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v-Ha-*ras* oncogene insertion: A model for tumor progression of human small cell lung cancer

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ABSTRACT **Small cell lung cancer (SCLC) manifests a range of phenotypes in culture that may be important in understanding its relationship to non-SCLCs and to tumor progression events in patients. Most SCLC-derived cell lines, termed "classic" SCLC lines, have properties similar to SCLC tumors in patients, including high expression of neuroendocrine markers and low c-myc oncogene expression. A significant number of SCLC lines characterized as "biochemical or morphologic variant" SCLC lines have decreased levels of endocrine differentiation markers associated with increased proliferative indices, amplification of the c-myc oncogene, and growth patterns and biochemical markers more typical of non-SCLCs. To delineate further the relationships between these phenotypes and the molecular events involved, we have inserted the v-Ha-*ras* gene in SCLC cell lines with (biochemical variant) and without (classic) an amplified c-myc gene. These two SCLC subtypes had markedly different phenotypic responses to similar levels of expression of v-Ha-ras RNA. No biochemical or morphologic changes were observed in classic SCLC cells. In contrast, in biochemical variant SCLC cells, v-Ha-*ras* expression induced features typical of large cell undifferentiated lung carcinoma, including adherent monolayer growth patterns, increased cloning efficiency, increased levels of non-SCLC cell markers, ultrastructural characteristics, and an acquired resistance to polyamine depletion typical of large cell carcinoma, but not SCLC, *in vitro*. Expression of v-Ha-*ras* in biochemical variant SCLC cells directly demonstrates that important transitions can occur between phenotypes of human lung cancer cells and that these may play a critical role in tumor progression events in patients. The findings provide a model system to study molecular events involved in tumor progression steps within a series of related tumor types.**

Human small cell lung cancer (SCLC) cells in culture exhibit differing phenotypes that reflect the clinical behavior of this tumor and that have generated interest in the cellular and molecular biology events involved (1–4). Most culture lines of SCLC, termed "classic" lines, have properties typical for the neoplasm in patients, including high expression of endocrine cell differentiation markers (1, 2), sensitivity to irradiation (5–7), and low levels of c-myc gene expression (8, 9). However, a significant number of cell lines, termed "biochemical variant" SCLC lines, have decreased levels of endocrine differentiation markers (1, 2), increased growth rates, relative resistance to irradiation (5–7), and amplification of *MYC* family genes (8, 9). Another subset, known as "morphologic variants," additionally exhibit features similar to the non-SCLC large cell undifferentiated carcinoma (1, 2, 10). The development of these morphologically variant cells is particularly important since it provides a model to study clinically observed links between SCLC and non-SCLC phenotypes (11–13) and to explore potential transitions between these phenotypes (11–17) that may play an important role in the development of treatment resistance in patients (11–14, 18).

The molecular events mediating the potential transitions between SCLC subtypes in culture are not known. Increased c-myc expression, seen in "variant" cells, has provided some insight into mechanisms that could convert classic to variant SCLC cells (8, 9). When classic SCLC cells were transfected with the c-myc gene, increased growth potential was observed and changes in growth patterns ensued (19). However, no reduction in neuroendocrine biochemistry occurred and transition to a large cell phenotype, including surface adherent growth patterns, was not observed (19). These results suggest that other events, including the expression of other genes, may be necessary for the observed transitions to occur.

In this regard, *RAS* family gene mutations have been reported in non-SCLC tumors (20), and increased *RAS* gene expression appears to be more characteristic of non-SCLC than SCLC tumors (21). Complementation is known between *MYC* oncogenes that code for nuclear proteins and *RAS* family genes whose products are involved in ligand signal transduction and fibroblast transformation (22). Since increased c-myc expression segregates with variant SCLC cell lines (8, 9, 23), we speculated that such an interaction of *RAS* and *MYC* gene expression might confer features of large cell undifferentiated carcinoma to SCLC. To explore this possibility, we infected representative cell lines of classic and variant SCLC with the Harvey murine sarcoma virus (Ha-MSV) that carries the v-Ha-*ras* oncogene. Our results demonstrate that such a mutated *ras* gene can supply critical functions for changing cell phenotypes in human lung cancer and also can provide a model system to study interrelationships among those phenotypes.

