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LONG-TERM CULTURE OF NORMAL AND CYSTIC FIBROSIS EPITHELIAL CELLS GROWN UNDER SERUM-FREE CONDITIONS

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SUMMARY

The understanding of pathways associated with differentiated function in human epithelial cells has been enhanced by the development of methods for the short-term culture of human epithelial cells. In general these methods involve the use of serum. The subculture and maintenance of epithelial cells in long-term culture has been more problematic. A serum-free medium developed for human bronchial epithelial cells was slightly modified and found to be useful for the subculture and long-term maintenance of not only bronchial epithelial cells, but also tracheal, nasal polyp, and sweat gland epithelial cells from either normal or cystic fibrosis individuals. The cells maintained epithelial-specific characteristics after multiple subcultures. Monolayers of epithelial cells showed junctional complex formation, the presence of keratin, and micro villi. Functional studies with Ussing chambers showed short circuit current (I_{sc}) responses to isoproterenol, bradykinin, or calcium ionophore (A23187) in subcultured tracheal and bronchial cells.

Key words: human epithelial differentiation; subculture; cystic fibrosis.

INTRODUCTION

Systems for the culture of human epithelial cells have evolved significantly in recent years (3,24). Techniques have been developed to culture epithelial cells from numerous organs including bronchus (12), trachea (8,27,28), mammary gland (21,22), esophagus (1), prostate (11,16), skin (3,17,19), nose (29,30), and kidney (6). Cells have been grown under conditions where the growth medium is supplemented with fetal bovine serum (FBS) (19,27,28,30) or under serum-free conditions (3,8,12,29). Many of the approaches taken involve primary culture of isolated cells. Few have been concerned with continued subculture of epithelial cells.

Current challenges in the area of epithelial cell culture are a) to develop methods for the continued propagation of epithelial cells and b) to establish cultures of epithelial cells from individuals with genetic disorders. This is particularly true for genetic disorders involving defects in epithelial function such as cystic fibrosis (CF). Epithelial cells in CF individuals have been shown to be defective in apical membrane chloride ion transport. The defect has been demonstrated in tracheal (13,20,28) nasal polyp (30), and sweat gland (18) epithelium. A number of approaches have been used to culture epithelial cells from the different organs mentioned above. Each approach is unique for the particular tissue used. This report shows that with very limited exposure to serum cultures of normal and CF epithelial cells, free of fibroblast contamination, can be initiated from various tissues and maintained for multiple subcultures in a defined medium originally developed for the culture of human bronchial epithelial cells. Furthermore, we show that subcultured airway epithelial cells retain the electrical properties observed in primary culture.

MATERIALS AND METHODS

Initiation of cultures. Tissue was obtained following biopsy, autopsy, or from terminated pregnancies under conditions outlined by National Institutes of Health (Bethesda, MD) and institutional human use guidelines. The tissues were approximately equally distributed between male and female. All airway tissue was washed at least twice with cold (4° C) phosphate buffered saline (PBS) containing penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (50 μg/ml). Cystic fibrosis nasal polyp cultures were initiated by enzymatic dissociation. Tracheal and bronchial cultures from adult tissue were initiated either by enzymatic dissociation or from tissue explants. Fetal tracheal and bronchial cultures were initiated from explants. Sweat gland cultures from the secretory coil or the reabsorptive duct were also initiated from tissue explants.

For enzymatic digestion, epithelium was dissociated with a solution containing DNAase (100 μg/ml), 1% bovine serum albumin (BSA) (fraction V powder), and 0.02% type I crude collagenase (27) or by overnight incubation in a PBS solution containing antibiotics and 0.1% pronase (29). Explants of airway tissue were cut into 1- to 2-mm squares with a sterile scalpel. Individual explants were placed onto 60- or 100-mm petri dishes that had been scored after coating with a solution containing 1 mg human fibronectin (Collaborative Research Inc., Bedford, MA), 1 ml Vitrogen 100 (Collagen Corp., Palo Alto, CA), and 10 ml of BSA (1 mg/ml stock solution) (Biofluids, Rockville, MD) made up to 100 ml with LHC basal medium (FN-V-BSA) (Biofluids) (9). The explants were covered with 1 drop of dialyzed FBS. This exposure to serum promotes attachment to the dish. Culture dishes containing explants were then incubated at 37° C under 5% CO_2 in air overnight. After 24 h explants were supplemented with at least 2 vol of serum-free growth medium. The limited exposure to serum was terminated after another 24 to 48 h incubation at 37° C. All serum-containing medium was removed and replaced with serum-free medium as described below. Explants from sweat gland secretory coil and reabsorptive duct were initiated in a fashion similar to that of the airway and attached to the culture dishes with a drop of dialyzed FBS (J. Wine, personal communication) (10).

