Video Article

Isolation of Mouse Respiratory Epithelial Cells and Exposure to Experimental Cigarette Smoke at Air Liquid Interface

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Abstract

Pulmonary epithelial cells can be isolated from the respiratory tract of mice and cultured at air-liquid interface (ALI) as a model of differentiated respiratory epithelium. A protocol is described for isolating and exposing these cells to mainstream cigarette smoke (CS), in order to study epithelial cell responses to CS exposure. The protocol consists of three parts: the isolation of airway epithelial cells from mouse trachea, the culturing of these cells at air-liquid interface (ALI) as fully differentiated epithelial cells, and the delivery of calibrated mainstream CS to these cells in culture. The ALI culture system allows the culture of respiratory epithelia under conditions that more closely resemble their physiological setting than ordinary liquid culture systems. The study of molecular and lung cellular responses to CS exposure is a critical component of understanding the impact of environmental air pollution on human health. Research findings in this area may ultimately contribute towards understanding the etiology of chronic obstructive pulmonary disease (COPD), and other tobacco-related diseases, which represent major global health problems.

Video Link

The video component of this article can be found at https://www.jove.com/video/2513/

Protocol

The overall protocol requires 2 days for cell isolations from animal tissue, 5-10 days for cell proliferation, and an additional 10-14 days for cell differentiation at air-liquid interface. An additional day is required for cell exposures and harvesting of samples.

1. Isolation of Mouse Tracheobronchial Epithelial Cells (MTEC).

Note: All procedures described below have been reviewed and approved by the Institutional Animal Care and Use Committee at Brigham and Women's Hospital/ Harvard Medical School Area.

Before getting started:

- Prepare Ham's F12 Media containing antibiotics. To 250 mL Ham's F12 basal media (Cellgro) add 2.50 mL of a 100 X Penicillin/Streptomycin (10^2 U/mL-10^2 mg/mL) solution and 250 µL of a 1000 X Fungizone solution. Store at 4°C for up to 4 weeks.
- Prepare a solution of 0.15% Pronase. Add 15 mg Pronase to 10 mL of Ham's F12 Media containing antibiotics. Make fresh and keep on ice until use.
- Prepare a clean work surface and sterile surgical instruments appropriate for small animal surgery, and a lamellar tissue culture hood for cell isolations. All other equipment is considered standard for animal cell culture including humidified CO_2 incubators, cold room, tabletop cell centrifuges, inverted microscope, and disposable plastic pipette and culture ware.
- Prepare collagen I solution (50 µg/mL in 0.02 N acetic acid). Pipette 1.0 mL collagen solution into each well of a 12 well transwell plate (Corning). Wrap in parafilm and let stand overnight on a flat surface at room temperature.

1. Using a commercially-available mouse strain (i.e., C57Bl/6, male 6-8 weeks old), euthanize mice using an approved method of euthanasia such as CO_2-induced narcosis. Typically 6 mice will yield enough cells (1.5-2.0 x 10^5 cells/mouse) to seed a 12-well transwell plate.
2. Spray the animal carcasses with 70% EtOH solution to sterilize the field.
3. With clean surgical scissors and scalpel, remove the skin around the tracheal area, and expose the trachea. Open the abdomen, cut along the sternum, and remove the rib cage. Remove tissue until the end of the trachea is exposed.
4. Place tracheas into a 50 mL conical tube containing 30 mL Ham's F12 media containing antibiotics, on ice.
5. In a sterile lamellar flow hood, transfer tracheal tissue to a sterile 100 mm Petri dish containing 10 mL Ham's F12 media containing antibiotics.
6. Gently dissect connective tissue with sterile forceps and surgical scissors.

