Chapter 24

In Vitro Model of Invasive Pulmonary Aspergillosis in the Human Alveolus

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Abstract

Cellular bilayer models can be used to simulate many biological compartments. Here, we describe a cell culture model of the human alveolus that enables the study of early invasive pulmonary aspergillosis. The cellular bilayer is constructed with human alveolar epithelial cells and human pulmonary artery endothelial cells. The cells are grown on a semipermeable polyester membrane. This model can be used to study the pathogenesis, immunobiology and pharmacology of invasive pulmonary aspergillosis.

Key words: *Aspergillus*, Aspergillosis, Tissue culture

1. Introduction

Transwells® are semipermeable synthetic membranes that enable the growth of various cell types and the simulation of biological compartments. Here, we describe a cellular bilayer grown on a polyester insert that mimics the alveolar-capillary barrier. This bilayer model can be used to study many aspects related to the pathophysiology and pharmacology of invasive pulmonary aspergillosis.

This bilayer model was originally developed to investigate the pathogenesis of *Mycobacterium tuberculosis* (1, 2). The model was subsequently adapted to study *Aspergillus fumigatus*, including the kinetics of clinically relevant biomarkers, the invasion of hyphae, the effect of antifungal agents and the antifungal effect of immunological effectors (3, 4).

Human alveolar epithelial cells are grown on the upper surface of a polyester membrane that is perforated with 3-μm pores. Human pulmonary artery endothelial cells are grown on the under surface. The cellular bilayer defines two compartments: (1) an upper

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compartment that simulates the alveolar airspace and (2) a lower compartment that simulates the pulmonary capillary. The alveolar compartment is inoculated with *A. fumigatus* conidia, where they germinate to form hyphae. These tissue invasive forms penetrate the cellular bilayer, thus mimicking invasion in the human lung. Antifungal agents can be applied to the endothelial compartment to simulate the systemic administration of these compounds. Immunological effectors can be added to the alveolar or endothelial compartment. Invasion can be assessed directly (e.g. by confocal microscopy) or via the measurement of fungal-related biomarkers.

2. Materials

Prepare solutions at room temperature and store at 4°C, unless otherwise stated. Media containing Foetal bovine serum (FBS) can be used for up to 1 month.

2.1. Construction of the Cellular Bilayer

1. Human pulmonary artery endothelial cells (HPAECs, see Note 1).
2. EGM-2 BulletKit (medium; see Note 1) is prepared following the manufacturer's instructions by adding the provided supplements required to support the growth of HPAECs (ascorbic acid, heparin, hydrocortisone, human endothelial growth factor, 2% FBS, vascular endothelial growth factor, human fibroblast growth factor-B and R3-insulin-like growth factor-1) to the supplied basal medium EBM-2 (see Note 1).
3. Human alveolar A549 epithelial cells (see Note 2).
4. EBM-10%: 10% FBS (see Note 1) in EBM-2 basal medium (see Note 1).
5. Hank's buffered salt solution (HBSS) with phenol red without calcium and magnesium (see Note 1).
6. 0.25% Trypsin-EDTA solution.
7. Transwell® permeable supports (inserts), 3-μm polyester membranes, 6.5-mm (24-well) inserts (see Note 3).
8. Vented T75 and T162 flasks.

2.2. Experimental Components

1. Phosphate buffered saline (PBS).
2. Potato dextrose agar (PDA); prepared according to the manufacturer's instructions.
3. EBM-2%: 2% FBS (see Note 1) in EBM-2 basal medium (see Note 4).
4. EBM-2 basal medium (see Note 1).
5. Green fluorescent protein (GFP)-expressing strain of *A. fumigatus* (3, 5, 6).

6. Antifungal compound, for example, dissolve pure posaconazole powder in dimethyl sulfoxide, to produce a stock solution at 1,600 mg/L. Stocks of drug to be stored at −80°C until use.
7. Vented T25 flasks.
8. Non-woven sterile gauze/swabs 7.5 × 7.5 cm, 4-ply.

3. Methods

Carry out all procedures at room temperature in a class II safety cabinet unless otherwise specified. Decontaminate cabinet (and equipment to be used within) before and after use with 70% industrial methylated spirits or 70% alcohol. Incubate at 37°C in 5% CO_2 unless otherwise stated.

