Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40

(immortalization/ion transport/phenoype/differentiation)

D. C. GRUENERT*,†, C. B. BASBAUM*‡, M. J. WELSH§, M. LI§, W. E. FINKEINER*¶, AND J. A. NADEL*§

*Cardiovascular and †Cancer Research Institute, and Departments of ‡Anatomy, †Pathology, and §Medicine, University of California, San Francisco, San Francisco, CA 94143; and ¶Laboratory of Epithelial Transport, Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242

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ABSTRACT To facilitate understanding of the mechanisms underlying pulmonary diseases, including lung cancer and cystic fibrosis, we have transformed and characterized cultures of human tracheal epithelial cells. Cells were transfected by calcium phosphate precipitation with a plasmid containing a replication-defective simian virus 40 (SV40) genome. Colonies of cells with enhanced growth potential were isolated and analyzed for transformation- and epithelial-specific characteristics. Precrisis cells were observed to express the SV40 large tumor antigen, produce cytokeratins, have microvilli, and form tight junctions. After crisis, cells continued to express the SV40 large tumor antigen as well as epithelial-specific cytokeratins and to display the apical membrane microvilli. Apical membrane Cl channels were opened in postcrisis cells exposed to 50 μM forskolin. These channels showed electrical properties similar to those observed in primary cultures. The postcrisis cells have been in culture for >250 generations and are potentially "immortal." In addition to providing a useful in vitro model for the study of ion transport by human airway epithelial cells, the cells can be used to examine stages of neoplastic progression.

Recent studies investigating mechanisms of carcinogenesis have shown that cultured human epithelial cells from various organs can be transformed by exposure to carcinogens, viruses, and/or oncoproteins (for reviews, see refs. 1 and 2). Such cells possess altered growth properties that can be correlated with the neoplastic potential. Foreskin and mammary epithelial cells treated with chemicals or radiation show enhanced growth capacity (3, 4). Human keratinocytes (5) have been immortalized with Harvey murine sarcoma virus (v-Ha-ras). Human keratinocytes, immortalized with adenovirus type 12/simian virus 40 (Ad12/SV40) hybrid virus, become tumorigenic after subsequent exposure to the Kirsten ras (K Ras) oncogene or a chemical carcinogen (6, 7).

In contrast to the relatively large number of studies describing cells from other organs (recently reviewed in refs. 1 and 2), only three reports indicate transformation of respiratory tract cells (8-10). Bronchial epithelial cells immortalized with v-Ha-ras display anchorage-independent growth and form tumors in nude mice. However, these cells were poorly differentiated and did not contain tight junctions or keratins characteristic of differentiated epithelial cells. The epithelial origin of the cells was verified by the presence of keratin. A somewhat greater level of differentiation was retained by diethylnitrosamine-transformed fetal tracheal cells, which contained mucin. These cells also expressed carcinoembryonic antigen and wool merokeratin and had enhanced, although limited, growth capacity (9). A third study primarily described methods for transfection and showed expression of the SV40 large tumor antigen (T antigen) three to four subcultures posttransfection (10).

A goal of the present study was to develop a transformed human tracheal epithelial (HTE) cell line for detailed studies of the mechanisms regulating Cl ion transport. This aspect of epithelial function is essential for rehydration of airway mucus. Abnormalities in the Cl conductance pathway also appear critical to disease processes such as those occurring in cystic fibrosis (11, 12).

Phenotypic characteristics have been well maintained in SV40-transformed fibroblasts (13-15). In SV40-transformed epithelial cells (16-19), certain aspects of phenotype are retained better than others (17, 18, 20, 21). A plasmid containing a SV40 genome defective in the origin of replication (pSVori-) has been shown to enhance the transformation efficiency of human fibroblasts (22). The defective origin of replication precludes background virus replication and eliminates the possibility of cell death or interference with phenotypic expression in the host cell due to uncontrolled viral DNA synthesis or production of viral particles. We have used the pSVori - plasmid to transform HTE cells. Transformed cells pass through a period of "crisis," after which phenotype may change and loss of certain differentiated functions may occur (21, 23). We have analyzed both pre- and postcrisis cells. Biochemical and morphological analyses reveal that the cells retain some aspects of the original phenotype even after crisis. These include the presence of microvilli, responsiveness to neural agonists, keratin production, and Cl ion transport.