MATERIALS AND METHODS

Cell Culture and Virus Infection. Cells from well-characterized culture lines of SCLC, NCI-H82, OH-3, and OH-1, were cultured as described (1, 2, 23). NIH 3T3 cells infected with Ha-MSV and 1504A amphotropic murine leukemia helper viruses were kindly provided by Alan Rein (Frederick Cancer Research Facility, Frederick, MD) (24, 25). The v-Ha-*ras* oncogene was introduced into the SCLC lines by retroviral infection as described (26).

Growth Studies. The cells were seeded in 75-cm^2 polystyrene flasks at a density of 2×10^5 cells per ml. Appropriate SCLC lines that were uninfected and those infected with 1504A helper virus alone were used as controls. Cell numbers were determined by using an automated cell counter (Coulter Electronics) and periodically confirmed by hemocytometer counts.

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Abbreviations: SCLC, small cell lung cancer; Ha-MSV, Harvey murine sarcoma virus; F_2MeOrn, difluoromethylornithine.

Cloning Efficiency. Cells infected with Ha-MSV and the appropriate uninfected SCLC controls were suspended at a density of 20,000 cells per plate in 1.8% methylcellulose in growth medium. The methylcellulose cell suspension was layered over 3% (wt/vol) agar base and colonies were scored as positive if >16 cells were found at day 10. Cloning efficiency was calculated as the number of colonies counted per plate divided by the number of cells suspended. The data reported are the results of quintuplicate samples in three independent experiments.

DNA Probes. Recombinant plasmids containing human β-actin sequences (provided by Don Cleveland, Johns Hopkins University School of Medicine) and an *Sst* I–*Pst* I fragment (730 base pairs) containing only the v-Ha-*ras* gene p21-coding region (Oncor, Gaithersburg, MD) were labeled with [^{32}P]dCTP by nick-translation.

RNA Hybridization. All procedures used for analyzing RNA and DNA in our laboratory have been described (27, 28). Total RNA (6 μg) was electrophoresed on a formaldehyde 1.2% agarose gel and transferred to nitrocellulose. After hybridization with the appropriate probes, filters received a final wash at 65°C in 0.1× SSC/0.1% $NaDodSO_4$ for 30 min and were then autoradiographed with intensifying screens at −70°C for 30–48 hr. (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0.)

Electron Microscopy. Cells growing as floating aggregates were centrifuged at 20 × *g* for 5 min, pelleted, washed, and fixed as below. Cells induced to grow as attached cells were fixed while still attached to the flask in 2% (vol/vol) glutaraldehyde/0.1 M sodium cacodylate, pH 7.4, postfixed in 1% OsO_4/0.1 M sodium cacodylate, sectioned, stained with uranyl acetate/lead citrate, and viewed with a Phillips model 410 PEM electron microscope at 100 kV (29).

Immunoperoxidase Stains. Attached cells were harvested with trypsin/EDTA and pelleted at 20 × *g* for 5 min. The cells were then fixed, stained with hematoxylin and eosin, and stained with a monoclonal antibody against carcinoembryonic antigen or a monoclonal antibody against keratin (30, 31).

Neuroendocrine Markers. The neuroendocrine marker L-dopa decarboxylase was measured by a modification of the method of Beaven *et al.* (32). Creatine kinase was measured (33) by A. F. Gazdar, H. Oie, and I. Linnoila (National Cancer Institute, Naval Medical Branch, Bethesda, MD).

RESULTS

Characteristics of SCLC Lines Selected for Study. Two phenotypic classes of SCLC were selected for expression of v-Ha-*ras*. Lines OH-1 and OH-3 were chosen to represent classic SCLC cells (1, 2, 24). These lines have typical features of SCLC, which include early neuroendocrine markers (such as high L-dopa decarboxylase activity), no c-myc gene amplification, low c-myc gene expression, nonadherent growth patterns typical for SCLC cells (1, 2, 8–10, 23), and a cytotoxic response to inhibition of polyamine biosynthesis by the ornithine decarboxylase inhibitor 2-difluoromethylornithine (F_2MeOrn) (34, 35). Cell line NCI-H82 (1, 2) is an example of biochemical variant SCLC. These are characterized by decreased neuroendocrine markers, an amplified and highly expressed c-myc gene, increased growth potential, and increased cloning efficiency. However, these variant cells maintain SCLC cell features including small cell morphology, nonadherent growth patterns, and the cytotoxic response to polyamine depletion by F_2MeOrn (1, 2, 34–36).

