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Propagation of Rhinovirus – C strains in Human Airway Epithelial Cells Differentiated at Air-Liquid Interface

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Summary

Rhinovirus–C (RV-C) were discovered recently using molecular methods. Classical methods failed to detect them since they could not grow in standard cell culture. The complete genome sequences of several RV-C strains are now available but there is little information about their biological characteristics. HRV-C were first grown in organ culture, and more recently, we developed a system for culturing RV-C strains in differentiated epithelial cells of human airway at air-liquid interface (ALI). These cultures supported efficient replication of RV-C strains as determined by quantitative RT-PCR. This system has enabled study of the biological characteristics of RV-C strains, including quantitative research.

Keywords

Rhinovirus; air-liquid interface; differentiated cultures; human airway epithelium; quantitative RT-PCR

1. Introduction

Rhinoviruses (RV) are the causative agents of common colds and are the most frequent cause of acute asthma exacerbations in both children and adults (1,2). RV strains are phylogenetically classified into three species: A, B and C. RV-A and RV-B have been recognized for many years, and are characterized by cross-neutralization tests into 100 serotypes. RV-C strains were more recently discovered with the use of molecular techniques. They have circulated unrecognized in the population due to inability to culture them in standard cell lines.

Evidence from clinical data is accumulating that RV-C are more virulent than the other species since they cause more acute asthma exacerbation (3) and RV-A and RV-C cause more severe illness in infancy than RV-B (4). Hence it is important to study their biology. RV-C can be grown in organ culture of human sinus epithelium obtained as a byproduct of human sinus surgeries (5). Although this system provided an important tool for RV-C

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research, limited amount of tissue and variability between donor samples made it difficult to standardize for quantitative research. Therefore, we developed a cell culture system to grow RV-C strains that overcame these problems.

Human sinus or bronchial epithelial cells (HSEC/HBEC) grown at an air-liquid interface (ALI) differentiate as early as 21 days in culture to form a pseudo-stratified epithelium with cilia protruding from the apical surface, and containing goblet and mucus producing cells (Fig 1) (Reprinted from Virology, 436, Ashraf, S. et al, Biological characteristics and propagation of of human rhinovirus C in differentiated sinus epithelial cells, 143–149, Copyright 2013, with permission from Elsevier). These cultures support 1–2 logs replication of RV-C (C15 or A16) strains over 24–48 hrs of inoculation (Fig 2)(6). (Reprinted from Virology, 436, Ashraf, S. et al, Biological characteristics and propagation of of human rhinovirus C in differentiated sinus epithelial cells, 143–149, Copyright 2013, with permission from Elsevier).

HSEC/HBEC-ALI cultures have several advantages over the organ culture system. The undifferentiated cells from the tissue snippets can be expanded to form a larger pool of cells, enough for making multiple plates from the same donor. Secondly, the stocks of undifferentiated cells can be cryopreserved and used later on allowing multiple tests done on different days. These cultures yield a uniform number of differentiated cells that consists entirely of epithelial cells thus providing a more uniform substrate for viral replication. Moreover, the ALI replication system generates reproducible and reliable data since the amount of virus replication observed from a given donor with the same inoculating dose is consistent over time. Altogether, this system permits experiments involving quantitative studies of RV-C replication, pathogenic mechanisms, host-virus interactions and testing of anti-viral drugs.

2. Materials

2.1. Reagents

Penicillin/streptomycin (10,000 U pen/10 mg strep)
Human placental collagen, Type VI (5% solution) (Sigma)
BEBM™ (bronchial epithelial cells growth medium) (Lonza)
SingleQuots™ supplements and growth factors (Lonza)
Insulin (100 units/ml).