Culture conditions. All cultures were grown in LHC-8e medium [LHC-9 medium with glutamine and without retinoic acid (12)] that has been slightly modified. The stock 4 and trace elements were increased by 20%, and glutamine was added to a final concentration of 4 m*M*. Some cultures were initiated in the presence of serum, so cells were put into serum-free medium as soon as possible after isolation to minimize the squamous differentiating effects of tumor growth factor-β (TGF-β) (14) and the growth of contaminating fibroblasts. The presence of TGF-β in blood-derived serum results in an inhibition of cell growth and minimizes the proliferative potential of cultured epithelial cells.

Purification of epithelial cells. Cultures derived from biopsy or autopsy tissue are often mixed populations of epithelial cells and fibroblasts. These mixed cultures must be further purified to generate cultures that are exclusively epithelial. The steps involved in the establishment of epithelial cultures are schematically depicted in the flow diagram in Fig. 1 and the description below. Growth in serum-free medium inhibits but does not totally eliminate fibroblast proliferation. Not all cultures were contaminated with fibroblasts. However, those that did show evidence of fibroblast outgrowth were subjected to selective trypsinization (15).

The mixed cultures were washed twice with a HEPES buffered saline (HBS) [18.3 m*M* HEPES, 121 m*M* NaCl, 2.7 m*M* KCl, 9.4 m*M* glucose, 7.2 m*M* $Na_2HPO_4 \cdot 7H_2O$, phenol red (0.5%), 0.25 ml/liter; (pH 7.45) (12)]. The cells were then incubated at 37° C in a trypsinization cocktail containing 0.02% EGTA, 1% polyvinylpyrrolidine (PVP), and 0.02% trypsin, 0.0016% EDTA in Puck's saline A, and 80 mg/liter glucose. After a 2-min incubation the cells were observed every 30 s with an Olympus IMT-2 inverted microscope (Olympus Corp., Los Angeles, CA) by phase optics. At the point when fibroblasts were in the process of detaching, i.e. rounding up, the cultures were rinsed with the trypsinization solution once. This rinse was followed by two additional rinses with HBS. The cells remaining attached were then incubated at 37° C overnight in the presence of modified LHC-8e (MLHC-8e) medium under 5% CO_2 in air. The following day cultures were assessed for further fibroblastic contamination. If there was still morphologic evidence of fibroblasts in the culture, the selective trypsinization procedure was repeated within 48 h of the initial trypsinization.

Subculturing. After pure cultures of epithelial cells were established, the cells were subcultured using the same trypsinization solution as described above. Cells were grown to 80 to 90% confluence, rinsed twice with HBS, and then incubated at 37° C in the trypsinization solution, which was repeatedly aspirated and expelled into the dish until all the cells were detached. The trypsin solution containing the cells was transferred to a sterile conical centrifuge tube containing MLHC-8e medium supplemented with 10% FBS to neutralize the trypsin. The cells were then pelleted at room temperature by centrifugation at 1000 *g*. The supernatant was removed by aspiration, and the pellets were resuspended in MLHC-8e medium. New cultures were established at a split ratio of between 1:2 and 1:4. All cultures were grown on tissue culture plastic that had been precoated with FN-V-BSA as described previously (8,12). After trypsinization, the growth medium was replaced with medium within 24 h. Growth medium was routinely replaced with fresh MLHC-8e every other day. Plating efficiency was determined 24 h after trypsinization. A given number of cells

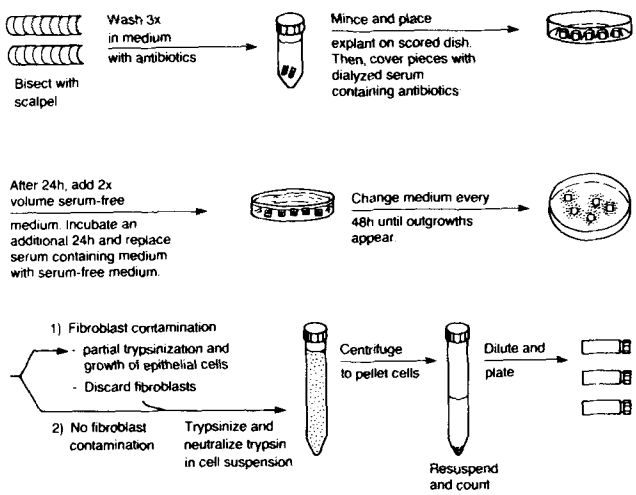

FIG. 1. A flow diagram representation of the steps involved in epithelial cell isolation, the initiation of primary cultures, and the cultivation of epithelial cells for multiple subcultures. The two modes of isolation and initiation, enzymatic dissociation and tissue explants, each with their advantages and drawbacks, can be effective for the establishment of long-term cultures of pure epithelial cells. Subculture of the epithelial cells is the same regardless of the method used for the establishment of primary cultures.