All media should be warmed to 37°C prior to use (see Note 5). Avoid creating bubbles in tissue culture media, particularly during mixing steps. Minimise the length of time that cells are out of the incubator.

3.1. Construction of the Cellular Bilayer

Refer to Fig. 1 (flow diagram of procedure) for timings throughout. Media volumes are approximate during construction of the bilayer, with the exception of cell adjustment and application to the polyester membrane. Use sterile forceps to handle inserts.

HPAEC and A549 cells are stored in liquid nitrogen, following the supplier's instructions.

1. On day 1, resurrect HPAECs from liquid nitrogen (see Note 6) into a vented T162 flask with 30 mL BulletKit medium and incubate for 24 h.
2. Change the HPAEC medium 24 h after resurrection and after a further 48 h (refer to Fig. 1). Do this by aspirating and discarding old medium with a serological pipette, then replace with fresh medium and re-incubate (see Note 7).
3. On day 4, resurrect A549 cells from liquid nitrogen (see Note 6) into a vented T75 flask with 15 mL EBM-10% medium and incubate for 24 h.
4. On day 5, change A549 medium.
5. On day 6, (Fig. 1) prepare HPAEC layer as follows: Aspirate medium from HPAEC flask with a serological pipette. Wash the surface of the flask with 20-mL of HBSS twice (see Note 8). Cover entire surface of cells with 6 mL trypsin, rock back and forth several times, and incubate at 37°C for ~5 min (see Note 9). Once detached, quickly re-suspend the cells in 20 mL BulletKit. Ensure maximal cell retrieval by gently rinsing the flask surface with the cell suspension and a serological pipette. Transfer the cells into a sterile 50-mL centrifuge tube

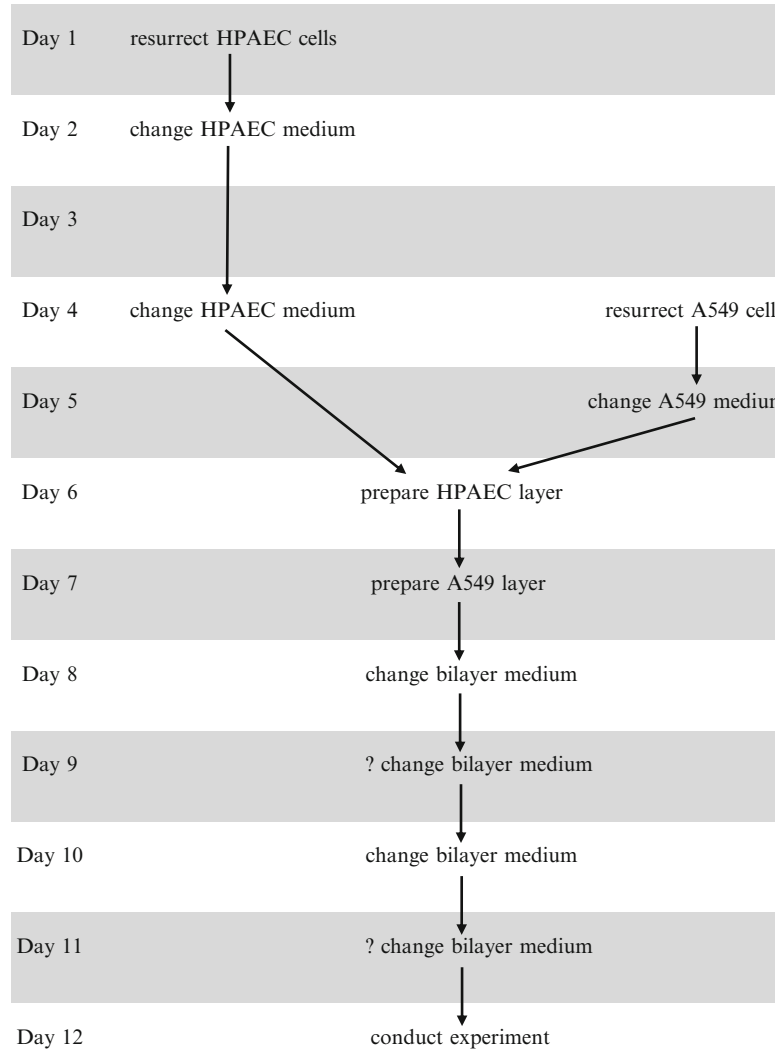

Fig. 1. Flow diagram of in vitro model preparation.

