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Analysis of cell surface proteins delineates a differentiation pathway linking endocrine and nonendocrine human lung cancers

[small (oat) cell cancer/large cell cancer/two-dimensional electrophoresis]

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ABSTRACT **We have previously determined that the cell surface protein phenotype distinguishes human small cell lung carcinoma (SCC), a neoplasm with endocrine properties, from non-SCC in culture. We now demonstrate that cloned cell cultures of human large cell undifferentiated lung carcinoma, established directly from a patient with mixed SCC and non-SCC, simultaneously express surface proteins characteristic of SCC and non-SCC lung cancer cells. Hence, SCC and a form of large cell carcinoma appear linked through a continuum of differentiation events that: (*i*) may explain clinically important transitions that occur between the major types of human lung cancer and (*ii*) provide evidence for a common cellular origin of endocrine and nonendocrine cells in the bronchial mucosa.**

Three major histologic forms of differentiated lung cancer arise in the complex bronchial epithelium of man: squamous, adeno-, and small cell carcinomas (1). A fourth major category, large cell undifferentiated carcinoma, encompasses a group of lung neoplasms that do not exhibit morphologic differentiation features of the other three (1).

Evidence has accumulated to suggest that these major forms of human lung cancer may be linked along a differentiation continuum (2, 3). Such a postulation is of particular biologic and clinical importance with respect to the relationship between small cell carcinoma (SCC) and the other histologic forms of lung cancer and for sorting out the precise cell lineages of large cell undifferentiated carcinomas. SCC is a virulent cancer that usually exhibits neuroendocrine properties, including the synthesis of biologically active peptide hormones (4), capacity for biogenic amine synthesis (3, 5, 6), presence of neuron-specific enolase (7, 8), and the presence of cytoplasmic neurosecretory granules (9, 10). These neural properties initially led to the speculation that SCC arises from lung endocrine cells that are embryologic derivatives of the neural crest (11), whereas non-SCC cancers arise from endodermally derived nonendocrine bronchial mucosal cells.

However, recent data have demonstrated that neuroendocrine features can be found in non-SCC lung cancers as well. Careful analyses of neuroendocrine-related biochemical properties in all the major histologic forms of lung cancers reveal the presence of immunoreactive peptide hormones and amine synthesis capacity in non-SCC lung cancers, although concentrations are usually less than those detected in SCC (12). Furthermore, morphological transitions between SCC and non-SCC lung cancers may occur. In some patients, with an initial diagnosis of SCC, a complete change from SCC to the other differentiated forms of non-SCC histology has been documented (13, 14). In established SCC cell cultures, Gazdar *et al.* have observed a time-dependent change in which SCC cells lose their endocrine features and, in *nude* mouse heterotransplants, change from SCC histology to typical large cell undifferentiated carcinoma (3).

In regard to the above cell culture changes described by Gazdar *et al.* (3), it is then of paramount biologic and clinical importance to determine whether: (*i*) the large undifferentiated carcinoma cells in SCC cultures are simply selected for with time and have no direct lineage relationship to SCC; (*ii*) these cells are linked directly to SCC cells through a process of differentiation; and (*iii*) such cells are capable of pluripotent expression with respect to the entire spectrum of differentiated human lung cancer cell types. In the present work, we address these questions by analyzing the cell surface proteins of a unique human cell culture line recently established in our laboratory. We have recently reported that a distinct cell surface protein phenotype distinguishes SCC from the non-SCC lung carcinomas in culture (15). We now report that such analysis of surface proteins may: (*i*) define differentiation pathways that may mediate clinically important transitions between the major forms of human lung cancer; and (*ii*) clarify the histogenesis of nonneoplastic endocrine cells residing in predominantly nonendocrine epithelial systems. We thus present evidence at a molecular level that SCC is indeed directly related to a form of large undifferentiated tumor cell that may have pluripotent differentiation capabilities for the major forms of differentiated lung cancer.

MATERIALS AND METHODS

Cell Cultures. Line OH-2 was established from the pleural effusion of a patient diagnosed as having SCC. The pleural fluid, obtained by thoracentesis, was heparinized, transferred under sterile conditions to 50-ml plastic centrifuge tubes, and centrifuged at 200 × *g* for 5 min. Cell pellets were then pooled and washed twice in phosphate-buffered saline and finally resuspended in growth media containing the following ingredients: RPMI 1640 medium, 16% fetal calf serum (B & B Research, Friskeville, RI), 2 mM glutamine, penicillin at 60 units/ml, and streptomycin at 60 μg/ml (GIBCO). Cells were grown at 37°C in 5% CO_2 in air. All other SCC and non-SCC cultures were maintained and characterized as described (3, 6, 15).

