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Small cell lung cancer cell lines: pure and variant types can be distinguished by their extracellular matrix synthesis

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Small cell lung cancer cell lines: pure and variant types can be distinguished by their extracellular matrix synthesis. S. Scarpa, G. Morstyn, D.N. Carney, A. Modesti, T.J. Triche.

ABSTRACT: Variant subclasses of cell lines derived from small cell lung cancers have previously been characterized, having distinctive biochemical, morphological and growth properties compared to the classic lines. Both types of small cell lung cancer express features suggesting that they are derived from neuroectodermal cells. We compared the capacity of these two types of lung cancer cell lines to synthesize the extracellular matrix glycoproteins, fibronectin and laminin, and also analysed a few other non-small cell lung cancer lines, as controls. We found that the cell lines of the pure type did not produce laminin or fibronectin, whereas the cell lines of the variant type synthesized laminin, and the non-small cell lung cancer lines produced either laminin or fibronectin. These findings suggest that the variant form of small cell lung cancer may be derived from a primitive neuroectodermal cell, with both neural and epithelial features, whereas the classic type is derived from a more mature cell with predominantly neuronal features. The differences in extracellular matrix synthesis, and laminin in particular, may explain some of the *in vitro* and *in vivo* characteristics of the tumour.

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Two major types of small cell (oat cell) lung cancer (SCLC) cell lines have been described: pure SCLC and a morphological and biochemical variant form (SCLC-V) [1–6]. Both SCLC-V and SCLC lines exhibit the small cell lung cancer properties of elevated levels of the brain form (BB isoenzyme) of creatine kinase and neuron-specific enolase [7–8], however, SCLC-V can be distinguished by their different histological and cytological features [1–6]. The SCLC-V can also be distinguished from pure SCLC by their failure to express typical SCLC amine precursor uptake and decarboxylation (APUD) markers such as L-dopa decarboxylase [3, 8–10]. The SCLC-V lack neurosecretory granules [11–12], have a faster doubling time, higher cloning efficiency and have higher extrapolation numbers on radiation survival curves [3, 8, 13, 14]. The SCLC-V usually have an amplified c-myc oncogene which is not amplified in SCLC [15]. The distinction between SCLC-V and SCLC is clinically important, as patients presenting with SCLC tumours having variant morphologies, respond less well to therapy and have shorter survival times than those with classic morphologies [2]. The relationship of SCLC-V and SCLC cell types to each other and to other neuroectodermal cells has not been clearly defined. However, the neural features of small cell lung cancers [7–8] suggest that they arise from a neuroectodermal cell. To determine the relationship of the two types of small cell lung cancer to neuroectodermal cells and to each other, we studied their ability to express and synthesize *in vitro* extracellular matrix proteins, in particular laminin (LM) [16] and fibronectin (FN) [17]. Other non-small cell lung cancers (NSCLC) were studied for comparison. We found that classic SCLC do not express or produce any extracellular matrix protein, whereas variant SCLC and non-small cell lung cancers do; in particular, SCLC-V constantly synthesize laminin, but not fibronectin, whilst NSCLC produce either LM or FN.

Materials and methods

Cell lines

The cell lines analysed in this study were established from patients with lung cancer at the National Cancer Institute (NCI) [1–2] (table 1). Thirteen cell lines were of the pure small cell lung cancer type (SCLC), as defined by the presence of dopa-decarboxylase, gastrin releasing peptide, neuron-specific enolase; six were of the variant form of small cell lung cancer (SCLC-V) that only expressed neuron-specific enolase; five were of non-small cell type (NSCLC) (four adenocarcinoma and one large cell carcinoma) and lacked amine precursor uptake

Table 1. – Lung cancer cell lines and L-dopa decarboxylase (DDC) activity.

