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Research Article

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v-*ras*^{H} Induces Non-Small Cell Phenotype, with Associated Growth Factors and Receptors, in a Small Cell Lung Cancer Cell Line

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Abstract

Small cell lung cancer (SCLC) tumor progression can involve partial or complete conversion to a more treatment-resistant non-small cell (NSCLC) phenotype. In a cell culture model of this phenomenon, we have previously demonstrated that insertion of the viral Harvey *ras* gene (v-Ha-*ras*) into SCLC cell lines with amplification and overexpression of the c-*myc* gene induced many NSCLC phenotypic features. We now report that the v-Ha-*ras* gene can also induce morphologic, biochemical, and growth characteristics consistent with the NSCLC phenotype in an N-*myc* amplified SCLC cell line, NCI-H249. We show that v-Ha-*ras* has novel effects on these cells, abrogating an SCLC-specific growth requirement for gastrin-releasing peptide, and inducing mRNA expression of three NSCLC-associated growth factors and receptors, platelet-derived growth factor B chain, transforming growth factor-α (TGF-α), and epidermal growth factor receptor (EGF-R). TGF-α secretion and EGF-R also appear, consistent with the induction of an autocrine loop previously shown to be growth stimulatory for NSCLC in culture. These data suggest that N-*myc* and v-Ha-*ras* represent functional classes of genes that may complement each other in bringing about the phenotypic alterations seen during SCLC tumor progression, and suggest that such alterations might include the appearance of growth factors and receptors of potential importance for the growth of the tumor and its surrounding stroma. (*J. Clin. Invest.* 1990. 85:1740–1745.) oncogenes • bombesin • progression • *myc* • epidermal

Introduction

Small cell lung cancers (SCLC)[1] are phenotypically distinct from all other forms of lung cancer, collectively known as non-small cell lung cancer (NSCLC) (1). Features that distinguish SCLC include the expression of a variety of neuroendocrine markers (2, 3), a characteristic histology (1), and a distinctive clinical behavior, including an initially high sensitivity to radiation and chemotherapy (4).

Unfortunately, chemoresistant tumors often recur. In autopsy series, approximately one-third of these recurrences exhibit an apparent transition towards non-small cell histology, with cells resembling NSCLC partially or completely replacing SCLC cells (5, 6). An analogous in vitro phenomenon has been observed, involving either the accrual of large cell undifferentiated NSCLC features when SCLC cell lines are maintained for extended periods of time in continuous culture (2), or the occasional manifestation of such features when SCLC is first cultured (7).

We sought to determine what characteristics predispose some SCLC tumors to undergo this apparent switch to the NSCLC phenotype during tumor progression, and what genetic events drive this transition. In a first study, we investigated the possible involvement of the *myc* and *ras* genes which have been reported to be activated in some lung tumors (8–10). We found that SCLC cell lines amplified for the c-*myc* gene undergo a transition towards the NSCLC phenotype after the insertion of an oncogenically activated *ras* gene, v-Ha-*ras* (11). We now demonstrate that v-Ha-*ras* has similar activity when inserted into an N-*myc* amplified SCLC cell line. In addition, we show that this transition toward the NSCLC phenotype includes a fundamental switch in the expression of growth factors and receptors to those characteristic of NSCLC.

Methods

Cell culture and virus infection. The SCLC cell lines NCI-H249 and NCI-H69, and the squamous-type NSCLC cell line U1752 have been described previously (12, 13), including their culture conditions. Infection with the 1504A amphotrophic helper virus and with the 1504A pseudotype of Harvey murine sarcoma virus (14) was performed as described previously (15).

Growth studies. Cells were plated at 10^6 cells/replicate T-75 flask (Bellco Glass, Inc., Vineland, NJ) and cell counts performed every 2 d in an electronic cell counter (Coulter Electronics Inc., Hialeah, FL).

Cloning efficiency was determined as described previously (11). For studies in defined medium, the previously described Hites medium was used (16). Gastrin-releasing peptide (GRP) came from Peninsula Laboratories, Inc., Belmont, CA.

