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A tobacco-specific *N*-nitrosamine or cigarette smoke condensate causes neoplastic transformation of xenotransplanted human bronchial epithelial cells

(tobacco smoke/human cells/lung/bronchus/carcinogenesis)

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ABSTRACT **Using a xenotransplantation system in which immortalized nontumorigenic human bronchial epithelial cells (BEAS-2B cells) are grown in deepithelialized rat tracheas that are subcutaneously transplanted into athymic nude mice, we exposed BEAS-2B cells either to cigarette smoke condensate or to the tobacco-specific *N*-nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone. After 6 mo the carcinogen-exposed BEAS-2B cells were neoplastically transformed to invasive adenocarcinomas. Cell lines obtained from xenografts exposed *in vivo* to chemicals exhibited several features typical of malignant lung cancer cells, such as increased *in vivo* invasiveness that correlated well with enhanced type IV collagenolytic activity, resistance to serum-induced growth inhibition, and increased expression of transforming growth factor α and its cellular-membrane receptor. Invasiveness, similar to that seen after exposure to phorbol esters, was also detected after *in vitro* exposure of BEAS-2B cells to cigarette smoke condensate. Collectively, these data indicate that cigarette smoke condensate and *N*-nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone induce *in vivo* phenotypic changes in BEAS-2B cells similar to the progressive changes that occur during human lung carcinogenesis.**

Epidemiological evidence indicates that cigarette smoking increases the risk of developing lung cancer (1, 2). Several components from tobacco smoke have proven tumor-initiating and/or tumor-promoting activities in experimental animals (3–5). For example, cigarette smoke condensate (CSC) and its fractions contain chemical carcinogens (3, 6, 7), including the tobacco-specific *N*-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is a potent animal carcinogen having produced respiratory-tract neoplasms in hamsters, rats, and mice (8). Extensive studies have shown that the neutral and weakly acidic fractions of CSC are potent tumor promoters in animal models (1, 7, 9). Although tobacco-derived carcinogens and tumor promoters have been identified as agents most probably involved in human lung carcinogenesis, human bronchial epithelial cells have not been neoplastically transformed experimentally by exposure to these chemicals. Previous studies with human bronchial epithelial cells *in vitro* have shown that CSC produces many genetic and epigenetic effects usually associated with carcinogens and promoters, such as alterations in cell growth and epithelial growth factor binding, increased plasminogen activator activity, formation of single-strand breaks, and induction of terminal squamous differentiation (10). In addition, Miyashita *et al.* (11) have shown that lung tumor-derived cell lines are less sensitive to CSC-induced inhibition of cell proliferation and terminal squamous differentiation than normal bronchial epithelial cells, indicating that tobacco smoke components may allow clonal expansion of preneoplastic or neoplastic cells during the process of lung carcinogenesis.

NNK is one of the most potent carcinogens in cigarette smoke and requires metabolic activation to exert its carcinogenic effects. Both human bronchial explants (11) and the immortalized human bronchial cells BEAS-2B (ref. 12 and P. Fowles and C.C.H., unpublished results) metabolize NNK. Several cytochrome P450s have been shown to enzymatically activate NNK to mutagenic metabolites (13, 14). The extensive debrisoquine metabolic phenotype associated with cytochrome P450 *CYP2D6* gene has been shown to be a lung cancer risk factor (15, 16).

In other experiments, exposure to phorbol 12-myristate 13-acetate (PMA) induces the nontumorigenic and noninvasive BEAS-2B cell line to express an advanced neoplastic phenotype characterized by increased type IV collagenolysis and *in vitro* invasion of basement-membrane material as well as *in vivo* penetration of surrounding tissues (17). Because CSC has tumor-initiating and tumor-promoting activities and NNK is one of the most potent tobacco-associated carcinogens in laboratory animals, a series of experiments was planned to determine whether *in vivo* exposure to these chemicals would induce changes of invasiveness and/or neoplastic transformation of BEAS-2B cells in a xenograft system that grows these cells in deepithelialized tracheas that are then transplanted subcutaneously into athymic nude mice (18).

