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A unique cell-surface protein phenotype distinguishes human small-cell from non-small-cell lung cancer

[small-cell (oat-cell) cancer/neuroblastoma/iodination/two-dimensional electrophoresis/neuroendocrine]

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ABSTRACT **We have used radioiodination (^{125}I) and two-dimensional polyacrylamide gel electrophoresis to determine that small- (oat) cell lung carcinoma (SCC)—a tumor with neuroendocrine features—possesses a surface protein pattern distinct from the other types of lung cancer cells (squamous, adeno-, and large-cell undifferentiated carcinoma). Twelve distinguishing proteins, 40 to 70 kilodaltons (kDal), characterized four separate lines of SCC; three of these, designated E (60 kDal; pI = 7.3), S (30 kDal; pI = 6.0), and U (57 kDal; pI = 5.6), may be unique SCC gene products and were identified only in [^{35}S]methionine labeling of SCC and not in non-SCC or human fibroblasts. Two lines of adeno-, one of squamous, and one of undifferentiated large-cell lung carcinoma exhibited similar surface protein patterns to one another. Nine distinguishing proteins (40 to 100 kDal) and at least five large proteins (>100 kDal) were unique to these lines. The surface protein phenotypes for SCC and non-SCC were distinct from those for human lymphoblastoid cells and fibroblasts. However, the neuroendocrine features of SCC were further substantiated because 6 of the 12 distinguishing SCC surface proteins, including E and U, were identified on human neuroblastoma cells. The proteins identified should (*i*) help define differentiation steps for normal and neoplastic bronchial epithelial cells, (*ii*) prove useful in better classifying lung cancers, and (*iii*) be instrumental in tracing formation of neuroendocrine cells.**

In man, four major types of lung cancer—squamous, adeno-, small-cell, and large-cell undifferentiated carcinomas—result from exposure to carcinogens. Each tumor expresses specific differentiation features of the different normal bronchial epithelial cells (1). The precise cell lineages involved in formation of normal and neoplastic bronchial epithelial cells have not been outlined (1). A better understanding of this process would not only clarify events in human epithelial cell differentiation but might also better define clinically important interchanges between different types of lung cancer (2). The present study seeks to define molecular markers that would be useful for these purposes.

Cell-surface membrane proteins can mark specific stages of cellular differentiation (3, 4). We have thus sought to determine whether (*i*) each major form of human lung cancer is associated with a characteristic cell-surface protein phenotype; (*ii*) the distinguishing neuroendocrine features of small-cell lung cancer (SCC) (2, 5, 6) are associated with a cell-surface phenotype characteristic of neuroendocrine cells; and (*iii*) specific surface proteins distinguish human lung cancer cells from nonlung cells. We describe cell-surface protein phenotypes that (*i*) distinguish SCC from non-SCC; (*ii*) should aid in identifying cell lineage relationships between normal and neoplastic bronchial epithelial cells; and (*iii*) may associate a specific pattern of surface proteins with the state of neuroendocrine differentiation.

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MATERIALS AND METHODS

Cell Culture Lines. The cell lines employed included (*i*) four SCC, O-H-1 (7), NCI-H69, NCI-H60, and NCI-N231 (2, 5); (*ii*) two human lung adenocarcinomas, NCI-H125 and NCI-H23 (2, 5); (*iii*) one large-cell undifferentiated lung cancer, NCI-H157 (2, 5); and (*iv*) one human squamous cell lung carcinoma, U1752 (8). The histology of each line was determined in *nude* mice (2, 5), and these lines have been used for studies of peptide hormone receptors (9) and monoclonal antibody recognition of lung carcinoma cells (8).

The following cells were also studied: (*i*) two human neuroblastomas—CHP-100 (10) and NB-H-1, a line developed 2 yr ago in our laboratory that has high L-dopa-decarboxylase activity, further documenting its neural properties; (*ii*) two established lymphoblastoid lines (NCI-H128BL and NCI-H209BL) from patients with SCC (8) and the Daudi B lymphoma (11); (*iii*) a line of human fibroblasts (NCI-H390SK3) from a patient with SCC (8).