7. Transfer tracheal tissue to a new 100 mm Petri dish containing 10 mL Ham's F12 media containing antibiotics to rinse. Cut tracheas along vertical axis to expose lumen.
8. Transfer tracheas to a 50 mL tube containing 10 mL 0.15% Pronase solution and incubate overnight at 4°C.
9. On the second day, have ready:
    - Prepare a DNAse I solution. To 18 mL of Ham's F12 Media containing antibiotics add 2 mL of a 10 mg/mL Bovine Serum Albumin (BSA) stock solution, and 10 mg of crude pancreatic DNAse I. Make 1 mL aliquots and store at -20°C (thawed on ice before use).
    - Prepare Ham's F12 media containing antibiotics with 20% fetal bovine serum (FBS). To 200 mL Ham's F12 basal media (Invitrogen) add 50 mL heat inactivated FBS, 2.5 mL of a 100 X Penicillin/Streptomycin (10^2 U/mL-10^2 mg/mL) solution, and 250 µL of a 1000 X Fungizone solution.
    - Prepare MTEC Basic Medium containing antibiotics. To 475.5 mL DMEM/F12 basic media (Cellgro) add 7.5 mL 1 M HEPES solution, 10 mL of 200 mM glutamine solution, 2 mL of a 7.5% $NaHCO_3$ solution, 5 mL of a 100 X penicillin/streptomycin solution, 500 µL of a 1000 X Fungizone solution
    - Prepare MTEC medium/10% FBS. To 45 mL of MTEC basic medium containing antibiotics, add 5 mL heat inactivated FBS.
10. Gently rock the tube (from Step 1.8) 10-12 times, and then let it stand for 30-60 minutes at 4°C.
11. Add 10 mL Ham's F12 media containing 20% FBS and antibiotics to the tube and rock 12 times.
12. Prepare 3 15 mL conical tubes containing 10 mL Ham's F12 media containing 20% FBS and antibiotics.
13. Remove tracheas from Pronase solution, setting aside this solution on ice. Transfer tracheas to first conical tube containing Ham's F12 and invert tube 12 times. Repeat this process two more times.
14. Combine Pronase solution with the three supernatants from step 1.13 into one 50 mL tube. Discard remaining tissue.
15. Centrifuge at 1400rpm (390 x g; Eppendorf 5810R centrifuge equipped with an A-4-62 rotor) for 10 min at 4°C, and discard supernatant.
16. Gently resuspend the pellet in 1 mL DNAse solution (100-200 µL/trachea) and incubate 5 min on ice.
17. Centrifuge at 1400rpm (390 x g) for 5 min at 4°C, and discard supernatant.
18. Resuspend the cell pellet in 8 mL MTEC medium containing 10% FBS
19. Plate cell suspension on Primaria Plates (Falcon). Incubate at 37°C in an atmosphere of 95% air, 5% CO_2 for 5 hrs (Note: this is a negative selection step for fibroblasts).
20. Collect cell suspension from plates and rinse plates twice with 4 mL MTEC containing 10% FBS. Pool cell suspension and washes together in a 50 mL conical tube.
21. Conserve 1 mL for cytospin and cell counting. Spin in a tabletop centrifuge for 5 min at 5,000 rpm (Eppendorf 5415D). Remove 500 µL and resuspend pellet in remaining supernatant. Use 100 µL for cell counting using the Trypan Blue vital staining method. Conserve 4 aliquots of 100 µL for cytospin analysis
22. Spin remaining 15 mL cell suspension at 1400rpm (390 x g), at 4°C for 10 min.

2. Propagation and Differentiation of MTEC At Air-Liquid Interface

Prepare retinoic acid stock solutions. Stock solution A: Weigh Retinoic acid in the dark (Mr 300.44 g/mol) to make a 5 mM stock solution in 95% EtOH. Store in a foil wrapped tube at -80°C. As needed, prepare Stock solution B (5 µM) by adding 50 µL Stock A, 500 µL BSA solution (100 mg/mL) and 49.5 mL Hank's balanced salt solution (HBSS). Store in a foil wrapped tube at -80°C for up to 4 weeks.