MATERIALS AND METHODS

Cells. BEAS-2B cells were obtained through infection of normal human bronchial epithelial cells with an adenovirus 12–simian virus 40 hybrid virus preparation (19). This cell line has been cultured continuously for >100 passages. In the present study passages 55 and 65 were used. The cells were cultured in serum-free LHC-9 medium (20) at 37°C in a humidified atmosphere of 5% CO_2 in air.

Invasion and Chemotaxis Assay. Subconfluent BEAS-2B cells were incubated in dimethyl sulfoxide containing CSC at 1 μg/ml, CSC at 3 μg/ml (from D. Hoffman, American Health Foundation, Valhalla, NY), or NNK at 1 μg/ml (Chemsyn, Lenexa, KS). Medium containing 200 mM PMA was used as positive control, and medium with dimethyl sulfoxide was used as negative control. The invasion assay

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Abbreviations: CSC, cigarette smoke condensate; NNK, *N*-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; EGFR, epithelial growth factor receptor; TGF-α, transforming growth factor α; PMA, phorbol 12-myristate 13-acetate.

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based on that designed by Albini (21) was done as described (17). Briefly, 3×10^5 BEAS-2B cells under different experimental conditions were placed in the upper portion of a modified Boyden chamber. The lower compartment of the chamber contained 5 μg of fibronectin as a chemoattractant and was isolated from the upper compartment by a Matrigel (50 μg per filter)-coated polycarbonate filter (8-μm-pore size, Nuclepore). In both compartments the incubation medium was Eagle's minimal essential medium/0.1% bovine serum albumin. The Boyden chambers filled with the cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 6 hr. The invasive cells—i.e., those able to move to the lower surface of the filter, were fixed by dipping the filters in neutral-buffered formalin, then staining the cells with Giemsa stain, and finally mounting the stained cells onto slides. The total number of cells per filter was counted under a light microscope.

Chemotaxis assays (noninvasive-migration assay) were done under the same conditions described for the invasion assay, except that type IV collagen (5 μg per filter) was used to coat the filters to permit cell attachment.

Preparation of Tracheal Transplants. Tracheal transplants were prepared as described (17, 18). The cultured cells of each cell line were harvested by incubation in 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid-buffered saline/0.05% trypsin/0.02% EDTA, washing, and resuspension in Eagle's minimal essential medium/20% fetal bovine serum/gentamicin. BEAS-2B cells (5×10^5) were inoculated into deepithelialized Fischer 344 rat tracheas. After cell inoculation, the tracheas were sealed and transplanted into the dorsal subcutaneous tissues of 8-week-old nude mice.

Four weeks after BEAS-2B cells were inoculated into the tracheal grafts, a simple columnar epithelium was reconstituted. At that time tracheal grafts were surgically opened, and the beeswax pellets (1-mm diameter and 18-mm length) containing 1 mg of CSC, 10 μg of NNK, or no carcinogen were introduced into the tracheal lumen; the transplants were then sealed, and the pellets were left inside for the entire experiment. Doses are of the same order of magnitude as those that produce tumors in experimental animals (7–9).

Explants and Cell Lines. After 6 mo of *in vivo* chemical response, tracheal xenograft tissues were explanted *in vitro* in LHC-9 medium. Epithelial outgrowths were cultured in monolayers, and after 5–10 *in vitro* passages, either 5×10^6 or 1×10^6 cells were inoculated s.c. into athymic nude mice.

Resistance to Serum-Induced Terminal Differentiation. Cells derived from xenografts exposed to blank and chemical-containing pellets were seeded at 250 cells per cm^2 in 60-mm dishes in LHC9 medium (20) with 0 or 8% fetal bovine serum. After 10 days of incubation, the cultures were fixed and stained, and colonies were counted. A mean colony-forming efficiency was calculated from duplicate experiments.