Presence and Expression of Ha-MSV Sequences. Integration and expression of the v-Ha-*ras* gene were efficiently achieved in each of the recipient SCLC lines. DNA from infected cells, when hybridized to a *Sst* I–*Pst* I restriction fragment containing only the v-Ha-*ras* coding region, showed a predicted 880-base-pair v-Ha-*ras* internal *Hin*dIII fragment, demonstrating presence of proviral DNA (data not shown).

The integrated Ha-MSV sequences were highly and similarly expressed (Fig. 1*E*) in each cell type, and no transcripts homologous to the v-Ha-*ras* p21-coding-region probe were detected in uninfected SCLC cells or cells infected with helper virus alone. Also, transcripts for endogenous *HRAS* were low or nondetectable in total RNA from the cells under study, using either the above v-Ha-ras probe or probes to the human *HRAS* protooncogene (data not shown).

Response of Classic SCLC to v-Ha-*ras* Expression. The classic SCLC lines OH-1 and OH-3 showed no discernible phenotypic changes in response to expression of v-Ha-*ras*. Despite the high levels of v-Ha-ras mRNA, no changes in morphology (Fig. 1 *A* and *B*), growth rate (see Fig. 3*A*), cloning efficiency (Table 1), biochemical markers (Table 1), or F_2MeOrn sensitivity (data not shown) were observed in the classic SCLC lines.

Response of Biochemical Variant SCLC Cells. Unlike classic SCLC lines, after Ha-MSV infection, the biochemical variant cell line NCI-H82 developed features typical of large cell undifferentiated lung carcinoma. Within 5–7 days after infection, heterogeneity of cell size was apparent, with many cells being larger than control cells, having an increased cytoplasm-to-nucleus ratio, and displaying a less pyknotic nuclear pattern with prominent nucleoli (Figs. 1 *C* and *D* and 2 *A* and *B*). A striking growth change appeared within 7–10

