TRANSFORMED HUMAN BRONCHIAL EPITHELIAL CELLS (BEAS-2B) ALTER THE GROWTH AND MORPHOLOGY OF NORMAL HUMAN BRONCHIAL EPITHELIAL CELLS *IN VITRO**

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Normal human bronchial epithelial cells (BE) and adenovirus-12 SV40 hybrid virus transformed, non-tumorigenic human bronchial epithelial cells (BEAS-2B) were cultured for 7 days in a serum-free hormone supplemented medium. BE cells after 3 days in culture were exposed to conditioned medium (CM_t) from confluent BEAS-2B cells. By day 7, CM_t-treated BE cells exhibited a lower colony forming efficiency (CFE), fewer cells per colony, and a reduced mitotic index (MI) and BrdU (bromodeoxyuridine) labeling index. CM_t also enhanced the expression of a terminally differentiated squamous phenotype in BE cells. Cell free lysates from BEAS-2B cells (CFL_t) had effects similar to CM_t on the MI and morphology of BE cells. In contrast, CM_t and CFL_t did not inhibit the growth, or alter the morphology of BEAS-2B cells. Conditioned medium from BE cells (CM_n) did not reduce the growth of BEAS-2B cells, and had little effect on the morphology of BE cells. In co-cultures,

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3. Abbreviations: BE = normal bronchial epithelial cells; BEAS-2B = adenovirus-12 SV40 hybrid virus transformed bronchial epithelial cells; CM_n = conditioned medium from BE cells; CM_t = conditioned medium from BEAS-2B cells; CFL_n = cell free lysate from BE cells; CFL_t = cell free lysate from BEAS-2B cells; BrdU = bromodeoxyuridine; KGM = keratinocyte growth medium; TGF-β = transforming growth factor type β; NCI-LHC = National Cancer Institute-Laboratory of Human Carcinogenesis

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BE cells in direct contact with BEAS-2B cells had a lower MI (0.4–0.7 vs. 1.6%) compared with colonies of BE cells in these co-cultures. The concentration of transforming growth factor beta (TGF-β) in conditioned media from BEAS-2B cells (CM_l) was increased 10-fold over that in CM_n. TGF-β is known to induce terminal differentiation in epithelial cells. These results suggest that the selective growth advantage of transformed cells over normal cells during human bronchial carcinogenesis may be related to the release of autocrine/paracrine factors (e.g., TGF-β) from transformed cells, which down-regulates and terminally differentiates the normal cells.

INTRODUCTION

Lung cancer has been one of the most prevalent causes of cancer deaths since the 1950s (Loeb et al., 1984). Many occupational (Vena et al., 1985), dietary (Byers et al., 1984), age (Filderman et al., 1986) and sex-related factors (Teeter et al., 1987) have been implicated in lung carcinogenesis. Cigarette smoking plays a significant etiologic role in lung carcinogenesis (Auerbach et al., 1957; Doll and Peto, 1976; Loeb, 1989). Cigarette smoking is implicated in the clonal growth of initiated cells (Gaffney and Altshuler, 1988), and in the occurrence of increasingly atypical squamous metaplasias over time, which precede the appearance of lung cancer (Saccomanno et al., 1974; Frost et al., 1984). These changes may be related to the effects of tumor promoting agents known to be present in cigarette smoke extracts (Van Duren and Goldschmidt, 1976). However, the actual biological mechanisms responsible for the establishment and subsequent proliferation of foci of initiated preneoplastic cells, and the role of interactions between adjacent normal and transformed bronchial epithelial cells in carcinogenesis are not well understood.

Many factors that regulate cell growth and differentiation in bronchial epithelium following acute cell injury (e.g., increased intracellular calcium, calmodulin antagonists, products of the phosphoinositol pathway, growth factors [Trump and Berezesky, 1987]), also affect the intercellular transfer of small molecular probes in several cell systems (DeMello, 1984; Davidson et al., 1985, Peracchia and Bernardini, 1984). Wounding and its associated inflammatory response increases the incidence of malignant transformation in initiated tracheobronchial epithelium *in vivo* (Keenan et al., 1989). Therefore, altered intercellular communication may be a key factor linking cell injury, growth, differentiation and carcinogenesis in bronchial epithlium.

The present studies were designed to measure the effects of conditioned media, cell-free lysates, and co-culture on the growth and differentiation of normal and transformed bronchial epithelial cells (BE) *in vitro*. The results indicate that

transformed bronchial epithelial cells can gain a growth advantage by "down-regulating" the growth of normal bronchial epithelial cells.

MATERIALS AND METHODS

Human Epithelial Cells. Normal human bronchial tissues were collected at autopsy from cases with a postmortem interval of 12 hrs or less (Resau et al., 1987). Tissue was not collected from deceased individuals who died of an acute systemic toxic cause (*e.g.*, carbon monoxide poisoning, drug overdose, etc.). Infectious cases and those with high risk exposure profiles were excluded from our population. Individuals with known drug pathology were also excluded. These protocols were reviewed and approved by the University of Maryland Human Volunteers Research Committee.

Bronchial epithelial cells were harvested using a previously described method (Jones and Elliget, 1987). Briefly, a segment of the main stem bronchus was incubated for 2 hrs at 37° in L-15 medium (GIBCO) containing pen-strep-fungizone, and supplemented with N-acetylcysteine, and neutral protease (type XIV, Sigma Chemical), clamped at both ends, and incubated overnight at 4°. The bronchial epithelial (BE) cells were then rinsed from the bronchus, diluted with culture medium, concentrated by centrifugation and resuspended in KGM culture medium (see below).

Adenovirus-12 SV40 hybrid virus transformed, immortalized human bronchial epithelial cells, strain BEAS-2B, were used in these studies. These cells have been shown to be non-tumorigenic in nude mice (Reddel et al., 1988; Bonfil et al., 1989). BEAS-2B cells were provided to Dr. Benjamin F. Trump by the NCI-LHC. Both BE and BEAS-2B cells were plated at a density of 5×10^3 cells/cm^2 in glass chamber slides (LabTek,® Miles Scientific, Naperville, Illinois). These cells were grown in serum-free Keratinocyte Growth Medium (KGM,™ Clonetics, San Diego, California) containing bovine pituitary extract (50 μg protein/ml), hydrocortisone (0.5 μg,ml), calcium 0.15 mM), pen-strep-fungizone, and supplemented with human transferrin (5 μg/ml).

