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Epithelial Cell Culture Protocols

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Human Lens Epithelial Cell Culture

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1. Introduction

Crystalline lens consists of epithelial cells, fiber cells, and a capsule. They originate from lens epithelial cells. Epithelial cells differentiate into fiber cells and produce collagen, which is the major compound of the capsule. The lens epithelial cells maintain normal physiology and homeostasis of the lens, so the cultures of human lens epithelial (HLE) cells provide important information concerning the role of epithelium in normal and cataract formation.

The difficulty of HLE cell culture is due to limited sources of the cells and a low viability and delicacy of the cells. HLE cells are easily damaged, resulting in the failure of the culture by mechanical injury, contamination, toxicity of reagents, and freezing for storage.

This chapter describes the procedures of HLE cell culture, so that scientists who are unfamiliar with this culture may carry it out successfully. The sources of HLE cells, explant culture, harvest, subculture, storage, and shipment are explained. The critical points for HLE cell culture are also stated in the note section.

2. Materials

1. Dulbecco's modified Eagle medium (DMEM).
2. Fetal bovine serum (FBS), qualified (*see* **Note 2**).
3. Gentamicin reagent solution.
4. Growth medium: DMEM supplemented with FBS is used as a standard medium. As the viability of HLE cells is very low (*see* **Note 3**), neither antibiotic nor antifungal agents should be used for HLE cell culture except cell line cell culture. Gentamicin reagent solution (10 µg/mL) can be used for HLE cell line cell

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culture. The concentration of serum should be at least 5%, and the best growth of HLE cells is observed in medium with 20% FBS ***(1)***. Store the medium at 4°C.

5. Cell dissociation solution: 0.05% trypsin, 0.02% EDTA.
6. Dulbecco's phosphate-buffered saline (PBS–), Ca^{2+}-, Mg^{2+}-free.
7. Dimethylsulfoxide (DMSO).
8. 35-, 60-, 100-, and 150-mm Tissue culture grade petri dishes (all tissue culture plastic is from Falcon).
9. 25- and 75-cm^2 Tissue culture grade flasks.
10. 6-, 12-, and 24-well Tissue culture grade plates.
11. Equipment for freezing: Mr. Frosty (Nalgene).
12. 1.2-mL Cryovials.
13. 0.2-µm membrane filter.
14. Incubator (*see* **Note 1**).
15. HLE cells. There are five possible sources of human lens epithelial cells. As most countries do not allow the use of tissue obtained from fetuses, four alternative sources are available.
    a. HLE cells from infants. The infant HLE cells can be obtained from patients with retinopathy of prematurity or congenital cataracts. The small fragments of the anterior capsule of the lens, where the HLE cells attach, can be collected during pars plana lensectomy. However, as these cases are not common, it is very hard to get HLE cells from infants ***(2)***.
    b. HLE cells from eye bank eyes. It is easiest to get HLE cells from eye bank eyes, if they are available for research purposes (some countries only allow use of eye bank eyes for keratoplasty). Take out the whole lens by cutting the zinn zonule, wash once in DMEM containing 50 µg/mL gentamicin, and dip into the growth medium. Peal off the posterior portion of the lens and remove the cortex and nucleous of the lens. HLE cells attached to the large anterior capsular flap are ready for explant culture.
    c. HLE cells from senile cataract patients. During senile cataract operations, the center part of the anterior capsule with HLE cells can be obtained. The capsular flap can be used for explant culture (usually HLE cells from elder patients over 60 yr do not proliferate when the cells are dissociated from the capsule) ***(3)***.
    d. HLE cell line cells. There are two HLE cell line cells. B3 cells are immortalized by infecting cells with adenovirus 12 containing an immortalizing gene derived from simian virus 40 (SV40) ***(4)***. The other cell line, SRA 01/04, is immortalized by transfecting cells with a plasmid vector containing a large T antigen (the immortalizing gene) of SV40 ***(5)***. Both cell lines have characteristics of human origin, normal epithelial cell morphology, and normal expression of lens epithelial cell specific proteins, α- and/or β-crystallin. These cell lines have a high proliferative potency, and thus a large number of cells are readily available to undertake a wide range of lens studies. When HLE cell lines are not commercially available, they are provided by each investigator.

3. Methods

3.1. Explant Culture

1. Culture HLE cells. If HLE cells attached to the anterior capsule of the lens are dissociated, the cells will not attach well to the culture vessels, because the cell number is very low, approx 1×10^5 per one large anterior flap, and the cell concentration does not reach a level at which the cells can survive.
2. Put the anterior capsular flap, with HLE cells on, onto a 60-mm culture petri dish and add growth medium to just cover the anterior capsular flap (*see* **Note 7**).
3. Put the petri dish in the incubator. After 1 d, increase the volume of growth media to 1 mL and incubate for a further 2 to 3 d. During this time, it is not necessary to change the medium (*see* **Note 4**).
4. After 2 or 3 d of culture, an outgrowth of HLE cells should be observed around the anterior capsule.
5. To obtain the maximum number of cells from the anterior capsular flap, the following procedure is followed (*see* **Note 5**).
6. Remove the growth medium from the culture.
7. Wash the culture with PBS– once.
8. Add 0.5 mL of trypsin-EDTA solution to the dish (*see* **Note 6**).
9. After a 5-min incubation in the incubator, shake the dish gently to dissociate the cells from the culture vessel.
10. Add growth medium to the dish without removing the trypsin-EDTA solution and put the dish back into the incubator (*see* **Note 7**).
11. Change the growth medium every other day. After 4 d in culture, HLE cells from infants or eye bank eyes of young donors will proliferate and cover the whole surface of the dish (confluence).