Zymogram. Zymograms for the detection of secreted type IV collagenolytic enzymes were done (22). Semiconfluent growing cells were cultured with serum-free Eagle's minimal essential medium for 24 hr; then the media were collected and centrifuged at 1000 rpm for 10 min. The supernatants were concentrated with Centricon 30 concentrators (Amicon) and mixed with SDS sample buffer. Electrophoresis in 7.5% polyacrylamide gel containing gelatin from swine skin at 1 mg/ml (Sigma) was done. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100/50 mM Tris·HCl, pH 7.5 and incubated at 37°C for 16 hr in 0.15 M NaCl/10 mM $CaCl_2$/50 mM Tris·HCl buffer, pH 7.5/0.05% NaN_3. The gels were stained with 0.05% Coomassie blue and destained in 10% (vol/vol) isopropanol/10% (vol/vol) acetic acid in H_2O. Gelatinolytic enzymes were detected as transparent bands of the blue background of Coomassie-blue-stained slab gel.

Molecular Analysis and Karyotypes. DNA extracted from various cell lines was used to determine point mutation in codons 12, 13, and 61 of Ki-*ras* and of exons 4–9 of p53, according to established sequencing techniques (23). Retinoblastoma protein was analyzed by using metabolic labeling and immunoprecipitation as described (24).

Chromosome studies were done by W. D. Peterson (Children's Hospital of Michigan, Detroit, MI). Exact counts on 30 metaphases were made on banded chromosomes, and at least eight karyotypes per cell line were prepared.

Immunohistochemistry and Flow Cytometry. Cells were seeded on tissue culture chamber slides (Nunc) and grown to subconfluency in LHC-9 medium. The monolayer cultures were fixed in cold ethanol for 30 min, rinsed in phosphate-buffered saline, and incubated with either anti-epithelial growth factor receptor (EGFR) or anti-transforming growth factor α (TGF-α) mouse monoclonal antibodies (Oncogene Sciences, Manhasset, NY) diluted 1/100 in phosphate-buffered saline for 1 hr. The primary antibodies were detected by using an avidin–biotin complex method with diaminobenzidine textrachloride as chromagen (Vectastain; Vector Laboratories). For quantitative determinations of EGFR and TGF-α, the cells were fixed and later incubated with the same primary antibodies in suspension at 4°C. A rabbit anti-mouse immunoglobulin G antibody labeled with fluorescein was used as secondary antibody. Controls incubated with the secondary antibody alone or with no antibody were used as negative controls to determine nonspecific binding and autofluorescence, respectively. The cells were analyzed by flow cytometry by using a FACStar Plus instrument (Becton Dickinson).

RESULTS

The ability of the BEAS-2B cells to penetrate and cross basement membrane material *in vitro* was markedly enhanced by treatment with CSC (Fig. 1). Although a dose-dependent increasing trend was seen, only the highest concentration of CSC (3 μg/ml) yielded statistically significant differences from the dimethyl sulfoxide-treated control. NNK-exposed cells were not different from the control (data not shown). Cell chemotaxis was not markedly affected by the exposures.

Six months after the intratracheal introduction of carcinogen-containing pellets, tumors measuring 2–12 mm in diameter were seen macroscopically in the tracheal transplants.

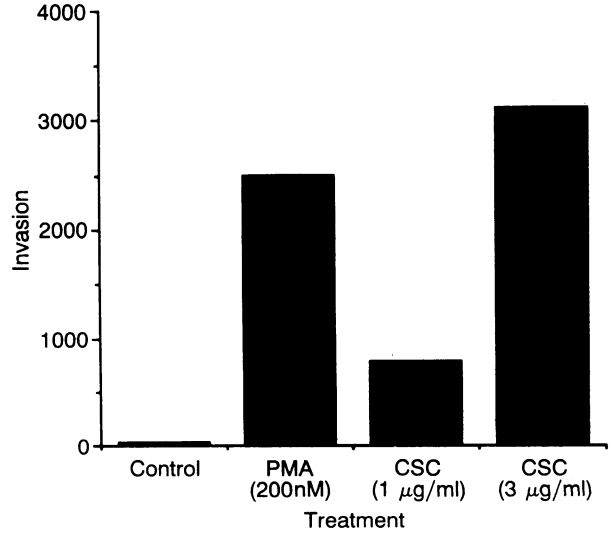

FIG. 1. *In vitro* invasiveness of BEAS-2B cells using Boyden chambers with porous filter coated with basement-membrane material (Matrigel). Cells were treated *in vitro* with either PMA or CSC and then assayed for their capacity to invade the coated filters.

