Identification of cytoskeletal structures
in hormone producing lung cancer cell cultures

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With 7 figures and 2 tables in the text

Abbreviations

SCLC: small cell lung cancer, APUD: amine precursor uptake and decarboxylation, DDC: L-dopa-
decarboxylase, CK-BB: The BB isoenzyme of creatine kinase, NSE: neuron-specific enolase.

Introduction

Ever since the detection of neuroendocrine granules in small cell lung cancer (SCLC; Bensch et al. 1968) this type of lung tumor has been designated neuroendocrine, and has been suggested to be derived from cells with amine precursor uptake and decarboxylation (APUD; Pearse and Polak, 1972) activity, for example the Kulchitsky cells.

Evidence to support this hypothesis has been found in the presence of many APUD-characteristics, such L-dopa-decarboxylase (DDC; Baylin et al. 1980), the BB-iso-
enzyme of creatine kinase (CK-BB; Gazdar et al. 1981), and neuron-specific enolase
NSE; Marangos et al. 1982) in such tumors.

Also several neuropeptides and hormones such as bombesin (Moody et al. 1981),
serotonin (Horai et al. 1973), ACTH (Horai et al. 1973, Ratcliffe et al. 1982) and calci-
tonin (Silva et al. 1974, Luster et al. 1982) have been demonstrated in SCLC. However,
elevated levels of ectopic hormones such as ACTH, calcitonin, beta-HCG, and parat-
hormone could also be demonstrated in sera of patients with non-SCLC (Gropp et al. 1980, Gropp et al. 1981).

Results in the examination of lung carcinoma cell lines sofar have confirmed that
cell lines derived from a certain type of tumor represent many of the characteristics
of the original tumor. Morphologically, similar histological types as the original tumor
recur in xenografts of cell lines in nude mice (Gazdar et al. 1980, Sorensen et al. 1981,
Carney et al. 1985, Luster et al. 1985). The production of many neurohormones is
retained in cell lines from SCLC (Sorensen et al. 1981, Carney et al. 1985, Luster et al. 1985)
and from non-SCLC (Luster et al. 1985). In addition, the presence of comparable
types of intermediate filaments (IF) in solid tumors and cell lines has been demonstrated

Intermediate filaments (IF) occur in almost all cell types and appear to have a
tissue-specific protein composition (Osborn and Weber 1981). Based upon their IF

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protein pattern the tissue origin of a given cell type can be determined, using biochemical and immunocytochemical techniques. For example, epithelial cells contain IF of the cytokeratin type while cells of mesenchymal origin contain vimentin as their IF protein. Vimentin is also expressed in many cell types when brought into tissue culture (Franke et al. 1978). Muscle tissue contains IF of the desmin type, glial cells contain glial fibrillary acidic protein (GFAP) as their IF-subunit and the IF in neural cells consist of the neurofilament (NF) protein triplet (Dahl and Bignami 1977). Normally, tumors derived from these tissues retain their typical intermediate filament protein pattern (Osborn and Weber 1983, Rammekers et al. 1983a), although some exceptions have been described (Holthöfer et al. 1983, Holthöfer et al. 1984). This also holds true for cell lines obtained from such neoplasms. Therefore, immunohistochemical staining of intermediate filaments in tumors and cell lines has proven to be a useful tool to determine their origin.

In order to characterize the IF patterns of a panel of hormone producing lung tumor cell cultures during early outgrowth, we have chosen a large panel of IF antisera, most of which directed against (cytokeratins, which appear to occur in most, if not all lung carcinomas; Blobel et al. 1984, Blobel et al. 1985, Kahn et al. 1983, Broers et al. 1985a, van Muilen et al. 1984). The cell biological significance of our findings is discussed. Furthermore antibodies to the neurofilament proteins were applied since recently we have described the expression of this type of IF in a certain subtype of pulmonary neuroendocrine tumors (Broers et al. 1985b).

Materials and methods

Cell cultures. Methods used for the establishment and characterization of the cell cultures used in this study and the determination of their hormone producing activity have been previously described (Luster et al. 1985). All cell cultures except MR 21 grew attached to the bottom of culture flasks. By selecting MR 21 cells which tended to grow attached to the bottom of culture flasks, subline MR 21B could be established, which grew as an adherent culture.