were plated and retrypsinized 24 h later. Cells were counted using a hemacytometer.

Cryopreservation. For frozen storage, approximately 10^6 cells were trypsinized as described above and pelleted by centrifugation. The pellet was resuspended in 1.5 ml of a solution containing MLHC-8e:FBS:dimethyl sulfoxide (Sigma, St. Louis, MO) in a ratio of 4:5:1. The cell suspension was placed in a 2-ml cryopreservation vial (Corning). The vial containing the cells was then placed at −20° C overnight and then transferred to a −70° C freezer. After several days to several weeks at −70° C, the cells were placed in liquid nitrogen for long-term storage.

Immunocytochemical staining. Cells were grown on well slides (Lab-Tek) coated with FN-V-BSA to various stages of confluence, washed 3 times with ice-cold PBS, and then fixed with −20° C acetone:methanol (2:3). After air drying, the cells were rehydrated in PBS and exposed to the primary antibody, either cytokeratin monoclonal antibodies AE1 and AE3 (Boehringer Mannheim, Indianapolis, IN) or a calcium-dependent cell adhesion molecule (cell CAM 120/80) monoclonal E9 (26) in a humidified chamber at 37° C for 30 min. Next, the slides were washed with 3 changes of PBS containing 1% goat serum for 30 min at room temperature. After washing, the cells were incubated in a humidified chamber with flourescein isothiocyanate labeled secondary antibody, goat antimouse (AE1/AE3), or goat antirat (E9) (Boehringer Mannheim) at 37° C for 30 min. The slides were then washed 3 times in PBS over 30 min and viewed under a fluorescent microscope.

Electron microscopy. Cells were fixed for transmission electron microscopy as described elsewhere (8). Confluent cultures on FN-V-BSA coated 22 × 22-mm glass cover slips were fixed with a solution containing 2.5% glutaraldehyde, 80 m*M* Na cacodylate, 5 m*M* $CaCl_2$, and 10% sucrose (pH 7.4, 4° C). After 12 to 18 h, the cells were postfixed with 1.5% osmium tetroxide in 200 m*M* phosphate buffer (pH 7.4) for 2 h. The cells were then rinsed in 25 m*M* sodium maleate buffer (pH 6.0) and stained with uranyl acetate (1.5% in 25 m*M* sodium maleate buffer, pH 5.2). The cells were placed in embedding capsules on top of the monolayers that were filled with Polybed 812. After polymerization, the resin and the cells were removed from the surface of the cover slip. Semithin (0.5 μm) 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 EM10 electron microscope.

Electrical measurements. Electrical measurements were taken as described previously (28). Briefly, cells grown on 12-mm diameter milli cell HA inserts (Millipore, MA) at a density of 10^6 cells/cm^2 in MLHC-8e. For Ussing chamber analysis, MLHC-8e medium was replaced with Eagle's minimal essential medium supplemented with 10% FBS 4 d before electrical analysis. The HA inserts were then mounted in Ussing chambers designed to accept the inserts without creating edge damage. Warm (37° C), HCO_3-buffered (pH 7.4) Krebs-Henseleit solution was circulated across both faces of the monolayer by gaslift oxygenators. Transepithelial potential difference (PD) was clamped to zero, and the current required (the short circuit current, I_{sc}) was continuously monitored on a pen recorder. Resistance was determined from current needed to displace the PD from zero to 0.5 mV. Current pulses were applied every 20 s for 200 ms.