3.2. Harvesting

Once the explant culture is confluent, harvest the HLE cells for the next step, which is either subculture or cell storage.

1. Remove the growth medium from the culture.
2. Wash the cells with 1 mL of PBS–
3. Incubate the culture at 37°C with 1 mL of trypsin-EDTA solution for 5 min. Almost all the cells should be dissociated from the culture vessel.
4. To remove all the cells, pipet up and down gently 3 to 5 times.
5. Transfer these cells to a centrifuge tube.
6. Stop the action of the trypsin-EDTA by adding 3 mL of growth medium.
7. Pipet up and down gently 2 to 3 times.
8. Count the cells in a hemocytometer and dilute with growth medium to an appropriate number of cells/mL, as determined by trypan blue exclusion. This cell suspension can be used for subculture or cell storage.

3.3. Subculture (see Note 8)

HLE cells (7000 ± 500 cells/cm^2) should be used for the initial seeding.

1. Centrifuge the cell suspension from the harvest at 1000*g* for 5 min.
2. Discard the supernatant and resuspend in growth medium.
3. Seed the cells out and incubate (*see* **Note 7**).
4. Change medium every other day.
5. When the culture becomes confluent, repeat the harvest, and subculture.

3.4. Storage (see Note 9)

The appropriate cell number for storage is 1×10^6 cells per mL.

1. Centrifuge the cell suspension from the harvest at 1000*g* for 5 min.
2. Discard the supernatant and resuspend in 1 mL of FBS containing 5% DMSO.
3. Transfer the suspension to a cryovial and put the vial into the freezing container (Mr. Frosty).
4. Store this at –70°C for 8 h. The temperature of the freezing container decreases 1°C/min automatically, when it is in the freezer.
5. Transfer the vial quickly to liquid nitrogen for storage in the liquid phase (–196°C).
6. To thaw the cells, warm the cryotube rapidly in a waterbath at 37°C to avoid cell damage by ice crystals.

3.5. Shipment

Shipment of frozen vials and monolayer cell cultures is possible. Frozen vials of cell cultures may be shipped in the package with dry ice. They do, however, require special handling. After delivery the vials must be placed in fresh dry ice or liquid nitrogen until they are thawed. If stored in a refrigerator (–4°C) or regular freezer (–20°C), the cells will be damaged.

The procedure for shipment of monolayer culture is as follows.

1. Subculture HLE cells onto a 25-cm^2 culture flask with a plug seal cap.
2. When the culture reaches confluency, remove the medium, and fill the flask completely with fresh growth medium.
3. Tape the screw cap in place and ship by mail at room temperature.
4. After delivery, the flask should be placed in the incubator overnight to permit recovery from trauma and shaking that may have dissociated some of the cells during the shipment.
5. The next day, open the flask and change the medium, or harvest and subculture the cells.

4. Notes

1. A CO_2 incubator is used for HLE cell culture. Five percent CO_2 and 100% humidity at 37°C are required. As antibiotic and antifungal reagents are not usually added to the medium, the culture is easily contaminated if the incubator is poorly

maintained. To avoid contamination, use the incubator only for HLE cells and clean it once a month by sterilizing the trays, wiping the inside with ethanol, and changing the water.

2. Cell attachment and proliferation are dependent on the serum condition. Batch testing should be performed before the purchase of the serum. If the serum tested is good, 80% of primary or secondary subculture of HLE cells from infants or eye bank eyes of young donors (below 40 yr) should attach to the culture vessels after a 3-h incubation. Also, FBS is a possible source of contamination. Filter the FBS before adding it to the DMEM.
3. HLE cells have a low proliferative potency and are very delicate. Usually antibiotic or antifungal reagents cannot be used for HLE cells, because they damage the cells. Keep the working area clean and use good aseptic technique.
4. For the first few days, especially on the first day of the explant culture, frequent observation of the culture should be avoided. The anterior capsular flap detaches easily from the surface of the culture dish if moved, and if the flap detaches, the cells cannot attach and outgrow on the dish.
5. Once the HLE cells outgrow on the dish in explant culture, the cells have the ability to attach and proliferate. Cell dissociation during explant culture, which is for loosening the contact inhibition of the cells around the capsular flap, is useful to get a large number of HLE cells.
6. Do not expose the cells to trypsin-EDTA solution for too long. This cell dissociation solution also damages the cells.
7. For at least 3 h after seeding HLE cells, do not move the culture vessel. The cells attach to the surface of the vessel during this 3-hour incubation.
8. The proliferative potency of HLE cells depends on their donor age. HLE cells from senile cataract patients, who are mostly aged around 60 yr, can be cultured with the anterior capsular flap (explant culture) and not be subcultured. HLE cells from donors aged between 20 and 50 yr can be subcultured once; under 20 yr, they can be passaged twice. Although HLE cells from infants can be subcultured through several passages, their proliferative potency is limited, and cytomegalic cells and cell degeneration in a long-term culture are observed.
9. During freezing and storage, damage of HLE cells is caused by mechanical injury by ice crystals, dehydration, pH changes, denaturation of proteins and other factors. These lethal effects can be minimized by: (*i*) adding DMSO, which lowers the freezing point; (*ii*) a slow cooling, which lets water move out of the cells before it freezes; (*iii*) storage in the liquid nitrogen (–196°C), which inhibits the growth of ice crystals; and (*iv*) rapid warming at the time of recovery, so that the frozen cells can pass rapidly through the temperature zone between –50° and 0°C, in which most cell damage is believed to occur.

