

Culture of Normal Human Airway Epithelial Cells and Measurement of Mucin Synthesis and Secretion

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1. Introduction

The plasticity of conducting airway epithelia is well recognized (1–3). Under normal conditions, the epithelia express mucociliary function, which is the first pulmonary defense mechanism against inhaled air pollutants. Aberrance in this function is either the cause or one of the major contributors to the pathogenesis of various pulmonary diseases, such as asthma and bronchitis. To exert this vital defense function, mucus-secreting cell types of surface epithelium and sub-mucosal gland synthesize and secrete a high-mol-wt mucous glycoprotein, mucin, which is responsible for the viscoelastic property of the surface mucus layer. Secreted mucus, which is able to trap air pollutants and microorganisms, is steadily removed from the airway surface by ciliary escalation. Overall, the coordinated mucociliary function helps to maintain homeostasis in airway lumen.

However, changes in airway epithelial cell (EC) differentiation are frequently observed (1–3), including the development of squamous and mucous cell metaplasia, as well as hypermucus secretion. The nature of these changes is not entirely clear. In addition, conducting airway epithelium also plays a pivotal role in the initiation and development of bronchogenic carcinoma (2). Most bronchogenic cancers are epithelial in origin. An uncontrolled cell proliferation of a certain EC type may lead to the development of a certain type of lung cancer. Because of the plasticity of epithelium, tracing the original cell type that initiates carcinogenic development is most difficult. These difficulties suggest a great need to understand the nature of airway EC differentiation and how it is regulated.

To achieve these goals, progress has been made in culturing differentiated airway ECs from human tissues in a well-defined culture environment (4,5),

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and for mucin quantitation (6). The primary culture system of hamster tracheal ECs is the first in vitro demonstration of new mucous cell differentiation (7,8) and ciliogenesis (9). This success mostly results from the development of serum-free, hormone-supplemented medium and the use of collagen gel substratum for cultivation. However, this similar culture condition was unable to allow primary human airway ECs to achieve new ciliogenesis in culture, except for mucous cell differentiation. It was not until the development of an air-liquid interface culture system that new ciliogenesis could be demonstrated in human cells (10,11). The first part of this chapter describes the procedures involved in the isolation of human airway ECs (12), the culture condition for serial cultivation of human airway ECs, and the air-liquid interface system to achieve mucociliary differentiation; the second half describes how mucin secretion and synthesis are quantified by a double-sandwich enzyme-linked, immunosorbent assay (ELISA) method.

Hypersecretion of mucin and the hypertrophy of mucous cell type are two clinical hallmarks associated with various airway diseases and infections (13,14). There are biochemical (15,16) and immunological methods (6,17) to measure these abnormalities: The biochemical method requires the fractionation of samples by gel filtration (15) and centrifugation, prior to the quantitation; for the immunological method, no preparation is needed. The biochemical separation method is based on the biochemical properties of mucin, which include the following characteristics: high mol wt, highly glycosylated and *O*-glycosidic linkage, and high buoyant density. The immunological approach is based on the specificity of the antibody (Ab), which must be able to recognize purified mucin and mucus-secreting granules at the morphological level (6,17,18). However, with few exceptions, Abs generated are specific for the carbohydrate portion of high-mol-wt mucous glycoprotein. The heterogeneous structure of mucous carbohydrate chains is well recognized, including differences in length, branching unit, and terminal sugar. Therefore, it is necessary to characterize the specificity of the epitope of Ab used in the study. The author and colleagues have extensively characterized both human mucin-specific 17B1 and 17Q2 monoclonal antibodies (MAbs), before the application of these Abs for mucin ELISA (6,18), and have observed that the epitopes for both Abs are not determined by blood group antigen or terminal sugar, nor are they affected by enzymes specific to various proteoglycans. However, the activity of the epitope was reduced by half by endo- β -galactosidase (6). The nature of this effect is not clear. Nevertheless, the result suggests that the epitope of these mucin-specific Abs may involve the structure at or near the nonsulfated galactosidic bond, such as Gal(β 1-4)Glc in lacto-*N*-tetraose, the structure of which is the major backbone structure in mucin. Thus, these studies confirm the specificity of these two Abs on the carbohydrate chains of human mucin.

Using these Abs, a double-sandwich ELISA method (6) was developed to quantify the amount of human mucin in various samples. The basic approach in this ELISA method is, first, to trap mucin antigen in the liquid sample by the purified immunoglobulin of these MAbs, then to quantify the amount of mucin trapped on the microplate with an alkaline phosphatase-conjugated, mucin-specific Ab. Because there are many epitope sites in mucin, a single mucin-specific Ab is used for both the trapping and detecting steps. The author and others have used this ELISA system to determine the amount of mucin secreted in culture and in various biological specimens. Some of these studies have led to the conclusion that the serum mucin level can be used as a diagnostic indicator that is correlated with the severity of the airway diseases, cystic fibrosis (19), and acute respiratory distress syndrome (20).