Prepare MTEC proliferation medium containing retinoic acid. To 45.7 mL MTEC basal media containing antibiotics, add 2.5 mL heat-inactivated FBS, 1 mL Retinoic acid Stock B, 250 µL Insulin solution (2 mg/mL insulin in 4 mM HCl), 250 µL Epidermal growth factor solution (5 µg/mL EGF in HBS containing 1 mg/mL BSA), 200 µL bovine pituitary extract (15 mg/mL in HBS containing 1 mg/mL BSA), 50 µL Transferrin solution (5 mg/mL transferrin in HBS containing 1 mg/mL BSA), 50 µL cholera toxin solution (100 mg/mL in HBS containing 1 mg/mL BSA). Filter sterilize final media preparation and use within 2 days of preparation.

1. Remove collagen solution from transwell plates, let stand under hood for 5 min, and wash with PBS 2 times.
2. Following centrifugation, resuspend the cell pellet in an appropriate volume of proliferation media (500 µL) to facilitate plating of 7.5 x 10^4-1.0 x10^5 cells per well. Pipette 500 µL cell suspension onto the apical surface of the transwell polycarbonate membrane insert. Add 1.5 mL of proliferation media to the basal compartment of the transwell.
3. Incubate the submerged MTEC cultures at 37°C in a humidified incubator containing 95% air, 5% CO_2 for 7-10 days.
4. Wait three days before changing media the first time. Change media every other day. Monitor cultures by visual inspection and measurement of transepithelial cell resistance using an electrode (EVOM Ohmvoltometer, World Precision Instruments, Sarasota, FL).
5. When cells appear confluent and epithelial resistance reaches 1000 Ω/cm^2, the cells are ready to differentiate at ALI.
6. Prepare MTEC basal media containing 2% NuSerum. Add NuSerum to MTEC basic medium to a final concentration of 2% V/V. Add retinoic acid Stock B to a final concentration of 1 X 10^{-7} M before using. Prepare fresh and use within 2 days.
7. Allow cell to differentiate for 10-14 days, by removing apical media and replacing the basal media with 750 µL MTEC basal media containing 2% Nuserum and retinoic acid. Change the basal media and wash the apical side with MTEC containing 2% NuSerum every other day.

3. Application of Cigarette Smoke to Cultured Epithelial Cells

1. Expose the apical side of the transwell cultures to mainstream cigarette smoke using a CS cell exposure system (EMI Instruments, Pittsburgh, PA). See Figure 1 for photograph of the apparatus. See Table of specific reagents and equipment for technical specifications. In brief, the apparatus consists of a peristaltic pump which smokes each cigarette in ~7-8 puffs, drawing in the mainstream smoke into a polypropylene line that is connected to a vented exposure chamber. The exposure chamber is maintained at 37°C by circulating water, and maintained at an atmosphere of 5% CO_2 on a gas line. The cells can be exposed to the smoke using Kentucky 3R4F research reference filter cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY). Typically cells are exposed to the smoke from 1-2 cigarettes, yielding 100-200 mg/m^3 of total particulate matter (TPM). Control cultures are exposed to room air for an equivalent exposure period.

2. The TPM is measured according to the manufacturer's protocol supplied with the TE-10c smoking machine (Teague Enterprises). In brief, a filter (Pallflex) is placed in an inline stainless steel holder on a sampling tube leading from the sampling port on the smoking chamber to a constant flow pump (sampling unit). An outflow tube connects the sampling unit to a gas meter (Dry Gas Meter, ONM61,67, AEM). The filter is weighed before and after gas sampling. The total material deposited on the filter in mg is normalized for the total volume of air sampled.
3. The cells can then be harvested at any time point (i.e., 0-24 hrs) post exposure for standard cell analytical procedures (i.e., Western immunoblot analysis, mRNA analysis, etc.) or microscopy (i.e., confocal or electron microscopy). The culture media can also be analyzed for cytokines or LDH release for cytotoxicity assays.

4. Representative Results

Successful epithelial isolation, proliferation, and differentiation at ALI should yield an intact monolayer with a cobblestone morphology. Transepithelilal cell resistance of healthy cultures should be approximately 1000-2000 Ω/cm^2. Figure 2 depicts a monolayer of mouse respiratory epithelial cells stained for epithelial cell and cilia markers under control conditions and after cigarette smoke exposure. Figure 3 shows representative electron micrographs of healthy MTEC cultures, depicting ultrastructure and cilia morphology.