Cell line	Site	Prior chemotherapy	DDC $nm \cdot mg^{-1}$	Media proteins SDS-PAGE	Cell layer proteins Immunofluorescence
			SCLC		
NCI-H146	Effusion	Yes	341	-	LM
NCI-H345	BM	No	198	-	FN
NCI-H432	SCN	Yes	9	-	-
NCI-H433	SCN	Yes	7	-	-
NCI-H434	SCN	Yes	3	-	-
NCI-H435	SCN	Yes	6	-	-
NCI-H449	BM	Yes	98	-	-
NCI-H450	LN	Yes	657	-	-
NCI-H478	Lung	No	71	-	-
NCI-H517	Effusion	Yes	101	-	-
NCI-H592	BM	Yes	204	-	-
NCI-H686	LN	No	200	-	-
NCI-H510	Adrenal	No	214	-	-
			SCLC-V		
NCI-H446	Effusion	Yes	0.1	LM	LM
NCI-H820	Effusion	No	0.6	LM	LM
NCI-H524	LN	Yes	0.1	LM	LM
NCI-H689	Effusion	No	0.1	LM	LM
NCI-H526	BM	No	0.1	LM, FN	LM, FN
NCI-H417	Lung	No	0.1	LM	LM
			NSCLC		
NCI-H157	Effusion	No	0.1	LM, FN	LM, FN
NCI-H441	Effusion	No	0.1	LM, FN	LM, FN
NCI-H520	Lung	No	0.1	LM	LM
NCI-H650	Effusion	No	0.1	LM	LM
NCI-H125	Effusion	No	0.1	LM, FN	LM, FN

and decarboxylation (APUD) characteristics. L-dopa decarboxylase (DDC) specific activity was measured for each lung cancer cell line and was expressed as nanomoles of CO_2 released from ^{14}C-L-dopa per hour per mg protein [2], as summarized in table 1. Nine of the cell lines were derived from pleural effusions, four from bone marrow (BM), three from lymph node metastases (LN), four from separate subcutaneous metastases in one patient (subcutaneous nodule, SCN), three from the lung parenchyma at thoractomy (lung), and one was from an adrenal metastasis. Thirteen cell lines were established from pre-treatment specimens (no prior therapy), as indicated; whereas eleven cell lines were from relapsed tumours and had received prior therapy.

Cell cultures were maintained in the log phase of growth in RPMI 1640 medium (Gibco) with 10% foetal calf serum and penicillin-streptomycin. All the pure SCLC lines grew as tight floating aggregates of cells; the variant SCLC lines grew as floating aggregates of cells which were loose and had a tendency to adhere; the NSCLC lines grew as adherent monolayers.

Immunofluorescence

Adherent cell cultures were first exhaustively washed with phosphate buffered saline (PBS), then fixed in cold absolute ethanol for 5 min; floating cell lines were first fixed and then cytocentrifuged. Fixed cells layers and cytocentrifuge preparations were incubated with monospecific rabbit antibodies to fibronectin and to laminin (Bethesda Research Laboratories, Bethesda, MD, USA) diluted 1:100, for 1 h at room temperature (RT) in humidified atmosphere. Cell layers and cytospins were then washed with PBS and incubated with fluorescein-conjugated anti-rabbit IgG (Cappel), diluted 1:20, for 1 h at RT.

Control specimens lacked the primary antiserum, for which normal rabbit serum at 1:10 dilution was substituted.

Protein radiolabelling

The confluent cultures were preincubated for two days in growth arrest medium: RPMI 1640 with decreasing concentrations (5 to 1%) of dialysed foetal calf serum. One hour before labelling, the cultures

were incubated with Selectamine RPMI 1640 (Gibco) lacking leucine and precursors, without foetal calf serum, with 3.5 $mg \cdot ml^{-1}$ of glucose. One hour later 50 $\mu Ci \cdot ml^{-1}$ of L-(4,5-^{3}H) leucine (120–190 $Ci \cdot mmol^{-1}$), (Amersham, Braunschweig, FRG) were added. Fourteen hours later the radiolabelled media were harvested and protease inhibitors were added (1 mM phenyl methyl sulphonyl fluoride, 5 mM ethylenediamine-tetracetic acid). Incorporation of ^{3}H-leucine was quantified in a Beckman liquid scintillation counter after precipitation of small amounts of radiolabelled medium with 10% cold trichloroacetic acid (TCA).