All cell cultures were maintained in Eagle's Modified Minimum Essential Medium (Flow Laboratories, Irvine, Scotland, U.K.) containing 16.6% fetal bovine serum (GIBCO, Paisly, U.K.) or in Roswell Park Memorial Institute Medium 1040 (RPMI-1040, GIBCO, Paisly, U.K.) supplemented with 15% fetal bovine serum (GIBCO, Paisly, U.K.) and maintained in a humidified incubator at 37 °C in 5% CO₂.

Flow cytometry. For flow cytometric DNA analysis cells were sedimented at room temperature at 500 × g for 5 minutes. The supernatant was discarded, but fluid sticking to the wall of the tubes was allowed to cover the pellet. The pellet was vortexed vigorously and the cells were resuspended in the small volume of medium. Then 70% ethanol (−20 °C) was added rapidly under constant vortexing. The final cell concentration was about 1 million cells per ml ethanol. At this stage the cells could be stored at −20 °C for several days.

A sample of approximately 1 million cells was taken from this suspension and after centrifugation the cell pellet was resuspended in 1 ml of a PI solution (20 µg/ml Agrade in 150 mM sodium phosphate buffer pH 7.4; Calbiochem, Boehringer Corp., La Jolla, CA, USA). To 1 ml of this cell suspension 0.1 ml of a stock solution of RNase (1% type A in sodium phosphate buffer; Sigma St. Louis, Mo., USA) was added and the suspension was incubated for exactly 10 min at 37 °C. Then the cell suspension was filtered through a 100 micron nylon filter (Ortho Diagnostic Systems, Beeve, Belgium). Cells were kept in the dark at room temperature prior to flow cytometry analysis.

Cell analysis was performed using a Cytofluorograph 50H (Ortho Instruments, Westwood, Mass., USA). The fluorochrome PI was excited at 488 nm with an argon ion laser (Spectra Physics, Mountain View, Ca., USA). Fluorescence was measured using a 630 nm longpass filter. All data were stored on a PDP 11/34 computer (Digital Equipment Corporation, Maynard, Mass., USA) for subsequent data analysis. For determining the DNA index chicken red blood cells served as an internal standard and human lymphocytes as an external standard (Jakobsen 1983). Cell cycle analysis was done for the characterization of the G1, S and G2M phases (Buischer et al. 1975).
Intermediate filament antibodies. The following antibodies were used in this study:

A) A panel of antibodies directed against cytokeratins.

1. An affinity purified polyclonal antibody directed against human skin keratins (pKer). This antibody reacts with virtually all epithelial tissues, but not with non-epithelial tissues (Ramaekers et al. 1983a).

2. The monoclonal antibody RGE 53 directed against cytokeratin 18, specifically recognizes columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues and mesothelial cells. No reaction is found in squamous epithelial or non-epithelial tissues (Ramaekers et al. 1983b). In immunoblotting assays RGE 53 was shown to react with a 45 kD cytokeratin protein (cytokeratin 18 according to the catalogue by Moll et al. 1982) only present in some epithelial tissues or epithelial cell cultures.

3. The monoclonal antibody CK16-2 raised against human mesothelial cells and also directed against cytokeratin 18. This antibody gives a similar staining pattern as RGE 53 when tested on human tissues. In immunoblotting assays this antibody only recognizes a 45 kD cytokeratin polypeptide and no other cytokeratins, nor does it react with non-epithelial cytoskeletal proteins.

4. The monoclonal antibody RKSE 66, directed against human skin keratins and specific for keratinizing stratified squamous cells. No reaction is found with columnar epithelial cells, non-keratinizing squamous cells or non-epithelial cells (Ramaekers et al. 1983a).

5. The monoclonal antibody PKK1 (Holthöfer et al. 1983, Holthöfer et al. 1984) kindly provided by Dr. I. Virtanen (Dept. of Pathology, University of Helsinki, Finland), was obtained after immunization of mice with cytokeratin polypeptides from a pig kidney epithelial cell line (LLC-PK).