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Human Airway Epithelial Cell Culture

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1. Introduction

Development of methods to culture airway epithelial cells has been needed to carry out research into various lung diseases, such as cancer, cystic fibrosis, and bronchial asthma. However, the culture of airway epithelial cells remained difficult. We have improved the culture conditions of these cells, so that these cells can now be used to better understand the mechanisms underlying cystic fibrosis ***(1–4)***, for characterizing viral infections ***(5–7)***, and for advancing our knowledge of airway inflammation.

In order to improve the conditions under which cultured human tracheal epithelial cells can retain their ion transport properties and ultrastructure of the original tissue, we have developed the following protocol. Briefly, human tracheal epithelial cells are isolated by digestion with protease overnight ***(1,2,8,9)***. The isolated epithelial cells are plated on vitrogen gel-coated porous-bottomed inserts in media containing Ultroser G serum substitute (USG). Cells are grown with an air interface (i.e., no medium added to the mucosal surface). These culture conditions, the vitrogen gel, USG-supplemented medium, and the air interface, lead to the appearance of cilia, an increase in the depth of the cell sheets (50 μm), longer and more frequent apical microvilli, and increased interdigitations of the basolateral membrane (**Fig. 1**). Protein and DNA content are also significantly increased. Secretory granules are present, which stain with antibody to goblet cells, but serous or mucous gland cells are not seen (**Fig. 2**) ***(1)***.

Acini of human tracheal submucosal glands are isolated by digestion with various enzymes ***(5–7,10)***. The isolated gland acini are incubated in flasks coated with human placental collagen in media containing USG and a variety of growth factors. The attached gland acini make confluent cell sheets after

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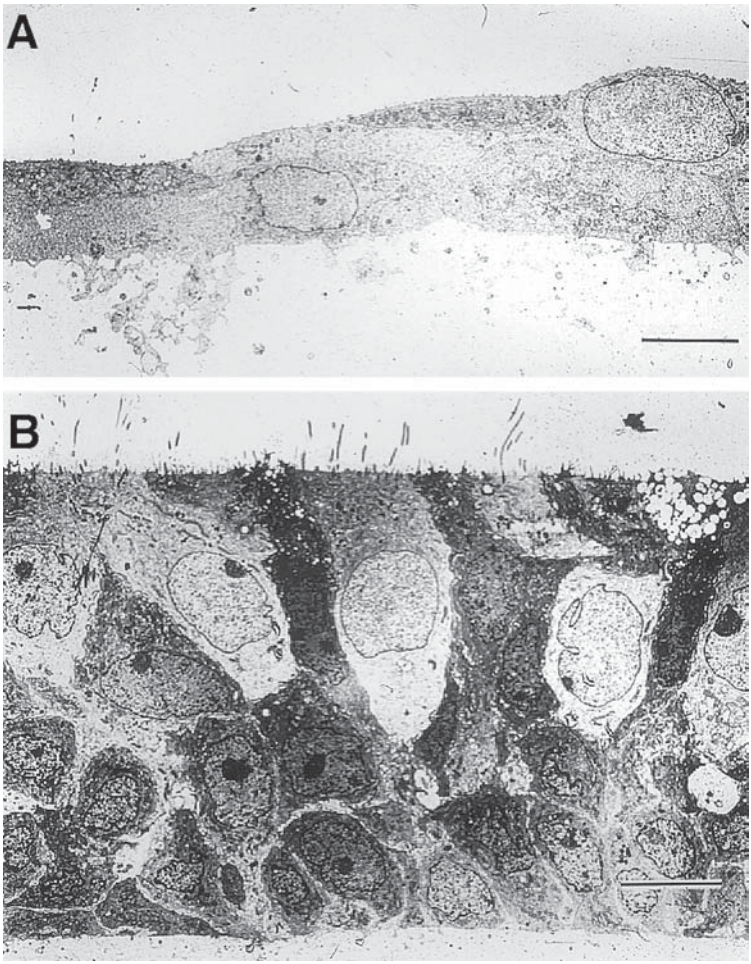

Fig. 1. Low power electron micrographs of cultured human tracheal epithelial cells. **(A)** human placental collagen, FCS-medium, immersed feeding. **(B)** vitrogen gel, USG medium, air interface feeding. Cells are multilayered, and the luminal surface contains cilia and secretory granules. Scale bars = 10 μm.