RESULTS

Establishment of epithelial cell cultures is dependent on several factors: a) the degree of fibroblast contamination in the primary cultures, b) the amount of time between the removal of the tissue sample and the establishment of the primary culture, c) the plating density of the cells after subculture, and d) the degree of confluence at the time of subculture. In terms of viability, enzymatically dissociated cells are much more sensitive than explant-derived cells to time elapsed between autopsy or biopsy (unpublished observations). Viability

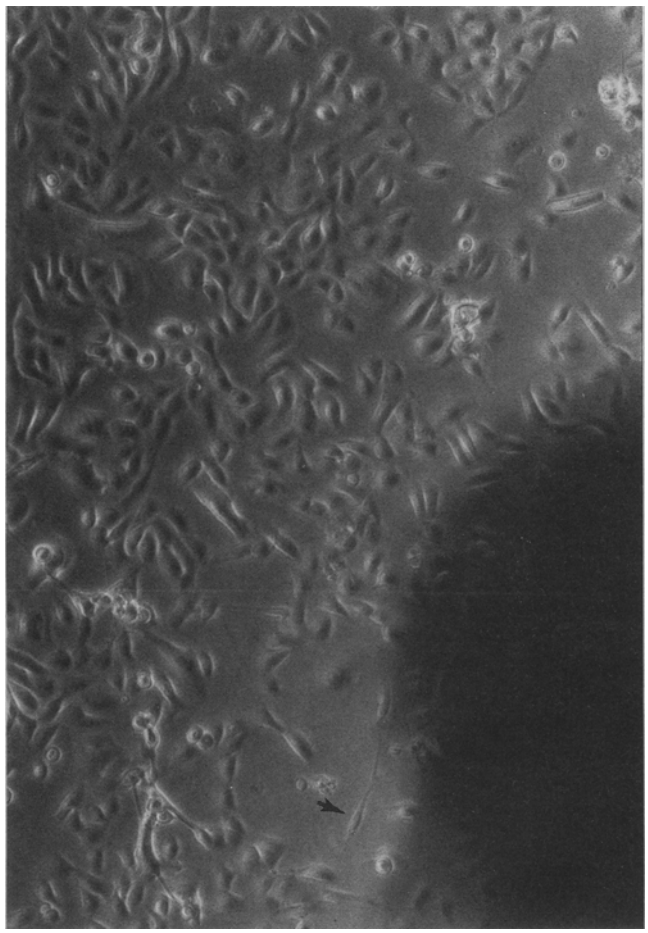

FIG. 2. Typical cell outgrowth from a tissue explant from a cystic fibrosis bronchial explant. Both elongated fibroblastic cells and the cuboidal epithelial cells can grow in the serum-free medium. Because of their morphologic differences it is possible to readily distinguish between epithelial cells and fibroblasts. *Arrow* indicates fibroblasts.

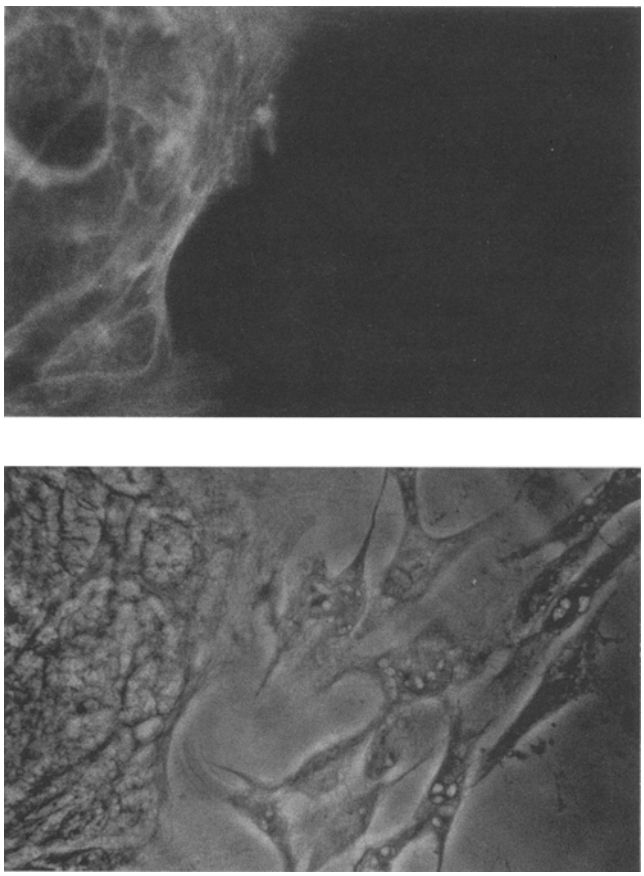

FIG. 3. Cytokeratin staining of airway epithelial cells correlates with the morphologic differences indicated from light microscopy. Analysis is performed on primary cultures of adult tracheal epithelial cells from normal individuals.

was assessed qualitatively as the ability of dissociated cells to attach to the culture dish (plating efficiency) and time before cell outgrowth from explants. Although it is possible to establish cultures as much as 24 h postmortem or removal of the tissue, the best results are achieved if primary cultures are established within 6 h postmortem or postbiopsy.