Co-Culture Assay. BE and BEAS-2B cells were plated at ratios of 2:1, 1:1, or 1:2 to give a final plating density of 5×10^3 cells/cm^2, and were grown in KGM as described above. After 7 days, the cells were fixed for morphologic, histochemical and immunocytochemical evaluation.

Conditioned Media. Conditioned medium was obtained 24 hrs after the addition of fresh medium to confluent monolayers of BE or BEAS-2B cells. The medium was aspirated from the cultures, centrifuged at 2000 rpm at room temperature for 10 min, pooled, filtered through a 0.22 μm sterile filter and stored frozen at −20° until used. To measure the effects of conditioned medium, BE and BEAS-2B cells were plated as described above, and conditioned medium obtained from

BE cells (CM_n) or from BEAS-2B cells (CM_t) was added to the cells after 3 days in culture. All cultures were fixed on day 7 for morphologic evaluation as described below.

Cell Free Lysates. BE and BEAS-2B cells at confluence were trypsinized and 0.5–1.0 $\times$ 10^5 cells, in 10 mls of KGM, were transferred to sterile NUNC tubes. These cells were freeze-thawed in liquid nitrogen 3 times, centrifuged at 4000 rpm for 10 min, filtered through a 0.22 μm sterile filter, and then the undiluted complete filtrate (CFL) was stored frozen at $-20°$ until used. To quantify the effects of CFL, BE and BEAS-2B cells were plated in KGM, transferred to KGM containing CFL after 3 days in culture and fixed for morphologic studies on day 7.

Papanicolaou Staining. Following 7 days in culture, BE and BEAS-2B cells were fixed in 95% alcohol, stained using a modified Papanicolaou technique (Gill et al., 1974), mounted in Permount (Clay-Adams, Parsippany, New Jersey) and covered with a No. 1 thickness coverglass.

Mucous Cytochemistry. After 7 days in culture, representative cell cultures were fixed in 4FIG (4% formaldehyde, 1% glutaraldehyde), stained with PAS-Alcian blue (pH 2.5), with and without diastase treatment, and counterstained with Gill hematoxylin (Polysciences, Inc., Warrington, Pennsylvania).

Intermediate Filaments Cytochemistry. Cytocentrifuge preparations of BE and BEAS-2B cells were fixed in absolute alcohol, and the presence of keratin and vimentin were demonstrated using the ABC technique (Vector Laboratory, Burlingame, California).

Quantitation of DNA Synthesis. BE and BEAS-2B cells on day 7 of culture were incubated with 100 μM BrdU for 10 min at 37° and then fixed in absolute alcohol. The slides were incubated in pepsin in 0.1 N HCl for 30 min, and then hydrolyzed in 1 N HCl for 20 min at room temperature. After rinsing in PBS ($\times$3), the slides were incubated with a monoclonal, mouse anti-BrdU antibody (Becton Dickinson, Mountain View, California) at a dilution of 1:40 in PBS overnight at room temperature. Immunoreactive sites were amplified using the ABC method; the slides were counterstained with Gill hematoxylin. These slides were coded, randomly ordered and the number of labeled cells was determined from counts of the first 100 cells in randomly selected microscope fields (10$\times$ ocular, 40$\times$ objective). The labeling index (LI) was defined as the ratio of labeled to unlabeled cells. Positive cells contain a brown reaction product localized to the nucleus (Gratzner, 1982).

Assays for Colony Growth. The mean number of population doublings per day (PD/D) was determined by dividing the log2 of the number of cells/colony in 10 colonies/culture by the number of days in culture, according to the method of Lechner and Kaign (1979). The mean colony forming efficiency (CFE) was determined by dividing the number of colonies/culture in 10 randomly selected

microscope fields (10× ocular, 20× objective) by the number of cells plated in 10 fields.

Morphologic Studies: Light Microscopy. Since differentiation in the cell biology sense is usually reserved for the presence of overt, grossly observable phenotypic features (*e.g.,* cilia, desmosomes, intermediate filament staining, synthesis and secretion of glycoproteins), we have classified these cells according to methods described for cells *in vitro* (Boone et al., 1986; Frost, 1986; Albright et al., 1989, 1990). The Papanicolaou stained slides were coded, randomized, and the percentages of basal-, secretory-, or squamous-type cells present on day 7 were determined from differential cell counts of 200–400 cells per slide. Transformed cells were discriminated from normal cells by specific nuclear and cytoplasmic features as previously described (Boone et al., 1986; Frost, 1986; Albright et al., 1989).

Electron Microscopy. Representative BE and BEAS-2B cells were fixed in 4F1G, post-fixed in OsO4, stained with uranyl acetate lead citrate, and examined with a JEOL-CX transmission electron microscope.

TGF-β. The mink lung epithelial cell line (ATCC CCL-64) growth inhibition assay was performed as described by Danielpour et al. (1989). The sensitivity of CCL-64 cells has been routinely used as a screen for TGF-β (Needleman et al., 1990). In brief, CCL-64 cells were cultured in 24 well plates containing low glucose DMEM supplemented with 2% fetal bovine serum, 10 mM HEPES, and 1% penicillin/streptomycin. Either the BE or the BEAS-2B conditioned media or measured amounts of acid activated and neutralized TGF-β (R & D Systems, Minneapolis, Minnesota) was added to the CCL-64 cells. Then, 8 to 10 two-fold serial dilutions of either the conditioned media or prepared concentrations of purified TGF-β antibody (R & D Systems, Minneapolis, Minnesota) were added to the first dilution of either the conditioned media or the known concentration of TGF-β to assure that inhibition of cellular growth was due to the presence of TGF-β. The CCL-64 cells were incubated for 22 hrs, pulsed for 2 hrs with 0.5 μ Ci ^{3}H-thymidine per well, then fixed, washed, and solubilized. Aliquots were counted in a β scintillation counter. The amount of TGF-β in each sample of KGM conditioned media was calculated from the standard curve of CCL-64 cell growth inhibition derived from the effects of purified TGF-β added to DMEM.