14–21 d ***(5–7,10)***. The cells are then isolated by trypsinization and replated in media containing USG and growth factors on porous-bottomed inserts coated with human placental collagen and grown with an air interface ***(5–7)***. Cells cultured under these conditions have high transepithelial electrical resistance and high short-circuit current. The human tracheal epithelial cells and gland cells can secrete chloride ions in response to bradykinin, α- and β-adrenergic and cholinergic agents, and ATP.

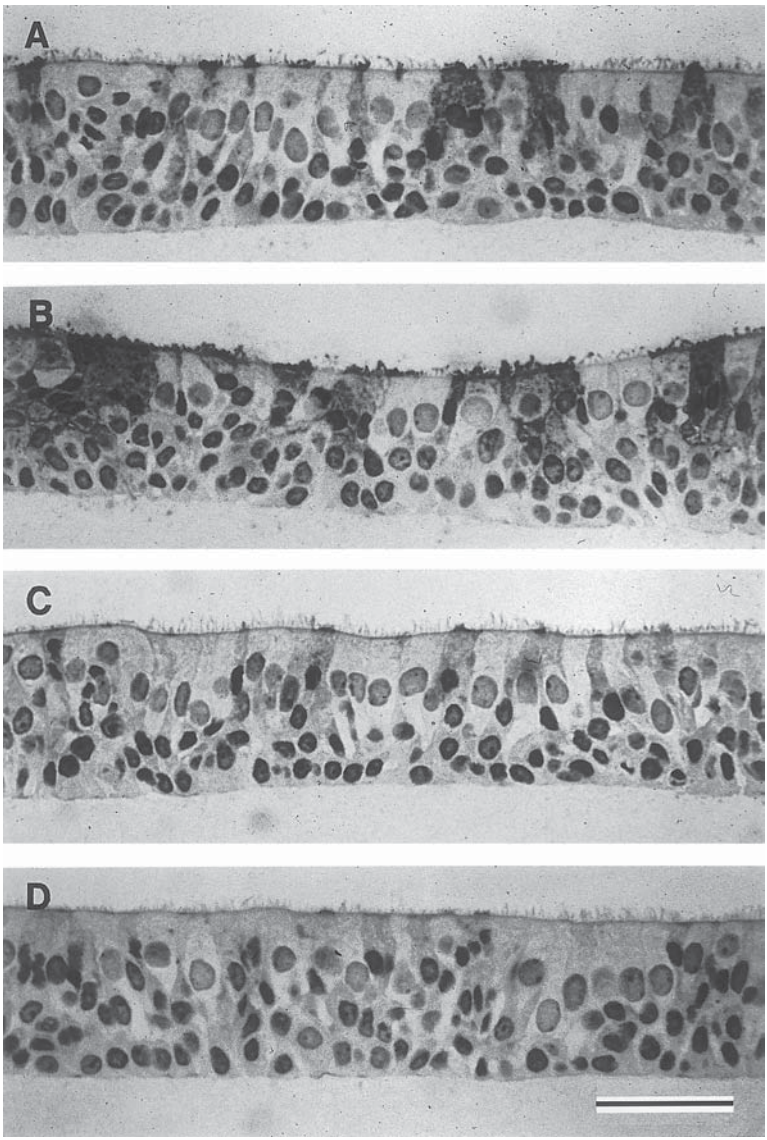

Fig. 2. Expression of goblet cell antigens by cultured human tracheal epithelial cells. Glycomethacrylate secretions were incubated with monoclonal antibodies and stained using an avidin-biotin-peroxidase procedure. **(A)** antibody A3G11 and **(B)** antibody B6E8. Both antibodies recognize tracheobronchial goblet epithelial and mucous gland cell antigens and stain cells throughout the cultured tracheal epithelial cells. **(C)** antibody A8E4. This antibody recognizes a tracheobronchial mucous gland cell antigen. Staining is absent. **(D)** antibody B1D8. This antibody recognizes a tracheobronchial serous gland cell antigen. Staining is absent. Scale bar = 50 μm.

Human tracheal epithelial cells and submucosal gland cells can be cultured in glass tubes, coverslips, slide glasses, and culture dishes as well as filter membranes. Cells cultured under these conditions can be used for studies on ion transport, intracellular calcium concentration, epithelial permeability, repair of epithelial cells after injury, and production of various enzymes and proteins, such as cytokines and intercellular adhesion molecules ***(1–10)***.