2.2. Media

MEM (Ca²⁺ and Mg²⁺ free)
DMEM/F12 (with L-glutamine and 15 mM HEPES).
DMEM (Dulbecco's modification of Eagle's medium with 4.5 g/L glucose & L-glutamine without sodium pyruvate).
1. Complete media. Add 2.5 ml of fetal calf serum, 0.5 ml of 100X MEM non-essential amino acids, 6 units of insulin (60 µL of 100 U/mL stock) to 47 ml of

DMEM-F12 with pen/strep (5 ml per 500 ml bottle of media). Keep refrigerated at 4°C.

2. Retinoic acid. Prepare 25 mg/ml stock solution in DMSO. Make a 1000 X dilution of the stock using PBS (with $\text{Ca}^{2+}/\text{Mg}^{2+}$) and aliquot 0.5 ml and freeze at -80°C .
3. ALI medium (BEGM/DMEM). Make complete BEGM by adding SingleQuots™ to bottles of BEBM and DMEM. Mix these two solutions in a 1:1 ratio. Keep at 4°C. Add μl retinoic acid per 10 ml medium (final RA concentration = 50 nM, see Note 1) right before use.
4. Pronase/DNase enzyme Solution. Add 56 mg Pronase and 4 mg DNase in a small beaker and then add 40 ml MEM (Ca^{2+} and Mg^{2+} free). Cover the beaker with parafilm and stir with magnetic stir bar until dissolved. Solution should be gassed with 5% CO_2 /95% O_2 for three min until the solution turns bright red color. Sterilize by passing through a $0.2\mu\text{m}$ filter and add 400 μl pen/strep. Cap tightly and keep at 4°C till used (see Note 2).
5. Human placental collagen solution. Dissolve 10 mg of human placental collagen in 20 ml sterile water with 40 μl glacial acetic acid. Cover the beaker with parafilm and stir until collagen is dissolved. Dilute collagen solution 1:10 with sterile H_2O (20 ml in 180 ml), filter sterilize ($0.2\mu\text{m}$ filter) and store at 4°C (see Note 3).

2.3. Supplies

Transwell-Clear 12-well plates with inserts (12mm diameter, $0.4\mu\text{m}$ pore size, sterile polyester membrane)

12-well tissue culture plates.

75 cm^2 Cell Bind® flasks.

Filter units ($0.2\mu\text{m}$)

50 mL conical tubes

Scalpels

Forceps (sterilized)

Tissue culture dishes ($100 \times 20 \text{ mm}$)

Shaker and stir bar

Serological pipets (sterile)

Pasteur pipets (sterile)

Hemocytometer

Trypan blue solution

¹Retinoic acid is very sensitive to UV light, air, and heat, so work quickly in tissue culture hood in low light to limit its oxidation and keep aliquots on ice while working.

²Pronase/DNase solution should be prepared fresh each time right before use.

³Collagen coated inserts can be stored at 4°C.

Micropipets and tips

Cryovials (2ml)

Gloves

3. Methods

Carry out all procedures using aseptic technique in a tissue culture hood.

3.1. Organ Specimen Preparation

1. Aspirate the storage media from the tube containing the tissue. Place the airway tissue specimen in 30 ml cold, sterile PBS in a 50 ml conical tube to rinse away red blood cells, mucus, and debris. Aspirate PBS with the help of a sterile Pasteur pipette. Wash the specimen three times (see Note 4).
2. Place the tissue into a sterile tissue culture dish and observe under the microscope to check the epithelium and ciliary beating. Trim away cartilage or undesired tissue using a disposable sterile scalpel and loosen the blood clots as needed (see Note 5).
3. Wash gently with PBS again.