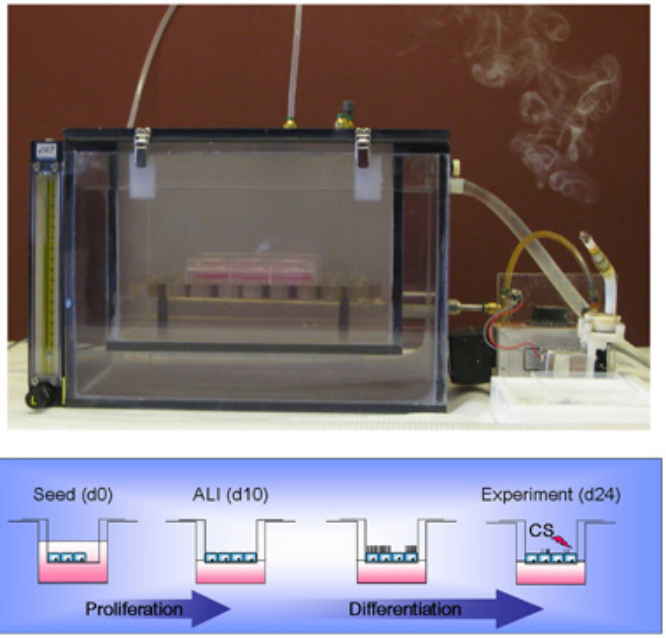

Figure 1. Preparation of epithelial cells proceeds through three phases, an isolation step, a propagation stage, and a differentiation stage at air-liquid interface (scheme). Epithelial cells are isolated from mouse trachea, seeded onto transwells where they proliferate in submerged culture, and then converted into air-liquid interface where they fully differentiate. Experiments are typically conducted on the 24th day of continuous culture. The picture depicts a model system for exposure of cultured cells to mainstream cigarette smoke. A transwell ALI culture system is placed inside a custom modular cigarette smoke delivery system which is controlled for temperature, humidity and CO_2.

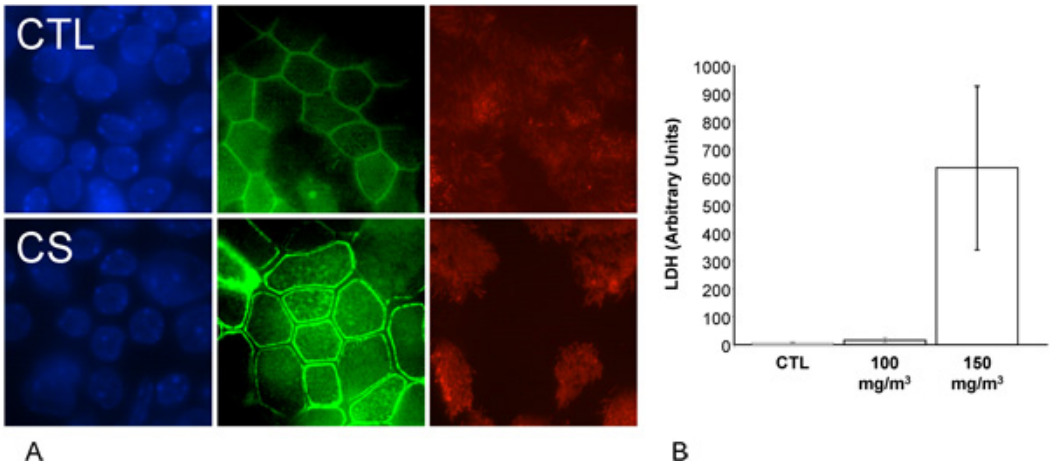

Figure 2. Epithelial cell cultures were fixed and stained for nuclei (Hoechst 33258), F-actin (green; Alexa-Fluor 488-conjugated Phalloidin) and the cilia marker acetylated α-tubulin (red; Cy3-conjugated secondary antibody). The lower panels show equivalent images of epithelial cells taken 4 hours after exposure to cigarette smoke (2 cigarettes, 150 mg/m^3). (B) Lactate dehydrogenase (LDH) release was measured in the basal medium 24 hours after exposure to varying doses of cigarette smoke as indicated.