2. Materials

2.1. Coating of Culture Vessels

2.1.1. Vitrogen Gels

1. Minimal essential media (MEM) (GIBCO BRL Life Technologies).
2. 0.1 mol/L Sodium hydroxide (NaOH).
3. Vitrogen solution (Collagen).
4. Millicell-CM or Millicell-HM inserts (Millipore): 0.45 μm pore size, 0.6 cm^2 area.

2.1.2. Collagen

1. Human placental collagen (Sigma).
2. 0.2% Glacial acetic acid in double-distilled water.
3. 12-well Tissue culture plates (Falcon).
4. Millipore-CM inserts with 0.45 μm pore size, 0.6 cm^2 area.
5. Transwell inserts (Corning Costar): 0.4 μm pore size.
6. Phosphate-buffered saline (PBS) (GIBCO BRL Life Technologies) supplemented with 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B (all from Sigma).

2.2. Human Tracheal Epithelial Cell Culture

1. Dissection kit.
2. Dissection tray.
3. PBS.
4. 5 mol/L Dithiothreitol (DTT) (Sigma) in PBS.
5. Protease solution: 0.4 mg/mL protease Sigma type XIV (Sigma), 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B in PBS.

   Dissolve 20 mg protease in 40 mL PBS, which already contains the penicillin, streptomycin, and gentamicin, in a 50-mL tube. Shake by hand until dissolved. Sterilize by passing through a 0.45-μm filter and then add the amphotericin B.
6. Fetal calf serum (FCS) (GIBCO BRL Life Technologies).
7. F-12-DMEM-FCS Mix I: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL Life Technologies) mixed 1:1 with Ham's F-12 medium (GIBCO BRL Life Technologies) and supplemented with 5% FCS.
8. 0.4% Trypan blue (Sigma).

9. Ultroser G serum substitute (USG) medium: 1:1 DMEM:Ham's F12 supplemented with 2% USG (BioSepra), 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B.

   Dissolve the USG in distilled water to make a stock solution according to the manufacturer's instructions. Mix 10 mL of the USG stock solution with 480 mL DMEM:Ham's F-12, to give a final concentration of 2% USG. The medium should then be supplemented with the antibiotics ***(1)***.
10. Vitrogen gel-coated Millicell inserts.
11. Collagen-coated Transwell inserts.
12. Millipore-CM inserts with 0.45 µm pore size, 0.6 cm^2 area.
13. Hemocytometer.
14. 50-mL Conical centrifuge tubes (Corning Costar).
15. T_{25} Tissue culture flasks (Corning Costar).
16. Glass tubes with round bottoms, 15 mm in diameter, 105 mm long (Iwaki Glass), coated with human placental collagen. To coat the tubes, add 1 mL of collagen working stock solution to the tubes. Keep the tubes stationary at a slant of 5° and incubate for at least 2 h. Remove the collagen solution and air-dry the tubes.
17. Roller culture incubator (HDR-6-T; Hirasawa, Tokyo, Japan).

2.3. Human Tracheal Submucosal Gland Culture

1. PBS.
2. Enzyme Solution: Hanks' buffered salt solution (HBSS) (GIBCO BRL Life Technologies) supplemented with 20 m*M* HEPES buffer, pH 7.4 (Sigma), 500 U/mL collagenase type IV (Sigma), 6 U/mL pancreatic porcine elastase (Sigma), 200 U/mL hyaluronidase (Sigma), 10 U/mL deoxyribonuclease (Sigma).

   Dissolve collagenase, pancreatic porcine elastase, hyaluronidase and deoxyribonuclease in 50 mL HBSS, containing 20 m*M* HEPES , penicillin, streptomycin and gentamicin. Sterilize by passing through a 0.45-µm filter and then add the amphotericin B ***(5)***.
3. F-12-DMEM-FCS Mix II: 40% Ham's F12, 40% DMEM, 20% FCS.
4. Growth medium: 1:1 DMEM:Ham's F12 supplemented with 0.1% USG, 10 µg/mL insulin (Becton Dickinson), 5 µg/mL transferrin (Becton Dickinson), 20 ng/mL triiodothyronine (Becton Dickinson), 0.36 µg/mL hydrocortisone (water soluble) (Sigma), 7.5 µg/mL endothelial cell growth supplement (Becton Dickinson), 25 ng/mL epidermal growth factor (Becton Dickinson), 0.1 mol/L retinoic acid (Sigma), 20 ng/mL cholera toxin (Sigma), 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B.

   Stock solutions of growth factors:
   a. Insulin: 20 mg in 4 mL distilled water.
   b. Transferrin: 10 mg in 4 mL distilled water.
   c. Triiodothyronine: 20 mg in 10 mL distilled water.
   d. Hydrocortisone: 10 mg in 10 mL distilled water.
   e. Endothelial cell growth supplement: 15 mg in 4 mL distilled water.

f. Epidermal growth factor: 100 μg in 10 mL distilled water.
g. Retinoic acid: Dissolve in 100% ethanol to give a stock solution of 1 m*M*. Dilute to 10 μ*M* in distilled water.
h. Cholera toxin: 0.5 mg in 5 mL distilled water.