PKK1 antibodies do not react with epidermal cells, but strongly stain all glandular and simple epithelial cells in human tissues. In immunoblotting assays PKK1 reacts with several cytokeratin polypeptides, including cytokeratins 8 and 18. PKK1 does not react with non-epithelial tissue.

6. The monoclonal antibodies AE1, AE2 and AE3 (Woodcock-Mitchell et al. 1982, Eichner et al. 1984, Cooper et al. 1985) were kindly provided by Dr. T.-T. Sun (Dept. of Dermatology, New York University Medical Centre) and were obtained after immunization of mice with human epidermal keratin.

The AE1 antibody reacts with cytokeratins 10, 14/15, 16 and 19 (Cooper et al. 1985).

In immunofluorescence the AE2 antibody stains the super-basal cell layers in the epidermis, but is negative in other epithelia (for example esophagus, urinary bladder or pancreas).

AE3 uniformly stains all epithelia tested and reacts with cytokeratin 1 to 8 (Cooper et al. 1985). Thus, the cytokeratins recognized by AE1 are the most acidic polypeptides (type I cytokeratins) within this protein family, while those recognized by AE3 represent the more basic polypeptides (type II cytokeratins). Using the three different antibodies no reaction was found with fibroblasts, muscle cells, fat cells, neural tissues, endothelial cells and blood cells.

7. The monoclonal antibodies LE41, LE61 and LE65 (kindly provided by Dr. E.B. Lane, IRCF, London) were obtained after injection of mice with cytoskeleton preparations of cultured PtK1-cells (Lane 1982). The monoclonal antibody LE41 is monospecific for cytokeratin 8, while LE61 and LE65 are both monospecific for cytokeratin 18. In frozen sections, LE61 and LE65 give tissue staining patterns comparable with biochemical data on the distribution of cytokeratin 18, while LE41 sees only some of the tissues known to contain cytokeratin 8, presumably recognizing a more variable aspect of the protein's conformation. No reaction is seen with any of these three antibodies in any stratified squamous epithelia. The antibodies LE41, LE61 and LE65 do not react with non-epithelial cells.

8. The monoclonal antibodies LP1K, LP2K, LP3K and LP6K (kindly provided by Dr. E.B. Lane, IRCF, London) were raised against detergent insoluble material from SV40-transformed human keratinocytes. LP2K is monospecific for cytokeratin 19, and stains most simple epithelia and basal cells in stratified squamous epithelia which are not keratinizing (Lane and Peckis, manuscript in preparation). LP1K recognizes many of the basic type II cytokeratins, and stains simple epithelia but not epidermis in unfixed frozen sections. LP2K and LP6K also recognize predominantly simple epithelia, and react principally with cytokeratin 8.

9. The monoclonal antibody LP34 (kindly provided by Dr. E.B. Lane, IRCF, London) recognizes all human epithelia tested by immuneroxidase on unfixed frozen sections, and by immunoblotting binds to several cytokeratin polypeptides of both type I (acidic) and type II (basic) groups. It was raised by immunizing mice with human stratum corneum material (Lane et al. 1985).


B) Antibodies directed against vimentin.

1. A polyclonal antibody (pVTM) to bovine lens vimentin. Purification and characterization of the polyclonal rabbit antibody have been described in detail elsewhere (Ramekers et al. 1983a).

2. A monoclonal antibody (mVIM) directed against bovine lens vimentin, purchased from Euro-Diagnostics B.V. (Apeldoorn, The Netherlands). This antibody stains tissues of mesenchymal origin, such as fibroblasts, endothelial cells, some blood vessel smooth muscle cells, glomeruli in the kidney etc., but does not stain most epithelial tissues. In cultured cells (bovine lens cells, BHK-21 cells, PtK2-cells) the monoclonal antibody shows a filamentous staining pattern, identical to the pattern observed with the polyclonal antibody to vimentin. In immunoblotting assays this monoclonal antibody gives a specific positive reaction with the vimentin band in cytoskeletal extracts from human leiomyosarcoma, bovine lens, BHK-21 and LLCMK-2 cultures.

C) A panel of antibodies directed against the neurofilament proteins.