The cell lines were grown at 37°C in 5% CO_2 in air in RPMI 1640 (GIBCO) with 10–16% fetal calf serum, penicillin at 50 units/ml, and streptomycin at 50 μg/ml (2, 5–7). The lines are free of mycoplasma (Microbiological Associates, Walkersville, MD) and free of HeLa cells by glucose-6-phosphate dehydrogenase isoenzyme testing (12).

Radiolabeling of Cell-Surface Proteins. Intact cells were radioiodinated by the procedure of Fraker and Speck (13) as adapted by Markwell and Fox (14) which employs 1,3,4,6-tetrachloro-3,6α-diphenylglycoluril (chloroglycoluril; Pierce) as the oxidizing agent. Single cell suspensions were prepared from SCC and lymphoid lines by trituration in a pipette and from adherent cell lines by release of cells with 2 mM EDTA in phosphate-buffered saline. Cells (1×10^7) from stationary phase cultures were then washed three times, suspended in 1 ml of phosphate-buffered saline, and exposed to 500 μCi of ^{125}I (carrier-free, Amersham/Searle; 1 Ci = 3.7×10^{10} becquerels) for 12–15 min at 20°C in glass vials coated with chloroglycoluril at 100 μg (14). The reaction was terminated by diluting the cells in phosphate-buffered iodide and immediately removing them from the reaction vessel.

Metabolic Labeling of Total Cell Proteins. Cells (5×10^6) (as aggregates for SCC cells and monolayers for non-SCC cells) were incubated for 12 hr in 2 ml of methionine- and serum-free media with 250–500 μCi of [^{35}S]methionine (Amersham/Searle).

Abbreviations: SCC, small-cell lung carcinoma; kDal, kilodaltons.

Polyacrylamide Gel Electrophoresis of Proteins. The labeled cell preparations were analyzed by the two-dimensional polyacrylamide gel electrophoresis procedure of O'Farrell (15). Cells were sonicated in 250 μl of O'Farrell sonication buffer and pancreatic DNase (40 μg) was added for 5 min at 4°C. Sonicates were brought to a final concentration of 9 M urea and an equal volume of O'Farrell lysis buffer was added. Labeled precipitable protein was assessed on aliquots adjusted to 25% trichloroacetic acid. Approximately 300 μg of protein or 200,000 cpm was isoelectric focused (15) by using 2% Ampholine, pH 3.5–10 (LKB). Tube gels were equilibrated (15) and run in the second dimension on 10% $NaDodSO_4$ gels (16). Second-dimension slab gels were stained with Coomassie blue and exposed to Kodak X-Omat AR film in a Kodak X-Omatic cassette for 1–7 days at −70°C to visualize labeled proteins.

Partial Purification of Cell Membranes. Selectivity of cell surface iodination was assessed by comparing ^{125}I-labeled proteins with Coomassie blue-stained proteins in partially purified nuclei and membranes (17, 18) of SCC cells.

RESULTS

Selectivity of Cell-Surface Labeling. We validated the selectivity of iodination for surface protein as follows. (*i*) The ^{125}I-labeled proteins were larger than 30 kilodaltons (kDal), as compared to whole cell preparations in which smaller histone proteins predominated. Membrane preparations showed enrichment of proteins seen in the ^{125}I-labeling studies. (*ii*) The surface labeling, as shown for O-H-1 SCC cells (Fig. 1*B*), was selective for only a small portion of total cellular protein (Fig. 1*A*). However, each protein visualized by iodination (Fig. 1*B*) was visualized in the metabolic labeling studies (Fig. 1*A*) and thus was not from fetal calf serum. Some proteins (A, B, C, and O regions) were equally well visualized in both ^{125}I and [^{35}S]methionine studies, whereas others—such as I, J, K, L, and G—form a small portion of the [^{35}S]methionine proteins (Fig. 1*A*) but label intensely with ^{125}I due to their surface location (Fig. 1*B*). (*iii*) A known intracellular protein, actin, was well visualized by [^{35}S]methionine labeling (Fig. 1*A*, arrow) but not with iodination (Fig. 1*B*). (*iv*) Iodination of broken cell preparations yielded a far more complex pattern than seen in Fig. 1*B* with an increase especially of acidic proteins. (*v*) Finally, because most iodination studies used cell monolayers *in situ*, we tested the chloroglycoluril procedure (14) on suspensions of a well-characterized cell. We obtained the expected labeling of 3T3 fibroblasts (19), including a predominance of a 240-kDal protein, fibronectin, and of a less prominent 90-kDal protein (data not shown).