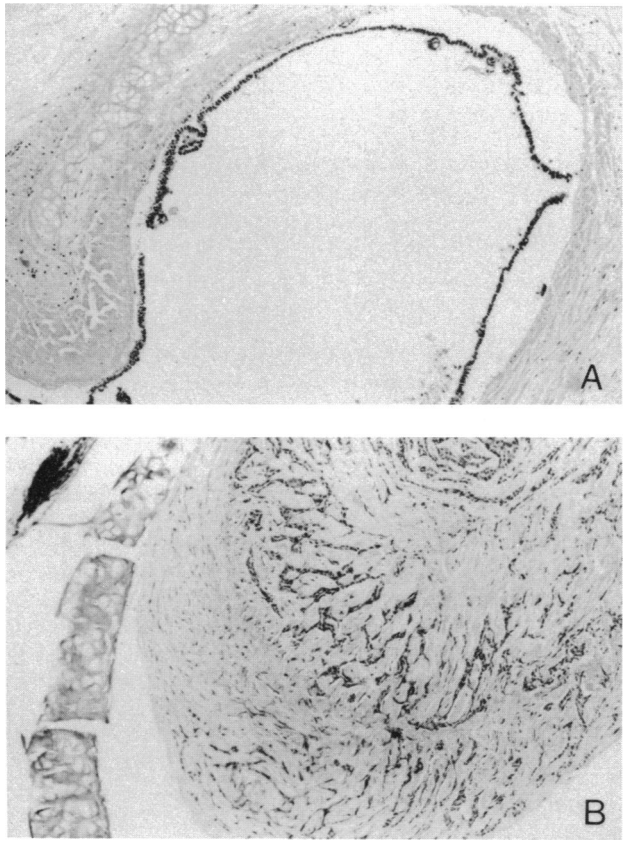

FIG. 2. Tracheal xenografts containing BEAS-2B cells were exposed for 6 mo to either a beeswax blank pellet containing no carcinogen or a beeswax pellet containing 10 μg of NNK. (*A*) When the cells are exposed to the control pellet, no neoplasm is seen; BEAS-2B cells form a simple columnar epithelium showing no outgrowth. (*B*) After exposure to NNK, a neoplasm composed of sheets and islands of epithelial cells is surrounded by reactive connective tissue. (Hemotoxylin/eosin stain; ×30.)

Histological observation confirmed that NNK produced two tumors in seven xenografts, that CSC caused two tumors in five xenografts, and that the five xenografts exposed to blank pellets did not develop any neoplasms. These tracheal grafts not exposed to chemicals were characterized by a simple columnar epithelium that occasionally produced small exophytic papillary projection but did not show any epithelial downgrowth or invasion of the tracheal wall (Fig. 2*A*). Conversely, most xenografts exposed to either NNK or CSC exhibited a multilayered epithelium that in four tracheal transplants invaded the tracheal wall and also totally obliterated the lumen with a typical adenocarcinomatous growth (Fig. 2*B*). Tissue fragments from the tracheal walls and tumors were established in explant cultures, and several cell lines were produced from CSC, NNK, and blank beeswax pellet-exposed xenografts. After these cell lines were expanded in culture for 20–28 passages and their capacity to grow *in vitro* in high concentrations of serum was established, two different experiments to determine tumorigenicity were done. Five athymic nude mice were inoculated s.c. with 1×10^6 cells per animal to determine cellular tumorigenicity in one experiment; in the other experiment 5×10^6 cells were inoculated into the s.c. tissue of similar animals (Table 1).

From the *in vitro* studies, it became evident that all cell lines derived from chemically exposed xenotransplanted epithelia could grow well in serum-containing medium (Table 1). Although all carcinogen-exposed cell lines had acquired resistance to serum, not all lines proved tumorigenic. Both tumorigenicity studies yielded very similar results, although the NNK cell lines required a larger inoculum to show tumorigenicity. Four cell lines derived from tumors (BEAS-2B-CSCA, BEAS-2B-CSC 1170-I, BEAS-2B NNKA, and BEAS-2B NNKB) were very tumorigenic, whereas two cell lines derived from CSC-treated tracheal transplants that contained no detectable tumors (BEAS-2B-CSC 1196 and BEAS-2B-CSC 1198) were not tumorigenic (Table 1). The cell line BEAS-2B 1170-II was derived from an apparently normal area of tracheal transplant wall distant from a primary tumor and was marginally tumorigenic. Histopathological evaluation of the tumors showed that irrespective of the cell line of origin all neoplasms were moderately differentiated invasive adenocarcinomas. Nevertheless, the tumors derived from CSC-transformed cells were more invasive, penetrating deeper into the s.c. tissue, than those derived from NNK-

