Differential expression of intermediate filament proteins distinguishes classic from variant small-cell lung cancer cell lines

(cytokeratins/intermediate filaments/vimentin/immunoblotting/immunofluorescence)

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ABSTRACT The expression of intermediate filament proteins in characteristic and variant-type small-cell lung carcinoma (SCLC) cell lines was studied using immunocytochemical techniques, two-dimensional gel electrophoresis and immunoblotting assays. Classic SCLC cell lines contain cytokeratin proteins but no neurofilament proteins. In contrast, variant cell lines do not contain detectable amounts of cytokeratins but partly express neurofilaments and vimentin. These results explain apparent discrepancies on the intermediate filament content of SCLC described in the recent literature. The application of antibodies to fresh biopsy specimens of SCLC may in the future allow the identification of the variant type of small-cell lung carcinoma (SCLC) cells (1–8). Results so far have been inconsistent, probably because of the use of different antisera and detection methods. Furthermore, it has been recognized that SCLC is often a very heterogeneous tumor in which in a considerable number of cases a large-cell type can be distinguished (9–12).

SCLC accounts for 20–25% of all new cases of primary lung cancer, and unlike the other major forms of lung cancer, it is highly sensitive to both chemotherapy and radiation therapy (13). Recently, several laboratories have reported on their success in the establishment of continuous cell lines of SCLC (1, 14–16). In general, SCLC cell lines grow as floating aggregates of cells, show electron-microscopically detectable dense core vesicles, and express elevated levels of a variety of biomarkers including the key amine precursor uptake and decarboxylation (APUD) enzyme, L-3,4-dihydroxyphenylalanine decarboxylase (dopaDCase), bombesin-like immunoreactive substances (BLI), neuron-specific enolase (NSEase), and the BB isoenzyme of creatine kinase (CK-BB). With few exceptions these markers are not expressed in cell lines of non-SCLC (14, 17–19).

In a recent study of a large panel of SCLC cell lines, it has been shown that these cell lines can be subdivided into two major categories, i.e., classic (70% of all lines) and variant (30%) cell lines (20). Variant cell lines do not express elevated levels of dopaDCase or BLI but continue to express elevated levels of NSEase and CK-BB (11, 20). In addition and in contrast to classic cell lines, variant cell lines have a much looser morphology in vitro, a higher cloning efficiency, and faster doubling time and are radioresistant in vitro (11, 20, 21). Finally, amplification of the c-myc oncogene (20 to 25-fold) at the DNA level and increased c-myc mRNA expression have been observed in variant but not in classic SCLC cell cultures (22).

These data suggest a more malignant behavior for the variant SCLC cell lines, which may in part be related to c-myc amplification and expression in these cells. As clinical correlates of this variant phenotype (most probably represented by the large-cell type within SCLC) may exist and be associated with a worse prognosis (12, 23), the development of assays that can distinguish classic and variant phenotypes of SCLC in biopsy specimens may be of therapeutic significance.

Since different types of intermediate filament proteins have been shown to occur in different types of tissues and tumors (24–26), detection of such constituents in SCLC was likely to give an indication about the nature of the different cell types present in such malignancies. In light of previously-published data (1–5), antibodies directed against cytokeratins and the neurofilament proteins are especially interesting in this respect.

MATERIALS AND METHODS

Cell Lines. The establishment and characterization of the NCI cell lines have been described in detail elsewhere (10, 11, 14, 15, 17–22). NCI-H69, NCI-H128, NCI-H449, GLC-1-M13, GLC-7, GLC-8, and GLC-12 have been classified as classic SCLC cell lines, while NCI-H82, NCI-N417, NCI-H524, GLC-1, GLC-2, and GLC-4 have been characterized as variant SCLC cell lines. The characterization of GLC-1, GLC-2, GLC-4, GLC-1-M13, GLC-7, GLC-8, and GLC-12 will be described elsewhere. GLC-1-M13 was obtained by limiting dilution of GLC-1 in serum-free medium.