1. A monoclonal antibody to neurofilaments (MNF) purchased from Euro-Diagnostics B.V. (Apeldoorn, The Netherlands). This antibody corresponds to the monoclonal antibody 2F11 described by Kliš et al. (1984). In immunoblotting assays we could show that this antibody reacts strongly with the 210 kD neurofilament polypeptide and to a somewhat lesser extent with the 68 kD neurofilament polypeptide. No reaction was found with vimentin from a bovine lens extract.

Using frozen sections and paraffin sections from human brain, peripheral nerves and some ganglioneuromas a positive staining reaction with this monoclonal antibody was found only in the neural tissue.

2. Two monoclonal antibodies BF10 and RT97 directed against different neurofilament polypeptides (Anderson et al. 1982), were kindly provided by Dr. Brian Anderson (St. Georges Hospital Medical School, London). In immunoblotting assays BF10 was shown to react only with the 155 kD neurofilament protein, while RT97 reacted mainly with the 210 kD protein. Both antibodies were shown to react exclusively with neural tissues.

D) A rabbit antibody to chicken gizzard muscle desmin.

Preparation and specificity of this antibody have been described in detail elsewhere (Ramekers et al. 1983c).

E) A rabbit antibody to glial fibrillary acidic protein (GFAP) from human spinal cord, which has been described earlier (Ramekers et al. 1983a).

Other antibodies and reagents used in this study.

F) For the detection of microtubuli a monoclonal antibody against beta-tubulin was used. This antibody has been described in detail elsewhere (van Bergen en Henegouwen et al. 1985).

G) For staining of microfilaments 7-nitrobenz-2-oxa-1,3-diazole-phallacidin (Molecular Probes Inc., Junction City, Oregon, USA) was used. This conjugate has been described elsewhere (Barak et al. 1980).

H) As second antibodies in the indirect immunofluorescence technique either fluorescein isothiocyanate (FITC)-conjugated goat anti rabbit IgG (1:25, Nordic, Tilburg, The Netherlands) or FITC-conjugated rabbit anti mouse IgG (1:25, Nordic, Tilburg, The Netherlands) were used. In control experiments PBS was used instead of the primary antibody.

Immunofluorescence- and immunoperoxidase techniques. For indirect immunofluorescence microscopy all cell cultures except MR21 were grown on glass cover slips (18x18 mm) in plastic petri dishes. Cells from the cell cultures MR21, which grows as tight floating aggregates, were spun down onto glass slides using a Cytospin centrifuge (Shandon Southern, 800 rpm for 10 min). To retain a good preservation of the cell shape and to prevent possible denaturation of cytokeratins and other IEP upon fixation cells were routinely fixed by dipping in methanol for 5 sec (−20=DBC) and acetone (3 times 5 sec), air dried and thereafter incubated with the primary antibody for 30 to 45 min at room temperature.

After repeated washing in PBS the appropriate FITC-conjugated antibodies were applied to the cells. After incubation for 30–45 min the cells were washed again in PBS, and mounted in Gelvatol (Mosaic, USA) containing 1,4-Diazolityclo-2,2'-oxane (DACO; Janssen Pharmaceutica, Beerse, Belgium) as described before (Langanger et al. 1983). Slides were viewed with a Leitz Dialux 20 EB microscope equipped with epifluorescent illumination. Pictures were taken using a 400 ASA Tri-X film (Kodak) with an automatic camera.
In the indirect immunoperoxidase technique fixation and incubation with the primary antibodies were performed in the same manner as described above for the indirect immunofluorescence technique.

Swine anti-rabbit conjugated to peroxidase (DAKOpatTs, Denmark) diluted 1:25 in PBS containing 10% normal goat serum, or rabbit anti-mouse conjugated to peroxidase (DAKOpatTs, Denmark) diluted 1:25 in PBS with 5% human AB-serum were used in the second step, and incubated for 30—45 min.

After washing in PBS, peroxidase activity was detected with 3-aminophenyl diamine substrate (AEC; Aldrich Chem. Comp., USA). 40 mg of AEC was dissolved in 10 ml N-N-dimethylformamide (Merck, Darmstadt, FRG) and added to 190 ml of sodium acetate buffer (0.65 M, pH 4.88). Hydrogen peroxide was added to a final concentration of 0.01%. After incubation for 10 min cells were rinsed with tap water, counterstained with hematoxylin and mounted with Aquamount (Gurr, BDH chemicals Ltd, Poole, UK).