SDS-PAGE

A volume of medium equivalent to 100,000 cpm TCA-precipitable counts was cold ethanol (-20°C) precipitated. The resulting pellet was redissolved in electrophoresis sample buffer (65 mM Tris-HCl pH 6.8, 4 M urea, 20% sodium dodecyl sulphate (SDS), 10% glycerol, bromophenol blue), boiled in water for 3 min, with or without reduction using 50 mM dithiothreitol (Dtt), and then separated by molecular weight in SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using 3.5% stacking gel and 4% separating gel, as described by LAEMMLI [18]. Gels were fixed in methanol-acetic acid, treated with fluorographic solution (Autofluor, National Diagnostics, USA), dried and exposed to photographic emulsion (Kodak XAR-5) at -70°C.

Immunoprecipitation

Radiolabelled media (500,000 cpm TCA-precipitable counts) were alternately incubated with the antibodies to laminin and to fibronectin for 12–14 h at 4°C. Next, 50 µl of goat anti-rabbit IgG (Cappel, Malvern, PA, USA) were added and incubated for 2 h at 37°C. The antigen-antibody complexes were precipitated by adding 1 ml of cold saline and pelleted in a Beckman microfuge. The pellets were resuspended in electrophoresis sample buffer and analysed by SDS-PAGE as described previously.

Results

Extracellular matrix proteins, laminin (LM) and fibronectin (FN) expression was analysed by immunofluorescent staining of different lung cancer cell lines. LM and FN synthesis and release into the medium was further demonstrated by immunoprecipitation of ^{3}H-leucine metabolically radiolabelled tumour cell culture media. In general, agreement between these two sets of results was high: all data are summarized in table 1 for each tumour cell line studied.

Among the five non-small cell lung cancer lines (NSCLC), all five expressed LM and three also FN. Figure 1 illustrates an adenocarcinoma cell culture (H157), as a representative example of NSCLC; intracytoplasmic accumulation of fibronectin (panel

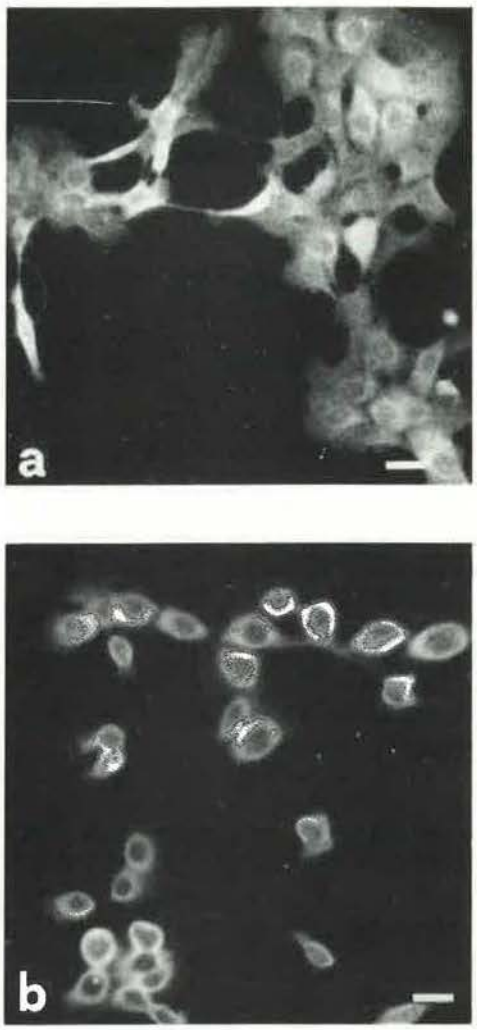

Fig. 1. Immunofluorescence of an adenocarcinoma adherent cell culture with a) anti-fibronectin and b) anti-laminin antibodies. Both antibodies express a strong cytoplasmic positivity. Bar 20 µm.