2. Materials

2.1. Human Airway EC Culture

2.1.1. Serial Cultivation

1. Minimum essential medium (MEM) from Gibco-BRL (Grand Island, NY) containing 50 U/L penicillin and 50 $\mu\text{g/L}$ streptomycin (Sigma, St. Louis, MO), 50 mg/L gentamicin (Irvine Scientific, Santa Ana, CA), 1.98 g/L NaHCO_3 , and 15 mM HEPES, pH 7.2.
2. 0.1% Protease solution: 0.1 g Sigma's type 14 protease in 100 mL MEM medium. This solution should be sterilized by filtering through 0.2- μm sterile filter membrane, then stored at -20°C until use.
3. Fetal bovine serum (FBS) (Sigma, or any other qualified commercial company).
4. Equal volumes of Dulbecco Modified Eagle's Medium (DMEM) and Ham's F12 nutrient medium are mixed, containing similar concentrations of penicillin, streptomycin, gentamicin, and 15 mM HEPES buffer as MEM, except NaHCO_3 at 2.45 g/L.
5. Airway serum-free, hormone-supplemented medium: DMEM-F12 medium is supplemented with 5 $\mu\text{g/mL}$ insulin (Sigma), 5 $\mu\text{g/mL}$ transferrin (Sigma), 10 ng/mL epidermal growth factor (Upstate Biotechnology, Lake Placid, NY), 0.5 μM dexamethasone (Sigma), 20 ng/mL cholera toxin (List Biochemical, Campbell, CA), 15 $\mu\text{g/mL}$ bovine hypothalamus extract, (30 nM all-*trans*-retinoic acid, 5 mg/mL bovine serum albumin (BSA) (Sigma), 0.3 mM MgCl_2 , 0.4 mM MgSO_4 , and 1.05 mM CaCl_2 (see **Note 1**).
6. 0.1% Trypsin (Sigma)-ethylene diamine tetraacetic acid (EDTA) (1 mM), stored at 4°C .
7. 1 mg/mL soybean trypsin-inhibitor (Sigma), stored at 4°C .

2.1.2. Differentiation of Human Airway ECs in Culture

1. Collagen gel substratum preparation: Collagen gel solution is prepared by mixing 3 mg/mL Vitrogen solution (Collagen, Palo Alto, CA) with an alkaline-F12

solution at 4:1 vol ratio under cold (4°C) conditions. The alkaline-F12 solution is prepared by mixing 1 N NaOH with 5X F12 medium at a ratio of 1:2.

2. Transwell chamber (ICN).
3. Humidified CO₂-37°C incubator.

2.2. Mucin Quantitation

2.2.1. Preparation of Standard Human Mucin Antigen

1. Protease inhibitor solution: 200 mM *p*-phenylmethylsulfonyl fluoride (PMSF) dissolved in methanol, toxic, and stored at 4°C.
2. CsCl density gradient centrifugation: 250,000g, for 48 h.
3. Sepharose CL-2B column (Pharmacia, Piscataway, NJ)
4. Elution buffer: Phosphate-buffered saline (PBS) solution is added with 0.1% sodium dodecyl sulfate and 3% β-mercaptoethanol.
5. Dialysis solution: PBS and water.

2.2.2. Quantitation of Mucin by Double-Sandwich ELISA Method

1. 17Q2 (or 17B1) immunoglobulin G (IgG) solution (Babco, Berkeley, CA): IgG of 17Q2 (17B1) is purified by affinity chromatography in a protein G-agarose column. Briefly, 2–5 mL 17Q2 (17B1) ascite fluids are passed through a protein G-agarose column at pH 8.0. The column is then washed several times with 0.01 M Tris-Cl, pH 8.0. After extensive washing, IgG of 17Q2 (17B1) is eluted from the column by a pH 3.0 buffer. After extensive dialysis against 2–3 changes of cold PBS, IgG concentration is adjusted to 1 mg/mL, and stored at –20°C.
2. Alkaline phosphatase-conjugated IgG solution: Conjugation is carried out by mixing 2 mg (2 mL) 17Q2 (17B1) IgG and 5 mg alkaline phosphatase (Sigma) in the presence of 0.06% glutaraldehyde. After an overnight conjugation at cold temperatures, the mixture is extensively dialyzed against PBS. After dialysis, the mixture is adjusted to a final solution containing 200 µg/mL IgG and 2 mg/mL BSA, and stored at 4°C.
3. Coating solution: 0.05 M sodium carbonate, pH 9.0, stored at 4°C.
4. Washing solution: PBS–Tween-20 (0.05%), filtered, and stored at room temperature.
5. Phosphate substrate solution: *p*-nitrophenyl phosphate, disodium (Sigma) at 1 mg/mL in 10% diethanolamine solution (pH 9.8). Freshly prepared at room temperature.
6. Immulon II 96-well plate (Dynatech, Alexandria, VA).
7. 3 N NaOH.
8. MR600 Microplate reader (Dynatech) or equivalent model from other manufacturer.