RESULTS

Morphology of BE Cells. In Papanicolaou stained BE cultures (Fig. 1), the percentages of basal-, small round secretory-, large flat secretory-, and squamous-type cells on day 7 were 2.2 ± 1.6, 4.6 ± 2.5, 37.8 ± 22.7, and 55.4 ± 23.2 percent, respectively. Basal-type cells had scanty cytoplasm, rounded hyperchromatic nuclei and occasional small nucleoli. The nuclei in secretory-type cells contained granular chromatin and typically had prominent, rounded eosinophilic nucleoli. Large flat

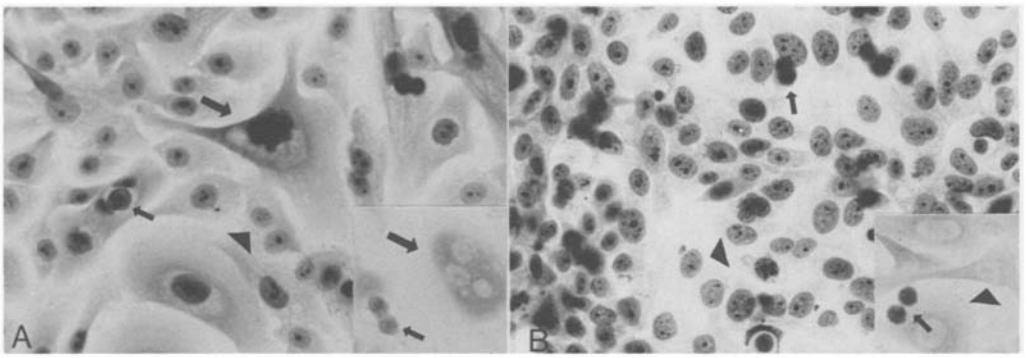

FIGURE 1. The light microscopic morphology of (A) BE cells and (B) BEAS-2B cells after Papanicolaou staining showing the morphology of basal-type cells (small arrow), secretory-type cells (large arrow), and squamous-type cells (arrowhead). The inset shows the pattern of PAS-AB staining: basal-type cells (small arrow), secretory-type cells (large arrow), and squamous-type cells (arrowhead). (A–B) × 400 (inset ×400).

secretory cells had more prominent secretory vacuoles, which often contained eosinophilic secretory-type granules, and the cytoplasm exhibited a biphasic texture: densely basophilic centrally *vs.* a granular texture at the periphery. Squamous cells had a textured, and more acidophilic cytoplasm when compared with secretory cells. Cells which were classified as secretory by phase contrast criteria and by Papanicolaou morphology, contained PAS(+) or AB(+) mucous substances (Fig. 1). Ultrastructurally, BE cells expressed intermediate filaments, and by LM showed uniformly positive staining for keratin (Fig. 2) and an absence of vimentin (not shown), thus confirming the epithelial origin of these cells.

Morphology of BEAS-2B Cells. In Papanicolaou stained BEAS-2B cultures on day 7, compared with BE cultures, basal-type cells often had a prominent nucleolus, the secretory-type phenotype was poorly developed, and the larger flattened cells expressed a less well differentiated squamous phenotype (Fig. 1). Diffuse and punctate AB(+) staining was seen in about one third (36.2 ± 20.4 percent) of the squamous cells and PAS(+) reactivity was rarely observed.

BEAS-2B cells co-expressed keratin and vimentin. Keratin staining (Fig. 2) was evident mainly in large flat cells, while vimentin filaments (not shown) were present in the smaller cells. A significant proportion of the cells expressed both classes of intermediate filaments. Junctional-like complexes between BEAS-2B cells were observed by EM (Fig. 2).

BEAS-2B cells, compared with normal BE cells (Table 1), grew at a faster rate (PD/D = 0.8 *vs.* 0.6), and thus formed larger colonies that were nearly confluent after 7 days in culture. BE cell cultures had a higher MI compared with BEAS-2B cells (2.3 *vs.* 1.03 percent) and contained mitotic basal- and secretory-type cells, whereas BEAS-2B cultures contained mitotic basal- and squamous-type cells.

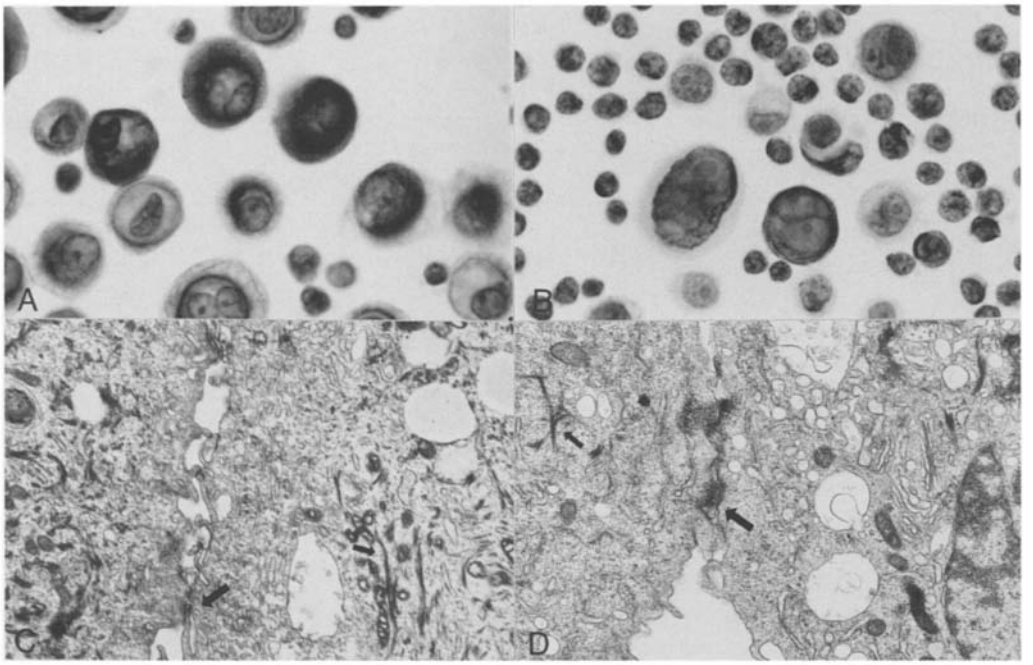

FIGURE 2. Immunocytochemical staining for keratin in (A) BE cells and (B) BEAS-2B cells. Electron micrographs of (C) BE cells, and (D) BEAS-2B cells showing intermediate filaments (small arrow) and junctional-like complexes (large arrow). Uranyl acetate and lead citrate. (A–B) ×787; (C–D) ×5000.