3.2. Digestion with Pronase/DNase

1. Cut the tissue specimen into small 4×4 mm size pieces and carefully place the pieces in a new tube with freshly prepared Pronase/DNase solution. Incubate these pieces at 4°C from 24–96 hrs with occasional shaking to dissociate the cells (see Note 6).
2. After incubation, add fetal calf serum to a final concentration of 10% to the tube containing the tissue specimen and shake it vigorously to dislodge cells. Let the big chunks settle down and remove the cell suspension carefully with a Pasteur pipet without disturbing the larger chunks and place in a new 50 ml tube.
3. Rinse the remaining tissue chunks with 10 ml DMEM/F12 to collect additional cells and pool them with the other cells. Centrifuge pooled cells for 10 min at 200 × g at 4°C.
4. Aspirate off medium and resuspend cell pellet in 10 ml of complete media. Transfer cells to an uncoated tissue culture plate and incubate at 37°C, 5% CO₂ for 1–6 hrs to remove fibroblasts.
5. After incubation, carefully collect unattached cell suspension. Rinse off the plate gently with some DMEM/F12 and pool together in a 50 ml tube. Centrifuge for 10 min at 200 × g at 4°C.

⁴Organ specimens should be freshly harvested and have viable ciliary beating. All specimens should be free from respiratory viruses and we recommend screening using a multiplex PCR for all common viruses.

⁵Tissue specimens can be placed at 4°C for up to 30 min to loosen blood clots if necessary.

⁶For our purposes the optimal time for dissociation is 48 hrs as cell viability decreases with longer incubations. If the tissue sample is larger than normal size, the amount of Pronase/DNase solution should be increased.

6. Aspirate off medium, resuspend cells in 10 ml DMEM/F12 and count the viable cells using trypan blue on a hemacytometer.
7. Centrifuge cells again, aspirate medium and resuspend in BEGM at the desired concentration for plating or freezing (see Note 7).

3.3. Freezing cells

1. Re-suspend cells in BEGM at a concentration of 500,000 cells per ml (see Note 8).
2. Add 1ml cell suspension, 0.6ml fetal calf serum and 0.18ml DMSO to each cryovial and freeze them at -80°C overnight and transfer to a liquid nitrogen tank until needed.

3.4. Thawing Cells

1. Quickly thaw cells by transferring a frozen vial from -80°C to a water bath at 37°C . Aseptically transfer cells to a 50 ml tube. Add 10ml of warmed DMEM/F12 medium drop-wise to the cells with a Pasteur pipette while gently agitating the tube.
2. Centrifuge tube for 10 min at $200 \times g$ at 4°C .
3. Carefully remove supernatant and resuspend the cell pellet in 10 ml of warmed BEGM. Transfer cells to a T-75cm² Cell Bind[®] flask and incubate flask at 37°C incubator supplied with 5% CO₂. The cells should attach and begin to grow after 24 hrs; aspirate and replace BEGM at this time.
4. Replace medium in the flask every two days as needed depending on the growth of the cells. Split or freeze the cells when they are 80% confluent.

3.5. Collagen coating of Trans-well inserts

1. Add 300 μl of 5% human placental collagen Type VI to each insert in a 12-well plate and incubate at 37°C , 5% CO₂ for 24 hrs.
2. Remove collagen solution from inserts and air dry completely in tissue culture hood for 30 min.
3. Rinse dried inserts twice with 500 μl PBS and once with 250 μl BEGM.

3.6. Culture of human airway epithelial cells at air liquid interface (ALI) on collagen-coated Transwell inserts

1. Remove medium from 80% confluent monolayers of human sinus or bronchial (HSE/HBE) cells from a T-75cm² Cell Bind[®] flask. Wash the flask gently with 2.5 ml trypsin, aspirating it off quickly.

⁷Recommended cell concentrations: for plating 150–300K in 10 ml per T-75 cm² flask; and for freezing, $5 \times 10^5 - 1 \times 10^6$ cells per vial.

⁸Freeze $3 \times 10^5 - 1 \times 10^6$ cells, depending on number of cells.