Electron microscopy. Transmission electron microscopy was performed as described previously (11, 15). Staining was with uranyl acetate/lead citrate.

Measurement of cellular GRP levels and dopa decarboxylase (DDC) activity. GRP levels in cell lysates were measured by RIA using a rabbit polyclonal antibody recognizing the NH_2-terminal 15 amino acids of GRP, as described previously (17).

DDC activity was determined as described previously (18). 1 U of

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1. *Abbreviations used in this paper:* DDC, L-dopa decarboxylase; DFMO, 2-difluoromethylornithine; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; GRP, gastrin-releasing peptide; NSCLC, non-small cell lung carcinomas; PDGF, platelet-derived growth factor; 249-*ras*, 249 infected with Harvey murine sarcoma virus; RRA, radioreceptor assay; SCLC, small cell lung carcinoma; TGF-α, transforming growth factor-α; v-Ha-*ras*, viral Harvey *ras* gene; 249, SCLC cell line NCI-H249.

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enzymatic activity is defined as 1 nm of $^{14}CO_2$ released from decarboxylated [^{14}C]L-dopa per hour of incubation.

RNA extractions and Northern analysis. RNA was extracted and poly A$^+$ selected as described elsewhere (19), electrophoresed on 1.2 or 1.5% agarose-formaldehyde gels which were alkali treated, and transferred to nylon membranes (Gene Screen; New England Nuclear, Boston, MA). Hybridizations were performed at 42°C for 20 h in buffers containing 50% formamide, 5× standard saline citrate (SSC), 2× Denhardt's solution, 150 μg/ml salmon sperm DNA, and ~ 2.5 × 10^6 cpm/ml labeled probe. Most stringent washes were in 0.1× SSC/1% SDS at 55°C except for the v-*sis* hybridization, which was washed at 42°C.

DNA probes were labeled to ~ 10^9 cpm/μg (20) and included coding regions of the following genes: v-Ha-*ras*, 730-bp Sst I–Pst I fragment (Oncor, Gaithersburg, MD); human β-actin, recombinant plasmids containing human β actin sequences (provided by Dr. Don Cleveland, Johns Hopkins University, Baltimore, MD); human EGF receptor, pE7, 2.4-kb Cla I fragment derived from A431 cells (21); transforming growth factor-α (TGF-α), 1.3-kb Eco RI fragment (22); and v-*sis*, 1.2-kb Pst I fragment (23) used at reduced stringency to probe for human platelet-derived growth factor B (PDGF-B) chain gene (24).

EGF receptor studies. For detection of specific cell surface EGF receptors, binding studies with ^{125}I-epidermal growth factor (EGF) were performed as described (25), with the following modifications: for cells in suspension, bound counts were separated from unbound counts by centrifuging the cells through an oil cushion, as described previously (26). All binding studies were performed in triplicate at 37°C for 1 h in binding buffer containing 0.5 nM ^{125}I-EGF (110 μCi/μg; Amersham Corp., Arlington Heights, IL). For assessment of nonspecific binding, replicate binding reactions were performed in binding buffer containing a 500-fold molar excess of unlabeled EGF (Collaborative Research Inc., Lexington, MA). Specific bound counts were calculated as total counts minus nonspecific counts.

Metabolic labeling of cells and immunoprecipitation. Cells were labeled for 4 h with [^{35}S]cysteine by previously described methods (27), then washed with PBS, harvested by scraping (adherent cells), and lysed in 1 ml of RIPA buffer (300 mM NaCl, 100 mM Tris-HCl, 2% Triton X-100, 2% Na-deoxycholate, 0.2% SDS, 0.4% BSA, and 2 mM PMSF). After a 30-min incubation on ice, the lysate was clarified by centrifugation (30 min at 2,100 *g*). ^{35}S-Labeled proteins were immunoprecipitated with 10 μg of partially purified rabbit preimmune serum or rabbit antiserum that had been raised against recombinant human TGF-α (a gift of Dr. Rik Derynck, Genentech, Inc., South San Francisco, CA).[2] After solubilization, the immunoprecipitates were analyzed by 15% SDS-PAGE gel (28) and subsequently fluorographed and autoradiographed at −70°C for 48 h.

