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Cell culture models using rat primary alveolar type I cells

Charles A. Downs [a,b,*], David W. Montgomery [a,b], Carrie J. Merkle [a,b]

[a] The University of Arizona, College of Nursing, Tucson, AZ 85721-0203, United States
[b] The Southern Arizona VA HealthCare System, Tucson, AZ, United States

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ABSTRACT

There is a lack of cell culture models using primary alveolar type I (AT I) cells. The purpose of this study was to develop cell culture models using rat AT I cells and microvascular endothelial cells from the lung (MVECL). Two types of model systems were developed: single and co-culture systems; additionally a 3-dimensional model system was developed. Pure AT I cell (96.3 ± 2.7%) and MVECL (97.9 ± 1.1%) preparations were used. AT I cell morphology, mitochondrial number and distribution, actin filament arrangement and number of apoptotic cells at confluence, and telomere attrition were characterized. AT I cells maintained their morphometric characteristics through at least population doubling (PD) 35, while demonstrating telomere attrition through at least PD 100. Furthermore, AT I cells maintained the expression of their specific markers, T1α and AQ-5, through PD 42. For the co-cultures, AT I cells were grown on the top and MVECL were grown on the bottom of fibronectin-coated 24-well Transwell Fluroblok™ filter inserts. Neither cell type transmigrated the 1 μm pores. Additionally, AT I cells were grown in a thick layer of Matrigel® to create a 3-dimensional model in which primary AT I cells form ring-like structures that resemble an alveolus. The development of these model systems offers the opportunities to investigate AT I cells and their interactions with MVECL in response to pharmacological interventions and in the processes of disease, repair and regeneration.

1. Introduction

The alveolus, or gas exchanging surface of the lung, is composed of alveolar epithelial type I (AT I), type II (AT II) and microvascular endothelial cells. AT I cells are large flat squamous epithelial cells that cover 95–99% of the alveolar surface area and participate in gas, ion and water exchange. AT II cells are cuboidal shaped cells that produce surfactant, are more numerous than AT I cells, and account for only ~1% of the alveolar surface area [1–3]. The alveolar epithelium and endothelium share a common basement membrane which reduces the distance gases must diffuse from the alveolar space to the vasculature and vice versa. However in the setting of many disease states, such as emphysema and acute respiratory distress syndrome, the alveolus is damaged leading to hypoxia and hypercapnia, or elevated carbon dioxide.

Our understanding of the roles that AT I cells play in the development and in the response to treatments for lung disease is hindered by a lack of cell culture model systems using primary AT I cells. The predominant reason is that primary AT I cells have been considered terminally differentiated—that is, incapable of mitosis—and difficult to isolate and maintain in culture. As a result AT I cells differentiated in vitro from alveolar type II cells, termed AT I-like cells, are used as a surrogate [4]. However, the similarities and differences of AT I cells and AT I-like cells are not fully understood. The purpose of this paper is to describe the development of model systems using rat primary AT I cells and microvascular endothelial cells from the lung (MVECL).

In our studies, we find that rat primary AT I cells undergo mitosis while maintaining the expression of AT I specific markers, T1α (RT140, rat podoplanin) [5] and aquaporin-5 (AQ-5) [6], through at least population doubling (PD) 42. Furthermore, we describe the development of a co-culture model in which rat primary AT I cells express T1α, AQ-5, and zona occludin-1 (ZO-1) when grown on the top, and MVECL express VE-cadherin when grown on the bottom of fibronectin-coated 24-well Transwell Fluroblok™ filters. In addition, when grown in a 3-dimensional matrix, rat primary AT I cells form ring-like structures that resemble an alveolus. These model systems offer opportunities to investigate the structural and functional properties of AT I cells, while the establishment of a co-culture model system allows for the study of interactions between AT I cells and MVECL in response

* Corresponding author. The University of Arizona, College of Nursing, Tucson, AZ 85721-0203, United States. Tel.: +1 520 626 7523; fax: +1 520 344 9076.
E-mail addresses: cdowns@nursing.arizona.edu (C.A. Downs), david.montgomery2@va.gov (D.W. Montgomery), cmerkle@nursing.arizona.edu (C.J. Merkle).

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to pharmacological interventions and in the processes of disease, repair and regeneration.

2. Materials and methods

2.1. Materials

2.1.1. Animals

Pathogen-free 3-month-old male Fischer 344 rats (~280 g) were obtained from Harlan Laboratories (Indianapolis, IN) and used as the source of primary AT I cells and MVECL. Protocols for animal use were approved by the Institutional Animal Care and Use Committees of The University of Arizona and The Southern Arizona VA HealthCare System (SAVAHCS).

2.1.2. Reagents and assay kits

Unless stated otherwise, all laboratory chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Vybrant® CFDA SE cell tracer kit, Vybrant® DiI cell tracer, 4′,6-diamidino-2-phenylindole (DAPI), rhodamine-conjugated phalloidin, Dynabeads® M-280 sheep anti-rabbit IgG, the CELLection™ Biotin Binder Kit, and DNA primers were purchased from Invitrogen (Carlsbad, CA). The CellTiter® AQ_{ueous} One Solution Assay, an MTS assay, was purchased from Promega (Madison, WI). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Elastase was purchased from Worthington (Lakewood, NJ). Matrigel was purchased from BD Life Sciences (Franklin Lakes, NJ). The mitochondria staining kit, modified papanicolaou staining kit, alkaline phosphatase staining kit, leukocyte esterase staining kit, and RPMI 1640 media were purchased from Sigma–Aldrich (St. Louis, MO). EGM-2MV media were purchased from Lonza (Basel, Switzerland). The Qiagen QIAamp DNA Blood Midi kit was purchased from Qiagen (Valencia, CA). Brilliant SYBR green qPCR reagent kit was purchased from Stratagene (Cedar Creek, TX).