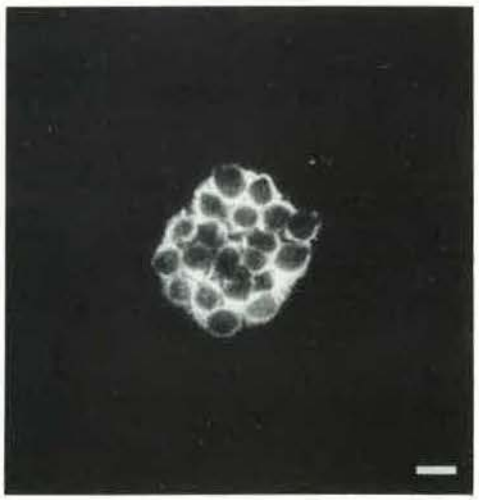

Fig. 2. Immunofluorescent positive staining of a variant small cell lung cancer culture cytospin with an anti-laminin antibody. Bar 20 µm.

a) and laminin (panel b) is demonstrated by immunofluorescent staining of the clearly adherent fixed cell layers. All six SCLC-V lines constantly expressed variable amounts of LM. Immunofluorescence with anti-LM antibody on a cytospin of a variant small cell lung cancer (SCLC-V) line (H446) is shown in figure 2: all the cells show a diffuse intracellular staining for laminin.

Of the thirteen pure SCLC lines listed in table 1, only one had scant immunofluorescent staining for FN (H345) and one for LM (H146) (data not shown). Also, no extracellular matrix proteins were detectable from metabolically radiolabelled media of all thirteen SCLC cultures (fig. 3). Figure 3 shows global extracellular matrix protein production by cell cultures representative of each tumour subtype studied. It is evident that none of the three SCLC synthesized any extracellular matrix constituent (lanes 1–3). In contrast, both the SCLC-V and the NSCLC synthesized abundant extracellular matrix in different patterns. Among the six SCLC-V lines, all six synthesized LM and only one FN (table 1). Radiolabelled media from three SCLC-V lines are illustrated in lanes 4–9: in every lane a LM band is evident, migrating in the unreduced form (Dtt-) at the very top of the gel around 900,000 Daltons (900 kDa) (lanes 4, 6, 8); the reduced 200 kDa LM subunit is also easily visible (lanes 5, 7, 9), while the 400 kDa subunit is detectable only in two lines (H526 and H524) [16]. Only one SCLC-V line produced very low amounts of FN (H526), visible as a weak band migrating unreduced at 480 kDa (lane 6). Of the five NSCLC, all five synthesized LM (lanes 10–15) and three also FN (table 1), detectable as a thick band migrating at 480 kDa (lane 14) when unreduced, and at 240 kDa in reduced form (lane 15) [17]. The radiolabelled medium from each tumour cell line was then immunoprecipitated respectively with anti-LM and anti-FN antibodies in order to confirm the identity of each band. Figure 4 shows a typical result of a SCLC-V line (H524) immunoprecipitated with anti-LM (lanes 1, 2), and a NSCLC line (H441) precipitated with anti-FN (lanes 3, 4). The immunoprecipitated laminin band migrates unreduced at around 900 kDa, and once reduced two subunits at 200 kDa and 400 kDa are easily visible; immunoprecipitated fibronectin appears as a thicker band migrating at 480 kDa unreduced, and at 240 kDa after reduction.

Discussion

Summarizing the above results, the variant and the pure form of SCLC cell lines could be distinguished by their different ability to produce laminin, whereas NSCLC synthesized abundant extracellular matrix glycoproteins, both laminin and fibronectin.