Gel electrophoresis. Cells were harvested from 75 cm culture flasks using plastic cell scrapers, spun down using an MSE Minor centrifuge at 1000 rpm for 5 min, washed once with PBS and stored at —20°C until use.

Cytoskeleton preparations were made essentially as follows: After pelleting, the cells were resuspended in PBS containing 1% Triton X-100 (BDH Chemicals, Poole, U.K.) and 1 mM phenylmethanesulfonyl fluoride (PMSF, Merck, Darmstadt, FRG) and extracted for 10 minutes at 0°C. After centrifugation (5,000g for 10 min) and washing in PBS the cytoskeletal preparation was dissolized by boiling in SDS-sample buffer during 5 min (Laemmli 1970). One-dimensional gel electrophoresis was performed in 10% polyacrylamide slab gels containing 0.1% SDS, as described by Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R250 (Gurr, Hopkin and Williams, Chadwell Heath, Essex, U.K.; 30 min, 60°C). Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (1975). In the first dimension isoelectric focusing (IEF) was performed in 4% polyacrylamide (Biorad, California, USA) rod gels containing 2% ampholines pH 3.5—10 (LKB, Bromma, Sweden). For the second dimension the rod gels were applied directly onto the stacking gel of 8% polyacrylamide gels and covered with a 1% agarose solution in running buffer.

Results

Characterization of the cell cultures. The cell cultures used in this study have been described before (Ohara and Okamoto, 1977, Luster et al. 1985). In short, cell cultures OAT 1975, MR 21 and MR 86 were derived from patients with small cell lung carcinomas (SCLC), MR 8 and MR 103 were derived from patients with large cell (LC) carcinomas, and MR 32 and MR 85 were derived from patients with squamous cell carcinomas. Except for MR 86 and OAT 1975, all cell cultures have been shown to

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*) Adapted from Luster et al. (1985).
Fig. 1. Flow cytometric analysis of five of the lung cancer cell cultures used in this study. DI = DNA index. Abscissa indicates channel number corresponding to the amount of DNA measured per cell. Ordinate indicates the number of cells counted.
produce a panel of different ectopic hormones into the culture fluid during early outgrowth as summarized in Table 1.

**Flow cytometry.** The results of the flow cytometric analyses of five of the cell cultures are indicated in Fig. 1 and Table 1. As can be seen all five cell cultures examined appeared to be aneuploid with the DNA-index ranging from 1.693 in MR 68 to 3.100 in MR 103. It should also be noted that all cell cultures contain relatively high proliferative fractions, with \((S + G_2 + M)\)-fractions ranging from 35—50\%. 

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*Fig. 2. Immunofluorescence photo micrographs showing the expression of different types of cytokeratins in small cell lung cancer cell cultures. A) Cell culture MR 21B reacting with Fker (×300), B) Cell culture MR 21B reacting with RGE 53 (×300). C) Cell culture OAT 1975 reacting with the monoclonal antibody PKK2 (×300). D) Cell culture MR 21B reacting with the monoclonal antibody LF1K (×250). E) Cell culture MR 86 reacting with the pKer antibody (D, ×250) and with the monoclonal antibody RGE53 (E, ×250) respectively. Note the filamentous staining reaction in all cell cultures and the relatively large proportions of the small cell lung cancer cell culture MR 86.*
Fig. 3. Immunofluorescence micrographs of the large cell carcinoma cell culture MR 103 (A–D) and the squamous cell carcinoma cell cultures MR 33 and MR 06 (E–H) stained with different cytokeratin antisera.
Table 2. Intermediate filament protein expression in hormone producing human lung cancer cell cultures as concluded from immunofluorescence and immunoperoxidase studies

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**Cytokeratins**

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**Neurofilaments**

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**Immunofluorescence studies with a panel of cytokeratin antibodies** (Table 2 and Figs. 2 and 3). The monoclonal and polyclonal cytokeratin antibodies can be subdivided into five groups, each with a distinct pattern of staining and immunoblotting reactions.