A Specific Combination of Proteins Is Common to Four Lines of Human SCC. Approximately 25 SCC surface proteins were consistently identified with ^{125}I-labeling (Fig. 1 *B* and *C*) and 12 proteins, ranging from ≈30 to 70 kDal, distinguished SCC from non-SCC cells (Table 1 and spots in brackets, Fig. 1 *B* and *C*). Three of these 12 proteins may be unique gene products for SCC; proteins E (see legends to Figs. 1 and 2) and S were found only in [^{35}S]methionine labeling (Fig. 1*A*) for SCC cells among all cell lines studied. Region U (Fig. 1*A*) also contains a protein heavily labeled only in SCC (Fig. 1*A*).

SCC cells had only trace labeling of ^{125}I-labeled proteins larger than 100 kDal; this was unaltered by addition of the protease inhibitor phenylmethylsulfonyl fluoride (2 mM) to all steps after initial iodination. The only variation in the labeling

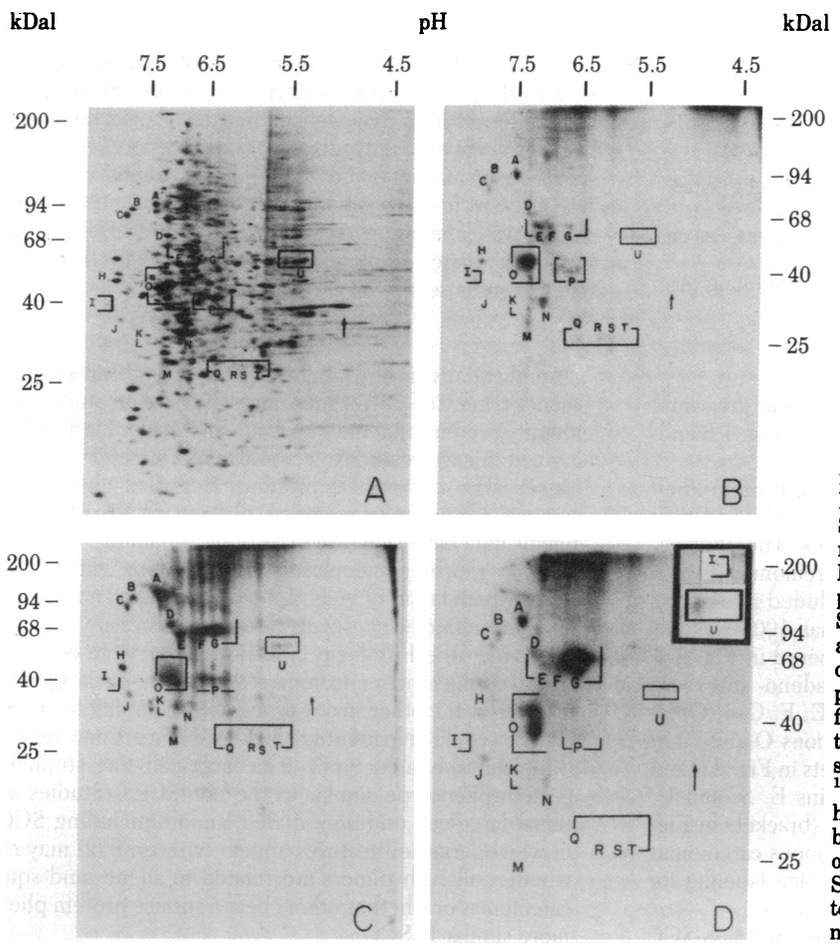

FIG. 1. Two-dimensional gel electrophoresis patterns for labeled proteins in cultured human SCC and neuroblastoma cells. The SCC surface proteins are lettered and used as reference points in the subsequent figure. Boxes have been placed around those lettered proteins found to distinguish SCC from non-SCC cells. The arrow marks the position of actin in each gel. (*A*) [^{35}S]Methionine labeling of O-H-1 line SCC cells. Letters indicate the positions of consistently visualized SCC surface proteins. (*B*) ^{125}I-Labeling of surface proteins on O-H-1 SCC cells. (*C*) ^{125}I-Labeling of surface proteins on NCI-H69 SCC cells. (*D*) ^{125}I-Labeling of surface proteins on CHP-100 human neuroblastoma cells. *Inset* shows labeling for proteins I and U that were seen only on heavier exposures of the autoradiographs. Such exposures did not reveal these two proteins to be iodinated on the non-SCC and non-neuroblastoma lines shown in Fig. 2.

Table 1. Summary of ^{125}I and [^{35}S]methionine labeling patterns

Proteins	^{125}I-Labeled proteins*	[^{35}S]Methionine-labeled proteins
Distinguishing SCC cells from non-SCC cells (Fig. 1 *A–C*)	E (68; 7.3); F (68; 6.9); G (68; 6.6); I (43; 7.7); O (56; 7.4);† P (53; 6.5–6.9); Q (30; 6.4); R (30; 6.2); S (30; 6.0); T (30; 5.8); U (57; 5.3–5.5)	E;‡ S;‡ U§
Common to SCC and neuroblastoma (Fig. 1 *B–D*)	E; F;¶ G;¶ I; O;¶ U	