Collection, preparation, size fractionation, radioreceptor assay (RRA), and RIA of conditioned media. Conditioned media were collected, concentrated, and dialyzed against acetic acid as described previously (29), clarified by centrifugation at 2,100 *g* for 30 min at 4°C, lyophilized, and dissolved in 1 M acetic acid to 25 mg total proteins/ml. Insoluble material was removed by centrifugation at 10,000 *g* for 15 min. The sample was chromatographed in 1 M acetic acid on a 2.6 × 90-cm Sephadex G-100 column (Pharmacia Fine Chemicals, Piscataway, NJ) with an upward flow of 30 ml/h to process 100 ng of protein from 4 ml of 100-fold concentrated medium. Fractions containing 3 ml of eluate were lyophilized and resuspended in 300 μl PBS. RRA of these fractions for assay of EGF-competing activity was performed on A431 membranes by published methods (27). For detection of TGF-α, samples were reduced with 40 mM dithiothreitol, denatured by immersion for 1 min in a boiling water bath, and assayed with a kit (Biotope, Inc., Seattle, WA) according to the manufacturer's protocol.

2. Lupu, R., A. Wellstein, J. Sheridan, B. W. Ennis, G. Zugmaier, D. Katz, R. B. Dickson, and M. E. Lippman, manuscript submitted for publication.

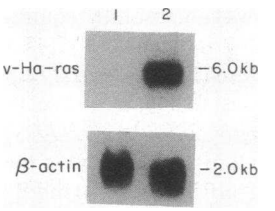

Figure 1. Northern blot of 13 μg of poly A$^+$ RNA from NCI-H249 cells (lane *1*) and NCI-H249 cells infected with v-Ha-*ras* (lane *2*) hybridized to ^{32}P-oligolabeled probes from the coding regions of the v-Ha-*ras* and human β-actin genes.

Results

Morphologic features of v-Ha-ras–infected cells. Retroviral insertion of v-Ha-*ras* into the N-*myc* amplified SCLC cell line NCI-H249 (30) resulted in high levels of v-Ha-*ras* expression, as confirmed by Northern blot analysis (Fig. 1). 2–4 wk after infection, NSCLC features appeared (31), including prominent nucleoli, a reduced nuclear/cytoplasmic ratio, well-developed spot desmosomes, and, importantly, the appearance of a surface adherent growth pattern. This mass culture of v-Ha-*ras*–infected 249 cells was subsequently passaged as a surface-adherent culture, referred to hereafter as 249-*ras*.

Increased growth rate, cloning efficiency, and tolerance to 2-difluoromethylornithine (DFMO). Relative to SCLC, NSCLC in culture exhibits a shorter population doubling time and a higher soft agar cloning efficiency (2). After *ras* insertion, 249 exhibited a reduction in its population-doubling time from 3.3 to 2.4 d, during log phase growth (Fig. 2, *249* vs. *249-RAS*), and an increase in soft agar cloning efficiency from 3.2 to 5.3%.

A cytocidal response to the drug DFMO, an inhibitor of polyamine biosynthesis, has been consistently observed in SCLC but not NSCLC (32, 33). In a previous study, v-Ha-*ras* insertion converted c-*myc* amplified SCLC cells to a DFMO response pattern typical of NSCLC cells (11). We now report similar observations in an N-*myc* amplified SCLC cell line. Before v-Ha-*ras* insertion, 249 cells displayed a typical cytocidal response to DFMO (Fig. 2, *249/DFMO*), with death of all cells occurring within 10 d. By contrast, the 249-*ras* cell line resembled NSCLC cell lines in demonstrating no evidence of cytotoxicity and only a mild growth suppression in the presence of DFMO (Fig. 2, *249 RAS/DFMO*).