Line OH-2 was found to be free of mycoplasma (test performed by Microbiological Associates) and HeLa cell contam-

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Abbreviation: SCC, small cell carcinoma.

ination as determined by glucose-6-phosphate dehydrogenase isoenzyme studies (16).

Cell Cloning. Single cell suspensions were obtained by treating confluent flasks of OH-2 cells with 0.25% trypsin. In sterile Costar Petri dishes (35 × 10 mm), 1×10^3 viable cells were plated in 1 ml of 0.85% SeaKem 15/45 agar (Marine Colloids, Rockland, ME) (clones 1–5) or 0.3% GIBCO agar (clones 6–19) made up with RPMI 1640 medium and 16% fetal calf serum (B & B Research), 2 mM glutamine, penicillin at 60 units/ml, streptomycin at 60 μg/ml, and 25% conditioned medium from OH-2 cells plus human placental extract prepared exactly as described (17) at 0.1 ml per plate. Clones, consisting of >50 cells, were carefully removed from agar under phase-contrast microscopy by using sterile plastic Pipetman tips (20–200 μl; Rainin) attached to a Pi Pump (2 ml; Glasfirn). Individual explanted clones were then placed in 96-well culture plates (Costar) with 0.05 ml of medium until cells reached confluency. Cells were then transferred to 24-well Costar plates and, when confluent, to 75-cm^2 flasks for stock cultures.

Analysis of Cell Surface Proteins. Materials and experimental procedures are as described (15). Briefly, the cell surface proteins of intact viable stationary-phase cells were radioiodinated ($Na^{125}I$, carrier-free, Amersham/Searle) by the procedure of Fraker and Speck (18) as modified by Markwell and Fox (19). Metabolic labeling of cells was accomplished by incubating 5×10^6 cells for 12 hr in 2 ml of serum-free medium containing 250–500 μCi (1 Ci = 3.7×10^{10} Bq) of [^{35}S]methionine (1,100 Ci/mmol; Amersham) as the only source of methionine. Trichloroacetic-acid precipitable protein of both ^{125}I- and [^{35}S]methionine-labeled cells was then determined and aliquots were analyzed by the two-dimensional polyacrylamide gel electrophoresis procedure of O'Farrell (20). Labeled proteins were then visualized by radioautography of the second-dimension slab gels (15).

***nude* Mice Heterotransplants.** *nude* mice heterotransplants were obtained by inoculating cultured OH-2 cells ($2–5 \times 10^6$ viable cells) into the rear flanks of BALB/c athymic *nude* mice. Tumors were excised after 3–4 weeks, portions were immediately fixed in 10% phosphate-buffered formalin for hematoxylin and eosin histologic analysis, and portions were homogenized in 0.1 M sodium phosphate buffer, pH 6.8, for subsequent biochemical assays (12).

Histaminase and L-Dopa Decarboxylase Radioassays and Calcitonin and β-Endorphin Radioimmunoassays. All assays were conducted on cell sonicates and tumor tissue homogenates exactly as described in ref. 12.

RESULTS

Characterization of Culture Line OH-2. The cells taken from the pleural fluid adapted readily to cell culture, showing evi-