Distinguishing non-SCC cells from SCC cells (Fig. 2 *A–F*)	1–6 (68; 6.0–6.7); 7 (57; 7.6); 8 (58; 6.6); 9 (60; 7.0) 10 (40; 6.6);‖ 11 (37; 6.6);‖ 5 proteins (100–200; 7.0–7.7)	Increased labeling of proteins >100 kDal above proteins A–C; pI 6.5–7.5
Characterizing lymphoblastoid cells	12 (90; 7.4);** 13 (90; 7.2);** 14 (94; 6.7);** 15 (94; 5.7);** F; G; O; HLA (40; 6.0–7.3)	Not tested
Characterizing fibroblasts	Trace of 1–6; fibronectin (180–240; 6.4); 5 proteins (100–200; 7.1–7.7)	No distinguishing proteins
Similarly labeled on most of cell types studied	A (100; 7.5); B (94; 7.6); C (90; 7.7); D (70; 7.3); H (50; 7.7); J (38; 7.7); K (35; 7.5); L (33; 7.5); M (30; 7.0); N (35; 6.9)	Most proteins except those listed above

* kDal (approximate) and pI in parentheses.
† Three peptides.
‡ Seen only in metabolic labeling pattern of SCC among cells tested.
§ Increased area of [^{35}S]methionine labeling in SCC cells.
¶ Proteins similarly labeled on SCC and neuroblastoma but also on lymphoblastoid cells.
‖ Seen only for ^{125}I-labeling of large-cell undifferentiated lung carcinoma cells.
** Proteins unique to lymphoblastoid cells.

pattern between different SCC lines and passages of the same line was in labeling intensity of proteins such as G (Fig. 1 *B* and *C*).

Non-SCCs Are Distinct from SCCs in Surface Protein Phenotype. Cultured non-SCC tumors (Fig. 2 *A–F*) showed distinctly different surface patterns from SCC cells. The patterns for adeno- (Fig. 2 *A* and *B*) and squamous carcinomas (Fig. 2 *C* and *D*) were identical to each other and included intense labeling of approximately five proteins larger than 100 kDal and of nine proteins smaller than 100 kDal (numbered in Fig. 2 *B* and *D*) not found in SCC cells (Table 1). The adeno- and squamous carcinomas also either lacked (proteins E, F, G, I, Q, R, S, T, and U) or had a marked reduction (regions O and P) in surface labeling of the 12 SCC proteins (brackets in Fig. 2 *B* and *D*). Interestingly, however, except for proteins E, S, and U, spots for distinguishing SCC surface proteins (brackets in Fig. 2 *A* and *C*) were detected in adeno- and squamous carcinomas by metabolic labeling despite the lack of surface labeling for these proteins.