Loss of GRP growth requirements, and reduced neuroendocrine markers in ras-infected 249 cells. GRP has been reported to be growth stimulatory for many SCLC cell lines in defined (HITES) medium (34), including line 249. By contrast, bombesin/GRP has not been reported to be an essential growth factor for the propagation of NSCLC cells in defined medium (35). We therefore looked for *ras*-induced alterations in the

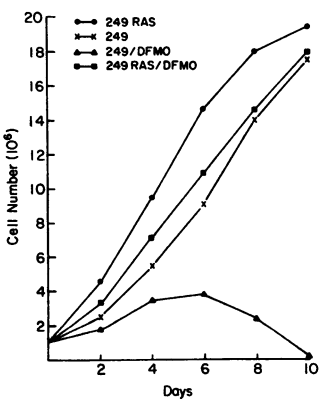

Figure 2. Growth study of 249 vs. 249-*ras* (*249 RAS*) cells in the presence or absence of the drug DFMO. Cells were plated at day 0 at 1 × 10^6 cells/T-75 flask. All data points represent averages of quintuplicate cell counts. Standard errors for all data points are ≤ 15%.

GRP requirements of 249 cells. GRP was an absolute requirement for both the clonal growth of 249 in semisolid HITES medium and for growth in liquid culture (Fig. 3, *249 HITES* vs. *249 HITES/GRP*). In marked contrast to 249, 249-*ras* cells demonstrated significant growth (Fig. 3, *249 RAS HITES*) and soft agar colony formation (3.8% cloning efficiency) in the absence of GRP. In HITES, 50 mM GRP was only mildly growth stimulatory for 249-*ras*, increasing soft agar cloning efficiency to 4.6%, and increasing cell counts by 50% at the end of a 10-d growth study (Fig. 3, *249 RAS HITES* vs. *249 RAS HITES/GRP*). By RIA (17) GRP was detectable in lysates of 249 cells, but was undetectable in 249-*ras* cell lysate. Thus, v-Ha-*ras* insertion was associated with the abrogation of an absolute growth requirement for exogenous GRP by a mechanism other than autocrine stimulation by enhanced endogenous GRP production.

The decline in GRP production in 249-*ras* relative to 249 was accompanied by a decrease in DDC activity, the key decarboxylating enzyme of amine precursors in neuroendocrine cells, and a marker of the SCLC phenotype (3). Activities of this enzyme were 2,100 and 1,200 U/mg protein (18) for 249 and 249-*ras*, respectively.

Induction of EGF receptor (EGF-R) gene expression and specific EGF cell surface binding. Because 249-*ras* had acquired a number of NSCLC features and had escaped from an SCLC-specific growth factor requirement, we next tested 249-*ras* for the expression of growth factors and receptors characteristic of NSCLC but not SCLC (25, 36–38), including EGF-R, TGF-α, and PDGF.

In Northern blot studies, no EGF-R transcripts were detected when 13 μg of poly A$^+$ 249 RNA was probed with a human probe spanning the transmembrane domain of the EGF-R gene (21) (Fig. 4, lane *1*). By contrast, an equal amount of 249-*ras* RNA exhibited intense bands for EGF-R of ~ 9.5 and 6 kb and a minor species of 1.7 kb (lane *2*), corresponding exactly in size to bands seen in poly A$^+$ RNA from U1752 (lane *3*), an epidermoid-type NSCLC cell line known to express large numbers of EGF receptors (37). Additional minor species of 4.6 and 2.9 kb were also seen in U1752.

In binding studies performed with 0.5 nM ^{125}I-EGF, specific cell surface binding of EGF was not observed in the parent 249 cells, 249 cells infected with helper virus, or NCI-H69, a cell line previously shown to lack EGF receptors (37). By contrast, 249-*ras* was observed to bind 33 fmol of ^{125}I-EGF/10^6 cells, nearly half of the binding observed in positive control U1752, which bound 70 fmol/10^6 cells. The specific binding of ^{125}I-EGF by 249-*ras* provides evidence for the appearance of significant numbers of specific cell-surface EGF receptors.