In general, for cultures derived from explants, tissue is removed within 6 h postmortem or postbiopsy and then immediately incubated in MLHC-8e medium at 4° C in the presence of antibiotics. We have initiated viable cultures from explants as much as 48 h postmortem or postbiopsy. In the case of the airways it is crucial that the explants are incubated and rinsed in the presence of antibiotics, which can generally be removed within 3 d of initiating an explant culture. The presence of antibiotics is particularly important for CF tissue because most of this tissue has come from individuals with upper respiratory infections. A primary cause of these respiratory infections has been *Pseudomonas aeruginosa* (23).

The outgrowth of cells from an explant is readily observable by light microscopy and it is possible to distinguish between epithelial cell and fibroblastic outgrowth (Fig. 2). Epithelial cells have a typical "cobblestone" morphology, whereas the fibroblasts are elongated and tend to grow in parallel "streams." Some explant outgrowths will be devoid of fibroblast contamination, however if there are multiple explants per dish it is often necessary to preferentially trypsinize away the fibroblasts before pure epithelial cultures can be generated. Selective trypsinization relies on the preferential removal of fibroblasts during exposure to trypsin. Fibroblasts are more sensitive to enzymatic detachment than epithelial cells, and can be selectively detached while a substantial portion of the epithelial cells remain attached (15). Excessive fibroblast contamination will complicate this procedure, and therefore alternative approaches to the isolation of pure epithelial cultures are required. An alternative to selective detachment is the trypsinization of patches of epithelial cells with cloning rings. If the patch is large enough, several rings may be used to selectively remove the epithelial cells and allow for plating of the cells at a high enough density to ensure the viability of the trypsinized cells. The correlation of morphology with the epithelial origin of these cell types was confirmed by cytokeratin staining (Fig. 3). All tissue samples are routinely tested for the presence of keratin.

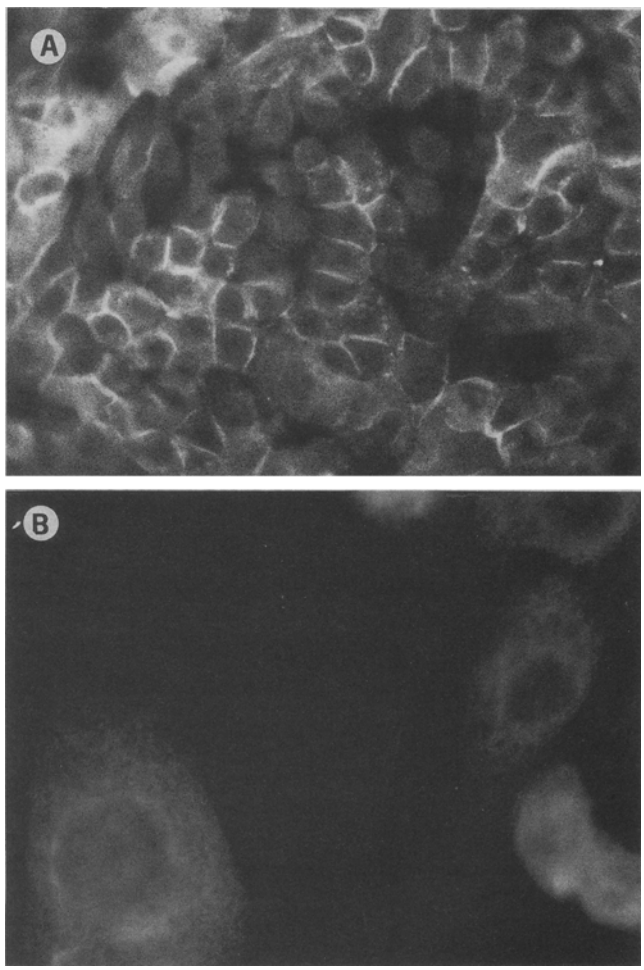

FIG. 4. *A*, immunocytochemical staining for the presence of calcium-dependent adhesion molecule, cell CAM 120/80 in normal adult bronchial epithelial cells, Passage 3. This antigen is expressed in the junctional complex and is present in the cultures of epithelial cells throughout their cell culture life-time. *B*, keratin staining in normal adult tracheal epithelial cells, Passage 4.