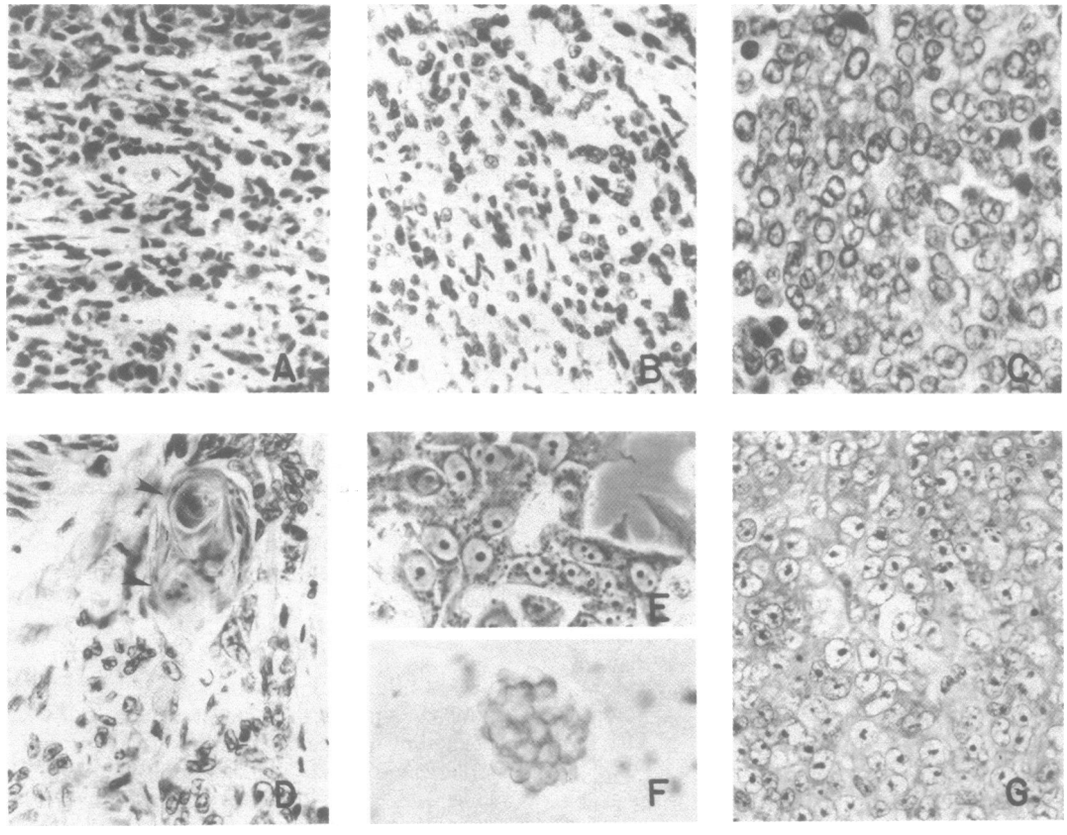

FIG. 1. Biopsy, autopsy, and heterotransplanted *nude* mouse tumor tissues from a patient with an initial diagnosis of SCC of the lung. (*A*) Original biopsy of lung mass showing SCC. Note the characteristic appearance of the cells, including small hyperchromatic nuclei and scant cytoplasm. (*B*) Autopsy tissues obtained 7 months after the original biopsy again revealed SCC histology in both primary and metastatic tissues. (*C*) Careful examinations of multiple histological sections at autopsy revealed foci of large, undifferentiated cells characterized by large vesicular nuclei with occasional nucleoli and moderately abundant cytoplasm. (*D*) Infrequent areas of tumor tissues demonstrated features of squamous cell carcinoma differentiation with well-developed keratin pearls (arrows). (*E*) Appearance of cells cultured from the pleural effusion of this patient obtained 4 weeks prior to autopsy. Note the comparison of these cells to the more typical suspended aggregate growth of SCC shown in *F* (line OH-1). Note also the large nuclei and nucleoli similar to those of the large undifferentiated cells in autopsy material (*C*) and *nude* mouse explants described below (*G*). (*G*) Histology of *nude* mice heterotransplants from the parent and five of five OH-2 cloned cultures, revealing large cell undifferentiated carcinoma morphology, characterized by large vesicular nuclei, prominent nucleoli, and abundant cytoplasm. (*A*, *B*, *C*, *D*, and *G*, hematoxylin and eosin, ×550; *E* and *F*, phase contrast, ×1,200.)

dence of growth within 1–2 weeks. After several days, these cultured cells failed to demonstrate the suspended cell aggregate growth pattern characteristic of SCC *in vitro* (3, 6); but rather, they grew as an anchorage-dependent monolayer (Fig. 1 *E* and *F*). Furthermore, OH-2 cells completely lacked the high levels of L-dopa decarboxylase activity that typically distinguish SCC cells *in vitro* from non-SCC lung cancer (5, 6). In *nude* mouse heterotransplants, both early (passage 6) and late (passage 37) passages of OH-2 cells grew typical large cell undifferentiated lung carcinomas (Fig. 1*G*).