METHODS

Cells and Culture Conditions. Cells were isolated from trachea, as described, by enzymatic digestion (24) or as outgrowths from tracheal tissue explants. Cells were plated onto dishes coated with fibronectin (Collaborative Research, Waltham, MA), Vitrogen (GIBCO), and bovine serum albumin. Cultures were grown in LHC-8e medium (25) at 37°C under 5% CO2/95% air.

Pure cultures of epithelial cells were generated by eliminating contaminating fibroblasts through selective detachment (26). Pure cultures of epithelial cells were grown to 70% or 80% confluence before subculturing into 100-mm Petri dishes.

Transformation. The pSVori - plasmid was amplified in bacteria and then isolated after banding on CsCl density gradients (27). Since the transfection efficiency is increased in linearized plasmids (22), pSVori - was linearized with Xho I at a unique restriction enzyme site distal to the transforming genes in the SV40 portion of the plasmid. The plasmid was cut in a region distant to the SV40 genes to minimize endogenous exonuclease damage to genes essential for transformation.

Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; HTE, human tracheal epithelium.
Gene transfer was achieved by calcium phosphate \((\text{CaPO}_4)\) precipitation (28) of plasmid DNA. Cells that were subcultured one to three times were grown to 80% confluence on 100-mm dishes and then transfected with linearized pSVori' DNA. LHC-8e growth medium was replaced with hypotonic medium [Dulbecco's modified Eagle's medium (DMEM) diluted with sterile \(\text{H}_2\text{O} (5.5:6.0)\)] before the plasmid was added. Cultures were incubated in the presence of 15 \(\mu\)g of plasmid per 100-mm dish. After 3–4 hr the incubation medium was removed. Cells were washed three times with LHC-8e medium and grown in LHC-8e for 48 hr before subculturing. Cultures were then grown for 3–4 weeks until colonies of cells with altered growth properties appeared on a background of terminally differentiated cells. Colonies were isolated and grown for further analysis. All clones of transformants were designated as HTE ori' (HTEO'). Numbers preceding the designation indicate clone number and those following indicate tissue sample.

**Immunofluorescence.** Cells grown on well slides (Lab-Tek, Naperville, IL) to various stages of confluence were washed three times with ice-cold phosphate-buffered saline (PBS) and then fixed with -20°C acetone/methanol (2:3). After drying in air at room temperature, cells were rehydrated in PBS and individual wells were exposed to the L19 monoclonal antibody for the SV40 T antigen (diluted 1:5 in PBS) (29) or the antibody for 50- and 56.5-kDa keratins (Hybritech, San Diego, CA) (diluted 1:40 in PBS). The slides were washed with three changes of PBS containing 1% normal goat serum (30 min) before exposure to the secondary antibody (goat anti-mouse IgG–fluorescein isothiocyanate conjugate) diluted 1:40 in the buffer described above and viewed with a Zeiss fluorescence microscope.

**Electron Microscopy.** Cells were fixed and processed for transmission electron microscopy as described elsewhere (30). Briefly, confluent cultures were fixed on glass four-well microscope slides (Lab-Tek) with a solution containing 2.5% glutaraldehyde, 0.08 M sodium cacodylate, 5 mM CaCl\(_2\), and 10% (vol/vol) sucrose (pH 7.4; 4°C). After 12–18 hr, the cells were postfixed with 1.5% osmium tetroxide in 0.2 M phosphate buffer (pH 7.4) for 2 hr. The cells were rinsed in 0.025 M sodium maleate buffer (pH 6.0) and stained with uranyl acetate (1.5% in 0.025 M sodium maleate buffer (pH 5.2)). The cells were embedded by placing embedding capsules on top of the monolayers and filling them with Epon 812. After polymerization was complete, the resin and cells were separated from coverslips. Semi-thin (0.5 \(\mu\)m) sections were cut with glass knives on an LKB Nova ultramicrotome, mounted on microscope slides, and stained with toluidine blue before examination with a light microscope. Specific areas were selected, and thin sections having a silver interference color were cut with a diamond knife and mounted on Formvar-coated copper slot grids. These sections were stained with uranyl acetate and lead acetate before examination in a Zeiss EM 10 electron microscope.