In these studies, cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 15% fetal bovine serum (GIBCO) and maintained in a humidified incubator at 37°C in 5% CO2/95% air.

Estimation of NSEase activity has been described (18). dopaDCase specific activity was calculated as nmol of 14C released from L-3,4-dihydroxyphenylalanine decarboxylase.

Abbreviations: SCLC, small-cell lung carcinoma; NSEase, neuron-specific enolase; dopaDCase, L-3,4-dihydroxyphenylalanine decarboxylase.
**Antisera.** Antisera used in this study were as follows: (i) An affinity-purified polyclonal antiserum directed against human skin keratins (pK): This antiserum reacts with virtually all epithelial tissues but not with nonepithelial tissue (25). In most cases of SCLC frozen sections, this antiserum stains the tumor cells when used in combination with cytochemical procedures (3). (ii) Monoclonal antibodies RGE53 and CK18-2, both directed against cytokeratin 18, which specifically recognize columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues, and mesothelial cells: No reaction is found in squamous epithelial or nonepithelial tissues (28). The antibodies react with SCLC in frozen sections (3). (iii) Monoclonal antibodies directed against different neurofilament polypeptides, BF10 and RT97, were provided by Brian Anderton (London): In immunoblotting assays BF10 was shown to react only with the 155-kDa neurofilament protein, while RT97 reacts mainly with the 210-kDa neurofilament protein (29). A third monoclonal antibody to neurofilaments (MNF) was purchased from Euro-Diagnostics (Apeldoorn, The Netherlands). In immunoblotting assays, we could show that this antibody reacts strongly with the 210-kDa neurofilament polypeptide and to a somewhat lesser extent with the 68-kDa neurofilament polypeptide. No reaction was found with vimentin, cytokeratins, or with human callus keratins. When frozen sections and paraffin sections from human brain, peripheral nerves, and some neural tumors were used, a positive reaction of monoclonal antibodies to neurofilament proteins (BF10, RT97, and MNF) was found only in the neural tissue. No positive reaction of these neurofilament antibodies was seen in frozen sections of SCLC. (iv) A rabbit antiserum to bovine lens vimentin: Preparation and specificity of this serum have been described (25, 28). (v) Antisera to chicken gizzard desmin and human glial fibrillary acidic protein were prepared as described before (25).

**Indirect Immunofluorescence Technique.** The cell lines, which grow as loose or tight floating aggregates, were either spun down in growth medium using a cytosin centrifuge (Shandon Southern Instruments, Sewickley, PA; 450 rpm for 5 min) or brought onto microscope slides by using the Cytopress system. Cells were fixed by dipping in methanol (−20°C) and acetone (three times), air dried, and incubated with the primary antiserum for 30 min. After repeated washing in phosphate-buffered saline (Pi/NaCl), either goat anti-rabbit antibody coupled to fluorescein isothiocyanate (FITC) (Nordic, Tilburg, The Netherlands) diluted 1:25 in Pi/NaCl or rabbit anti-mouse antibody coupled to FITC (Nordic, Tilburg, The Netherlands) was used as a secondary antibody. After incubation for 30 min, the cells were washed again in Pi/NaCl, mounted in Gelvatol (Monsanto) containing 1,4-diazobicyclo[2.2.2]octane at 100 mg/ml (DABCO, Jansen Pharmaceutica, Beerse, Belgium).

Slides were viewed with a Leitz Dialux 20 EB microscope equipped with epifluorescent illumination. Pictures were taken on 400 ASA Tri-X film (Kodak) with an automatic camera.

**Gel Electrophoresis and Immunoblotting Assays.** Cytoskeletal preparations from the cultured cell lines were made by extracting cell pellets with 1% Triton X-100 in Pi/NaCl. One-dimensional gel electrophoresis was performed in 10% NaDodSO4/polyacrylamide slab gels as described by Laemmli (30). Two-dimensional gel electrophoresis (31) was performed by applying the 4% polyacrylamide rod gels after isoelectric focusing directly onto the stacking gel of the 10% NaDodSO4/polyacrylamide gel. Gels were stained with Coomassie brilliant blue R250.