Although most of the pure SCLC lines were from patients pretreated with chemotherapy, this did not appear to be the cause of the lack of extracellular matrix production by the cell lines, since 70% of the cell lines from untreated patients also failed to produce matrix proteins.

However, only one SCLC line was derived from a primary tumour in the lung because SCLC are not

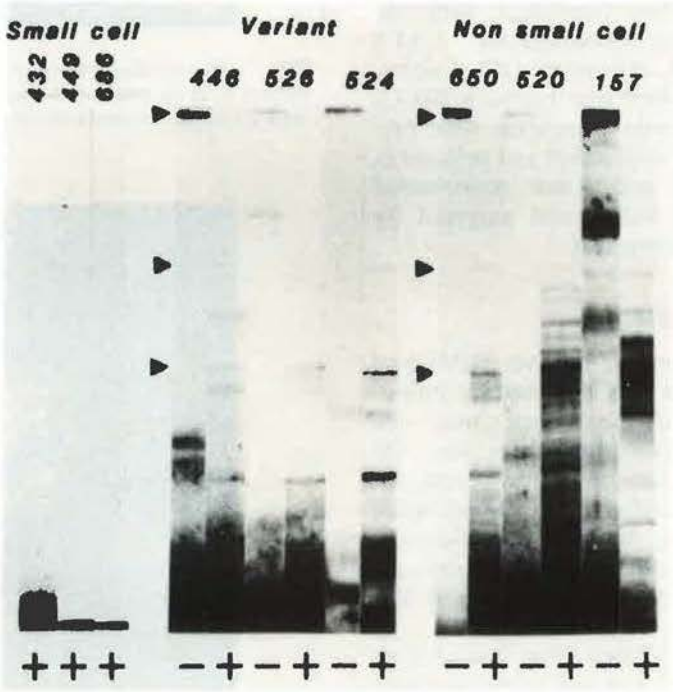

Fig. 3. Autoradiogram of a 4% SDS-PAGE representing extracellular matrix proteins synthesized and released into the media by different lung cancer cell lines. Three representative cell lines are shown for each class of lung cancer listed as pure SCLC (small cell), variant SCLC and NSCLC (non-small). Proteins were analysed with (+) and without (−) reduction.

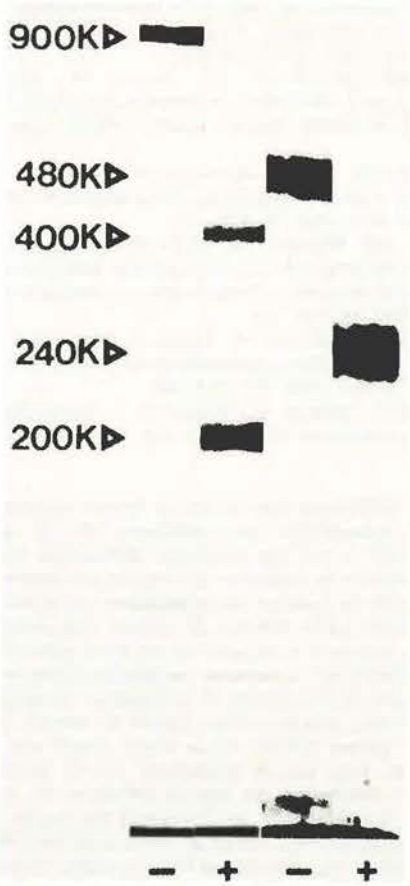

Fig. 4. Immunoprecipitation of radiolabelled media of a variant SCLC with anti-LM antibody (lane 1, 2); and of a NSCLC with anti-FN antibody (lanes 3, 4). Immunoprecipitates were separated on 4% SDS-PAGE reduced (+) and unreduced (−).

usually resected and so the primary tissue is not available. It is, therefore, possible that the lack of synthesis of matrix proteins by SCLC lines is predominantly a feature of metastatic cells since it has been found that invasive tumours lose basement membrane components [19]. However, this seems unlikely, because the cell line from the primary lung tumour did not synthesize laminin or fibronectin and only one of the SCLC-V lines was also from a primary tumour, and yet all of the SCLC-V lines, derived from metastatic sites, retained the capacity to synthesize laminin. We also investigated whether a particular metastatic site such as the marrow or the pleural space might favour the growth of cells with the capacity to synthesize laminin or fibronectin: but this did not appear to be the case.