Table 1. Analysis of xenograft-derived human bronchial epithelial cells

Cell line	Colony-forming efficiency,* %		Expression†, %		Tumorigenicity, %	
	0% FBS	8% FBS	TGF-α	EGFR	Experiment 1‡	Experiment 2§
Beeswax control						
BEAS-2B-BW1799	100	22	7	28	0	0
CSC exposed						
Tumor derived						
BEAS-2B-CSC A	147	159	81	89	40 (20)	56 (12)
BEAS-2B-CSC 1170-I	80	127	75	94	60 (24)	70 (16)
BEAS-2B-CSC 1170-II	92	122	3	7	40 (24)	20 (32)
Nontumor derived						
BEAS-2B-CSC 1196	112	64	2	23	0	0
BEAS-2B-CSC 1198	54	122	2	2	0	0
NNK exposed						
BEAS-2B NNKA	141	153	46	35	0	40 (24)
BEAS-2B NNKB	59	100	4	50	0	60 (15)

*A mean colony-forming efficiency was calculated from duplicate experiments. Colony-forming efficiency is expressed as percentage of the BEAS-2B control cell line. FBS, fetal bovine serum.

†After incubation with the primary antibody, cells were incubated with the fluorescein isothiocyanate-labeled secondary antibody. Cells were evaluated by flow cytometry, and the values were expressed as percentage of fluorescent cells.

‡Experiment 1: Tumorigenicity was determined after s.c. inoculation of 1×10^6 cells into athymic nude mice of BALB background (Fox Chase Cancer Center). Tumors 5 mm or larger were recorded for 6 mo. Five animals per cell line were used. Data are expressed as percent of animals with tumor. The latency period in weeks is enclosed in parentheses.

§Experiment 2: Tumorigenicity was established after s.c. inoculation of 5×10^6 cells into athymic nude mice of BALB background (National Cancer Institute). Only tumors 10 mm or larger that appeared 6 mo after inoculation or earlier were assessed. Nine to ten animals per cell line were injected. The latency period in weeks is enclosed in parentheses.

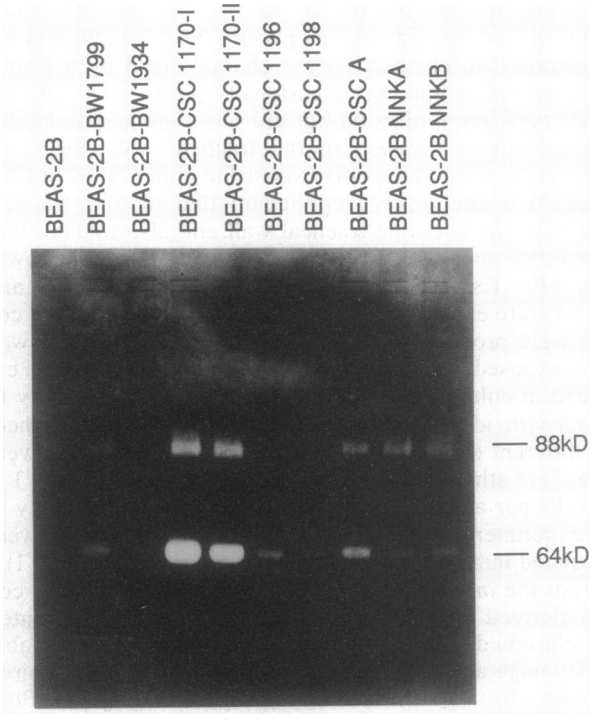

FIG. 3. Gelatinase zymogram shows an intense band corresponding to the lower-molecular mass-species (64/72 kDa) in the most invasive and tumorigenic cell lines BEAS-2B-CSC A, CSC 1170-I, and CSC 1170-II.

transformed cells. This result correlated well with the gelatinolytic activity detected in zymograms that showed intense activity of the high-molecular-weight form of type IV collagenase (gelatinase) in the BEAS-2B CSC and NNK cells as compared with control BEAS-2B BW. The lower-molecular-weight species (64/72 kDa) was absent in the control cell lines but present in the CSC- and NNK-exposed cell lines (Fig. 3).