Immunoblotting assays were performed essentially as described by Towbin et al. (32). Non-washing steps between incubations were performed with Pi/NaCl containing 0.5% Triton X-100. The immunoblots were incubated overnight with the primary antibodies, CK 18-2, undiluted culture supernatant, or MNF, diluted 1:20 in Tris-HCl buffer (pH 7.6) containing 0.3% gelatin and 0.5% Triton X-100. They were then incubated 1 hr in rabbit anti-mouse antibody coupled to peroxidase (DAKO patts, Denmark) diluted 1:200 in Pi/NaCl containing 0.5% gelatin and 0.5% Triton X-100. Peroxidase activity was detected by its reaction with 0.015% 4-chloro-1-naphthol (Merk), 0.06% H2O2 in Pi/NaCl for 10–30 min.

**RESULTS**

**Immunofluorescence.** The intermediate filament characteristics of seven classic and six variant SCLC cell lines and two adenocarcinoma (non-SCLC) cell lines were studied by use of a panel of monoclonal and polyclonal antibodies. The results of these studies are shown in Table 1 and Fig. 1. All seven classic SCLC cell lines contained cytokeratins, as demonstrated by a polyclonal antiserum to human skin keratins and a monoclonal antibody to human cytokeratin 18 (RGE53). Both these antisera reacted with intracellular filaments. In most cases not all cells of the classic SCLC cell lines reacted with the cytokeratin antibodies used and no neurofilament proteins were detected (Fig. 1 A and B). In addition, desmin, glial fibrillary acidic protein, and vimentin could not be demonstrated in classic cell lines.

The intermediate filament characteristics of variant SCLC cell lines were different from those of classic SCLC cell lines in that none of them were positive for cytokeratins (Table 1 and Fig. 1 C). In contrast, MNF, and MNF variant SCLC cell lines, while carrying low amounts of cytokeratins, were positive for vimentin. The staining pattern with the vimentin antiserum varied considerably within the variant SCLC cell lines. In NCI-H82 and NCI-H524, only a few cells reacted weakly positive. In NCI-N417, some cells reacted strongly with the vimentin antiserum while most of the other cells were only weakly stained or were negative. In contrast, GLC-1 and GLC-2 exhibited a strong filamentous staining pattern in all cells with the vimentin antiserum. The variant SCLC cell lines showed considerable heterogeneity when stained with antibodies to neurofilaments. Of the six variant SCLC cell lines evaluated, three cell lines, NCI-H82, NCI-H524, and GLC-2, were found to give a filamentous staining reaction with the neurofilament antibodies in a considerable percentage of the cells (Fig. 1 D) but the number of cells stained with the different monoclonal neurofilament antibodies varied considerably. For example, the antibody MNF stained almost all cells in NCI-H82 and GLC-2 while the antibody BF10 stained only a small number of cells in these cell lines. The remaining three cell lines, NCI-N417, GLC-1, and GLC-4, were negative for both cytokeratin and neurofilaments.

In both adenocarcinoma cell lines a strong filamentous staining reaction with the antisera to cytokeratins and the antiserum against vimentin was observed. No reaction was seen with the antisera to neurofilament proteins, glial fibrillary acidic protein, or desmin.

**Gel Electrophoresis and Immunoblotting.** In two-dimensional gel electrophoresis, all cell lines appeared to contain the microfilament protein actin (indicated as A in Fig. 2 a, b, and c). In classic SCLC cell lines, variable but always low amounts of cytokeratins 7, 8, 18, and 19 could be demonstrated (Fig. 2 a and b). In contrast, no cytokeratin spots were observed in two-dimensional gels of the variant SCLC cell lines (Fig. 2 c). In classic and variant SCLC cell lines, two striking protein spots occurred at the level of vimentin (57 kDa; pi 5.7); however, the presence of vimentin could not be confirmed by immunoblotting assays of cytoskeletal preparations of these cells (results not shown). When taking into account the negative reaction of classic SCLC cell lines with antibodies to vimentin in the immunofluorescence technique, it is very unlikely that these protein spots represent vimentin. The two protein spots do not occur in...
two-dimensional gels of non-SCLC cell lines (results not shown).