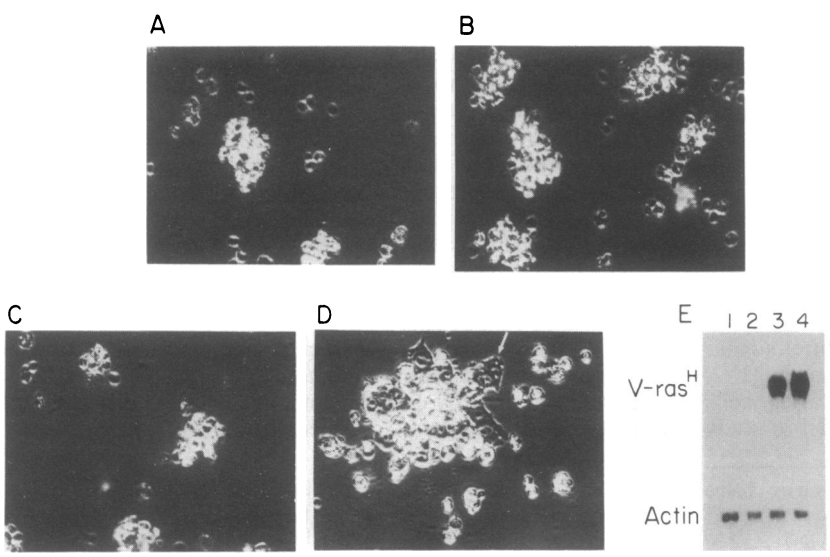

FIG. 1. Morphology of OH-3 control (*A*) and OH-3 v-Ha-*ras*-expressing (*B*) cells. Cells expressing v-Ha-*ras* (*B*) continue to grow as suspended aggregates of small homogenous birefringent cells. NCI-H82 (*C*) normally grows as a suspension culture of loosely aggregated cells. In cultures of v-Ha-*ras*-infected NCI-H82 (*D*) many adhere to polystyrene after v-Ha-*ras* expression. Note increased cell size and prominent nucleoli (arrow). These morphologic changes are maintained in long-term culture. (×130.) (*E*) RNA blot hybridization of total cytoplasmic RNA (6 μg) from control OH-3 (lane 1), NCI-H82 (lane 2), v-Ha-*ras*-infected OH-3 (lane 3), and v-Ha-*ras*-infected NCI-H82 (lane 4) cells. A *Sst* I–*Pst* I fragment (730 base pair) (Oncor) containing the v-Ha-*ras* p21-coding region was used as the probe. Note similarly high levels of expression in v-Ha-*ras*-infected OH-3 and v-Ha-*ras*-infected NCI-H82. β-Actin hybridization demonstrates equivalent loading of RNA in each lane.

Table 1. Characteristics of Ha-MSV-infected and noninfected SCLC cells

Cell line	Cloning efficiency	L-Dopa decarboxylase activity	Creatine kinase BB activity
OH3	2.1	190	ND
OH3/v-Ha-*ras*	1.8	198	ND
NCI-H82	4.1	6.01	906
NCI-H82/v-Ha-*ras*	7.2 ($P < 0.05$)	5.02	387

Cloning efficiency values are means of quintuplicate samples from three experiments. L-Dopa decarboxylase activity is reported as (mol of $^{14}CO_2$) $\times 10^{-2}$ per hr per 10^7 cells. Creatine kinase BB is measured as units/mg of protein. ND, not determined. OH3/v-Ha-*ras* and NCI-H82/v-Ha-*ras*, v-Ha-*ras*-infected OH3 and NCI-H82 cells, respectively.

days in which 60–70% of the cells formed adherent monolayers (Fig. 1 *C* and *D*), which is typical for non-SCLC cells in culture but unusual for most lines of SCLC (1, 2, 10, 23). The growth potential of the cells was affected by increased v-Ha-*ras* expression. As shown in Fig. 3, *ras*-infected NCI-H82 cells had a decreased doubling time, and cloning efficiency was significantly increased (Table 1). Doubling time and cloning efficiency were unaffected by infection of SCLC cells with helper virus alone.

The growth and morphologic changes induced after v-Ha-*ras* expression in biochemical variant cells were accompanied by biochemical changes, consistent with a transition from the SCLC phenotype to a large cell lung cancer phenotype. The L-dopa decarboxylase activity, already low in these cells, did not change in infected cells (Table 1). However, the level of creatine kinase BB, a marker that is elevated in SCLC (1, 37), was reduced sharply in variant cells with v-Ha-*ras* expression (Table 1). Infected cells also developed additional features typical of non-SCLC but not SCLC. Poorly developed desmosomes typical of large cell carcinoma (38-43) were detected at cell junctions (Fig. 2*C*). Immunohistochemistry also revealed an increase in carcinoembryonic antigen content of the *ras*-infected cells (Fig. 4 *A* and *B*), consistent with the higher levels of this marker in non-SCLC vs. SCLC tumors in patients (44). Altered keratin expression, suggesting conversion to non-SCLC (45), was also found in v-Ha-*ras*-infected variant cells (Fig. 4 *C* and *D*).

Importantly, v-Ha-*ras* infection changes the response of the variant cells to F_2MeOrn to that of non-SCLC cells. F_2MeOrn depletes cells of polyamines by inhibiting ornithine decarboxylase, the first and rate-limiting enzymatic step in polyamine biosynthesis (46, 47). Polyamine depletion causes a cytotoxic response in SCLC cells in culture, as opposed to the typical cytostatic response seen in most cultured tumor cells, including non-SCLC cells (34–36). Large cell undifferentiated lung carcinoma cells are especially resistant to F_2MeOrn (36, 48). Line NCI-H82 has proven (35, 36) to be particularly sensitive to F_2MeOrn, as is again seen in this study in the control cells prior to *ras* infection (Fig. 5). However, after introduction and expression of v-Ha-*ras* and acquisition of large cell lung cancer features, the NCI-H82 cells became resistant to the cytotoxic effects of F_2MeOrn treatment (Fig. 5).