Karyotypes showed that all cell lines had BEAS-2B chromosomal markers (25) in addition to additional markers acquired during *in vivo* growth and exposure to chemicals. Monosomies of chromosomes 4, 13, and 19 were seen in the carcinogen-exposed cell lines. We also investigated possible changes in all cell lines by using mutation-specific oligonucleotide hybridization for Ki-*ras*, direct DNA sequencing of exons 4–9 of the p53 tumor-suppressor gene, and immunoprecipitation analysis of retinoblastoma protein. No alterations were found (data not shown). In addition, we investigated the possible alteration of an autocrine loop involving EGFR and TGF-α. With immunohistochemical techniques and flow cytometry, the cell lines derived from CSC-exposed transplants had significantly higher levels of EGFR and TGF-α than the lines derived from beeswax blank-exposed controls (Table 1) (Fig. 4).

DISCUSSION

In vivo exposure of the immortalized bronchial epithelial cells, BEAS-2B, to either NNK or CSC induced the formation of invasive neoplasms. CSC, in particular, seemed more effective because it could induce invasive behavior of BEAS-2B cells both *in vivo* and *in vitro*. This behavior was associated with an increased type IV collagenolytic activity similar to the one induced by PMA in the same cell system (17). This change was detected long after exposure to CSC had finished, indicating that this is a persistent characteristic of the BEAS-2B CSC cell lines that originated in tumors produced in xenotransplants exposed to CSC for several months.

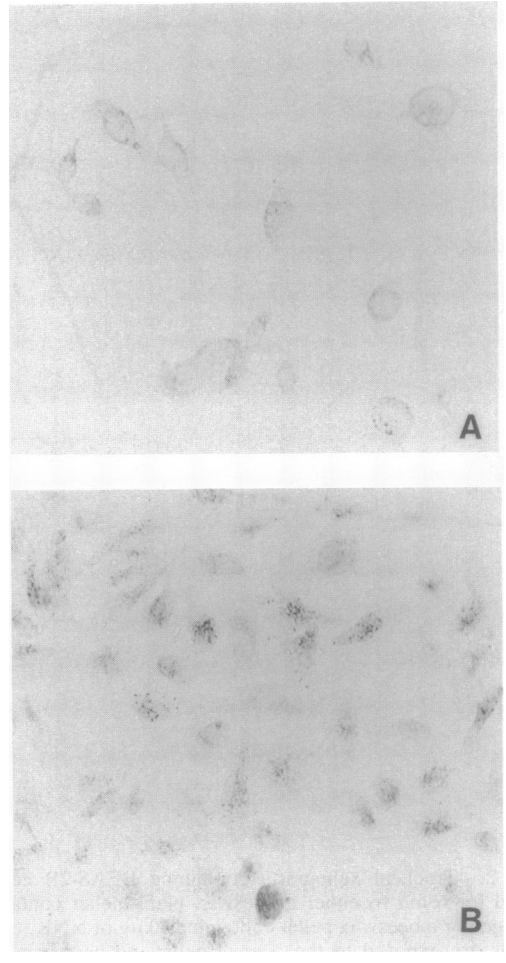

FIG. 4. By using indirect immunohistochemical detection of EGFR, a very low immunoreactivity is seen in the control cell lines (BEAS-2B-BW1799) that were originated from xenografts exposed to the beeswax blank pellets (*A*), whereas the cell line derived from a xenograft exposed to CSC (BEAS-2B-CSC A) exhibits a much higher immunoreactivity (*B*). (×325.)

