markers [der3(t3;?) and 11q-], indicating their relationship and common origin. The small arrows indicate chromosomal abnormalities, and the large ones indicate the abnormal chromosome 3.
lines were derived from: (a) the untreated tumor (Biopsy 1, GLC-14); (b) the recurrent tumor, which was sampled immediately after the only partly successful reinduction chemotherapy (Biopsy 3, GLC-16); and (c) persistent tumor, which was sampled after additional radiotherapy later on in the disease (Biopsy 4, GLC-19).

Characterization of these cell lines indicated a slight difference in culture morphology and doubling time between GLC-14 on the one hand (more tight aggregates, 65 h, respectively) and GLC-16 and GLC-19 on the other (more loose aggregates, 40 h, respectively). No clear differences between the different lines were noted in ultrastructural morphology (all cell lines showed moderate amounts of dense core vesicles), cloning efficiency (a high cloning efficiency of 10% was found with all lines), and DDC and CK-BB content (moderately elevated amounts of these enzymes were present in all lines). Thus, despite the different clinical status of the biopsies from which they were derived, all three established cell lines met the type 2 description put forward by Carney (8) and, accordingly, can be characterized as “classic-type” SCLC-derived cell lines. In all three cell lines we found abnormalities of the short arm of chromosome 3, resulting in a 3p-. This result extends the findings of Whang-Peng et al. (30, 31) and our previous finding with three “variant-type” SCLC-derived cell lines (23). Although del 3p has not been found in all assessed SCLC-derived cell lines by karyotypic procedures (32, 33), deletion of parts of

Fig. 4. Electron microscopic appearance of tumor cells of GLC-14 (A), GLC-16 (B), and GLC-19 (C and D). Dense-core vesicles can be appreciated in GLC-14, GLC-16, and GLC-19. Uranyl acetate and lead citrate stain, × 23,290, × 17,000, and × 23,290, respectively. In GLC-19 (D), desmosomes with associated tonofilaments can be identified. Uranyl acetate and lead citrate stain, × 43,766.
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Fig. 5. Fast green assay of cultured GLC-14, GLC-16, and GLC-19 cells after incubation with etoposide (0.625, 3.125, 6.25, 31.25, 62.5 μg/ml), doxorubicin (0.1, 0.5, 1.0, 5.0 μM), melphalan (1.0, 1.75, 2.5, 5.0, 10.0, 25.0 μg/ml), actinomycin (0.05, 0.1, 0.2, 0.3, 0.4, 0.5 μg/ml), vincristine (25, 100, 150, 250, 500, 1000 ng/ml), and vinblastine (150, 250, 500, 1000, 1500, 2000, 2500, 5000 ng/ml) for 1 h. Viable cells versus chicken RBC are counted in the assay and expressed as a percentage of the viable cells versus chicken RBC counted in the control experiment in which the cells are incubated in PBS. For doxorubicin there is a significant difference between GLC-14 and -16 at a drug concentration of 1.0 μM (P < 0.0025). For etoposide there is a significant difference between GLC-14 and GLC-16 at drug concentrations of 0.625 (P < 0.025), 6.25 (P < 0.01), 31.25 (P < 0.005), and 62.5 μg/ml (P < 0.005), and for GLC-14 and GLC-19 at drug concentrations of 0.625 (P < 0.0005), 3.125 (P < 0.01), and 6.25 μg/ml (P < 0.01). For melphalan there is a significant difference between GLC-14 and GLC-16 at drug concentrations of 1.0 (P < 0.0005), 1.75 (P < 0.0025), 2.5 (P < 0.0005), 5.0 (P < 0.05), 10.0 (P < 0.05), and 25.0 (P < 0.0125), and for GLC-14 and GLC-19 at drug concentrations of 1.0 (P < 0.0005), 1.75 (P < 0.0025), 2.5 (P < 0.0005), 10.0 (P < 0.05), and 25.0 (P < 0.005). For vinblastine there is a significant difference between GLC-14 and GLC-16 at drug concentrations of 500 (P < 0.025) and 1000 (P < 0.025), and for GLC-14 and GLC-19 at drug concentrations of 1000 (P < 0.005), 2500 (P < 0.005), and 5000 (P < 0.025). For actinomycin there is a significant difference between GLC-14 and GLC-16 at drug concentrations of 0.2 (P < 0.0125), 0.4 (P < 0.025), and 0.5 (P < 0.05), and for GLC-14 and GLC-19 at drug concentrations of 0.1 (P < 0.0025), 0.2 (P < 0.005), 0.3 (P < 0.025), and 0.5 (P < 0.005).