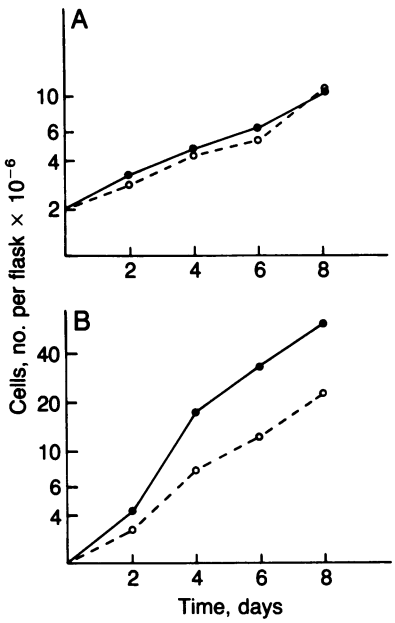

FIG. 3. (*A*) Growth curves for OH-3 (●) and v-Ha-*ras*-infected OH-3 (○). (*B*) Growth curves for NCI-H82 (●) and v-Ha-*ras*-infected NCI-H82 (○). Cells were counted by using an automated cell counter (Coulter). Values presented are the mean of quintuplicate samples in three experiments. Standard errors were <15%. Note decreased doubling times upon v-Ha-*ras* expression in NCI-H82 (*B*). No changes in growth were observed after infection of OH-3 with Ha-MSV (*A*).

DISCUSSION

Introduction of the v-Ha-*ras* oncogene into cultured human SCLC cells has produced a dramatic change in cell phenotype that is dependent upon the subtype of the cell in which the mutated *RAS* gene is expressed. This change may involve complementation events between this gene and the c-myc gene. In classic SCLC, which exhibits typical neuroendocrine features and low c-myc expression, v-Ha-*ras* expression exerted no discernible phenotypic effect. However, in variant SCLC lines that have lost the endocrine biochemistry of SCLC and have increased c-myc expression, identical levels of v-Ha-ras mRNA induced dramatic phenotypic changes, resulting in cells that, by several criteria, modeled

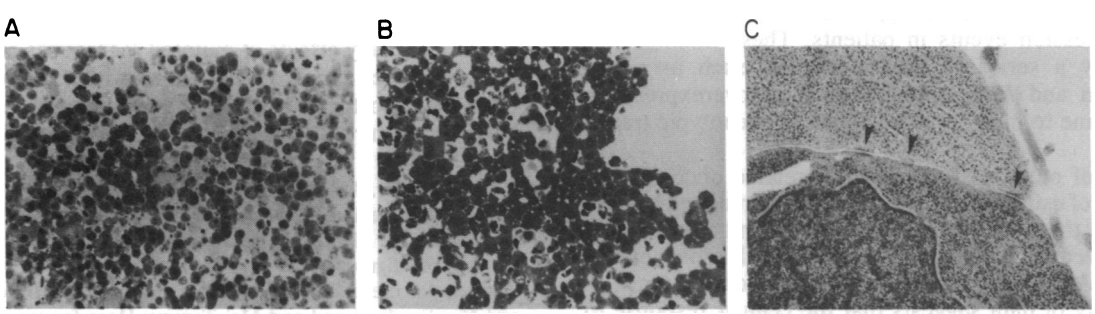

FIG. 2. Thick-section (0.5 μm) micrographs of NCI-H82 (*A*) and v-Ha-*ras*-infected NCI-H82 cells (*B*) grown to confluence and prepared for electron microscopy. (×180.) Note that the adherent v-Ha-*ras*-infected NCI-H82 (*B*) cells are larger with more prominent nucleoli and increased cytoplasmic/nuclear ratios. (*C*) Poorly developed desmosomes (arrows), which are found in cell–cell attachment areas of v-Ha-*ras*-infected NCI-H82, are not found in NCI-H82. (×12,600.)