After pure epithelial cultures have been established, it becomes necessary to subculture the cells so that they do not become confluent and terminally differentiate (12). Plating efficiency of cells ranges from 10 to 50% and does not seem to vary with respect to cell origin; whether fetal or adult, CF or normal, or with regard to the organ of origin (unpublished observations). The condition of the cells at the time of subculture seems to play a more important role in the plating efficiency than where the cells originate. Plating efficiency is highly sensitive to the density of the cells when they are trypsinized and is significantly lower (<1%) when the cells are trypsinized after they have been maintained at confluence for several days or if they have been trypsinized at too low a cell density. We found the optimal range for trypsinizing the cells is between 60 and 90% confluence. Plating efficiency after trypsinization is also dependent on several other parameters, e.g., plating density and trypsinization time. The cell growth medium should be changed within 24 h of trypsinization to neutralize residual

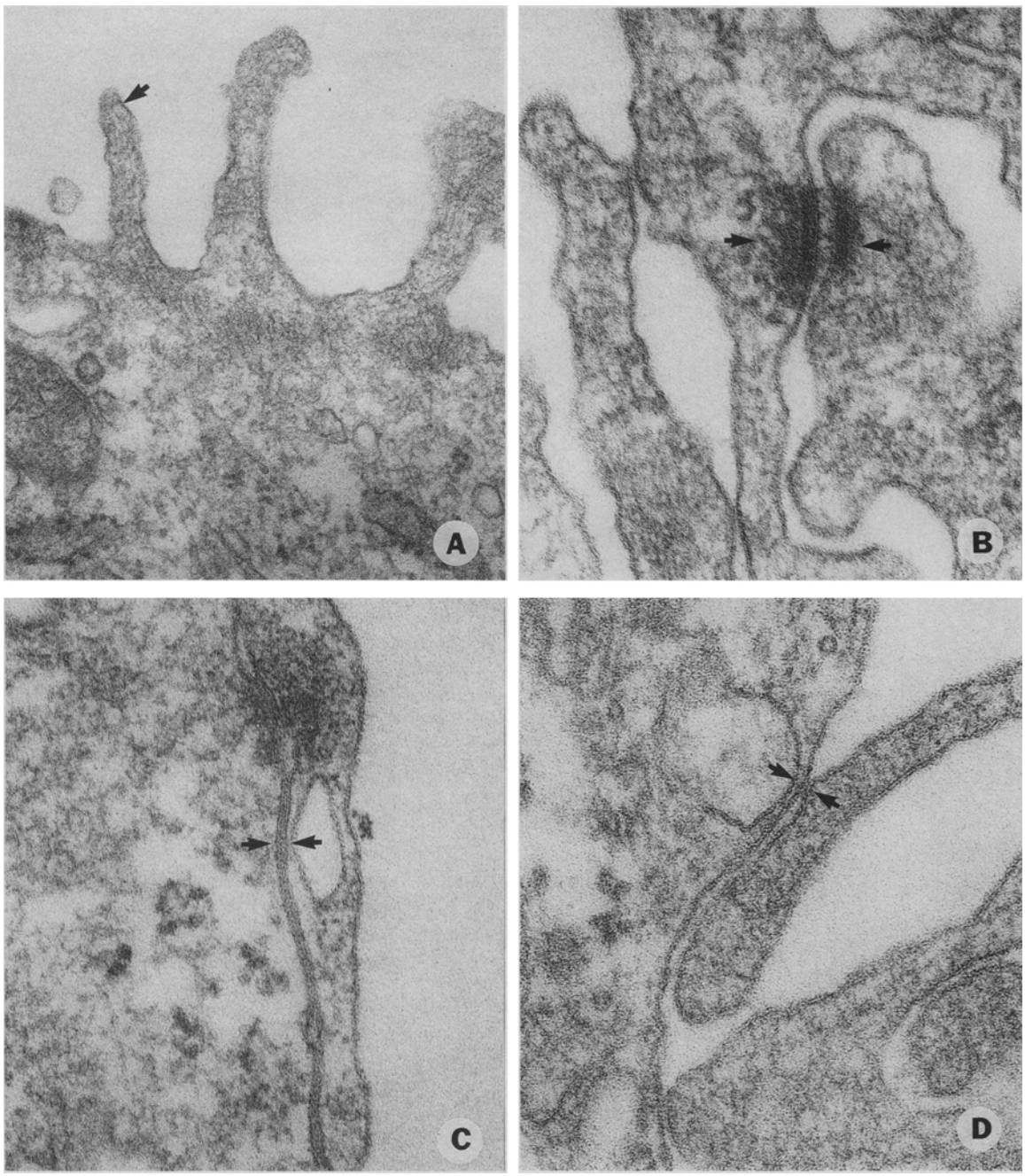

FIG. 5. Transmission electron micrograph of features characteristics in cultured airway epithelial cells. Typical subcultured epithelial cells show the presence of *A*, microvilli with actinlike filaments (*arrow*) ×67 888. *B*, desmosomes (*arrows*) ×137 931. *C*, gap junctions (*arrows*) ×137 931. *D*, tight junctions (*arrows*) ×140 000. The cells analyzed are normal fetal tracheal epithelial cells that have been subcultured 4 times (1:3).

trypsin. The growth medium can then be changed every other day until the cells are again ready to subculture.