Eight surface proteins were common to SCC and non-SCC lines (A, B, C, D, H, J, K, L, M, and N; Figs. 1 *B* and *C* and 2 *B* and *D*). In general (Table 1), these proteins were also found on human lymphoid cells and fibroblasts.

A line of large-cell undifferentiated lung cancer (Table 1; Fig. 2 *E* and *F*) had a surface protein pattern similar to the adeno- and squamous carcinomas including heavy labeling of the high molecular weight proteins above the A region, absence of the 12 SCC proteins, and trace labeling in most of the specific non-SCC proteins. Two proteins (10 and 11; Fig. 2*F* and Table 1) appeared to be unique to the large-cell carcinoma line.

Human SCC and Neuroblastoma Share a Similar Cell-Surface Protein Phenotype. The neuroendocrine features that distinguish SCC from non-SCC cells (2, 5) were further substantiated by our finding that 6 of the 12 distinguishing SCC surface proteins, including proteins E and U, were identified on neuroblastoma cells (Fig. 1*D*; Table 1). Two neuroblastoma lines differed from SCC only in that proteins of 100–200 kDal labeled lightly (seen above the A and D proteins; Fig. 1*D*) and proteins in the P and Q–T regions were not visualized.

Relationships Between the Cell-Surface Phenotype for Lung Cancer Cells and Other Human Cells. *Lymphoid cells.* Two B lymphoblastoid lines and a B lymphoma had some similarities to SCC and neuroblastoma (Table 1) including minimal ^{125}I-labeling of proteins >100 kDal and distinct labeling of the O, F, and G regions. However, most of the SCC proteins, including E, I, S, and U, were not present (Table 1). The lymphoid cells were also distinguished by a series of 40-kDal proteins correlating to the expected position of HLA histocompatibility antigens (20) and an increased number of proteins of 15–30 kDal (pH 6–7). None of the proteins that distinguished non-SCC cells (Table 1) was visualized in the B lymphoblastoid lines.

The B lymphoma (Daudi) line (Table 1) resembled the B lymphoblastoid lines, except for reduced numbers of the 40-kDal proteins. The known loss of HLA surface determinants from the Daudi line (21) would be consistent with this finding.

Fibroblasts. Labeling patterns for human fibroblasts were also different from SCC cells (Table 1). The 12 distinguishing SCC proteins were not iodinated, and proteins E, S, and U were not seen during metabolic labeling. Also, extensive ^{125}I-labeling was present in the position expected for fibronectin (Table 1). There was labeling of those proteins >100 kDal seen in the non-SCC. Trace labeling in non-SCC proteins 1–6 (Table 1) could also be seen.

DISCUSSION

Implications of the Data for Human Lung Cancer and Neuroendocrine Cell Differentiation. The present study sought to identify proteins that might provide molecular markers for tracing cell lineage relationships between the major forms of lung cancer. The cell-surface proteins identified already provide some important information in this regard. First, recent histologic data have suggested that lung squamous and adenocarcinomas represent neoplastic differentiation within a closely related population of cells (1). Our findings lend further support, at the molecular level, to this hypothesis.

Second, the histologic classification of large-cell undifferentiated lung cancer encompasses those tumors lacking distinct features of the other major types (22). Mapping of cell-surface proteins could help identify how these cancers may relate to the three differentiated types. The large-cell line studied had a protein phenotype similar to the non-SCCs. Studies of additional large-cell undifferentiated tumors including SCC lines in which large-cell features appear with time (2) may confirm whether all such tumors are related to adeno- and squamous carcinomas or whether others bear a surface protein phenotype more similar to SCC.