We found heterogeneity in the dopa decarboxylase expression in tumours of different origin, but there was no relationship between dopa decarboxylase expression and extracellular matrix synthesis, when comparing different SCLC, with very low enzyme values, and all SCLC-V, with totally negative enzyme values.

Our studies demonstrate that all SCLC lines fail to synthesize extracellular matrix constituents, irrespective of their site of origin; and the major determinant of laminin production seems to be whether the cell line is of the pure or variant SCLC type.

In addition, all the pure SCLC lines grew as tight floating aggregates of cells: this is in agreement with the lack of extracellular matrix synthesis. In contrast, the variant cell lines grew as floating aggregates of cells which were loose and had a tendency to adhere, and they did synthesize extracellular matrix proteins, in particular laminin. Finally, the NSCLC lines, which produced either LM or FN, grew as adherent monolayers.

Synthesis of extracellular matrix by cells of neuronal origin has been studied previously. Mature neurons in the central nervous system completely lack adjacent basal laminae, and do not secrete matrix proteins such as laminin and fibronectin, whereas ectodermally derived cells do [20]; in the peripheral nervous system Schwann cells are responsible for the production of the extracellular matrix [21, 22]. Neuroblastoma [23, 24] and epithelial cell lines usually secrete laminin and fibronectin. Our finding that the pure SCLC lines do not usually secrete laminin or fibronectin suggests that they are more closely related to mature neurons whereas the variant cells may be related to a less mature cell with predominantly ectodermal rather than neuronal features. The presence of neuron-specific enolase supports the concept that both pure and variant SCLC arise from cells with neuronal features, and the absence of neurosecretory granules and APUD markers in the variant cells suggests that they arise from cells that are less mature. This study of nineteen well characterized human small cell lung cancer lines shows that they maintain one of two distinct phenotypes with the pure SCLC form appearing to arise from a more mature neuronal cell type than the SCLC variant. The difference in biological phenotype appears to be reflected in the different clinical behaviour of the two cell types with the variant form appearing to grow more rapidly and to be less responsive to therapy and, by these criteria, more malignant.

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RÉSUMÉ: Différentes sous-classes de lignées cellulaires dérivant de cancers pulmonaires micro-cellulaires ont été caractérisées antérieurement et ont des propriétés distinctives biochimiques, morphologiques et de croissance par rapport aux lignées classiques. Les deux types de cancers micro-cellulaires pulmonaires ont des traits suggérant qu'ils dérivent de cellules neuro-ectodermiques. Nous avons comparé la capacité de ces deux types de lignées de cancers pulmonaires à synthétiser des glycoprotéines de la matrice extra-cellulaire, la fibronectine et la laminine, en analysant aussi, comme contrôle, quelques autres lignées de cancers pulmonaires autres qu'à petites cellules. Nous avons trouvé que les lignées cellulaires du type pur ne produisent pas de laminine ou de fibronectine, tandis que les lignées cellulaires du type variant synthétisent la laminine, et que les lignées des cancers autres qu'à petites cellules produisent soit de la laminine soit de la fibronectine. Ces observations suggèrent que la forme variante du cancer micro-cellulaire pulmonaire peut être dérivée d'une cellule primitive neuro-ectodermique ayant à la fois des caractéristiques neurales et épithéliales, alors que le type classique est dérivé d'une cellule plus mûre à traits principalement neuronaux. Les différences dans la synthèse de la matrice extra-cellulaire, et en particulier de la laminine, peuvent expliquer quelques-unes des caractéristiques de la tumeur *in vitro* et *in vivo*.