Analysis of Autopsy Tissues. Four weeks after these cells were obtained, the patient expired and autopsy revealed predominantly SCC histology as shown in Fig. 1*B*. However, a careful reexamination of multiple sections of primary and metastatic tumor revealed small foci of cells with histologic properties very similar to those seen in the *nude* mouse explants,

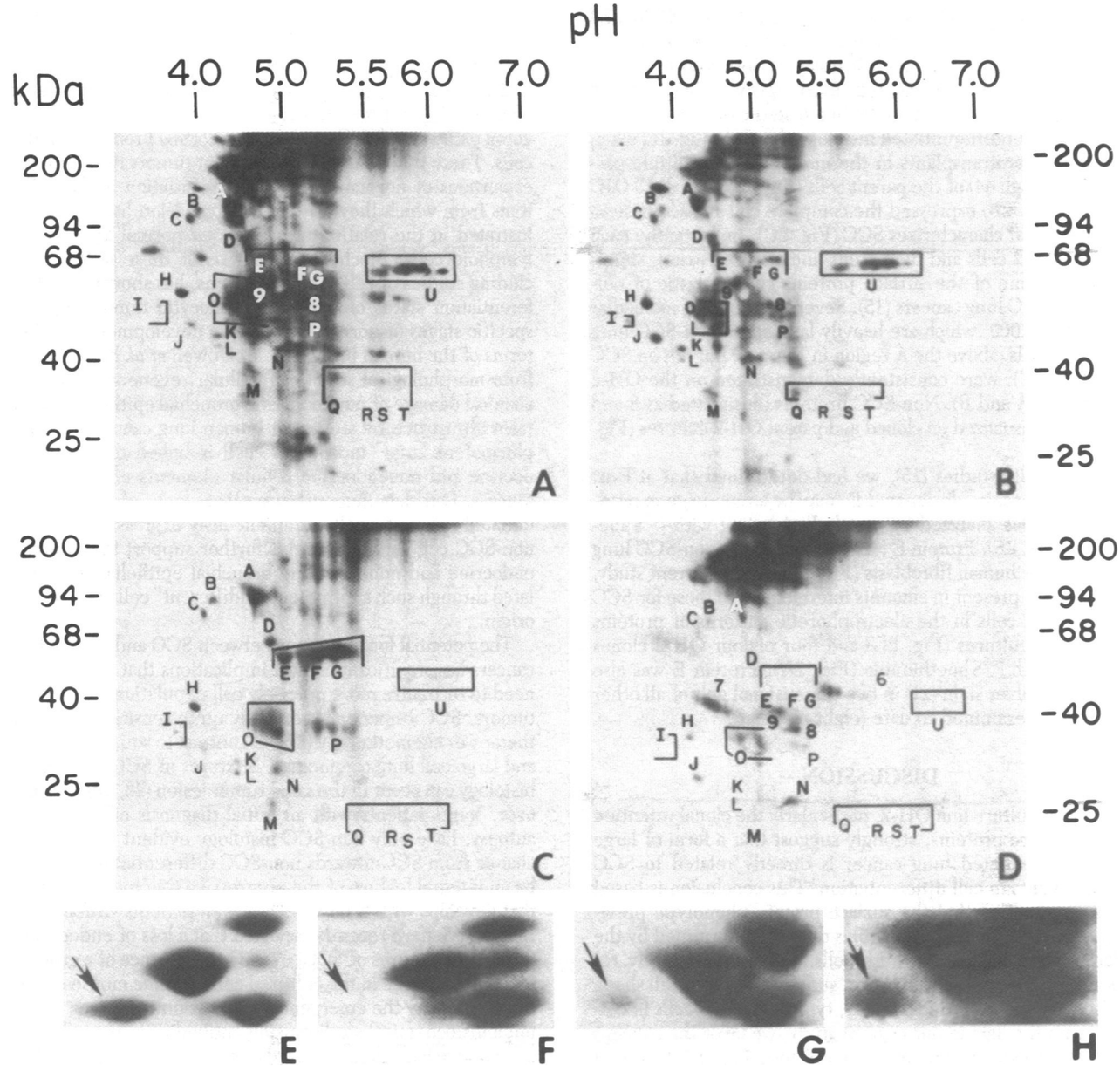

FIG. 2. Two-dimensional polyacrylamide gels of ^{125}I-labeled surface proteins and [^{35}S]methionine-labeled total cellular proteins from cultures of SCC, non-SCC, and parent and cloned cultures of line OH-2. kDa, Kilodaltons; pH values are shown across the top. (*A* and *B*) The surface labeling pattern typical of the parent (*A*) and cloned cells (*B*) of line OH-2 exhibited all of the SCC proteins (15) that distinguish SCC (bracketed regions in *C*) from non-SCC lung cancers (*D*). These SCC proteins are in regions E, F, G, U, I, Q, R, S, and T. Some surface proteins characteristic of non-SCC lung cancer cells (*D*) are also visualized on OH-2 cells (*A* and *B*) and include the high molecular weight proteins seen above the A region and proteins numbered 8 and 9. (*C*) The ^{125}I surface labeling pattern from line OH-1 (2), which is characteristic of the pattern found for six established lines of SCC lung cancer. (*D*) The gel shown for the surface proteins of non-SCC lung cancers is that for line NCI-HUT 125 of lung adenocarcinoma. This pattern is identical to that for one additional line each of lung adenocarcinoma and squamous carcinoma. Note the absence of the SCC-specific proteins from the bracketed areas and the presence of proteins 1–10 and the high molecular weight proteins above protein A. (*E–H*) The [^{35}S]methionine-labeled protein synthesis patterns for SCC, non-SCC lung cancer cells, and OH-2 cultured cells are shown. The regions shown are restricted to protein regions E and F of the second-dimension gel. Note the presence of protein E (arrow) in the protein synthesis pool of SCC (*E*), its absence from non-SCC lung cancers (*F*), and its presence in intermediate amounts in both parent and cloned cultures of OH-2 (*G* and *H*). The intermediate levels of E correlated with a more variable intensity of ^{125}I labeling for this protein on the surface of OH-2 cells (*A* and *B*) than on typical SCC cells (*C*).

including large vesicular nuclei and abundant cytoplasm (Fig. 1*C*). Importantly, foci of poorly differentiated squamous cell carcinoma (Fig. 1*D*) with well-developed squamous pearls were also seen. Multiple biochemical analyses of the autopsy tumor (most of which contained typical SCC histology) failed to reveal significant concentrations of four biochemical markers (L-dopa decarboxylase activity, diamine oxidase activity, β-endorphin, calcitonin), one or more of which are characteristically present in high quantities in SCC tissues (12).