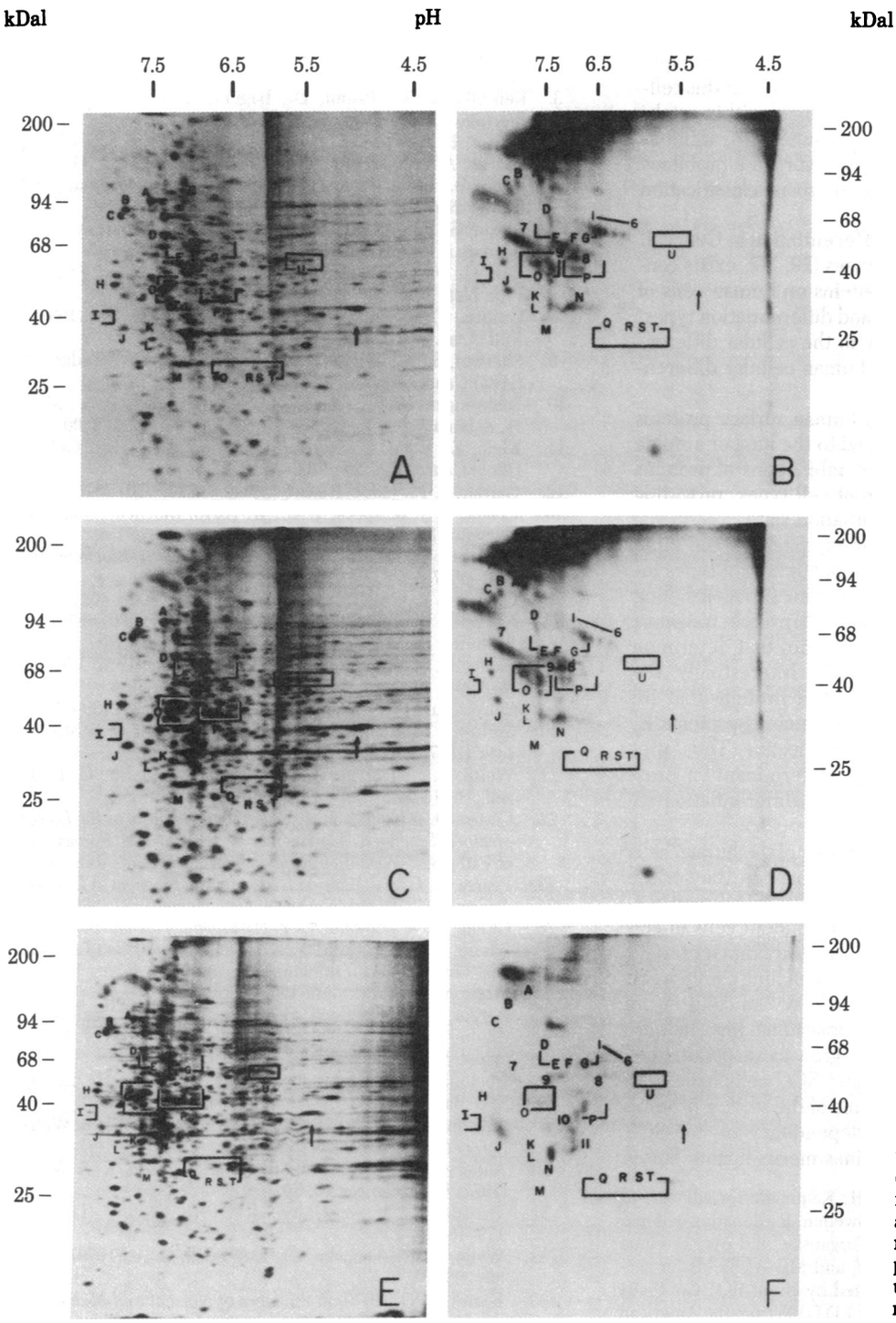

FIG. 2. Two-dimensional gel electrophoresis patterns for labeled proteins in cultured human non-SCC cells. Surface proteins that distinguish these lines from the SCC lines are numbered. Letters indicate the positions of all SCC surface proteins and boxes indicate the distinguishing SCC surface protein positions. The arrow marks the position of actin in each gel. (*A*) [^{35}S]Methionine labeling of line NCI-H23 lung adenocarcinoma. (*B*) ^{125}I-Labeling pattern for same cells shown in *A*. (*C*) [^{35}S]Methionine labeling of line U1752 of human lung squamous cell carcinoma. (*D*) ^{125}I-Labeling pattern for same cells shown in *C*. (*E*) [^{35}S]Methionine-labeling pattern for line NCI-H157 of large-cell lung carcinoma. (*F*) ^{125}I-Labeling pattern for same cells shown in *E*. A lighter exposure is given for this line to show proteins 10 and 11. Proteins 1–9 and A, B, C, K, L, and M could be better seen on heavier exposures. SCC surface proteins E, F, G, I, P, Q, R, S, T, and U are absent in *B*, *D*, and *F*; labeling of proteins in the O region is also diminished. Proteins E and S are absent in *A*, *C*, and *E* and protein U is much diminished (absence of protein E can be appreciated by noting in Fig. 1*A* that proteins E and F form the left and right bases, respectively, of a triangle of three proteins; in *A*, *C*, and *E* of this figure the left base of the triangle is missing).