and centrifuge for 5 min at 125 × *g*. Remove the supernatant (taking care not to disturb the cellular pellet); re-suspend the pellet in BulletKit (approximately 1 mL per HPAEC flask) by gently mixing up and down with a Gilson pipette. Count the cells using a haemocytometer and adjust to 1×10^6 cells per mL in BulletKit (see Note 10). Invert the required number of inserts in a sterile tray (see Note 11), pipette 100 µL 1×10^6 cell/mL HPAECs onto the undersurface of each inverted insert (giving 1×10^5 cells per insert) and incubate inverted for 2 h. Flip inserts (to the correct way up) and place in 600 µL BulletKit in a 24-well tissue-culture-treated plate (see Note 12). Add 100 µL EBM-10% into the top compartment of the insert and incubate overnight.

6. On day 7, (Fig. 1) prepare the A549 layer as follows: Transfer the inserts into fresh BulletKit (600 µL). Aspirate the previous days EBM-10% from the top compartment and incubate until required. Aspirate the medium from the A549 flask. Quickly wash the surface of the flask with 15 mL HBSS twice (see Note 8). Cover the entire surface of the cells with 6 mL trypsin, rock back and forth several times, and incubate at 37°C for ~5 min (see Note 9). Once detached, add 15 mL EBM-10% and gently wash the flask surface with the cell suspension. Transfer cells into a sterile 50-mL centrifuge tube and centrifuge for 5 min at 125 × *g*. Remove the supernatant whilst avoiding the cell pellet. Re-suspend the pellet in EBM-10% (approximately 1-mL per A549 flask). Count cells with a haemocytometer and adjust to 5.5×10^5 cells/mL in EBM-10%. Add 100 µL 5.5×10^5 cells/mL A549 onto the top layer of each insert (giving 5.5×10^4 cells per insert) and incubate (see Note 13).
7. Change insert medium every 24–48 h (Fig. 1). Do this by transferring the inserts into a new 24-well plate containing 600 µL fresh BulletKit whilst aspirating any medium from the top compartment.

3.2. Experiment

1. Subculture the *A. fumigatus* GFP strain onto PDA in a vented T25 flask. Incubate at 37°C (atmospheric CO_2) for 7 days.
2. In a class II safety cabinet, harvest *A. fumigatus* spores by adding approximately 20-mL PBS and gently agitate the surface with a sterile swab or loop. Aspirate the fluid and place in 50-mL sterile centrifuge tube. Vortex vigorously for ~1 min. Aseptically place sterile gauze over a 50-mL sterile centrifuge tube, transfer the spore suspension through the gauze (see Note 14). Centrifuge the spore suspension at 1,000 × *g* for 10 min. Gently discard the supernatant. Re-suspend the pellet in 20 mL PBS to wash. Vortex for approximately 1 min and centrifuge at 1,000 × *g* for 10 min. Wash spores one further time in PBS. Store at 4°C until required (for up to 1 week).
3. Transfer inserts into 600-µL of EBM-2% whilst aspirating any residual medium from the top compartment. Vortex harvested spores vigorously for approximately 1 min. Count spores with a haemocytometer and dilute inoculum to 1×10^4 cfu/mL in EBM-2 basal medium. Ensure the inoculum is vigorously vortexed before each dilution step and prior to use. Inoculate the top compartment of the inserts with 100-µL 1×10^4 cfu/mL spore suspension (1×10^3 spores per insert). Incubate at 37°C in 5% CO_2 for 2 h. Remove the liquid from the top compartment (spores will have attached to the A549 monolayer) to maintain an air/liquid interface. Apply the antifungal drug of interest in EBM-2% to the lower compartment 6 h post-inoculation.

4. Downstream analysis was performed 24 h after inoculation using galactomannan detection by commercial kit to determine the fungal burden and HPLC measurement of antifungal drug levels.