To make up growth medium (500 mL): mix 484 mL 1:1 DMEM:Ham's F12 (242 mL of each), 1 mL insulin stock solution, 1 mL distilled water containing 5 μL triiodothyronine stock solution, 1 mL distilled water containing 35 μL hydrocortisone stock solution, 1.25 mL epidermal growth factor stock solution, 5 mL 10 μ*M* retinoic acid. Sterilize by passing the solution through a 0.45-μm filter. Then add 1 mL transferrin stock solution, 1 mL endothelial cell growth supplement stock solution. Add 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B, and 0.5 mL USG stock solution.

Because retinoic acid is photosensitive, the growth medium and stock solution of retinoic acid should be made up in the dark, as much as possible. Wrap the bottle of growth factor medium in foil and store at 4°C.

5. 0.25% trypsin-EDTA solution (Sigma).

3. Methods

3.1. Coating of Culture Vessels

3.1.1. Vitrogen Gels

1. Mix 10% 10X MEM with 10% 0.1 *M* NaOH and 80% vitrogen solution, at 4°C (v/v/v).
2. The solution will be yellow.
3. Add 0.1 *M* NaOH until the color changes to red.
4. Add 0.15 mL/cm^2 of this solution to the Millicell inserts.
5. Place these at 37°C for 1 h.
6. Use within 2 h of manufacture.

3.1.2. Collagen

1. Make a stock solution of human placental collagen by dissolving 50 mg collagen in 100 mL 0.2% glacial acetic acid, using a magnetic stirrer.
2. Sterilize by passing the solution through a 0.45-μm filter.
3. Dilute the stock solution 1:5 with double-distilled water. This will give a working concentration of 20 μg of collagen per cm^2 of surface area, when added to the culture vessels.
4. Coat 35-mm dishes, wells, or coverslips in 6-well plates with 2 mL of working stock solution, 12-well plates with 1 mL/well, Millicell or Transwell inserts with 0.5 mL or glass tubes with 1 mL.
5. Incubate for at least 2 h, or preferably overnight, at room temperature.
6. Remove the collagen solution and allow to air-dry.
7. Prior to use, rinse dishes, plates or inserts with PBS containing antibiotics, and allow to dry.

3.2. Human Tracheal Epithelial Cell Culture

1. Open tracheas for cell culture longitudinally along the anterior surface.
2. Mount in a stretched position, with the epithelium uppermost, in a dissection tray.
3. Score the surface of the epithelium in longitudinal strips.
4. Clamp the end of one of these mucosal strips and pull off the entire length from the submucosa ***(1,11)***.
5. Rinse the tissue strips 4× in 5 m*M* DTT in PBS. The DTT is important to prevent the formation of mucus globs.
6. Rinse the strips twice in PBS alone.
7. Incubate at 4°C overnight in 40 mL of protease solution in a 50 mL conical centrifuge tube.
8. The following day, add FCS to a final concentration of 2.5%, to stop the action of the protease solution.
9. Remove 20 mL of the solution and add the same volume of F-12-DMEM-FCS Mix I.
10. Dislodge the smaller sheets of cells from the epithelial strips by vigorous agitation.
11. Remove the denuded strips.
12. Disperse the remaining sheets of cells by repeated aspiration using a 10-mL pipet.
13. Pellet cells at 200*g* for 10 min.
14. Resuspend the pellet in F-12-DMEM-FCS.
15. Count the cells using a hemocytometer and estimate viability using trypan blue, counting cells with blue-stained nuclei as dead ***(1,11)***.

3.2.1. Preparation of Cells for Further Analyses

1. To measure the electrical properties or enzyme production of epithelial cell sheets or transepithelial permeability, plate cells at 10^6 viable cells/cm^2 onto Millicell-CM or Millicell-HA inserts coated with Vitrogen gel (*see* **Subheading 3.1.**) ***(1,2)***.
2. One day after plating the cells out, replace medium with USG medium.
3. The cells should be grown with an air interface by removal of fluid on the mucosal side.
4. Culture cells at 37°C in 5% CO_2-95% air incubator, and change the media every day for the first 7 d, and then every 2 d thereafter.
5. The epithelial cells will form a confluent cell sheet about 5 d after plating. At this point, the cells can be used for experiments.
6. To measure the intracellular calcium concentrations $[Ca^{2+}]i$ of the human tracheal epithelial cells, plate cells onto collagen-coated membranes and culture as above (*see* **Subheading 3.2.**) ***(2)***.
7. To examine the repair and proliferation of epithelial cells, plate cells onto Millicell-CM inserts at 10^6 viable cells/cm^2, or plate onto 6-well culture dishes or T_{25} flasks at 1 to 2×10^5 viable cells/cm^2, and culture as above ***(3)***.
8. To examine the repair of epithelial cells in T_{25} flasks touch the epithelial cells with a pipet tip to introduce defects in focal contacts.
9. Observe cell growth every day.