Immunoblotting assays could confirm our findings with the immunofluorescence technique. The presence of cytokeratin 18

Table 1. Intermediate filament protein expression by immunofluorescence, presence of NSEase and dopaDCase activity, and c-myc oncogene expression in classic and variant SCLC cultures and in adenocarcinoma cultures

<table>
<thead>
<tr>
<th>Cell line</th>
<th>NSEase</th>
<th>dopaDCase activity*</th>
<th>c-myc expression</th>
<th>Cytokeratin</th>
<th>Neurofilaments</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pK</td>
<td>RGE53 BF10 RT97 MNF Vimentin Desmin GFAP</td>
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<td>Classic SCLC</td>
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<tr>
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<td>264</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
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<td>174</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>98</td>
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<td>Non-SCLC</td>
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- no reaction in immunofluorescence test; +, filament staining in a varying number of cells; ++, strong filament staining in virtually all cells.

GFAP, glial fibrillary acidic protein.

*Expressed as nmol of [14C] released from L-[14C]dopa per hr per mg of protein assayed.
could be demonstrated by using monoclonal antibody CK18-2 but only after heavily overloading the gels with cytoskeletal proteins (Fig. 3, lanes 1, 2, and 3). No reaction was seen with CK18-2 in immunoblotting of the variant SCLC cell lines (results not shown). Immunoblotting assays with the variant SCLC cell lines using the antibody MNF resulted in a clearly positive reaction with NCI-H82 at the 68-kDa level (Fig. 3, lane 5), while a very faint reaction was observed with NCI-H524 (Fig. 3, lane 6), and no reaction could be seen with NCI-N417 (Fig. 3, lane 4). These immunoblotting data agree with the results of the immunofluorescence experiments in which NCI-H82 was completely positive for antibody MNF, NCI-N417 was completely negative, and in NCI-H524 only a small number of cells were stained with this antibody. Classic SCLC cell lines did not react with antibody MNF (results not shown).

**DISCUSSION**

In the past few years, several investigators have tried to elucidate the origin of SCLCs. After the demonstration of neuroendocrine granules in these neoplasms by Bensch et al. (33), the presence of other neuroendocrine markers was described (34). The nature of the intermediate filament proteins in these tumors is still a matter of discussion. Antibodies to intermediate filament proteins have become available that react in a tissue-specific manner (24); for example, antibodies to cytokeratins react only with epithelial tissues and carcinomas while antibodies to neurofilament proteins react with tissues of neural origin and some neural tumors (25, 26). Application of such antibodies to SCLCs has resulted in contradictory findings (1-8). Lehto et al. (4) have reported the expression of neurofilament protein and no cytokeratin in such neoplasms while others (2, 3, 5) have obtained the opposite result; i.e., only cytokeratin intermediate filaments and not neurofilaments are identified.

Our findings explain these discrepancies and have led us to suggest the following hypothesis. The SCLC solid tumors and SCLC cell lines that have been described as containing neurofilaments but not cytokeratin (1, 4) most probably represent the variant type of SCLC, while those tumors and cell lines described as having cytokeratin and no neurofilaments (2, 3, 5) most probably represent the classic type of this neoplasm. However, the inability to detect the presence of cytokeratins in SCLCs (1, 4, 6, 8) may be due in part to the low amount of cytokeratins present in SCLCs as demonstrated by our immunoblotting assays and two-dimensional gel electrophoresis. Furthermore, fixation procedures can result in the destruction of cytokeratin antigenic sites. Finally, the type of cytokeratin antiserum used is very critical in the detection of these intermediate filaments.