4. Notes

1. We use HPAEC cells, EGM-2 BulletKit medium, EBM-2 basal medium, FBS and HBSS supplied by Lonza. Omit gentamicin/amphotericin from the EGM-2 medium if the latter may confound the experimental results. Alternatively, 100 units/mL penicillin and 0.1 mg/mL streptomycin can be used to reduce bacterial contamination, if required.
2. We use A549 cells from LGC Standards.
3. We use Transwells® produced by Costar Corning.
4. Use 2% FBS in the lower compartment during the experiment to simulate the human protein concentrations of the pulmonary capillary.
5. Ideally, media should be warmed on the day of use, but in our experience, it may be warmed for up to 24 h prior to use. Also, the appropriate volume of media should be aliquoted to avoid warming media more than twice.
6. Cells resurrected from liquid nitrogen should be defrosted swiftly to minimise damage from dimethyl sulfoxide (DMSO) in the storage media; warming in the crease of a gloved hand is preferable to a water bath to reduce the risk of contamination.
7. Aspirating or dispensing media by pipetting is preferable to pouring to minimise the risk of infection.
8. Gently rock the flask back and forth approximately six times, aspirate the HBSS and discard. Washing removes any traces of FBS which inhibits the action of trypsin.
9. Detachment of cells can be monitored by checking under a microscope (with experience detachment can also be visualised macroscopically when the flask is held up to a light source). The length of time the cells are outside the incubator should be minimised. Move to next step once approximately 95% cells have detached. Do not agitate the A549 cells by tapping or shaking whilst waiting for the cells to detach as this can cause clumping and atypical growth.
10. A small aliquot of the cell suspension may be diluted to facilitate counting. Optionally, the cells can be diluted in trypan blue (Sigma) to exclude counting non-viable cells.

11. Invert the inserts, for example, in 140-mm glass sterile Petri dishes, which can be subsequently hot air–sterilised. Other sterile glass or plasticware can also be used, although caution should be taken with the height of the vessel, to avoid disrupting the cell suspension on inverted inserts.
12. Avoid spilling medium down the side of the inserts during flipping to minimise the risk of infection.
13. Removing EBM-2% after 2 h following addition of A549 cells creates an air/liquid interface, which is more physiologically appropriate for this pulmonary model. Alternatively, a liquid/liquid interface can be adopted by simply leaving the medium in contact with both sides of the bilayer.
14. The gauze filtration step removes large clumps of hyphae, medium and hydrophobic spores, which eases subsequent counting. We do not use Tween in our inoculum, but this could be tried (0.05% Tween 80) if excessive conidial clumping is seen.

References

1. Birkness, K. A., Deslauriers, M., Bartlett, J. H., White, E. H., King, C. H., and Quinn, F. D. (1999) An in vitro tissue culture bilayer model to examine early events in *Mycobacterium tuberculosis* infection. *Infect Immun 67*, 653–8.
2. Bermudez, L. E., Sangari, F. J., Kolonoski, P., Petrofsky, M., and Goodman, J. (2002) The efficiency of the translocation of *Mycobacterium tuberculosis* across a bilayer of epithelial and endothelial cells as a model of the alveolar wall is a consequence of transport within mononuclear phagocytes and invasion of alveolar epithelial cells. *Infect Immun 70*, 140–6.
3. Hope, W. W., Kruhlak, M. J., Lyman, C. A., Petraitiene, R., Petraitis, V., Francesconi, A., Kasai, M., Mickiene, D., Sein, T., Peter, J., Kelaher, A. M., Hughes, J. E., Cotton, M. P., Cotten, C. J., Bacher, J., Tripathi, S., Bermudez, L., Maugel, T. K., Zerfas, P. M., Wingard, J. R., Drusano, G. L., and Walsh, T. J. (2007) Pathogenesis of *Aspergillus fumigatus* and the kinetics of galactomannan in an in vitro model of early invasive pulmonary aspergillosis: implications for antifungal therapy. *J Infect Dis 195*, 455–66.
4. Lestner, J. M., Howard, S. J., Goodwin, J., Gregson, L., Majithiya, J., Walsh, T. J., Jensen, G.M., and Hope, W.W. (2010). Pharmacokinetics and pharmacodynamics of amphotericin B deoxycholate, liposomal amphotericin B, and amphotericin B lipid complex in an in vitro model of invasive pulmonary aspergillosis. *Antimicrob Agents Chemother 54*, 3432–41.
5. Wasylnka, J. A., and Moore, M. M. (2002) Uptake of *Aspergillus fumigatus* conidia by phagocytic and nonphagocytic cells in vitro: quantitation using strains expressing green fluorescent protein. *Infect Immun 70*, 3156–63.
6. Wasylnka, J. A., and Moore, M. M. (2003) *Aspergillus fumigatus* conidia survive and germinate in acidic organelles of A549 epithelial cells. *J Cell Sci 116*, 1579–87.