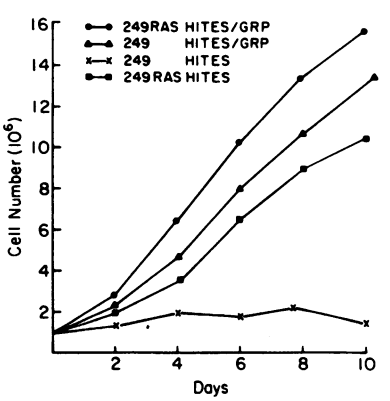

Figure 3. Growth of 249 and 249-*ras* (*249 RAS*) cells in defined (*HITES*) medium±50 mM GRP. Seeding density was 10^6 cells/T-75 flask. All data points represent averages of quintuplicate cell counts. Standard errors for all data points are ≤ 15%.

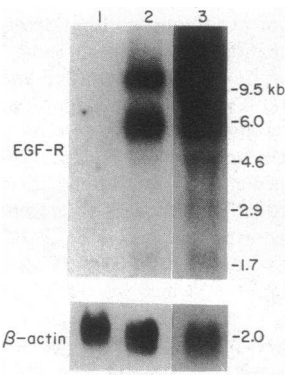

Figure 4. Northern blot of 13 μg poly A$^+$ RNA/lane from the following cell lines probed with the coding region of the human EGF receptor and β-actin genes: lane *1*, 249; lane *2*, 249-*ras;* lane *3*, U1752 squamous lung carcinoma cell line.

249-ras expresses TGF-α mRNAs and secretes TGF-α. Expression of the gene for a key ligand of EGF-R, TGF-α, has been found in most NSCLC cell lines but not in any SCLC cell lines tested to date (38). Therefore, 249 and 249-*ras* were tested for expression of this gene and its protein product. 10 μg of 249-*ras* and U1752 poly A$^+$ RNA probed with a 1.3-kb Eco RI fragment from the TGF-α coding region (22) detected a major 4.8-kb and a minor 1.6-kb species (Fig. 5, lanes *2* and *3*), corresponding in size to transcripts previously observed in NSCLC cell lines (38). TGF-α expression was not detected in 249 (Fig. 5, lane *1*).

Since 249-*ras* demonstrated TGF-α gene expression, it was next tested for the biosynthesis of TGF-α. Lysates of [^{35}S]cysteine-labeled 249-*ras* cells immunoprecipitated with a rabbit anti–human TGF-α antiserum and electrophoresed on SDS-PAGE gels (28) revealed a single 6-kD species (Fig. 6 *A*) corresponding exactly in size to a TGF-α species (39) observed in lysates of MCF-7 breast carcinoma cells, known producers of TGF-α (29), immunoprecipitated by the same antiserum under identical conditions.[2] No bands were seen in lysate precipitated with rabbit preimmune serum or in anti-TGF-α immunoprecipitates of 249 cells. Furthermore, EGF RRA and TGF-α RIA (Fig. 6 *B*) of medium conditioned for 48 h by 249-*ras* and column-chromatographed detected ~ 220 ng/liter of a 6-kD species of TGF-α. No TGF-α was detected by identical methods in medium conditioned by control 249 cells. Thus, 249-*ras* cells not only produced TGF-α, but also processed it to its biologically active form and secreted it into the medium.

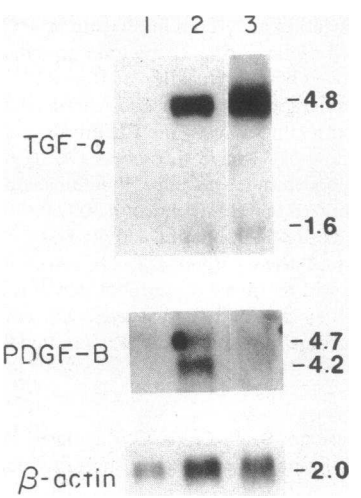

Figure 5. Northern blot of 10 μg poly A$^+$ RNA, probed with the coding regions of human TGF-α and β-actin genes. PDGF-B chain gene was probed at reduced stringency with a v-*sis* probe. Lane *1*, 249; lane *2*, 249-*ras;* lane *3*, U1752 squamous lung carcinoma cell line. Size markers are in kilobases.