1) The group of antibodies with a broad cross-reactivity recognizing all epidermal as well as non-epidermal epithelial cells includes the monoclonal antibodies AE1,

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Fig. 4. Immunofluorescence micrographs of cell cultures stained with antiserum against vimentin. A) OAT 1975 with pVIM (x \(\times\) 300). B) MR 21B with pVIM (x \(\times\) 350). C) MR 86 with pVIM (x \(\times\) 300). D) MR 8 with pVIM (x \(\times\) 260). E) MR 168 with mVIM (x \(\times\) 150). F) A lung fibroblast cell line MR 33.
Fig. 5. Immunofluorescence photo micrographs of some of the cell cultures tested with an actin marker NBD-phallacidin (A—C), and with a monoclonal antibody to beta-tubulin (D—F).

with mVIM (×200). G) MR 32 with pVim (×350). H) MR 65 with pVIM (×360). Note that all cell cultures growing as adherent cultures express vimentin. The floating cell culture MR 21 did not express vimentin (not shown).
pKK1, LP34 and the polyclonal cytokeratin antibody (pKer). All three SCLC cell cultures reacted positively with these antibodies. The large cell (LC) cell culture MR 8 did not show a positive reaction with any of these antibodies. The LC carcinoma cell culture MR 103 and the two squamous cell carcinoma cell cultures MR 32 and MR 65 did, however, react strongly.

2) The group of antibodies recognizing stratified squamous (keratinizing) epithelia, includes the monoclonal antibodies RKSE 60, AE2, and AE3. None of the cell cultures did react clearly with these antisera, although there was some faint reaction with AE2 and AE3 in all types of cell cultures.

3) The group of antibodies reacting with simple epithelia and not with squamous epithelia, recognizing only cytokeratin 18 by immunoblotting analysis. This group includes the monoclonal antibodies RGE 52, CK18-2, LE31, and LE66. All SCLC cell cultures, the two squamous cell carcinoma cell cultures and the LC culture MR 103 reacted very strongly with these antisera. The LC carcinoma cell culture MR8 was negative.

4) The group of antibodies recognizing cytokeratin 8 as their major or exclusive target, i.e., the monoclonal antibodies LE41, LP1K, LP3K and LP5K. A clear positive reaction could be observed in the two SCLC cell cultures tested, the LC culture MR 103 and both squamous cell carcinoma cell cultures tested. No reaction was seen in the LC carcinoma cell culture MR8.

5) The antibody to cytokeratin 19 (LP2K) whose tissue distribution appears to be distinct from the other cytokeratins since it occurs in most simple epithelia and in basal cells of stratified squamous epithelia. The two SCLC cell cultures and the LC culture tested with this serum reacted in a clear positive manner.

In conclusion the antisera to the different types of cytokeratins show that cell cultures OAT 1975, MR 21, MR 86, MR 103, MR 32 and MR 65 do contain cytokeratins and that in the large cell carcinoma cell culture MR 8 no cytokeratins could be demonstrated during early outgrowth.

Fig. 6. One-dimensional SDS-polyacrylamide gelelectrophoretic separations of cytoskeletal preparations from OAT 1975 (lane B), MR 21 (lane C), MR 21B (lane D), MR 8 (lane E), MR 103 (lane F), pR 32 (lane G) and MR 65 (lane H). V indicates the position of vimentin and A indicates the position of actin. Note the absence of a band at the vimentin position in MR 21 (lane C).
Fig. 7. Two-dimensional gel electrophoresis of cytoskeletal preparations from six different lung cancer cell cultures. Vimentin and actin are indicated by V and A respectively. The brackets embrace the typical degradation products of vimentin. Cytokeratins 7, 8, 18, and 19 are indicated as such by arabic numerals. Note the two pronounced protein spots at the level of vimentin in the SCLC cell cultures OAT 1975 and MR 21B, which is also present in the large cell carcinoma cell culture MR 8.
Immunofluorescence studies with a panel of other (intermediate) filament antibodies (Table 2 and Figs. 4 and 5). All cell cultures except MR21 reacted clearly with the antibodies to vimentin in a filamentous manner. Cells from MR 21B, which tend to attach to the bottom of culture flasks express vimentin in the same manner as the other cell cultures.