Effects of Conditioned Media. The morphology of BE cells was unaffected by exposure to CM_n. The addition of CM_t to BE cells was associated with an increase in the percentage of basal-type cells, and a corresponding decrease (−50 percent of controls) in the percentage of large flat secretory-type cells present (Table 2). CM_t-treated BE secretory-type cells showed an increased cytoplasmic basophilia, nucleoli were less often eosinophilic, and there were fewer nucleoli per cell (1.6 $\pm$ 0.05 in controls *vs.* 1.07 $\pm$ 0.06 in CM_t, $p < .05$). In 2 of 5 normal BE specimens, exposure to CM_t increased the percentage of large flat squamous cells (84.3 $\pm$ 21.8 percent of controls) by day 7. In the remaining 3 of 5 CM_t-treated specimens, there was expression of an atypically keratinized metaplastic squamous phenotype (2.4 $\pm$ 1.6 percent of all cells) as evidenced by the presence of orangeophilic and hyaline basophilic cytoplasm in squamous cells and keratinized squamous pearl formations (Fig. 3).

CM_t significantly reduced the growth measured by the number of cells per colony, PD/D, MI, and the BrdU labeling index of BE cells (Table 2; Fig. 4). The percentage of multinucleated BE cells was 6.5 $\pm$ 2.4, 7.3 $\pm$ 2.6 and 13.9 $\pm$ 4.5 percent in control. CM_n and CM_t treated cultures, respectively. In contrast to the effects on BE cells, BEAS-2B cells treated with CM_t showed an increased proliferation

TABLE 1
The Effects of Conditioned Media or Cell Free Lysates on the Growth of Normal BE and Transformed BEAS-2B Cells in Culture

		Growth Parameter[a]			
Treatment	n	CFE	Cells/Colony	PD/D	MI
BE Control	10	14.2 ± 8.7	24.4 ± 4.2	0.61 ± 0.15	2.3 ± 0.6
BE + CM_n	3	14.2 ± 5.4	49.1 ± 4.2[b]	0.76 ± 0.10	1.7 ± 0.7[b]
BE + CM_t	5	10.2 ± 1.9	18.9 ± 5.9[b]	0.51 ± 0.07[b]	0.5 ± 0.3[b]
BE + CFL_t	5	6.7 ± 3.3[b]	11.0 ± 3.9[b]	0.48 ± 0.07[b]	0.3 ± 0.2[b]
BEAS-2B Control	10	14.5 ± 4.5	61.6 ± 17.9	0.80 ± 0.95	1.0 ± 0.3
BEAS-2B + CM_t	4	15.4 ± 4.7	224.9 ± 38.0[c]	0.97 ± 0.09[c]	2.4 ± 0.7[c]
BEAS-2B + CM_n	4	29.0 ± 13.8	100.8 ± 36.8	0.91 ± 0.05	2.3 ± 0.7[c]
BEAS-2B + CFL_n	2	(cultures confluent)		0.85 ± 0.01	3.1 ± 1.0[c]

[a] Mean ± SE; n = number of replicates; CFE = colony forming efficiency; PD/D = population doublings per day; MI = mitotic index.
[b] *t*-test, $p \leq 0.05$ *vs.* control BE.
[c] *t*-test, $p \leq 0.05$ *vs.* BEAS-2B.

TABLE 2
The Effects of Conditioned Media or Cell Free Lysates on the Morphology of Papanicolaou Stained Be and BEAS-2B Cells After 7 Days in Culture

		Percent Cell Type[a]			
Treatment	n	Basal Cells	Round Secretory	Flat Secretory	Squamous
BE Control	10	2.4 ± 0.5	4.7 ± 7.4	36.3 ± 7.4	56.5 ± 7.5
BE + CM_n	3	2.5 ± 2.1	3.6 ± 0.1	30.0 ± 4.5	63.8 ± 2.2
BE + CM_t	5	5.0 ± 1.3[b]	6.3 ± 1.4	18.3 ± 4.6[b]	70.6 ± 4.9[b]
BE + CFL_t	5	0.7 ± 0.7[b]	5.6 ± 0.7	37.0 ± 13.7	56.7 ± 13.9
BEAS-2B Control	10	2.6 ± 0.3	2.6 ± 0.8	0.01 ± 0.01	94.8 ± 0.8
BEAS-2B + CM_t	4	2.8 ± 0.7	3.8 ± 0.6	0.0 ± 0.0	93.4 ± 1.0
BEAS-2B + CM_n	4	2.5 ± 0.6	2.7 ± 0.8	0.0 ± 0.0	94.7 ± 1.1
BEAS-2B + CFL_n	2	6.4 ± 1.1[c]	2.2 ± 0.7	0.0 ± 0.0	91.4 ± 1.0[c]

[a] Mean ± SE; n = number of replicates.
[b] *t*-test, $p \leq 0.05$ *vs.* BE control.
[c] *t*-test, $p \leq 0.05$ *vs.* BEAS-2B control.

(Table 2), and did not express an atypical keratinized squamous phenotype (Fig. 4). The morphology of BE and BEAS-2B cells was unaffected by exposure to CM_n, or to CM_t (Table 1; Fig. 2). The growth of BE was much less inhibited, and that of BEAS-2B cells was stimulated by CM_n (Table 1; Fig. 4), indicating that the effects of CM_t on BE cells were not due to simple nutrient depletion of the medium.