**cAMP Assay.** Transformed tracheal epithelial cells were grown to confluence in 35-mm dishes. The dishes were rinsed three times with PBS and incubated in growth medium. After 30 min, the medium was replaced with identical medium or with medium containing (–)isoproterenol (10–3 M). At 1, 5, 10, 20, or 30 min, the medium was removed and replaced with ice-cold 10% trichloroacetic acid. Cells were scrapped from culture dishes, sonicated, and centrifuged (2500 \(\times\) g; 4°C; 15 min). The supernatants were removed and the trichloroacetic acid was extracted in ether. The extracted samples were dried under a stream of air and the residue was dissolved in acetate buffer. cAMP concentrations were measured in the samples of \(\frac{1}{25}\) of radioimmunoassay (Riaden assay, DuPont), corrected for recovery after extraction (82% as determined with \[^3\text{H}\]cAMP), and normalized with respect to the protein content of the cell pellet as determined by the methods of Lowry et al. (31).

**Ion Transport.** The methods used for patch clamp are similar to those described (32). Currents were amplified with a List Electronics EPC-7 amplifier (Darmstadt, F.R.G.). The experiment was controlled and data were analyzed with a laboratory computer system (Indec Systems, Sunnyvale, CA). Currents were low-pass filtered (1000 Hz) by an eight-pole Bessel filter and sampled at 500 \(\mu\)s. We patched isolated cells or cells growing at the edge of a cluster of cells. Pipette resistance was 2.5–6 MΩ and seal resistance was 4–35 GΩ. During seal formation and when recording in the cell-attached mode, bath and pipette solutions contained 140 mM NaCl, 1.2 mM CaCl\(_2\), 1.2 mM MgCl\(_2\), and 10 mM Heps buffered to pH 7.4 with KOH. When the NaCl concentration was decreased, osmolality was maintained with sucrose. Junction potentials in the presence of asymmetric solutions were measured with a KC1 electrode and were used to correct measured reversal potentials. Experiments were performed at room temperature (21–23°C).

**RESULTS**

In the present study, we introduced the pSVori plasmid (22, 33) into HTE cells by CaPO\(_4\) precipitation. Although another study suggested that strontium phosphate (SrPO\(_4\)) precipitation protocol may reduce Ca\(^{2+}\)-induced squamous differentiation and lysis of epithelial cells (10), we detected no adverse effects of Ca\(^{2+}\). Before transfection, the plasmid was linearized by cleavage with Xho I to further enhance the efficiency of transformation. Exonuclease inactivation of genes was minimized by selecting a restriction site distal to the transforming genes.

After 3–4 weeks in culture, foci of proliferating cells appeared with an efficiency of \(\approx 10^{-5}\). The foci were distinct from a background of squamous cells or clear plastic (Fig. 1). Individual foci were isolated and expanded in culture. The isolated cells were subcultured numerous times and did not become squamous as did the nontransformed cells. Several colonies of cells went through crisis. The karyotype of the various pre- and postcrisis clones analyzed show an aneuploid character (data not shown). Although some clones of transformants eventually adapted to growth on uncoated plastic, cells generally had a higher plating efficiency and grew more rapidly on coated dishes.