Of the other antisera to IFP, i.e. neurofilament antisera, antisera to GFAP and desmin, none reacted with the cell cultures examined.

All four cell cultures examined with the antisera to beta-tubulin and actin (Fig. 5) reacted in a typical manner. As could be seen from the staining pattern using the antiserum to tubulin, high percentages of cells were in mitosis showing pronounced spindle cell figures.

Immunoperoxidase reaction patterns. The results obtained by the indirect immunofluorescence technique with the antibodies described above could be confirmed using the peroxidase technique (results not shown). In some cases, however, some differences in intensity between the two methods were noticed. For example, the intensity of the staining reaction with monoclonal antibody CK18-2 was clearly stronger when assayed in the peroxidase technique than with the immunofluorescence technique.

Gel electrophoretic identification of IF polypeptides. The results of the one-dimensional gel electrophoretic separations of cytoskeletal proteins from the lung cancer cell cultures are shown in Fig. 6. In all cell cultures the microfilament protein actin is demonstrable (denoted as A; 42 kD), while in all cell cultures except MR 21 a distinct protein band at the vimentin position can be seen (denoted as V, 57 kD). In the 40 to 56 kD region several protein bands, which might represent cytokeratins can be seen. In order to better analyse the cytokeratin content of the cell cultures, two-dimensional gel electrophoresis of cytoskeletal preparations from the cell cultures was performed (Fig. 7). From these biochemical analyses it became clear that the SCLC cell cultures OAT 1975 and MR 21B contain the cytokeratins 8, 18 and 19, while in MR 21B also traces of cytokeratin 7 are present. In the large cell carcinoma cell culture MR 8 no cytokeratins can be demonstrated, while the presence of vimentin with its typical degradation pattern is evident. The two SCLC cell cultures and the LC carcinoma cell culture MR 8 contain two protein spots at the level of vimentin, one of which could be shown to be vimentin, while the other spot could not be identified. These spots are located at the same level as has been shown earlier to occur in classic and variant SCLC cell lines, some of which did not contain vimentin as seen by immunocytochemical procedures (Broers et al. 1985b). Whether these two spots are typical for SCLC cell lines needs to be examined. The LC carcinoma cell culture MR 103 contains the cytokeratins 7, 8, 18 and 19, next to vimentin. In the squamous cell carcinoma cell culture MR 32 cytokeratins 7, 8, 18 and 19 next to vimentin are present, while in the other squamous cell carcinoma cell culture MR 65 only cytokeratins 7, 8 and 18 can be seen next to vimentin. Whether these cell lines contain still other cytokeratins could not be concluded on basis of these studies.

Discussion

Intermediate filaments (IF) consist of tissue-specific proteins (Osborn et al. 1981, Franko et al. 1981) which can be used to study the origin of cells and tissues. In general, neoplasms, derived from a certain type of tissue retain their IF characteristics, e.g. carcinomas express cytokeratins, sarcomas express vimentin or desmin, malignant gliomas contain glial fibrillary acidic protein (GFAP) and tumors derived from neural tissues can express neurofilaments. Conflicting results have been obtained using antisera to IFP in solid lung tumors, especially in the case of small cell lung carcinomas (SCLC). Several authors have been able to demonstrate the presence of cyto-
keratins in pulmonary adenocarcinomas and squamous cell carcinomas, although also false negative results have been published (Schlegel et al. 1980, Gusterson et al. 1982, Nagle et al. 1983, Blobel et al. 1984). Also SCLC have been described to contain cyto-keratins, albeit in some cases in a minor proportion of the cases examined (Gusterson et al. 1982, van Muijen et al. 1984, Blobel et al. 1985, Broers et al. 1985). Others (Lehto et al. 1983, Bergh et al. 1984) could not demonstrate cyto-keratins at all. Lehto et al. (1983) have shown a positive staining reaction in SCLC and in some of the carcinoind cases (Lehto et al. 1984) with a neurofilament antiserum but not with cyto-keratin antisera. Bergh et al. (1984) have demonstrated only neurofilaments and not cyto-keratins in cultured SCLC cell lines. Banks-Schlegel et al. (1985) have suggested that SCLC cell lines contain both cyto-keratins and neurofilaments. These apparent discrepancies in the literature may possibly be explained by our recent findings that classic SCLC cell lines contain cyto-keratins, while variant SCLC cell lines may express neurofilaments (Broers et al. 1985b, de Leij et al. 1985).