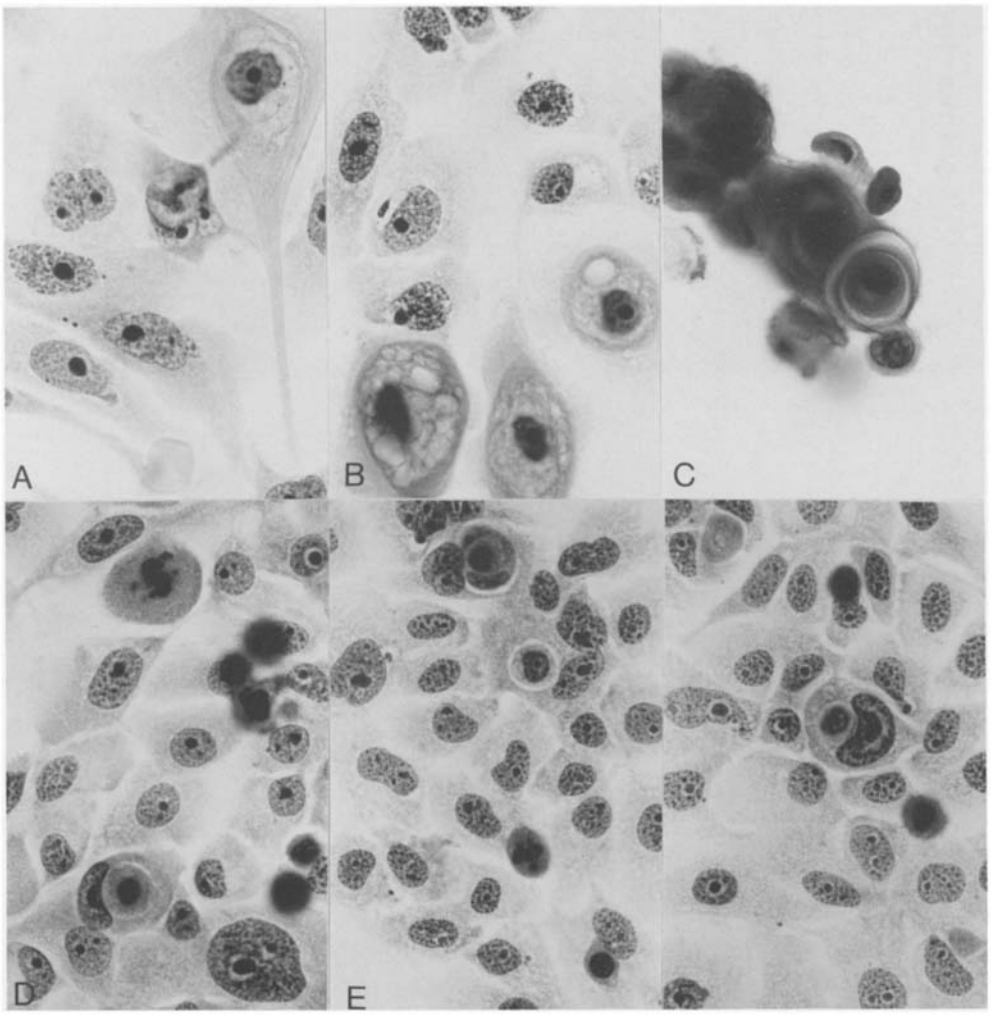

FIGURE 3. Effects of conditioned media on the morphology of BE (A–C) and BEAS-2B (D–F). (A) BE control (*i.e.*, KGM alone). (B) CM_n had no apparent effects on the morphology of BE cells. (C) CM_t induced an atypical metaplastic squamous phenotype, and increased the percentage of large flat squamous cells in BE cell cultures. (D) BEAS-2B control. (E) CM_n and (F) CM_t had little noticeable effect on the morphology of BEAS-2B cells. Papanicolaou stain, ×787.

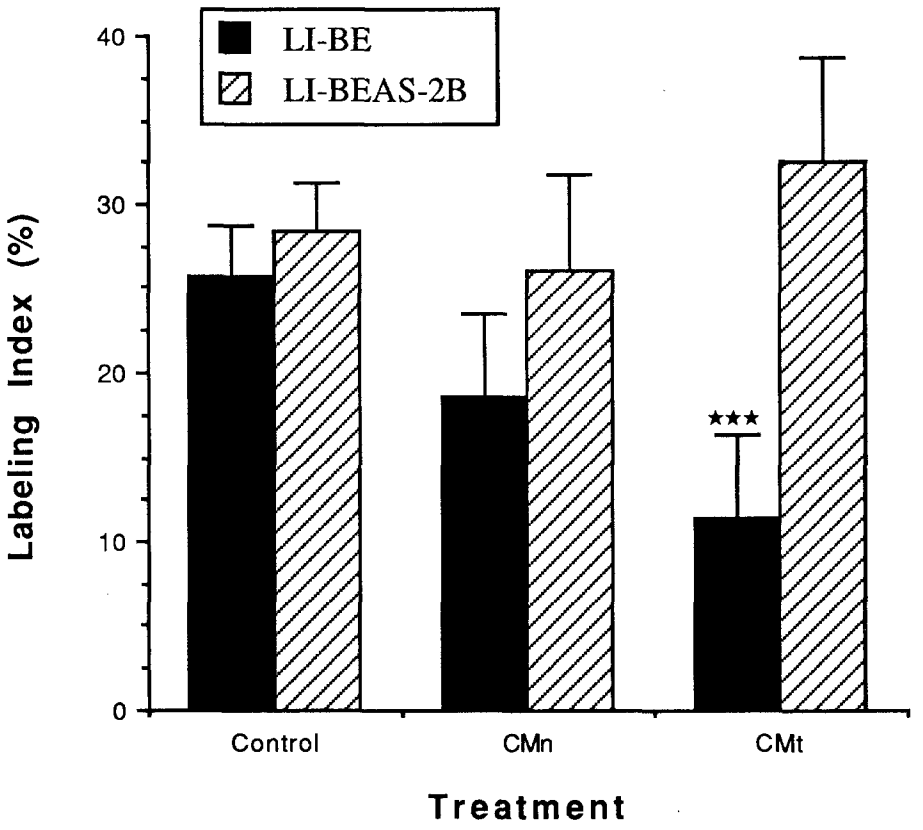

FIGURE 4. Effects of conditioned medium on the BrdU labeling index in BE (white bars) and BEAS-2B cells (black bars) on day 7 in culture. The height of the bars represents the mean ± SE of three experiments. (*t*-test: *** = $p < .01$.)