Since cells transformed by pSVori express the viral T antigen, we established the presence of this antigen by immunofluorescence to verify that the cells were in fact transformed. The T antigen was present in all pre- and postcrisis cells, indicating that this epithelial phenotype was maintained at different stages of transformation. The postcrisis cells also did not stain with their keratin antibody staining, lacked the degree of cytoskeletal organization observed in the precrisis cells. No keratin was detected in fibroblasts when the same antibody was used (data not shown). Electron microscopy revealed that both pre- and postcrisis cells contained microvilli (Fig. 3). Tight junctions, a feature unique to epithelial cells, were observed in precrisis cells by electron microscopy (Fig. 3).

The capacity of airway epithelial cells to secrete chloride ion from the submucosal to the mucosal surface is a distinctive feature of the tracheal epithelium. CI ion enters the cell at the basolateral membrane via electrically neutral cotrans-
porters and exits at the apical membrane via Cl channels (34). Apical Cl channels control, in part, the rate of transepithelial secretion. These channels are in turn regulated by β-adrenergic receptors through a cAMP-mediated pathway. The secretion of Cl is correlated with an intracellular accumulation of cAMP. To investigate whether the signal-transduction mechanisms mediating Cl secretion were present in transformed cells, cells were exposed to the β-adrenergic agonist isoproterenol, known to stimulate Cl transport in intact tracheal epithelium and primary culture. Stimulation with 10−5 M isoproterenol led to an accumulation of cAMP in several pre- and postcrosis cell lines within 1 min of exposure (Table 1). The HTE-8o−, 3/HTEo−, 8/HTEo−, and 9/HTEo− cell lines maintained increased levels of cAMP for up to 30 min during exposure to isoproterenol.

We were not able to study the transport properties of the postcrosis cells with Ussing chambers because the monolayer cultures did not develop an appreciable transepithelial electrical resistance (J. H. Widdicombe, D. B. Jacoby, and D. C. G., unpublished observations). This was expected on the basis of the paucity of tight junctions in cell monolayers. We were, however, able to detect Cl ion channels by the patch-clamp technique. We used the patch-clamp technique to study two of the postcrosis cell lines and found channels with properties similar to those observed in primary cultures of human airway cells (32). Previous studies in primary cell cultures show that these channels are responsible for the apical membrane Cl conductance based on (i) their activation by secretagogues, (ii) their anion selectivity, (iii) their location, and (iv) their inhibition by Cl channel blockers (32, 35, 36). Fig. 4A shows tracings from a cell-attached patch of 8/HTEo− (150 generations posttransfection) during baseline conditions and after addition of 50 μM forskolin to the bathing solution. Before addition, there are no channel openings, but after addition a channel is open the majority of the time. Forskolin also opened Cl channels in five cell-attached patches from 9/HTEo− cells (200 generations posttransfection). However, the channel was not strictly regulated by forskolin; it was also observed to open in the absence of forskolin when the patch was excised (three cells from 9/HTEo− and one cell from 8/HTEo−). This pattern is the same as that observed in primary cultures of human airway epithelium (32). The probability of finding a channel in any patch was 5–10%, similar to the probability in primary cell cultures.

The conductive properties of the channels observed in 9/HTEo− and 8/HTEo− cells were also similar to those found in Cl channels from primary cultures of airway cells. Fig. 4B shows the single channel current–voltage relationship of a Cl channel in an excited inside-out patch bathed in symmetrical 145 mM NaCl solutions. The relationship shows outward rectification—i.e., single-channel currents are larger at depolarizing than hyperpolarizing voltages. Replacement of the internal bathing solution with 72 mM NaCl shifted the reversal potential to positive voltages, indicating a relative Cl− to Na+ permeability (P_{Cl}/P_{Na}) of 8. In contrast, cation substitution did not appreciably change the reversal potential.

**DISCUSSION**

We have described transformed tracheal epithelial cell lines that retain phenotypic characteristics, permitting the study of
Cl ion transport. The cells contain other features such as keratin, microvilli, and tight junctions, which are also characteristic of the epithelial phenotype.