Fig. 6. The areas under the curve are expressed as a percentage of the area obtained for a presumed 100% survival at all drug concentrations. There is an increase in drug resistance in posttreatment cell lines (GLC-16, GLC-19) for etoposide (etop), doxorubicin (doxo), melphalan (mel), and vinblastine (vinbl). Drug resistance remained unchanged or decreased for actinomycin (act) and vincristine (vincr). The short arm of chromosome 3 can be demonstrated in all assessed SCLC cases by molecular-biological methods (34). Therefore this deletion might be causally related to the etiology of SCLC. All cell lines showed oncogene amplification (see Table 1).4

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The in vitro drug sensitivity of the different cell lines (Figs. 5 and 6) reflected the clinical development of drug resistance. The cell line GLC-14, which was established from the untreated tumor, turned out to be most sensitive, whereas GLC-16, which was obtained immediately after the first induction chemotherapy, was most refractory to both doxorubicin and etoposide. GLC-19, which was cultured from the tumor biopsy obtained after additional radiotherapy later on in the course of disease, was more sensitive to etoposide and doxorubicin than GLC-16. Since only radiotherapy (and no additional chemotherapy) was given, an in vivo partial reversal to a more drug-sensitive phenotype could have taken place, at least if a possible additional development of resistance to radiotherapy is accomplished by a different mechanism than the development of refractoriness to chemotherapy. In contrast, GLC-19 showed greater drug resistance to melphalan than GLC-16. This may be due to resemblances in mode of action between alkylating agents and radiation therapy.

Actinomycin, vincristine, and vinblastine were included to assess whether in these series of cell lines the development of a classic drug resistance phenotype (35) could be identified. Only vinblastine, however, showed greater drug resistance in GLC-16 and GLC-19 as compared to GLC-14.

The antigenic makeup of both biopsies and matching cell lines has been investigated with the aid of a panel of Moab (Table 1). In general, it was found that the different cell lines expressed the same set of antigens as the biopsies from which they were derived, indicating that in vitro culturing causes, at least for these tumor cell characteristics, no major changes. Concerning the expression of the SCLC-related neuroendocrine antigens, it was found that the pretreatment biopsy [as well as the matching cell line (GLC-14)] expresses the antigen detected by MOC-51, whereas the posttreatment biopsies and matching cell lines (GLC-16 and GLC-19) did not. The other neuroendocrine-related antigens (detected by MOC-1, MOC-21, MOC-32, and MOC-52) were equally well expressed in all assessed biopsies and matching cell lines. None of the cell lines expressed gastrin-releasing peptide (detected by 2A11). We have reported earlier a diminished presence of neuroendocrine differentiation antigens in SCLC, when recurrent disease biopsies were compared to pretreatment specimens (36). In the present case a similar change in (neuroendocrine) differentiation status appears to have taken place. This change was quite moderate, however, and did not imply also a diminished expression of DDC (Table 1) as has been reported in the “variant-type” cell lines. Intratumor heterogeneity was found when the tumor specimens and cell lines were stained with Moab directed against different intermediate filament proteins (Table 1). In agreement with the “classic-type” phenotype of the different cell lines, it was found that cytokeratin 18 was present in GLC-14, GLC-16, and GLC-19. A small percentage of the cells in both GLC-14 and GLC-16 turned out to express neurofilaments and vimentin as probed by Moab MNF and VIM. Until now, neurofilament expression in SCLC was found to be confined to “variant-type” SCLC cell lines (10). Therefore these cell lines may contain a minor “variant-type” component.

The series of cell lines described in this paper do not fit in the general distinction of therapy-sensitive “classic-type” and therapy-resistant “variant-type” cell lines. This distinction has been based on the characterization of over 50 established SCLC-derived cell lines, demonstrating an apparent digimomy in both gross morphological appearance in culture, the expression of a series of biomarkers, and sensitivity to radiation-induced cell kill (7-9, 11). About 70% of the cell lines could be classified as “classic-type” and about 30% as “variant-type” (8). “Variant-type” cell lines have been proposed to represent the in vitro counterpart of the poor prognosis, mixed small cell/large cell SCLC subtype (11). In addition, it appears that they become established from pretreated patients more often (8, 37). In contrast to “classic-type” SCLC-derived cell lines, “variant-type” lines contain no DDC (8), express no cytokeratins (10), have a shorter doubling time (7), and are relatively radioresistant (9). Although a conversion from “classic” to “variant” phenotype might explain the clinical development of resistance to therapy in some cases, other mechanisms are likely also to be operative in the apparently almost always occurring change from therapy responsiveness to nonresponsiveness in SCLC. This is so because only 10% of SCLC patients have tumors showing a histology in which large cell admixtures are present (38). At autopsy, the percentage of cases in which non-SCLC histologies are visible has only moderately increased to 13 to 28% (39-41). Despite this, the majority of these tumors must have been completely refractory to treatment.

The cell lines GLC-16 and GLC-19 described in this paper could be the in vitro counterpart of the therapy-resistant SCLC tumors in which no admixtures of non-SCLC histology are apparent, whereas GLC-14 reflects the therapy-sensitive form of SCLC. All cell lines can be clearly classified as “classic-type,” because of their culture morphology, DDC content, presence of dense core vesicles, and cytokeratin. The only phenotypical change detected between GLC-14 on the one hand and GLC-16 and GLC-19 on the other was the loss of a neuroendocrine differentiation antigen (defined by MOC-51). Since the in vitro drug sensitivity assays reflected the in vivo development of drug resistance, it is concluded that the series of cell lines described here, which were obtained during intradividual follow-up, might provide a relevant in vitro model to study the development of drug resistance in the clinic.

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