Conditioned media were screened for TGF-β in the CCL-64 assay. The concentration of TGF-β in CM_t was 8–10× increased over CM_n (Table 3), and CM_t inhibited the incorporation of ^{3}H-TdR in CCL-64 cells, as well as the proliferation (*e.g.*, MI, BrdU labeling index) of BE cells. This is consistent with the secretion of an active form of TGF-βB by BEAS-2B cells.

Effects of Cell-Free Lysates. Cell free lysates from BEAS-2B cells (CFL_t) reduced the MI, PD/D and the number of cells per colony in BE cells (Table 1). This correlated with the induction of an atypical squamous phenotype, representing 5.0 ± 3.7 percent of the cells in 3 of 5 replicates. In BE cell cultures, secretory-type cells and large flat squamous cells exhibited a variable but decreased range of cytoplasmic basophilia, which positively correlated with the degree of nucleolar eosinophilia. CFL_t did not inhibit the growth or alter the morphology of BEAS-2B cells.

TABLE 3
Quantitation of TGF-β in Conditioned Medium by CCL-64 Mink Lung Epithelial Cell Assay

Producer Cells	TGF-β (ng/ml)[a]
BEAS-2B	2.672
BE	0.336
KGM	0.194
KBM	0.039

[a]All data are reported as the means of multiple determinations.
[b]KBM™ (keratinocyte basal medium) (Clonetics Corp.) is identical to KGM, but is without growth factors or hormones.

Effects of Co-culture. Co-culture of BEAS-2B and BE cells enhanced the expression of a squamous phenotype and reduced the expression of a secretory phenotype in BE cells (Fig. 5), independent of the relative number of BE cells plated (Table 4). When the number of BE cells plated exceeded that of BEAS-2B cells, the MI, but not the BrdU labeling index, in BE cells was similar to control levels (*i.e.,*

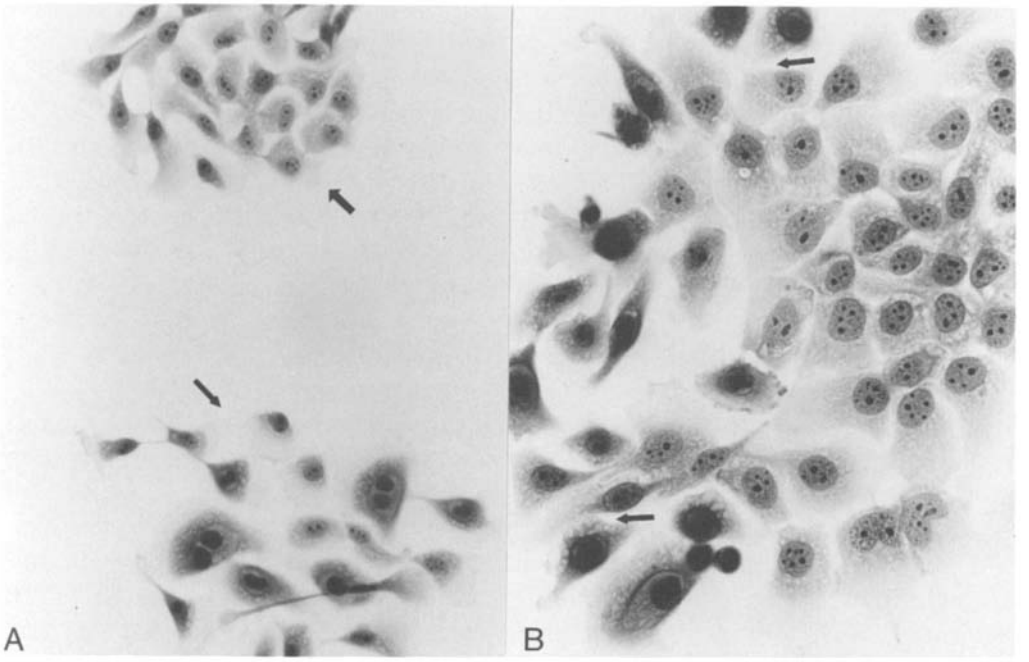

FIGURE 5. Effects of co-culture on the morphology of (A) BE and (B) BEAS-2B cells. (A) Individual colonies composed of BE (small arrows) and BEAS-2B cells (large arrows). (B) Heterologous colony showing direct contact (arrow) between BE and BEAS-2B cells. Papanicolaou stain: (A) ×300, (B) ×787.

TABLE 4
The Effects of Co-culture of BE and BEAS-2B Cells on the Morphology of BE Cells.

BE:BEAS-2B (n)	Percent Cell Type[a]			
	Basal Cells	Round Secretory	Flat Secretory	Squamous
1:0 (10)	2.4 ± 0.5	4.7 ± 0.8	36.3 ± 7.4	56.5 ± 7.5
2:1 (9)	8.1 ± 5.1[c]	6.0 ± 0.8[c]	19.1 ± 2.9[c]	66.8 ± 3.1[c]
1:1 (9)	2.5 ± 1.4	5.5 ± 1.9	15.0 ± 4.4[c]	76.4 ± 4.1[c]
1:2 (9)	2.9 ± 1.9	7.6 ± 2.2[c]	16.4 ± 10.2[c]	73.0 ± 6.4[c]

Note: Chamber slides were seeded with a total of 5×10^4 cells in the ratios indicated, and the cells were grown in serum-free KGM for 7 days. This tabular data is based on the analysis of Papanicolaou stained co-cultures.

[a]Mean ± SE.

[b]The percentages of BEAS-2B cells were not affected by co-culture and therefore are not shown in this table.

[c]*t*-test, $p < 0.05$ *vs.* control (BE cells cultured without BEAS-2B cells).

no BEAS-2B cells) (Fig. 6). Decreasing the ratio of BE:BEAS-2B cells resulted in a significant inhibition in both indeces of proliferation in BE cells. The effects of direct cell-cell contact on proliferation was assessed. Compared with controls (*i.e.,* BE cells cultured without BEAS-2B cells), the MI was significantly reduced (2.3 ± 0.6 *vs.* 1.6 ± 0.7; $p < .05$) in colonies containing only BE cells, but in co-culture with BEAS-2B cells. When the number of BE cells exceeded the number of BEAS-2B cells in an individual colony, the MI was 0.43 ± 0.38 in the BE cells, and 2.18 ± 1.76 in the BEAS-2B cells. When BEAS-2B cells exceeded the number of BE cells in any colony, the MI was 3.37 ± 0.50 in the BEAS-2B cells, and 0.72 ± 0.38 in the BE cells. Co-culture did not change the morphology of BEAS-2B cells.