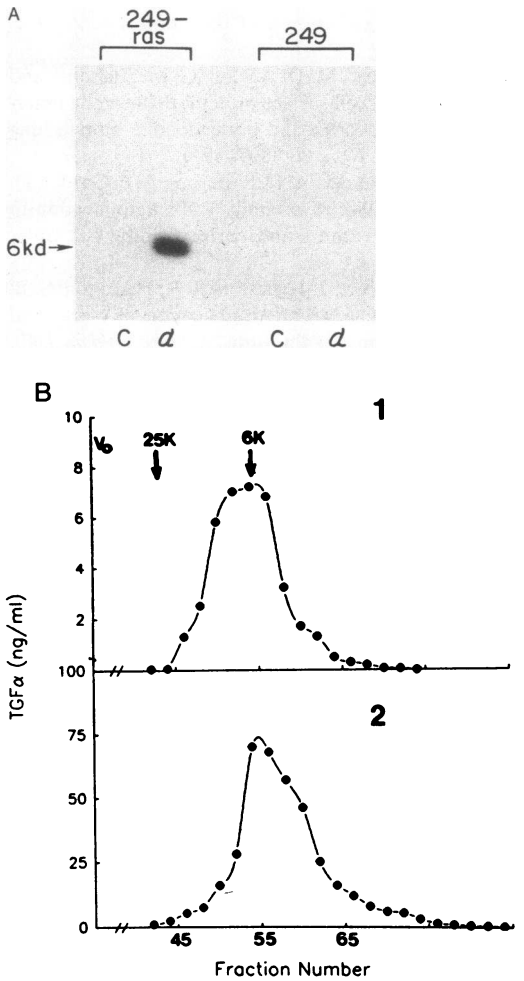

Figure 6. A, SDS/PAGE gel of cells metabolically labeled for 4 h with [^{35}S]cysteine, lysed, and immunoprecipated with either control normal rabbit serum (*C*), or rabbit anti-human TGF-α antiserum (α). *B,* Detection of TGF-α in conditioned medium from 249-*ras* cells after size fractionation by Sephadex G-100 column chromatography. *1,* RRA using A431 cell membranes; *2,* RIA (Biotope kit). *25 K* and *6K,* molecular weights as determined by marker proteins.

249-ras expresses PDGF-B chain mRNAs. As with TGF-α, expression of the genes for the two PDGF peptides, A and B chain, has been found almost universally in NSCLC cell lines but never in SCLC (38). No PDGF-B chain mRNA transcripts were seen when 10 μg of poly A$^+$ RNA from 249 cells was probed at reduced stringency with a probe from the coding region of v-*sis* (23), with which PDGF-B shares 87% homology (24) (Fig. 5, lane *1*). However, the characteristic human PDGF-B mRNA species of ~ 4.2 kb (40) was seen in 249-*ras* poly A$^+$ RNA (lane *2*), as well as a weaker 4.7-kb band, consistent with a previous report of additional PDGF-B chain mRNA species in some NSCLC cell lines (38). Thus, 249-*ras* also expresses the NSCLC-associated PDGF-B chain gene.

Discussion

Our results provide the most direct evidence to date that cells indistinguishable from NSCLC can rapidly evolve out of apparently homogeneous populations of SCLC cells. The factor inciting this progression, in our model system, involves the imposition of a single genetic event, the insertion of an activated *ras* gene. Mutational activation of *ras* genes may sometimes contribute to the NSCLC phenotype in vivo, as suggested by the detection of activating mutations of the Ki-*ras* gene in a high percentage of adenocarcinomas of the lung (10), in contrast to the lack of reports of these mutations in SCLC.