3. Methods

3.1. Growth and Differentiation of Human Airway ECs in Culture

Primary culture of human airway ECs is widely used as an in vitro model for various studies related to airway diseases, bronchogenic cancer, environmental air pollutant effects, and cell differentiation. ECs are dissociated from air-

Table 1
Effects of Culture Conditions
on Cell Differentiation of Cultured Airway ECs

Culture conditions	Mucous cell differentiation	Ciliogenesis
Tissue culture dish	+	–
Collagen gel substratum	++	–
Transwell chamber/air–liquid interface	+++	++
Transwell chamber/CG/air–liquid interface	++++	++++

way tissue by protease (*see Note 2*). These ECs rapidly adhere to the culture surface of various tissue culture wares. With the development of defined hormone-supplemented culture medium, human airway ECs can be serially cultivated. This procedure yields ECs obtained from the distal region of airway tree, which is isolated by microdissection. Despite serial cultivation, ECs are largely squamous ones, expressing keratinization and cornification. To achieve mucociliary differentiation, at least three additional culture conditions are needed. First, vitamin A or one of its retinoid derivatives is essential for all of the differentiation to occur in vitro. The second requirement is to maintain the culture under an air–liquid interface condition. Finally, the use of collagen gel substratum can further maximize the differential potential. **Table 1** summarizes the extent of EC differentiation under various culture conditions.

3.1.1. Serial Cultivation of Human Airway ECs

Human airway tissues can be obtained from local and national programs related to consent autopsy, organ transplant, and routine biopsy services. These tissues are immersed in serum-free MEM with various antibiotics, such as penicillin, streptomycin, and gentamicin, and shipped to the lab cold. Treating these tissues immediately upon arrival with further washing and cleaning is advisable, because it can further minimize the contamination in culture (*see Note 3*).

1. These tissues are immersed in 0.1% protease solution in MEM overnight at 4°C or for 1 h at 37°C (*see Note 4*).
2. After protease treatment, epithelial sheets are flushed away from tissue with ice-cold 10% FBS–MEM medium, and the cold cell suspension is then centrifuged at 200g for 5 min (*see Note 5*).
3. The cell pellets are then suspended in the airway serum-free, hormone-supplemented culture medium at $0.1\text{--}1 \times 10^6$ cells/mL. Normally, the initial seeding density is at least 1×10^4 cells/cm² of culture surface area. Dishes are incubated in a CO₂ incubator at 37°C and 5% CO₂ (*see Note 6*).
4. Medium change is carried out every other day.

5. A confluent culture with a cell density of approx $1-5 \times 10^5$ cells/cm² is achieved within 7–10 d of incubation (see **Note 7**).
6. For subculturing, confluent dishes are treated with trypsin–EDTA solution at room temperature, or at 37°C, until cell detachment occurs. An equal or slightly higher volume of trypsin-inhibitor solution is added to stop further trypsinization (see **Note 8**).
7. The cell suspension is centrifuged, and cell pellets are suspended in the culture medium with a density of 1×10^5 cells/mL.
8. Cells are plated at a density of 1×10^4 cells/cm². Human airway ECs can be routinely passaged 3–5×, with a total of approx 25 population doublings, until senescence is reached.

3.1.2. Expression of Mucociliary Differentiation in Culture (see **Note 9**)

1. ICN's Transwell chamber well is coated with freshly prepared collagen gel solution at 0.2 mL/cm² surface area. Incubate at 37°C for 30–60 min until gel forms.
2. ECs, obtained from protease-treated tissues, are suspended in the airway serum-free, hormone-supplemented medium, and pipeted on the chamber well.
3. After 1-d incubation, the medium at the upper chamber well of Transwell is removed and replaced with new serum-free, hormone-supplemented medium.
4. The outer and lower part of the Transwell chamber is also filled with airway culture medium.
5. Maintain the immersed culture condition with a periodic medium change for 5–7 d, then change the immersed culture condition to an air–liquid interface, by removing the apical culture medium and incubating the culture in a well-humidified CO₂ incubator.
6. After 7–10 d of air–liquid interface culturing, a mucociliary epithelium is formed in culture.