The presence of Cl ion transport in transformed HTE cells means that it will be possible to develop epithelial cell lines important to studies of basic defects in cystic fibrosis. The 9/HTEo- and 8/HTEo- cell lines accumulate cAMP in response to isoproterenol and contain Cl channels that open in response to an agent that stimulates cAMP. These observations indicate that the signal-transduction mechanisms involved in Cl secretion are retained in these two postcrisis cell lines. The differences in the amount of cAMP accumulated within 1 min of stimulation may reflect clonal variability or variability due to the stage of neoplastic progression. The differences in cAMP levels do not appear to affect the Cl transport properties of the transformants, since the Cl ion channels respond to stimulation in a manner similar to that observed in primary culture (32, 37). The present cultures provide an important tool for analysis of the mechanism by which Cl channels are regulated in normal individuals. This issue is of particular interest with respect to abnormalities in the regulation of Cl channels in cystic fibrosis (11, 12, 35, 36). Transformation of epithelial cells from cystic fibrosis patients could potentially provide cells that would be useful for studies of the Cl defect associated with this disease (38, 39).

In addition to their importance for studies of ion transport, the cells provide an in vitro system for studies to determine how tracheal cell function changes throughout neoplastic progression in the airways (2). Since the HTE-2o- and the HTE-8o- cells have not yet gone through crisis, they can be monitored for changes in function with respect to crisis, immortalization, and tumorigenicity. The cells can also be studied to determine possible correlations between changes in growth, oncogene expression, and differentiated function after exposure to carcinogens.

The postcrisis HTEo- clones are more advanced in the neoplastic process and will be useful for the study of the later stages of neoplastic progression. These cells appear to be immortalized since they have been in culture for >1 year and have now been subcultured >250 generations posttransformation. Although the 3/HTEo-, 8/HTEo-, and 9/HTEo- cell lines are no longer contact inhibited, they do not form colonies on soft agar (unpublished observations).

Changes in the expression of oncogenes have been correlated with variations in malignant potential in the airways. The MYC and RAS oncogenes are expressed in various forms of lung cancer. In small cell lung cancer, amplification of the MYC family of oncogenes appears to be related to a more malignant phenotype (40–42). Analysis of the expression of these oncogenes during different stages of neoplastic progression, such as immortalization and tumorigenicity, will

Table 1. cAMP levels

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transformed state</th>
<th>Control</th>
<th>Isoproterenol</th>
</tr>
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<tbody>
<tr>
<td>HTE-8o-</td>
<td>Precrisis</td>
<td>70 ± 14</td>
<td>306 ± 36</td>
</tr>
<tr>
<td>3/HTEo-</td>
<td>Postcrisis</td>
<td>102</td>
<td>1376</td>
</tr>
<tr>
<td>8/HTEo-</td>
<td>Postcrisis</td>
<td>99 ± 8</td>
<td>3204 ± 395</td>
</tr>
<tr>
<td>9/HTEo-</td>
<td>Postcrisis</td>
<td>178 ± 41</td>
<td>2172 ± 98</td>
</tr>
</tbody>
</table>

cAMP values in pmol per mg of protein were determined 1 min after exposure to 10^{-5} M isoproterenol and represent the mean of three samples. Results are expressed as mean ± SEM. cAMP values for 3/HTEo- are from a single measurement.
facilitate an understanding of how these oncogenes modulate neoplastic progression in lung cancer.

Genetic complementation analysis of candidate genes for cystic fibrosis (43) will require cells with an unlimited life span in culture to ensure stable expression of the gene. The feasibility of generating such cells is indicated by the results of this study. The ultimate availability of similarly transformed cells from cystic fibrosis patients will facilitate development of treatment regimens for this and other genetic disorders defective in epithelial cell function.

Note Added in Proof. Since this manuscript was submitted for publication, we have learned that Reddell et al. (44) have published results that support the use of SV40 oncogenes for the transformation of human airway epithelial cells.

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