All these results taken together suggested to us that (hormone producing) neuro-endocrine lung tumor cells may express cyto-keratins and/or neurofilaments, markers for an epithelial or neural differentiation, respectively. In some of the cases of variant SCLC cell lines none of these two types of intermediate filament polypeptides could be detected. We therefore wondered about the intermediate filament proteins expressed in other types of lung carcinoma cell cultures (i.e. squamous cell carcinoma, and large cell carcinoma), and focused special attention on such cell cultures which could also be shown to produce ectopic hormones.

Here we have shown that all three SCLC cell cultures examined in this study express cyto-keratins but not neurofilaments. Therefore, the suggestion that SCLC might be derived from stem cells of the hompoietic system (Raff and Perth, 1984) cannot be supported by our results, since intermediate filaments of the cyto-keratin type do not occur in cells of hemopoietic origin.

Also one LC carcinoma cell culture and the two squamous cell carcinoma cell cultures could be shown to contain cyto-keratin polypeptides. Interestingly, in one of the cell cultures derived from a large cell carcinoma we could not detect cyto-keratins or neurofilaments by neither immunocytochemical nor biochemical techniques. These reactivity patterns resembles those of some of the variant cell lines (Broers et al. 1985b). Especially since such variant SCLC tumor cell lines are mostly derived from SCLC which contain large cell components (Carney et al. 1985) we cannot exclude the possibility that these cell cultures actually represent variant SCLC cell cultures exhibiting large cell features. The presence of the two protein spots at the level of vimentin in MR 8, also evidently present in variant SCLC cell lines (Broers et al. 1985b), is a further indication for such a relation.

Vimentin expression is observed in most cell cultures with the exception of MR 21. This is not surprising since many cell types in tissue culture can co-express vimentin next to their original TFP-type (Franke 1970). The adhering cell culture MR 21B which was derived from MR 21 (growing in tight clusters) also expresses vimentin. It therefore seems that at least in this cell type substratum attachment and cell spreading is correlated with vimentin synthesis.

In conclusion we can state that the results described in this paper show that lung cancer cells can synthesize neurohormones and still contain the epithelial type of IF.

Summary

In order to investigate the intermediate filament protein content of hormone producing lung tumor cell cultures a panel of 18 different cyto-keratin antisera were tested using immunocytochemical and biochemical techniques on lung carcinoma cell cultures from different origin. These included
three cell cultures derived from small cell lung carcinoma, two large cell carcinoma cell cultures, and two cell cultures derived from squamous cell carcinomas.

Flow cytometric analysis of the cell cultures demonstrated that all cell lines examined were aneuploid with DNA-indices ranging from 1.7 to 3.1 times the DNA-content of normal human lymphocytes.

In both immunofluorescence and immunoperoxidase techniques six out of seven cell cultures reacted with most of the cytokeratin antisera used in a filamentous manner, while a large cell carcinoma cell culture did not react with any of the cytokeratin antisera used.

None of the cell cultures examined reacted with the antibodies to neurofilament proteins, suggesting that none of these (neurofilaments producing cell cultures were of neural origin.

All cell cultures which were growing as adherent cell cultures did express vimentin. The cell culture that grew with cells floating in aggregates did not express this intermediate filaments protein while a subline which did attach, expressed vimentin. This finding strongly indicates the relation between growth pattern in vitro (floating vs. adherent) and the expression of vimentin. No reaction was found with antisera to desmin and GFAP. The presence of cytokeratins and vimentin in most cell cultures could be confirmed using one- and two-dimensional gel electrophoresis. Cytokeratins 7, 8, 18, and 19 were most commonly present.

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References


Identification of cytoskeletal structures


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