Vimentin intermediate filaments could be detected only in the non-SCLC and the variant SCLC cell lines, not in the classic SCLC cell lines. It should be stressed, however, that in three of the variant SCLC cell lines, only a few cells contained these filaments. Coexpression of vimentin filaments with other types of intermediate filaments is often observed in cultured cells (35) and it seems to be the result of adaptation of the cells to the environment or to the survival of cells as individuals that are not part of a cohesive tissue or cell cluster (36). Our findings with the anti-vimentin antibody suggest a correlation between growth pattern and morphology of the cells in vitro and the expression of this intermediate filament type. Cells growing attached to the surface of the culture dish (such as the non-SCLC H23 and GLC-1 and GLC-2 lines) express vimentin, while those growing as floating aggregates express much less or no vimentin at all. Within this latter group, a subdivision can be made even between cells growing as loose clusters (most variant cell lines) that contain small amounts of vimentin in a few cells and cells growing as tightly packed floating aggregates (the classic cell lines) that are negative for vimentin.

The coincidence of the cell and tissue specificity of intermediate filament proteins with the current concepts of embryologic derivation of the different cell lineages allows us to speculate about some aspects of the ongoing discussion dealing with the histogenetic origin of SCLCs. Since primary SCLC and all classic SCLC cell lines contain cytokeratins (refs. 2, 3, and 5 and this paper), it is obvious that these cells

**Fig. 2.** Details of two-dimensional gels showing cytoskeletal proteins from classic SCLC cell lines (a and b) and a variant SCLC cell line (c). Note the presence of cytokeratins 7, 8, 18, and 19 (indicated as such) in the two classic SCLC cell lines (a, NCI-H128; b, NCI-H69) and the absence of cytokeratins in the variant SCLC cell line (c, NCI-H82). IEF, isoelectric focusing; A, actin.

**Fig. 3.** Immunoblots of a cytokeratin antibody and a neurofilament antibody on cytoskeletal preparations from classic and variant SCLC cell lines. Note the positive reaction of NCI-H449 (lane 1), NCI-H128 (lane 2), and NCI-H69 (lane 3) with the monoclonal antibody to cytokeratin 18 (CK18-2). This antibody was negative on heavily overloaded gel blots from variant SCLC cell lines. With the monoclonal neurofilament antibody MNF a protein band from the variant SCLC cell line NCI-H82 (lane 5) migrating in the 68-kDa region is immunostained. A very faint reaction was also seen with NCI-H524 (lane 6) while no reaction is seen with MNF and NCI-N417 (lane 4).
are epithelial in nature. Therefore, the origin of SCLC from a hematopoietic stem cell as suggested by Ruff and Pert (37) seems to be unlikely. Furthermore, the presence of cytokeratin filaments and absence of neurofilaments within a tissue or tumor does not deny its neuroendocrine nature (compare refs. 1 and 7) since several neuroendocrine tissues and tumors have been shown to contain cytokeratin intermediate filaments (38, 39). Other neuroendocrine tumors, such as Merkel cell tumors, may contain both cytokeratin and neurofilament types of intermediate filament proteins (40, 41). Van Muijen et al. (5) have reported the coexpression of cytokeratins and neurofilament proteins in an epidermoid cell carcinoma of the lung, while Bergh et al. (1) have described a cell line derived from a large-cell carcinoma of the lung that also shows this coexpression.

Therefore, the presence of neurofilaments and absence of cytokeratins in the variant SCLC cell lines raises several questions with respect to the origin and differentiated state of these cells. Future studies with other markers will have to show whether these cells are derived from cells that originally contain both cytokeratins and neurofilaments, as suggested by Bergh et al. (1) or whether they have (de)differeniated from cells positive for cytokeratin only via an intermediate cell type that is negative for both cytokeratins and neurofilaments. The fact that the variant SCLC shows a particularly low sensitivity to chemo- and radiotherapy (12, 21, 23) and that patients with such tumors have a significantly worse prognosis than patients with classic SCLC makes it particularly important to recognize the variant phenotype in diagnostic biopsies. The use of antibodies to cytokeratin and neurofilament proteins and other intermediate filament proteins in a rapid immunohistochemical technique may therefore become a valuable tool in the pathological diagnosis of these tumors.

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