2. Trypsinize the cells with just enough trypsin (4 mL) to cover the cells for 3–5 min in the 37°C incubator, checking frequently and tapping flasks firmly, just until cells are observed to float freely.
3. Transfer cells to a 50 mL conical tube containing 10 mL of DMEM/F12, 10% FCS to inactivate trypsin and centrifuge at 200 x g for 10 min.
4. Resuspend the pellet in 10 ml of DMEM/F12, remove an aliquot for counting cells, and then centrifuge the cells at 200 x g for 10 min (see Note 9).
5. Gently resuspend the cells in BEGM at a concentration of 750K cells/ml.
6. Add 200 µl of 750 K cells/ml cell suspension per well for a 12 well plate insert (1.13cm²).
7. Add 1 ml BEGM to the outer wells and incubate at 37°C, 5% CO₂ for 24 hrs (see Note 10).
8. Aspirate medium from both outer well and insert. Add 1ml of ALI medium to the outer well leaving cells at air-liquid interface and incubate at 37°C (see Note 11).
9. Remove media from outer well, and remove any seepage inside the insert every other day during the first week and three times a week after that (see Note 12).
10. Cultures should start developing cilia by day 17 in culture and will exhibit increased trans-epithelial resistance (see Note 13).

3.7. RV-C inoculation of differentiated human airway epithelial cells at air liquid interface

1. Observe mature ALI cultures under the microscope for actively beating cilia. If present, remove medium from the basal compartment of the ALI cultures and wash the cells gently three times with PBS (Ca⁺²/Mg⁺² free) to remove mucus.
2. Dilute RV-C to a desired concentration (1×10⁶ RNA copies) in BEGM with 0.01% BSA and apply 100 µl of the inoculum to the apical surface of the ALI culture. Next, rock the plate gently on a shaker for 15 min at room temperature and then transfer the plate to an incubator (34°C, 5% CO₂) for 4 hrs (see Note 14).
3. Wash the cultures thrice with PBS (Ca⁺²/Mg⁺² free) and collect samples to assess cell associated virus in 350µl RLT buffer (Qiagen).
4. Feed the remaining samples basally with 1 ml of ALI medium and incubate at 34°C for 24- 72 hrs.

⁹The cells obtained at P-0 and P-1 are frozen in liquid N₂ for future use. Cells obtained from later passages do not differentiate as well at ALI.

¹⁰If the cells do not form a confluent monolayer after first day of seeding on the inserts, they should be kept in BEGM and allowed to grow till they become confluent and then transfer to air-liquid interface.

¹¹Make sure no air bubbles are trapped underneath the inserts after addition of medium, and remove old media from the bottom of the wells every time.

¹²Cell debris and mucus will collect on the apical surface as the cells grow. These can be gently washed with warmed BEGM/DMEM once per week or as needed.

¹³Cultures become differentiated from 21 days onwards as observed by ciliary beating and increased trans-epithelial resistance.

Cultures allowed to differentiate for two months permit greater RV replication as compared to those allowed to differentiate for one month. Cultures can be maintained for 6 months by replenishing the medium regularly and washing mucus from the apical surfaces.

¹⁴The concentration of RV that gives the optimal replication ranges from 10⁵ – 10⁶ RNA copies/well.

5. After aspirating the media, collect the cells in 350 µl of RLT buffer for RNA extraction and qRT-PCR analysis.

3.8. RNA extraction and qRT-PCR

1. Extract total RNA from the ALI cultures using the RNeasy Mini kit (Qiagen).
2. RV-RNA concentrations are determined by qRT-PCR using RV-specific primers (5).

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Fig. 1.
Photomicrograph of human sinus epithelium (HSE) after differentiation at ALI. A cross-section of tissue was stained with haematoxylin & eosin at 600X magnification.

Fig. 2.

RV infection of ALI cultures. HSE ALI cultures (n=6) at 21 days of age were infected with RV-C15 (2.0×10^8 RNA copies) or RV-A16 (3×10^8 RNA copies) at 34°C. Cell-associated virus was analyzed 4 hrs and again 24 – 48 hrs post-infection (PI). *P=0.001, **P< 0.005 compared to viral RNA post-inoculation.