Our previous (11) and present findings suggest that oncogenically activated *ras* genes may act in concert with certain amplified or overexpressed *myc* genes in prompting phenotypic conversion of SCLC to NSCLC. Approximately 16% of small cell lung tumors have fourfold or higher amplifications of L-, N-, or c-*myc* (8). Additional studies suggest more subtle increases in copy numbers of *myc* genes in additional SCLC tumors (9). Evidence also suggests a greater preponderance of *myc* amplifications in tumors after combined chemotherapy (8), the period when partial or complete progression toward NSCLC has been observed (5, 6). Previous studies have found associations between particular types of *myc* gene amplifications and phenotypic features of SCLC lines. First, c-*myc* amplification has been associated with the "morphologic variant" SCLC features of increased growth rate, increased soft agar cloning efficiency, and growth in looser aggregates, compared with classic SCLC (41). In studies in our laboratory it was found that a c-*myc*-amplified SCLC cell line, NCI-H82, responded to v-Ha-*ras* insertion with the acquisition of growth characteristics, morphologic features, and a biochemical profile consistent with poorly differentiated NSCLC. By contrast, OH-3, a line not amplified for c-*myc,* appeared unaffected by v-Ha-*ras* insertion and expression (11). A permissive role for overexpression of c-*myc,* but not L-*myc,* in progression of SCLC to NSCLC was suggested by studies with SCLC cell line NCI-H209, which expresses high levels of L-*myc* (42). NCI-H209 was found to respond to v-Ha-*ras* insertion with phenotypic conversion only after its expression of c-*myc* had been augmented by the transfection of an exogenous c-*myc* gene (43) (Mabry, M., M. Borges, J. P. Falco, R. Casero, B. D. Nelkin, R. Jasti, and S. B. Baylin, manuscript submitted for publication). Our present study suggests that N-*myc* amplification/overexpression may similarly permit progression of SCLC to NSCLC in response to the appearance of an activated *ras* gene.

In the present study we show that, accompanying the morphologic and growth alterations prompted by v-Ha-*ras* insertion into NCI-H249, there is a fundamental shift in its growth factor/receptor profile. This shift is marked by the loss of an SCLC-associated growth requirement for GRP in defined medium (34) and by the appearance of growth factors and growth factor receptors characteristic of NSCLC. 249-*ras* resembles a number of NSCLC cell lines in expressing both TGF-α and its receptor, EGF-R (44). In two such lines, exogenous TGF-α was found to be growth stimulatory (44) and anti-TGF-α antibodies were found to be growth inhibitory (45). These data imply a role for an autocrine loop involving TGF-α and EGF-R in stimulating the growth of NSCLC.

In vivo evidence that EGF receptor and TGF-α expression may be involved in the pathogenesis of NSCLC includes the finding of increased expression of EGF receptors in pathologic specimens of NSCLC relative to the adjacent normal lung (46). Also, in epidermoid lung carcinoma a correlation has been observed between the amount of EGF receptor expression detected by immunohistochemical stains of tumor specimens and the clinical stage of the tumor (47). Finally, higher levels of TGF-α have been detected in the malignant pleural effu-

sions of NSCLC tumors compared with nonmalignant effusions (48).

Studies have suggested a paracrine role for TGF-α and PDGF, secreted by NSCLC but not by SCLC, in stimulating the proliferation of neighboring fibroblasts and thus inducing the prominent fibrous stromal reaction observed after the inoculation of NSCLC cells but not SCLC cells into nude mice (49). Thus, production of TGF-α and PDGF may have both autocrine and paracrine roles in influencing the behavior of NSCLC tumors and their surrounding stroma. Our data suggest that SCLC that has progressed to NSCLC may be influenced by the same factors.

Our cell culture model of tumor progression suggests the involvement of certain genes and genetic events in the progression of SCLC toward NSCLC. The hypotheses posed by this model can, to some extent, be tested in vivo by tracking tumor progression through the examination of pathologic specimens. It will be of interest to determine by this method whether c- or N-*myc* gene amplifications in vivo increase the likelihood of progression of SCLC toward NSCLC and whether tumors that have progressed to NSCLC have acquired new *ras* gene mutations, the expression of NSCLC-associated growth factor and growth factor receptor genes, or the capacity to specifically bind EGF.

Finally, our findings, which support the concept that SCLC can evolve into NSCLC, provide further evidence to suggest that endocrine cells of the lung, which bear features similar to SCLC (2, 3), may be linked to the other major types of bronchial epithelial cells through a common progenitor cell (2, 50, 51).

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