DISCUSSION

This study shows that transformed bronchial epithelial cells (BEAS-2B) are capable of altering the growth and differentiation of normal BE cells *in vitro*. The proliferation of BE cells was inhibited by: (1) BEAS-2B conditioned medium, (2) extracts of BEAS-2B cells reconstituted in fresh medium, and by (3) co-culture and the effects of direct cell-cell contact between BE and BEAS-2B cells. Under these conditions, BE cells showed an enhanced expression of a squamous phenotype. BEAS-2B factors (*i.e.,* CM_t, CFL_t) stimulated cell proliferation and never induced an atypical keratinized squamous morphology in BEAS-2B cells.

The morphology of the major cell types in human pseudostratified tracheobronchial epithelium (*e.g.,* basal, ciliated, mucous secretory cells) has been summarized elsewhere (Barrett et al., 1976; McDowell et al., 1978; Trump et al., 1980; Jeffery

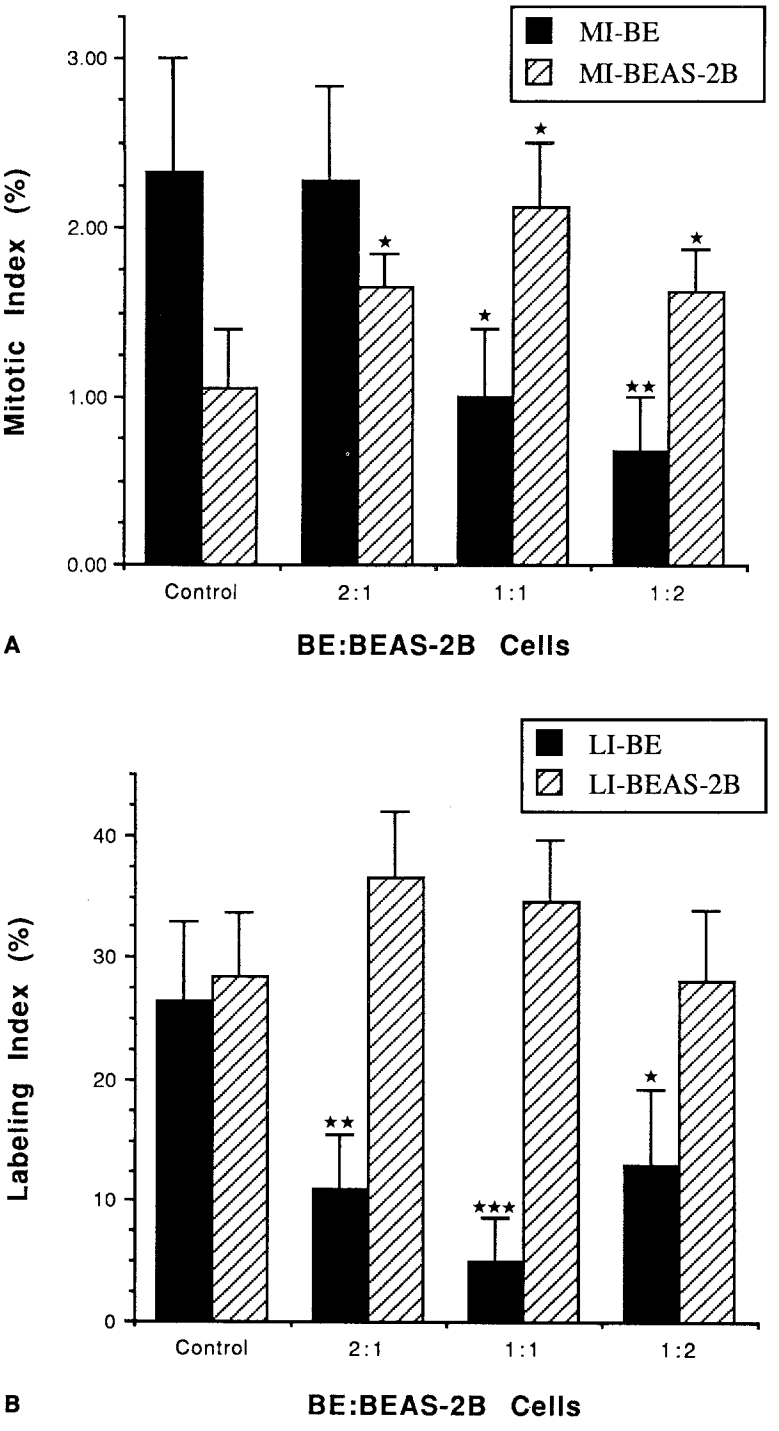

FIGURE 6. Effects of co-culture on the MI (A) and BrdU labeling index (B) in BE (black bars) and BEAS-2B cells (cross-hatched bars). The height of the bars represents the mean ± SE of at least three experiments. (*t*-test: * = $p < .05$; ** = $p < .02$; *** = $p < .01$).

et al., 1983; Müller and Müller, 1983). Human BE cells have been evaluated in a variety of primary cell culture systems (Trump et al., 1980; Lechner et al., 1981; Müller and Müller, 1983; Willey et al., 1984; Masui et al., 1986; Bonnard et al., 1988; Ke et al., 1988). The morphology of the cells which comprise these cultures, however, has not been as well-characterized.

Basal, mucous and ciliated cells have been identified in enzymatic cell isolates from rat and rabbit tracheas (Chang et al., 1985; Inayama et al., 1988). Basal cells in the intact human bronchus are PAS(−) (Chopra et al., 1987), and when exfoliated, these cells exhibit a high nuclear to cytoplasmic ratio (N/C), and a darkly stained nucleus surrounded by a scanty amount of cyanophilic cytoplasm after Papanicolaou staining (Johnston et al., 1986). Comparison studies by EM and Papanicolaou morphology on the same basal cell fraction isolated and purified from rabbit tracheas confirmed the reproducibility of the Papanicolaou criteria for the identification of basal cells (Inayama et al., 1988, 1989).