Epithelial cells that have been through multiple subcultures maintain numerous characteristics indicative of epithelial cells. Immunocytochemical analysis shows that the cells maintain epithelial cell differentiation markers after subculture. For example, positive staining for the presence of a calcium-dependent cell adhesion molecule (Fig. 4 *A*) and keratin (Fig. 4 *B*) indicates that junctional complexes and intermediate filaments, respectively, are maintained after the cells have been subcultured. Fibroblasts from the same original tissue continue to be negative for the presence of cell CAM 120/80 or keratin (data not shown). The absence of staining for cell CAM 120/80 is not unexpected, because the E9 monoclonal was isolated by screening using a choriocarcinoma cell line (positive) and a human lung fibroblast cell line (negative) (26). The lack of keratin in fibroblasts is consistent with the results for primary cultures. Electron microscopic analysis of tissue samples from one adult trachea (Passage 5), one fetal bronchus (Passage 4), two fetal tracheas (Passages 4 and 5), and three cystic fibrosis nasal polyps (Passages 1, 2, and 5) further supports epithelial differentiation by showing the presence of microvilli, desmosomes, gap junctions, and tight junctions characteristically found in primary cultures of epithelial cells (Fig. 5).

TABLE 1

CULTURED EPITHELIAL CELLS

Cell Type, Tissue Source	Maximum Subcultures[a]	Age Range Donor	Sample Number
Fetal			
Tracheal	8	16-24 wk(19)[b]	42
Bronchial	8	16-24 wk(19)	41
Adult			
Tracheal			
Normal	10	7-82 yr(41)	12
Cystic fibrosis	9	4 mo-45 yr(22)	7
Bronchial			
Normal	8	18-66 yr(46)	4
Cystic fibrosis	4	12 & 32 yr	2
Nasal polyp			
Cystic fibrosis	9	7-35 yr(19)	7
Sweat gland			
Secretory coil			
Normal	7	14-52 yr(34)	3
Cystic fibrosis	5	21 & 26 yr	2
Reabsorptive duct			
Normal	4	21 & 49 yr	2
Cystic fibrosis	4	21 & 46 yr	2

[a] The number of subcultures reflects the maximum for the number of epithelial cell samples tested.

[b] The numbers in parentheses indicate the median age in weeks or years as appropriate. There seems to be no substantial difference in cell culture life span between CF and normal cells or if the tissue is of adult or fetal origin.