3.2. Mucin ELISA

3.2.1. Preparation of Referenced Mucin

1. Sputum mucus or secreted culture media collected from human airway cultures are treated with DNase and hyaluronidase in the presence of 1–2 mM PMSF protease-inhibitor solution.
2. After overnight treatment, the mixture is heat-denatured in the presence of 1% SDS and 3% β-mercaptoethanol.
3. Powdered CsCl is added to the mixture until a density of 1.5 g/mL is achieved.
4. The mixture is centrifuged at 30,000 rpm for 48 h. Fractions having a density greater than 1.5 g/mL are collected and pooled. The collected mixture is dialyzed against PBS.
5. The mixture is further fractionated in a preparative Sepharose CL-2B column, which has been equilibrated with the eluting solution of PBS–0.1% SDS–3% β-mercaptoethanol.

6. Void volume peak fractions are collected and further fractionated in a new Sepharose CL-2B column. Void volume fractions are collected and dialyzed against PBS and water, with 2–3 changes.
7. A small but sufficient amount of solution is subjected to amino-acid-analysis. The amino acid analyzed results provide both necessary information regarding the mucin nature of the preparation and information regarding the content.
8. Based on this information, references of mucin are prepared at 0.5, 1, 2, 4, 8, and 16 ng/mL levels.

3.2.2. Double-Sandwich Mucin ELISA Method

1. Immulon microplate wells are coated with purified 17Q2 (17B1) IgG, at 0.2 µg/well in coating buffer, and incubated at 37°C for 1 h under an airtight cover (*see Note 10*).
2. After washing with PBS–Tween-20 (0.05%) solution, 200 µL of various standard mucin (0.5–16 ng/mL), and unknown samples at different dilutions, are added to each well. The reaction is carried out at 37°C under an airtight cover for 1–2 h (*see Notes 11 and 12*).
3. Microplate wells are washed with PBS–Tween-20 (0.05%), then each well is treated with 200 µL diluted alkaline phosphatase-conjugated 17Q2 (17B1) IgG solution at 1 µg/mL IgG and 10 µg/mL BSA in PBS–Tween-20 (0.05%).
4. After further incubation at 37°C for 1 h under an airtight cover, wells are washed with PBS–Tween-20, and 200 µL phosphate substrate solution is added to each well for color development.
5. The reaction can be stopped by the addition of 50 µL 3 N NaOH to each well.
6. Developed color in the plate is read at 405 nm wavelength in an MR600 microplate reader.

4. Notes

1. Bovine hypothalamus extract is prepared according to the procedure described by Maciag et al. (21). Commercial sources, such as endothelial cell growth supplement from Collaborative Research (Waltham, MA), are also suitable. The concentration used in the culture should be predetermined, because preparation of the extract can be variable, and the biological activity is variable from lot to lot.
2. General safety and ethical rules for acquiring human tissue for research should be followed.
3. Microorganism contamination in human airway tissues, especially those from autopsy, is a major problem in preventing the development of a successful and uncontaminated primary culture. Generally, the fresher the tissue from an organ donor patient, the less contamination. The initial step of cleaning and treatments with various antibiotics on tissues can vastly improve the contamination problem.
4. Tissues are viable for several days, when immersed in the culture medium under cold condition (4°C).
5. Protease-dissociated EC preparation has a viability greater than 95%, and tissues can be repeatedly treated with protease to ensure a complete recovery of all ECs from tissue.

6. Seeding density less than the recommended 1×10^4 cells/cm² has a difficult time in achieving confluency and in subsequently performing serial cultivation.
7. Low calcium medium, such as LHC-9 (5) and the commercial media from Clonetics (San Diego, CA), such as the bronchial EC growth medium and small airway EC growth medium, are suitable for serial cultivation of airway ECs. Within the low-calcium medium (<0.1 mM Ca²⁺), ECs multiply and maintain basal appearance. However, these low-calcium media are not suitable for airway ECs to grow on collagen gel substratum nor to express mucociliary differentiation.
8. Cells grown on tissue culture wares are easily used for serial cultivation by trypsin-EDTA solution; cells plated on collagen gel substratum are difficult to use.
9. Mucous cell differentiation in culture can be assessed at the level of mucin secretion, and the mucous cell population can be identified by mucin-specific Ab.
10. Primary IgG coating may be variable, depending on the quality of the microplate well. Excessive coating reduces the sensitivity of the assay; less coating traps less mucin and shortens the linearity of reference mucin.
11. The presence of detergents and reducing agents in the sample should be kept to a minimum, or $<0.1\%$ and 1 mM levels, respectively.
12. Protease inhibitor should be included during mucin ELISA for samples known to be contaminated with protease, such as sputum (19,20).

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