Cell Surface Protein Analysis of OH-2 Parent Cells and Clones. In order to assess the precise relationship of the cultured OH-2 cells to the tumor histologies observed in the patient, we obtained and expanded 19 clones from single cultured cells grown in soft agar. Five of five tested clones maintained the large cell undifferentiated histology shown in Fig. 1*G* when grown as heterotransplants in the *nude* mouse. Multiple passages (5 through 44) of the parent cells (Fig. 2*A*) and all 19 OH-2 clones (Fig. 2*B*) expressed the complete cell surface protein phenotype that characterizes SCC (Fig. 2*C*). Importantly, each clone of OH-2 cells and the parent line also expressed, simultaneously, some of the surface proteins characteristic of cultured non-SCC lung cancers (15). Several proteins of molecular weight >100,000, which are heavily labeled on non-SCC lung carcinoma cells (above the A region in Fig. 2*D*) but not on SCC cells (Fig. 2*C*), were consistently demonstrated on the OH-2 cells (Fig. 2 *A* and *B*). Non-SCC proteins (designated as 8 and 9) were also visualized on cloned and parent OH-2 cultures (Fig. 2 *A* and *B*).

In our initial studies (15), we had determined that at least one surface protein, designated E, may be a unique gene product for SCC, as analyzed by metabolic labeling with [^{35}S]methionine (Fig. 2*E*). Protein E was not detected in non-SCC lung cancer cells or human fibroblasts (Fig. 2*F*). In the current study, protein E was present in amounts intermediate to those for SCC and non-SCC cells in the electrophoretic patterns of proteins from parent cultures (Fig. 2*G*) and four of four OH-2 clones incubated with [^{35}S]methionine (Fig. 2*H*). Protein E was also detected by silver stain (21) in two-dimensional gels of all other OH-2 clones examined to date (eight of eight).

DISCUSSION

The data for culture line OH-2, particularly the clonal retention of SCC surface proteins, strongly suggest that a form of large cell undifferentiated lung cancer is directly related to SCC through events in cell differentiation. This conclusion is based on the assumption that the surface protein phenotype previously described for SCC cells (15) is directly determined by the differentiation lineage of these cells, rather than simply reflecting the growth characteristics, such as the round cell shape and lack of anchorage dependency, typical for SCC cells in culture. From the data in our current study, we favor the concept that differentiation events are the most important determinants because OH-2 cells are generally flat, grow as anchorage-dependent monolayers, and yet exhibit all of the surface proteins that constitute the unique SCC surface protein phenotype previously demonstrated (15).