In addition to these phenotypic changes, most tumor-derived cells also exhibited an altered capacity to grow in medium containing a high concentration of serum that inhibits its growth and induces terminal squamous differentiation of both normal human bronchial epithelial cells (26) and untreated BEAS-2B cells (27). Conversely, as was described (28), cell lines derived from human lung carcinomas grew well in high serum concentrations. Thus, this assay indicates that the cell lines derived from NNK- or CSC-treated epithelia acquire serum resistance similar to that seen in carcinoma cell lines. Previous studies have shown that transforming growth factor type β is the major negative growth factor in serum (26).

Because Ki-*ras*, p53, and retinoblastoma gene abnormalities have been described as the most common molecular abnormalities in lung cancer (29, 30), we analyzed these genes for molecular alterations. The lack of p53 and *Rb* mutations may reflect the inactivation of the respective proteins by simian virus 40 large tumor antigen in the BEAS-2B cells (23). The absence of changes in these genes does not exclude molecular alterations in other genes.

Interestingly, we could observe increased expression of both EGFR and TGF-α in some tumorigenic cell lines. This autocrine loop has been described as an important factor in the development of certain human and experimental tumors, including those of the lung (31–34). Increased TGF-α expression has also been associated with poor prognosis and

aggressive behavior of human lung adenocarcinomas (34). In the present group of cell lines, increased TGF-α and EGFR clearly correlated with tumorigenicity and invasive behavior of CSC-transformed cell lines. Consequently, this type of autocrine loop could have been activated by the *in vivo* exposure to tobacco-associated carcinogens, resulting in tumorigenic cells. Because similar TGF-α changes occur in lung adenocarcinomas, most of which develop in cigarette smokers, it is proposed that this mechanism could contribute to the development of human tumors as a consequence of chronic exposure to tobacco smoke.

Furthermore, NNK and other tobacco-related *N*-nitrosamines could have a direct mitogenic, or growth factor-like effect on lung cell subpopulations, by interacting at high affinity with nicotine receptors (35).

The *in vivo* malignant transformation of human bronchial BEAS-2B cells by tobacco smoke-derived chemicals offers the possibility of further investigating the mechanism that leads to the formation of the many human malignancies clearly associated with tobacco-product consumption in an *in vitro/in vivo* cell system.