In the present study, we identified small sized cells having a high N/C, hyperchromatic nucleus and an absence of both secretory granules and cilia as basal-type cells. Analysis of the same cell cultures by phase contrast and after Papanicolaou staining identified the same proportions of basal-type cells. We aspirated the media prior to fixation and Papanicolaou staining, indicating that these basal-type cells were not floating, rounded-up cells. The nucleoli of BEAS-2B cultured basal-type cells were prominent. These cells comprised from 3–5% of the BE cells, and about 2–3% of the BEAS-2B cells on day 7 in culture.

The mucus cells described in this study are similar to ones observed by phase contrast microscopy (DeBuysscher et al., 1984), and after PAS staining (Wu et al., 1985) in other animal species. The distribution of PAS/AB positive material was localized to perinuclear secretory-type vacuoles present in Papanicolaou stained human BE cells. The present morphologic classification of human BE secretory cells is further supported by recent reports correlating the patterns of lectin staining for secretory glycoproteins with the Papanicolaou staining (Albright et al., 1990) and ultrastructural morphology (Chopra et al., 1987).

The epithelial nature of transformed BEAS-2B cells initially described by Reddel et al. (1988), and later confirmed by Bonfil et al. (1989), has also been confirmed in the present study. In addition, this study showed that a low frequency of these cells exhibit a secretory-type phenotype by Papanicolaou and PAS/AB staining; that cultures contain a low percentage of squamous pearls, and that there is a retention of a basal-type cell phenotype. Thus, it is proposed that BEAS-2B cells express a poorly-differentiated epithelial phenotype with a limited ability to undergo secretory and terminal epidermoid differentiation when grown in serum-free medium. This is in agreement with observations by Bonfil et al. (1989) that BEAS-2B cells regenerate a poorly differentiated epithelium in repopulated tracheal grafts.

Secretion of diffusible factors and direct intercellular transfer of small molecules are important in establishing patterns of embryonic development (Caveny, 1985) and in epithelial regeneration during wound healing (Dvorak, 1986; Furcht, 1986; Mustoe et al., 1987; Schultz et al., 1987), and are the basis for the autocrine-paracrine hypothesis proposed by Sporn and Todaro (1980) for the control of cell growth and differentiation in the development of cancer.

Prior investigations demonstrated a reduction in the growth rate of lung carcinoma cells by lung homogenates (Houck et al., 1976), and by lung fibroblasts (Janik et al., 1980). Epithelial factors or mesenchymal factors in these cultures may down-regulate the growth of malignant cells. This is a controversial issue, since more recent studies indicate that the conditioned medium from a lung carcinoma cell line (Shiroeda et al., 1987) or from a mesothelioma (Van Der Meeren et al., 1988) inhibits the growth of normal cells.

Evidence from several studies indicates that primary cultures of normal BE cells grow to form a confluent monolayer rather than developing multilayered foci (Lechner et al., 1981, 1982, 1983). The present results indicate that BEAS-2B cells down-regulated the growth and enhanced the squamous differentiation of normal BE cells. This was reflected in the increase in cell size and the induction of multilayered foci of keratinized squamous cells. Since the growth of BEAS-2B cells was stimulated by their own conditioned medium, these results are consistent with an autocrine-paracrine type of mechanism which selects for the transformed phenotype.

Recent investigations have focused on the role of direct cell-cell contact on the suppression of the malignant phenotype. Miller et al. (1988) studied the effects of co-culture on two subpopulations from the same tumor. They observed that inhibition by the dominant population was proportional to the number of cells plated, and was not mediated by gap junctions, but was apparently due to a concentration gradient of paracrine factor(s). The suppression of tumorigenicity in HeLa-keratinocyte hybrid cells correlated with the expression of involucrin by these cells (Harris and Bramwell, 1987). This is in agreement with an earlier report by Pierce and Wallace (1971) which showed that squamous pearls isolated from a carcinoma were nontumorigenic. Empirical observations have revealed that the poorly differentiated or less well differentiated cells are the component of a tumor most capable of aggressive, independent growth. In our study, co-culture of BE and BEAS-2B cells inhibited the MI ($\approx$ cell division) of the normal in proportion to the ratio of normal to transformed cells plated. BEAS-2B cells produce an activated form of TGF-β. There was an increased expression of a squamous phenotype among BE cells co-cultured with BEAS-2B cells. These results are consistent with the effects of conditioned medium and cell free lysates on BE cells, and suggest that the responses of BE cells may be due to the TGF-β released by BEAS-2B cells. BE cells, however, did not suppress the growth and had little apparent effects on the morphology of BEAS-2B cells, consistent with the reports of Harris and Bramwell (1987) and Pierce and Wallace (1971).

The mechanisms involved in the development of lung cancer are not completely understood. In this report, we have documented that transformed BEAS-2B cells express autocrine/paracine factor(s) which select for the growth of transformed cells and inhibit the growth of normal cells. A transient induction of an epidermoid phenotype is one of the hallmarks of regeneration in tracheal epithelium. It is known that lung cancer cells produce but may not respond to the inhibitory effects of TGF-β (Lechner et al., 1983; Sporn et al., 1986). BEAS-2B cells in our study produced 8–10× the amount of TGF-β produced by BE cells in serum-free medium. Factors produced by BEAS-2B cells induce terminal differentiation in BE cells. The apparent bifunctional effects of conditioned medium from BEAS-2B cells paralled the known effects of TGF-β on normal and malignant BE cells. It is known that TGF-β antagonizes the mitogenic effects of EGF (Roberts et al., 1985) and insulin (Like and Massagué, 1986) and decreases the level of cAMP (Masui et al., 1986; Mioh and Chen, 1989) which precedes the inhibition of DNA synthesis. These studies are consistent with the hypothesis that transformed cells affect the growth and differentiation of normal cells and select for a transformed phenotype. The effects of co-culture indicate that direct cell-cell interactions may be involved in this process. Thus, it will be appropriate to determine if the selective growth advantage induced by TGF-β is due to an effect on intercellular gap junctional communication in human BE cells.

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