The concept that a form of large cell undifferentiated carcinoma is linked along a differentiation continuum with SCC raises the question whether this association is limited to the cell culture environment *in vitro* or whether the previous observations (3) of a time-dependent transition, in culture, of SCC to a form of large cell undifferentiated tumor also occurs *in vivo*. Two features of our OH-2 cells now suggest strongly that this transition can occur in the human host. First, the OH-2 cells grew immediately, in culture and in *nude* mice heterotransplants, as large cell undifferentiated tumors. Second, undifferentiated large tumor cells with morphologic similarities to the cultured and *nude* mouse heterotransplanted OH-2 cells were found in the autopsy tissues of the patient from whom the cells were obtained. We must then conclude that the time-dependent transitions from SCC to large cell tumors, observed in culture by Gazdar *et al.* (3), also occur *in vivo*.

Our data also suggest, although more indirectly, a differentiation link between SCC and squamous cell lung carcinoma. The simultaneous expression of non-SCC and SCC surface proteins in single clones of OH-2 cells (taken from a patient with both SCC and squamous carcinoma) supports such a possibility.

The close relationships demonstrated in this study between histologically distinct bronchial neoplasms probably reflect ongoing pathways of differentiation in normal bronchial epithelial cells. There is increasing evidence that tumors represent clonal expansions of normal stages of differentiation in the cell systems from which they arise. This conclusion has been best illustrated in the relationships between normal and neoplastic lymphoid cells. Biochemical analysis of these neoplasms, including studies of cell surface antigens, has shown that the differentiation status of various lymphocytic tumors represents specific stages of normal lymphocyte development (22–24). In terms of the human bronchus, McDowell *et al.* have proposed, from morphological studies of cellular regeneration after mechanical damage of nonneoplastic bronchial epithelium (25) and from ultrastructural studies of human lung cancers (26), that a pluripotent large "indifferent" cell is linked to both the endocrine and nonendocrine cellular elements of the bronchial mucosa. Our data demonstrating that clones of large undifferentiated neoplastic cells simultaneously express both SCC and non-SCC cell surface proteins further support the notion that endocrine and nonendocrine bronchial epithelial cells are related through such a totipotent "indifferent" cell of endodermal origin.

The potential for transitions between SCC and non-SCC lung cancers has significant clinical implications that emphasize our need to recognize more precisely cell populations in given lung tumors. SCC tumors are generally quite sensitive to radiation therapy or chemotherapy (27), in contrast to squamous, adeno-, and large cell lung carcinomas. Mixtures of SCC and non-SCC histology can occur in the same tumor lesion (28, 29) and, moreover, some patients with an initial diagnosis of SCC may, at autopsy, have only non-SCC histology evident (13, 14, 29). A change from SCC towards non-SCC differentiation could then be an integral feature of the progressive therapeutic resistance that develops with time in virtually all patients with SCC (27). Indeed, we have recently reported that a loss of endocrine features from cultures of SCC, even in the absence of a gross morphological change in the cultured cells or *nude* mouse explants, can accompany the emergence of radiation resistance (30). Immunohistochemical studies using antibodies to the cell surface proteins we have demonstrated could prove extremely valuable for identifying, in lung cancers from individual patients, cell populations at the specific stages of differentiation that may determine their therapeutic sensitivities.

Note Added in Proof. In ref. 15, we inadvertently labeled the pH gradients for figures 1 and 2 in the reverse direction. The corrected pI values for the major distinguishing proteins for SCC and non-SCC lung cancer cells, as determined from the gradient in Fig. 2 of the present manuscript, are as follows. (*i*) SCC proteins: E, pI = 4.5; F, pI = 5.2; G, pI = 5.3; I, pI = <4.0; O region, pI = 4.3–4.7; P region, pI = 5.3–5.5; Q, R, S, and T, pI = 5.3–6.0; and U, pI = 5.5–6.3. (*ii*) Non-SCC proteins: 1–6, pI = 5.3–6.0; 7, pI = 4.0–4.5; 8, pI = 5.3; 9, pI = 5.0; and the high molecular weight proteins above protein A, pI = 4.0–5.5.

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