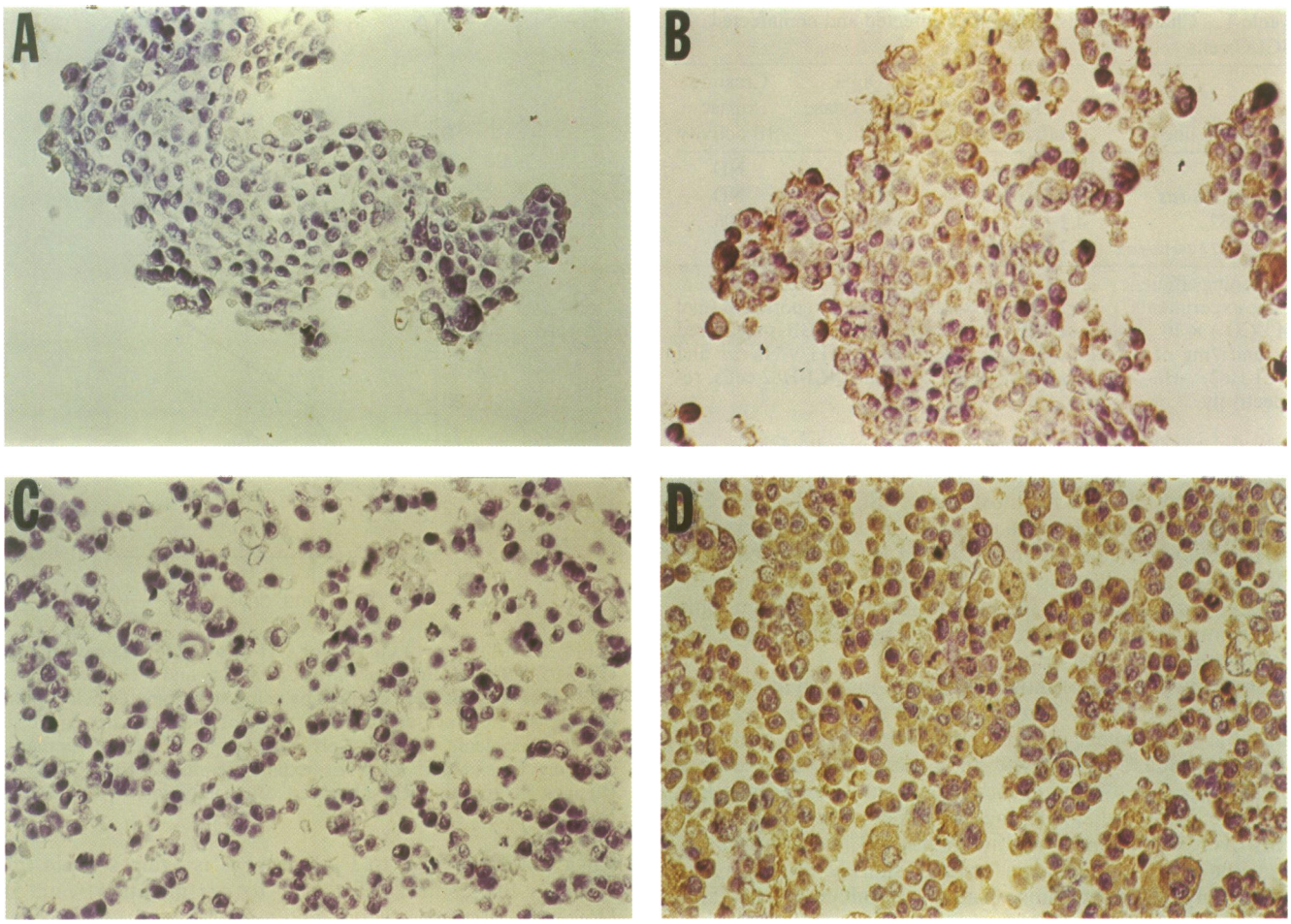

FIG. 4. Immunostaining of carcinoembryonic antigen in NCI-H82 (*A*) and v-Ha-*ras*-infected NCI-H82 (*B*) cells. Note increased intracytoplasmic staining in NCI-H82 cells expressing v-Ha-*ras* (*B*) when compared to NCI-H82 (*A*). Immunostaining with antibody to keratin of NCI-H82 (*C*) and of v-Ha-*ras*-infected NCI-H82 (*D*) shows an alteration in keratin expression in v-Ha-*ras*-infected NCI-H82 (*D*). Note in *B* and *D* that v-Ha-*ras*-infected NCI-H82 cells are larger and more heterogeneous in size and that nuclei are less pyknotic with an "open" chromatin pattern. (Hematoxylin counterstain; ×200.)

progression to large cell undifferentiated carcinoma. These findings have important implications for understanding the evolution of different cell populations in SCLC tumor progression (11–13).