Third, our findings further emphasize the neural features of SCC. Some workers have suggested that SCC might arise from a neural crest derivative in the bronchial mucosa rather than in the endodermal cells which give rise to the other cell types (23). However, recent data suggest an endodermal origin for some nonpulmonary endocrine cells (24, 25). Quantitatively, there is overlap for neuroendocrine biochemistry between SCC and non-SCC (26, 27). It is probable, then, that SCC arises within the endodermal cell lineage in the bronchial mucosa. If so, our present findings for SCC and neuroblastoma and previous findings that monoclonal antibodies to whole SCC cells specifically bind to neuroblastoma (8) emphasize that embryonically diverse cells with similar function or differentiation properties (or both) may share common or similar cell-surface protein patterns. It will be important to determine what other types of endocrine cells have a cell-surface protein phenotype similar to SCC and neuroblastoma.

Our findings might help refine the histologic categories upon which the treatment of lung cancer is based. Different histologic patterns may coexist within the same tumor; also changes during tumor growth and treatment from one histology to another occur (27, 28). These mixed histologies have included SCC and non-SCC cell types. SCC has both a greater metastatic potential and initial response to therapeutic modalities than the non-SCCs. A more precise determination of cell types within a given tumor may have important future implications for ther-

apeutic approach. Within the SCC classification, cellular heterogeneity exists for biochemical markers (26, 27). The surface protein patterns now identified seem to be constant for multiple culture lines of SCC. Preliminary data also suggest that this cell-surface protein phenotype is clonally maintained within established SCC lines. Immunohistochemistry studies with antibodies to specific surface proteins might help overcome problems of cellular heterogeneity which now hinder precise classification of individual lung cancers.

Implications of the Data for Cell Differentiation in General. Little information, except for lymphocytes (29, 30), exists concerning the distribution of surface proteins on human cells of widely divergent embryologic origins and differentiation types. Our present data then relate not only to the cellular differentiation of lung neoplasms but also to human cellular differentiation in general.

Most of the approximately 50 total human surface proteins identified in this study are not restricted to the lung or a single cell type (Table 1). Rather, different combinations of proteins within this group are found on different cell types, providing patterns characteristic of each differentiation state.

Most of the surface proteins identified appear in the same relative amounts during metabolic labeling of all cell types investigated. Thus, presence or absence of their surface labeling appears to be determined by factors such as (*i*) protein transport to the surface or (*ii*) membrane characteristics that determine external protein exposure to ^{125}I (or both). Interestingly, the metabolic labeling of a small group of surface proteins that are relatively restricted to a given cell type—such as proteins E, S, and U on SCC and neuroblastoma—is selective. Thus, protein synthesis may be a more important determinant for presence or absence of these more "tissue- or differentiation-restricted" surface proteins.

Finally, the *in vitro* growth characteristics of the human cells studied differ greatly and may depend in part on the surface proteins identified. Glycoproteins of 120–240 kDal may mediate substrate adhesion by anchorage-dependent cells in culture (31–33). Interestingly, the cells in our study that form tight monolayers (adeno-, large-cell, squamous cell lung carcinomas, and fibroblasts) are all characterized by heavy labeling in the 120–200-kDal region. The two neuroblastoma lines adhere much less tightly, and most SCC lines grow as suspended cell aggregates (2, 5, 7). Neuroblastoma and SCC cells have little labeling in proteins >100 kDal. The role of the surface proteins identified in mediating anchorage dependency of non-SCC lines and cell–cell adhesion in SCC lines merits further study.

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