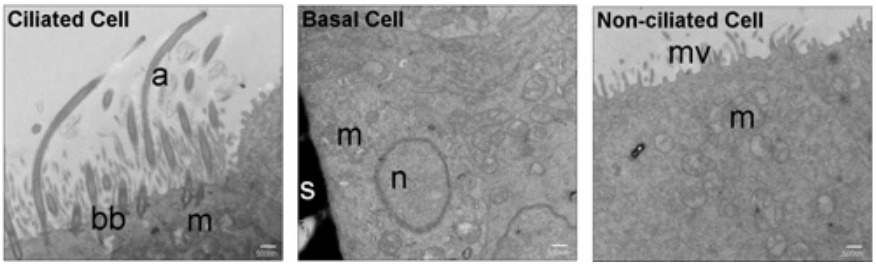

Figure 3. Air-liquid interface cultures of epithelial cells isolated from mouse tracheal epithelium differentiate in several subtypes that by transmission electron microscopy (TEM) have the characteristics of ciliated, basal, and non-ciliated cells [1]. These cells typify pseudostratified respiratory epithelium. Figure labels correspond to: bb: basal body, a: axoneme, m: mitochondria, n: nucleus, s: substrate (polycarbonate membrane), mv: microvili

Discussion

The protocol describing isolation of mouse tracheal epithelial cells is adapted from the protocols of You *et al.*, [1], and others [2-3] with modifications. As with any protocol describing cell isolations, the most critical aspect is to avoid contamination from bacterial or fungal pathogens by using strict aseptic techniques. A second critical step is to avoid fibroblast contamination of the cultures, which can be avoided by careful dissection of the tracheas, and negative selection as described in Step 1.19. Provided that the cultures are monitored, washed, and the culture media replenished every other day following the initial 72 hour incubation, the cultures should fully differentiate in approximately two weeks from the initiation of ALI.

Chronic obstructive pulmonary disease (COPD), which is largely caused by chronic cigarette smoke exposure, continues to represent a major global health problem [4-7]. COPD, is characterized by progressive airflow limitation, destructive alveolar loss (emphysema), and exaggerated inflammatory responses of the lung to cigarette smoke [4-7]. A large number of studies have used alveolar, bronchial, and airway epithelial cell systems to attempt to model lung cell responses to CS. Many of these studies have been conducted in epithelial cell or transformed lines (i.e., Beas-2b), see Refs [8-11] for examples. The ALI transwell culture system allows the culture of pulmonary epithelial cells in fashion that is closer to their physiological orientation in the airway, than that which can be provided by conventional liquid (submerged) culture [1-3]. Although the featured protocol describes the application of this system to mouse tracheal primary cultures [1], in principle other primary epithelial cells can be applied (i.e., mouse Type II epithelial cells, Human bronchial epithelial cells, etc.). The application of live mainstream CS to cell cultures represents a model that perhaps more closely approximates human exposure to CS than application of aqueous cigarette smoke extract (CSE), which is commonly used in cellular studies of CS exposure [12-15]. CSE represents a soluble fraction of mainstream smoke that lacks many chemical components of whole smoke. While both CS exposure systems have limitations, CSE has the advantage of being more easily calibrated, since a single preparation can be used for multiple experiments, and dosing is achieved by dilution [15-16]. On the other hand, we include here a method for quantifying mainstream smoke delivery based on TPM measurements. The elucidation of molecular and cellular responses to toxin exposure, in particular CS as exemplified in this article, will further the understanding of the impact of air pollution on human health, in particular the etiology of chronic diseases of the lung.

Disclosures

No conflicts of interest declared.

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