Loss of neuroendocrine features in SCLC, especially in conjunction with morphologic features of large cell undifferentiated carcinoma, is associated *in vitro* with increased cloning efficiency and radiation resistance (1, 2, 5–7, 10). In patients with SCLC, the appearance of such non-SCLC cell types has been noted after relapse (11–13), a setting characterized by resistance to therapy (3, 4, 18). Our data now provide experimental evidence that the direct transition from SCLC to the large cell phenotype can occur and may model tumor progression events in patients. These findings also suggest how a series of genetic events, such as c-myc amplification and *RAS* gene mutation or overexpression, might combine to mediate this important phenotypic transition.

The role of mutated *RAS* genes or related oncogenes in progression of established tumors has been predicted but less well studied than effects of such genes in initial cell transformation events. Our data are consistent with known events involving *RAS* genes in transformation model systems. A growing body of data suggests that the cellular response to mutated forms of the *RAS* oncogene depends upon a series of background events related to the state of cell growth, differentiation, and transformation (49–53). For example, transformation of primary normal fibroblasts by mutated *RAS* genes is dependent upon interaction with c-myc or similar genes (22).

In addition to the presently studied transitions between SCLC and large cell phenotypes, other features of the differentiation potential of SCLC may be studied by our models. For example, occasional lines of SCLC manifest features of well-differentiated endocrine cells. Work from our laboratory (26) and others (49–53) suggest that, in tumor cells with such mature endocrine features, mutated *RAS* genes induce differentiation rather than tumor progression events. We have preliminary results with Ha-MSV infection in one such SCLC line that suggest that differentiation ensues after integration and expression of v-Ha-*ras*. Our data now suggest a model to study effects of mutated *RAS* genes on neoplastic cells of related tumor types. Human lung cancer cell lines offer the opportunity to study the cellular and the molecular basis for each response.

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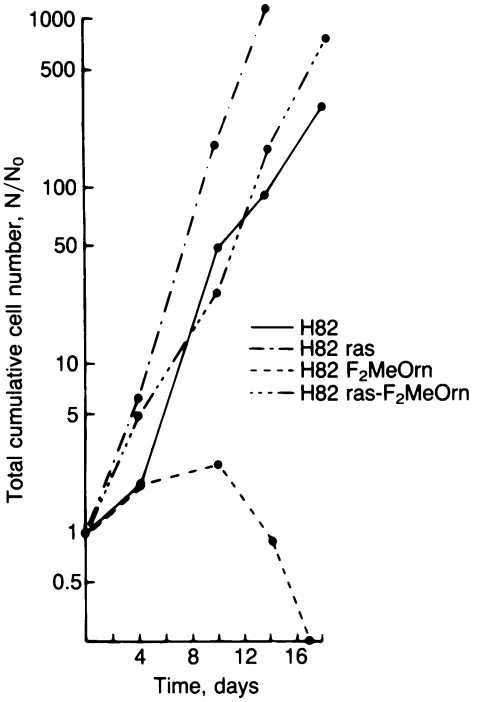

FIG. 5. Differential growth effects of 5 mM F_2MeOrn on NCI-H82 and v-Ha-*ras*-infected NCI-H82 cells. Values presented are means of quintuplicate samples from a representative experiment. Standard error was <20%. Note that F_2MeOrn is cytotoxic to NCI-H82 cells (as shown in previous studies) and that v-Ha-*ras*-infected NCI-H82 cells have become resistant to F_2MeOrn. As shown in Fig. 3*B*, v-Ha-*ras*-expressing NCI-H82 cells grow faster than H82 control cells in studies without F_2MeOrn added. N, cell number; N_o, original cell number.

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