10. To examine cell proliferation, culture cells in medium supplemented with [^{3}H] thymidine for 24 h and then measure radioactivity.
11. To examine the effects of virus infection on the production of inflammatory cytokines and intercellular adhesion molecules by the human tracheal epithelial cells, plate cells at 5×10^5 viable cells/mL (2×10^5 cells/cm^2) in glass tubes with round bottoms coated with human placental collagen ***(8,9)***.
12. Seal the glass tubes with rubber plugs, keep stationary at a slant of approximately 5°, and culture at 37°C.
13. When the epithelial cells have formed confluent sheets, infect the cells with rhinovirus, and culture at 33°C in a roller culture incubator.

3.3. Human Tracheal Submucosal Gland Cell Culture

1. Score the tracheal surface epithelium in longitudinal strips and pull away from the submucosa.
2. Dissect the gland-rich submucosal tissue away from the cartilage and the adventitia.
3. Immerse in fresh PBS.
4. Rinse the submucosal tissue 4 times in PBS.
5. Mince with scissors.
6. Centrifuge the tissue fragments at 200*g* for 10 min.
7. Resuspend the fragments in enzyme solution ***(5,6,10)***.
8. Place in a flask on an orbital shake (set to 240 rpm) and leave to disaggregate for 12–16 h at room temperature.
9. Decant the fluid, which should contain the disaggregated tissue.
10. Centrifuge at 200*g* for 10 min.
11. Wash once in a mixture of F-12-DMEM-FCS Mix II.
12. Wash twice in PBS.
13. Resuspend the disaggregated tissue in F-12-DMEM-FCS Mix II.
14. Plate acini out onto two T_{25} tissue culture flasks and incubate for 24 h at 37°C in 5% CO_2-95% air. These are both the attached and the unattached acini.
15. The fragments of submucosal tissue remaining in the trypsinizing flask should be exposed again to enzymatic digestion as above.
16. The cells collected from the second digestion are dispersed gland acini.
17 Combine both the unattached acini from the two T_{25} flasks with the dispersed gland acini.
18. Spin the combined acini from these two sources at 200*g* for 10 min.
19. Resuspend in fresh F-12-DMEM-FCS Mix II and plate in the two T_{25} flasks containing the attached acini from the first plating.
20. The following morning, replace the medium with growth medium ***(5–7,10)***.
21. It takes 14–21 d to achieve confluency, at which point the cells should be collected by trypsinization.
22. To trypsinize, wash twice with PBS and then add 5 mL trypsin.
23. Incubate for 10–20 min or until all the cells have detached.
24. Pellet the cells at 200*g* for 10 min.
25. Resuspended in F-12-DMEM-FCS Mix II.

26. Count cells using a hemocytometer and estimate the viability with trypan blue.

3.3.1. Preparation of Cells for Further Analyses

1. To measure the electrical properties of the human tracheal submucosal gland cells, plate the cells out in F12-DMEM-FCS Mix II at 10^6 cells/cm^2 on Millicell-CM inserts.
2. Cells will appear confluent after 24 h and should then be grown in growth media.
3. Culture the cells for 7–9 d, at which point they can be used in experiments.
4. Cells should be grown with an air interface; so no media is added to the mucosal surface.
5. To measure the intracellular calcium concentrations $[Ca^{2+}]i$ of the human tracheal submucosal gland cells, plate cells onto either collagen-coated Transwell membranes ***(2)*** or collagen-coated coverslips (*see* **Subheading 3.1.**) ***(7)***.
6. Culture the cells for 7–10 d before measurement of $[Ca^{2+}]i$.
7. To examine the effects of virus infection on the production of inflammatory cytokines and intercellular adhesion molecules by the human tracheal submucosal gland cells, plate the cells at 5×10^5 viable cells/mL (2×10^5 cells/cm^2) in glass tubes with round bottoms coated with human placental collagen ***(10)***.
8. When the submucosal gland cells have formed confluent sheets, infect the cells with rhinovirus, and culture at 33°C in a roller culture incubator.

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3

Rat Gastric Mucosal Epithelial Cell Culture

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1. Introduction

The stomach consists of many types of cells, including smooth muscle cells, mesenchymal cells, vessel forming cells, nerve cells, blood cells, including immune cells and gastric gland cells. Gastric epithelial cells can be further subdivided into at least 11 different cell types, ranging from highly differentiated cells to actively proliferating undifferentiated cells ***(1)***. Chief cells are characterized by production and secretion of pepsinogen, parietal cells have a specialized function as acid secreting cells, and neck cells and pit cells (surface mucous cells) are recognized as mucous producing cells. In addition, there are several kinds of endocrine cells producing gastrin, somatostatin, and histamine. These cells are considered to be terminally differentiated. On the other hand, premature forms of these cells such as pre-pit cells and pre-parietal cells also exist in the gastric gland. Interestingly and importantly, all of these different cell types are known to be originated from a single "stem cell".