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1. International Agency on the Research of Cancer (1986) *IARC Monograph on the Evaluation of the Carcinogenic Risks of Chemicals to Humans: Smoking* (IARC, Lyon, France), Vol. 38, pp. 199–243.
2. U.S. Department of Health, Education and Welfare (1979) *U.S. Smoking and Health: A Report to the Surgeon General* (Dept. of Health, Education, and Welfare, Washington), Publ. No. 79-50066, p. 1136.
3. Bock, F. G., Swain, A. P. & Stedman, R. L. (1970) *J. Natl. Cancer Inst.* **44,** 1305–1310.
4. Hoffmann, D., Hecht, S. S. & Wynder, E. L. (1983) *Environ. Health Perspect.* **50,** 247–257.
5. International Agency for Research on Cancer (1986) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco Smoking* (IARC, Lyon, France), Vol. 38, pp. 135–139.
6. Swain, A. P., Cooper, J. E. & Stedman, R. L. (1969) *Cancer Res.* **29,** 579–583.
7. Rivenson, A., Hoffmann, D., Prokopczyk, B., Amin, S. & Hecht, S. S. (1988) *Cancer Res.* **48,** 6912–6917.
8. Hecht, S. S. & Hoffmann, D. (1989) *Cancer Surv.* **8,** 273–294.
9. Hoffmann, D. & Wynder, E. L. (1971) *Cancer* **27,** 848–864.
10. Willey, J. C., Grafstrom, R. C., Moser, C. E., Ozanne, C., Sundfvit, K. & Harris, C. C. (1987) *Cancer Res.* **47,** 2045–2049.
11. Miyashita, M., Willey, J. C., Sasajima, K., Lechner, J., La Voie, E., Hoffmann, D., Smith, M., Trump, B. F. & Harris, C. C. (1990) *Exp. Pathol.* **38,** 19–29.
12. Castanguay, A., Stoner, G. D., Hecht, H. A. & Schut, J. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6694–6697.
13. Crespi, C. L., Penman, B. W., Gelborn, H. V. & Gonzalez, F. J. (1991) *Carcinogenesis (London)* **12,** 1197–1201.
14. Guo, Z., Smith, T. J., Ishizaki, H. & Yang, C. S. (1991) *Carcinogenesis (London)* **12,** 2277–2282.
15. Ayesh, R., Idle, J. R., Ritchie, J. C., Crothers, M. G. & Hetzel, M. R. (1989) *Nature (London)* **312,** 169–170.
16. Caporaso, N. E., Tucker, M., Hoover, R., Hayes, R., Pickle, L., Isaag, H., Muschik, G., Green-Gallo, L., Aisner, S., Resau, J., Trump, B. F., Tollerud, D., Weston, A. & Harris, C. C. (1990) *J. Natl. Cancer Inst.* **82,** 1264–1272.
17. Bonfil, R. D., Momiki, S., Fridman, R., Reddel, R., Harris, C. C. & Klein-Szanto, A. J. P. (1989) *Carcinogenesis (London)* **10,** 2335–2338.
18. Klein-Szanto, A. J. P., Pal, B., Terzaghi, M. & Marchok, A. (1984) *Environ. Health Perspect.* **56,** 75–86.
19. Reddel, R. R., Ke, Y., Gerwin, B., McMenamin, M., Lechner, J., Su, R., Brash, D., Pork, J. B. & Harris, C. C. (1988) *Cancer Res.* **48,** 1904–1909.
20. Lechner, J. & LaVeck, M. A. (1988) *Cancer Res.* **48,** 1904–1909.
21. Albini, A., Iwamoto, H. K., Martin, G. R., Aaronson, S. A., Kozlowoski, J. & McEwan, R. N. (1987) *Cancer Res.* **47,** 3239–3245.
22. Nakajima, M., Lotan, D., Baig, M., Corralero, R. M., Wood, W., Hendrix, M. J. & Lotan, R. (1989) *Cancer Res.* **49,** 1698–1706.
23. Lehman, T. A., Bennet, W. P., Metcalf, R. A., Welsh, J. A., Ecker, J., Modali, R., Ullrich, S., Romamo, J. W., Appella, E., Testa, J., Gerwin, B. & Harris, C. C. (1991) *Cancer Res.* **51,** 4090–4096.
24. Ruggeri, B., Caamano, J., Goodrow, T., Conti, C. J. & Klein-Szanto, A. J. P. (1991) *Cancer Res.* **51,** 6560–6563.
25. Kaighn, M. E., Gabrielson, E. W., Iman, D. S., Paul, S. E. & Harris, C. C. (1990) *Cancer Res.* **50,** 1890–1896.
26. Masui, T., Wakefield, L. M., Lechner, J. F., LaVeck, M. A., Sporn, M. B. & Harris, C. C. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2438–2442.
27. Miyashita, M., Smith, M. W., Willey, J. C., Lechner, J. F., Trump, B. F. & Harris, C. C. (1989) *Cancer Res.* **49,** 63–67.
28. Lechner, J. F., McClendon, I. A., LaVeck, M. A., Shamsuddin, A. & Harris, C. C. (1989) *Cancer Res.* **43,** 5915–5921.
29. Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C. C. (1991) *Science* **253,** 49–53.
30. Carbone, D. P. & Minna, J. D. (1991) in *Molecular Foundations of Oncology*, ed. Broder, S. (Williams & Wilkins, Baltimore), pp. 339–366.
31. Todd, R., Donoff, B. R., Gertz, R., Chang, A., Chow, P., McBride, J., Chiang, T., Gallagher, G. T. & Wong, T. W. (1989) *Carcinogenesis (London)* **10,** 1553–1556.
32. Liu, C., Woo, A. & Tsao, M. S. (1990) *Br. J. Cancer* **62,** 425–429.
33. Borton, C. M., Hall, P. A., Hughes, C. M., Gullick, W. J. & Lemoine, N. R. (1991) *J. Pathol.* **163,** 111–116.
34. Tateichi, M., Ishida, T., Milsudome, T., Kaneko, S. & Sugimachi, K. (1990) *Cancer Res.* **50,** 7077–7080.
35. Schuller, H. M. (1989) *Biochem. Pharmacol.* **42,** 3439–3442.