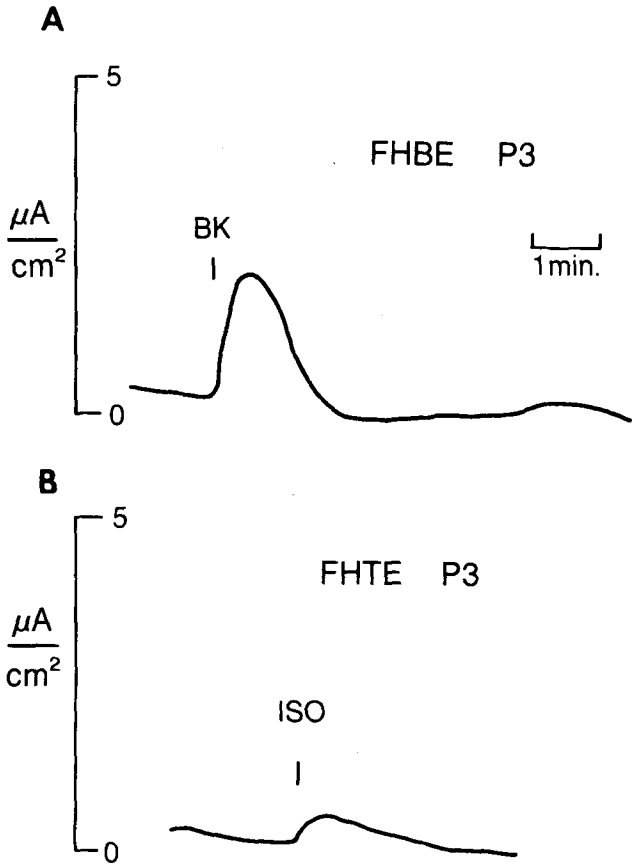

FIG. 6. Changes in I_{sc} in response to mediators. *A*, fetal bronchial epithelial cells, Passage 3, after 10^{-6} *M* bradykinin; R = 115 Ω·cm². *B*, fetal tracheal epithelium, Passage 3, after 10^{-5} *M* isoproterenol; R = 145 Ω·cm². Drugs were added to both sides of the tissues as indicated by the *arrowhead*.

Bronchial and tracheal epithelial cells at Passage 3 were studied in Ussing chambers. Electrical resistance was 100 to 150 Ω·cm² and baseline I_{sc} was zero. However, bradykinin increased I_{sc} across bronchial cells, and isoproterenol increased I_{sc} across tracheal cells (Fig. 6). A23187 also stimulated I_{sc} across tracheal cells by approximately 2 μA/cm², and control experiments with fibroblasts from the same original tissue showed negligible resistance (<5 Ω·cm²) and no baseline I_{sc} or response to isoproterenol (data not shown). These results are consistent with what has been shown in the analysis of primary cultures of airway epithelial cells (2,4,13,25,27).

DISCUSSION

We have shown in this report that it is possible to culture several types of epithelial cells, relevant to cystic fibrosis, in a defined growth medium developed for the culture of human bronchial epithelial cells. These epithelial cell cultures are derived from normal and cystic fibrosis individuals, using tissues from various organs (Table 1), either by enzymatic dissociation or from explant outgrowth.

Tracheal epithelial cells have been subcultured 10 times (approximately 70 generations with a 1:3 split ratio). In both normal and CF, the cells seem to maintain epithelial-specific characteristics after multiple subcultures and when analyzed immunocytochemically or by electron microscopy. Intermediate filaments indicated by keratin staining and microvilli with actinlike filaments show that these specific differentiated characteristics are maintained throughout the culture life of the epithelial

cells. The junctional complex formation, as indicated by the presence of the cell CAM 120/80 (26), is another feature that seems to be stable after subculture. Inasmuch as the cell CAM 120/80 belongs to the cadherin family, is associated with the zonula adherence, and is epithelial-specific (26); it is significant as an indicator of tight junction formation and that elements of intercellular communication are maintained in culture.

The high electrical resistance (100 to 150 $\Omega \cdot cm^2$) of cultures further confirms the presence of tight junctions. In addition, the changes in I_{sc} in response to mediators demonstrates not only that the relevant receptors are present, but that the cultures have retained the polarization of transport proteins typical of epithelia in general. The increase in I_{sc} of bronchial epithelial cultures in response to isoproterenol was considerably less than that of tracheal epithelial cultures to bradykinin. This difference may reflect different types of ion transport by the two cell types. Thus, the predominant active ion transport process of human bronchi is Na absorption (9), whereas the tracheal epithelium shows mainly active Cl secretion (Widdicombe, unpublished data), and it is well established that the ion transport processes of cultured airway epithelial cells reliably reflect those of the native tissue (2,4). Chloride secretion across airway epithelia is increased by a wide range of agents acting through either cAMP or Ca_i (25). In contrast, Na absorption across human bronchi (9) is virtually unaltered by elevation of these second messengers.

At high subculture numbers the rate of proliferation of the cells slows and the cell cycle seems to become protracted (M. Yezzi and D.C. Gruenert, unpublished observations). This observation correlates with previous findings for fibroblasts at or near senescence (5). The relationship between this slowing down of proliferation and the onset of senscence requires further study. It is, however, clear that epithelial cells lining human airways and human sweat gland cells can be effectively cultured for multiple generations when terminal differentiation is inhibited by maintenance of cells in serum-free medium at low calcium concentrations. These diploid cells are grown under conditions that promote growth, as opposed to conditions that enhance expression of differentiated function or lead to cell death. After sufficient proliferation has occurred (as much as a 70-fold increase from the original starting material), conditions can potentially be manipulated, as in the Ussing chamber analysis, so that differentiated characteristics are expressed. Optimization of differentiated properties will be important for studies assessing the functional characteristics of epithelial cells.

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