When researchers try to culture gastric epithelial cells, they are faced with at least two major problems. First, is the problem of the "purity" of the cells. As mentioned above, the stomach or gastric gland consists many different cell types. Therefore, it is difficult to get a highly purified culture, consisting only of a single cell type. Soll et al. ***(2)*** first solved this problem. They made isolated cell suspensions from canine fundic mucosa and fractionated the cells by their size, using a counterflow elutriation technique followed by a Percoll density gradient. Using this method, our knowledge of parietal cells, ECL cells, D cells, G cells, and chief cells has been greatly enhanced. Cell purity obtained by this method is sufficient for certain experiments such as acid output and gastrin or somatostatin secretion.

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However, for more precise experiments such as elucidation of intracellular signal transduction mechanisms, contamination of the culture by other cell types is a critical problem. Kinoshita et al. succeeded in culturing primary pit (surface epithelial) cells based on this method *(3)*. Fortunately, in the case of primary culture of gastric pit cells, they are present in high numbers and attach and grow on a culture plate relatively faster than other types of gastric gland cells *(4)*. The methods we describe here finely utilize these characteristics of gastric pit cells.

Secondly, is the problem of "differentiation". Immediately after gastric cells are separated and inoculated onto plastic plates, they start to de-differentiate and rapidly lose or decrease their terminally differentiated characteristics. Although culture conditions, such as cell density in culture medium, additives in the culture medium, and plate coating materials, can modify the tendency towards de-differentiation, careful observation of cultured cells and close comparison with in vivo models are indispensable.

Here, we introduce two methods of primary culture of rat gastric epithelial cells and two cell lines derived from nontransformed rat or mouse gastric glands.

1.1. Primary Culture of Gastric Epithelial Cells from Newborn Rat Stomach

There have been a number of attempts to establish primary cultures of gastric epithelial cells *(5–7)*, but the methods developed by Terano et al. *(8)* have been most widely adopted. Using this method, over 90% of the cultured cells have the characteristics of epithelial cells. Although previous researchers tried to reduce the number of fibroblasts by 20% by administration of pentagastrin *(7)*, this method using newborn rat stomach is more efficient. Even using this method, however, fibroblast overgrowth can be observed after subculturing on d 4. This overgrowth can be prevented by collagenase treatment or using F-12 medium containing D-valine. The mitotic index is maximum on d 2 (2.0%).

After the success of primary culture of gastric epithelial cells from newborn rat stomach, many attempts were made to obtain a large number of cells from a single procedure from adult rat stomach. Gastric epithelial cell culture systems from adult rat *(9)*, rabbit *(10,11)*, and guinea pig *(12)* were reported. Generally, epithelial cultures from adult stomach contain more fibroblasts than those from newborn stomach. To prevent contamination with a large number of fibroblasts, Matsuoka et al. *(10)* inverted the stomach to expose the mucosa to proteinase E to digest only surface epithelial cells efficiently and then scraped off the mucous layer before enzymatic digestion with collagenase *(12)*.

2. Materials

2.1. Primary Culture of Gastric Epithelial Cells from Newborn Rat Stomach

1. 1- to 2-wk-old Sprague-Dawley rats.
2. Hank's balanced salt solution (HBSS) containing 100 U/mL of penicillin and 100 µg/mL of streptomycin.
3. Enzyme solution: HBSS supplemented with 0.1% collagenase and 0.05% hyaluronidase (both from Sigma).
4. Nylon mesh 200 (Nakarai Tesque, Japan).
5. Growth medium: Coon's modified Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 15 mol/L HEPES, 100 U/mL fibronectin, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL gentamycin.
6. Sodium pentobarbitol: 5 mg/mL stock; use 10 µL/g bodyweight.

2.2. Primary Culture of Gastric Epithelial Cells from Adult Rat Stomach

1. 8-wk-old Wistar rats.
2. Perfusion solution: calcium- and magnesium-free HBSS supplemented with 50 m*M* EDTA.
3. Digestion solution: HBSS supplemented with 0.75% type IV collagenase, 0.1% hyaluronidase.

3. Methods

3.1. Primary Culture of Gastric Epithelial Cells from Newborn Rat Stomach

1. Sacrifice the rats by anethestizing with an ip injection of diluted sodium pentobarbital (5 mg/mL stock; 10 µL/bodyweight g).
2. Resect the stomachs from the rats (*see* **Note 1**).
3. Place in HBSS containing penicillin and streptomycin in a 10-cm plastic plate at room temperature.
4. Excise the fundic area (this is usually recognized as the area with folds) with fine scissors from the stomach.
5. Cut the fundic tissue into strips.
6. Rinse the strips 3 times with HBSS and then mince into 2- to 3-mm^3 pieces (*see* **Note 2**).
7. Place the minced tissue into enzyme solution.
8. Incubate this suspension at 37°C in a shaking water bath for 60 min (*see* **Note 3**)
9. Pipet the tissues up and down several times to complete dispersion of the cells.
10. Incubate for a further 15 min and then pipet again.
11. Filter through nylon mesh.
12. Centrifuge the filtrate, containing cell clumps, at 1000*g* for 5 min.
13. Wash the pellet in growth medium and centrifuge as before.