2.1.3. Antibodies

Rabbit anti-rat aquaporin-5 (Product No. sc-28628), rabbit anti-rat PECAM-1 (Product No. sc-28188) and rabbit anti-rat anti-von Willebrand factor (Product No. sc-14014) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-rat ZO-1 (Product No. 402200) and Alexa Fluor® 568 donkey anti-goat IgG secondary antibody (Product No. A11057) were purchased from Invitrogen (Carlsbad, CA). Rabbit anti-rat podoplanin (Product No. P2120) and rabbit anti-human VE-cadherin (Product No. V1514) and goat anti-vimentin (Product No. V4630) were purchased from Sigma–Aldrich (St. Louis, MO). Rhodamine (TRITC) conjugated affinity purified goat anti-rabbit IgG secondary antibody was purchased from Jackson ImmunoResearch (Product No. 111-025-003; West Grove, PA).

2.1.4. Cell isolation and culture

AT I cells were obtained from the lungs of pathogen-free 3-month-old male Fischer 344 rats (~280 g) using a previously published protocol [7]. In brief, rats were anesthetized with isoflurane and the lungs were extracted. The trachea was cannulated and the lungs were lavaged with PBS containing 5 mM EDTA and 5 mM EGTA. The lungs were lavaged three times with RPMI 1640 with 10% FBS, 10% Dextran 40, and 4.5 U/mL elastase for 10 min at 37 °C. After the trachea and large airways were removed, the lung tissue was minced into approximately 1 mm^3 pieces and mechanically ground (using a syringe plunger) through 160 and 37 μm filter mesh to produce a cell suspension. Following centrifugation (250*g* for 8 min) the cell suspension was resuspended in 0.5 mL of RPMI 1640 with 1% FBS, 25 mM HEPES, and 100 μg/mL DNase I. Dynabeads® M-280 sheep anti-rabbit IgG coated magnetic beads were incubated with 1 μg rabbit anti-rat podoplanin antibody for 30 min at 4 °C. Following conjugation, the beads were incubated with the cell suspension for 45 min at 4 °C with end-to-end rotation and were used to positively select for AT I cells. The MVECL were isolated using a previously published protocol [8] in which cells were positively selected by using the CELLection™ Biotin Binder Kit, a magnetic bead kit. AT I cells and MVECL were grown separately in 25 cm^2 tissue culture flasks and maintained in RPMI 1640 with 10% FBS (AT I) or EGM-2MV (MVECL) medium. Human dermal fibroblasts derived from normal neonatal foreskin, a gift from Dr. Steven Goldman at SAVAHCS, were grown in RPMI 1640 media with 10% FBS and used as a positive control for vimentin staining as discussed below.

2.1.5. Immunocytochemical markers and microscopy

Immunocytochemical staining was performed to assess the purity of freshly isolated cell preparations and to evaluate maintenance of T1α and AQ-5 expression at successively higher PDs. To assess cell purity, freshly isolated AT I and MVECL cell preparations were grown on gelatin-coated 8-well Permanox chamber slides (VWR) for 48 h. Then samples were washed with PBS without calcium and magnesium, fixed with 4% paraformaldehyde in PBS for 5 min, and then washed 5 times with PBS. Samples to be stained for AQ-5, VE-cadherin and von Willebrand factor were permeablized for 2 min with 0.5% Triton X-100 in PBS. To prevent non-specific binding, the samples were blocked with PBSAA (PBS with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide) containing 10% goat serum for 15 min at 37 °C. The respective primary antibodies diluted in PBSAA with 0.05% Tween-20, 10% goat serum and 1% BSA were added and incubated at 37 °C for 1 h. The samples were washed, and the secondary antibody in PBSAA with 0.05% Tween-20 was added and incubated at 37 °C for 1 h. Then the samples were washed, mounted using Vectashield, and images were taken using a SPOT™ camera (Diagnostic Instruments) attached to an inverted microscope (Leica DMIRB) with brightfield, phase contrast, differential interference contrast (DIC), and fluorescence optics. Image analysis was performed using either Image-Pro-Plus™ Software (Media Cybernetics) or Image J software (NIH).

To assess expression of T1α and AQ-5 at successively higher PD (6, 17, 30 and 42), AT I cells (2.0×10^4) were seeded on gelatinized 8-well Permanox chamber slides and grown in RPMI 1640 with 10% FBS. The samples were incubated overnight at 37 °C in 5% CO_2 then fixed with 4% paraformaldehyde, and immunocytochemical staining for T1α and AQ-5 was performed as described above. Fifteen images of cells at each PD were taken and used to quantify fluorescence intensity and percent of cells positive for T1α and AQ-5. Percent of positive cells was calculated as the percentage of cells having a measured fluorescence value of ≥2.5 standard deviations above the control. Samples stained with secondary antibody only were used as negative controls for each marker.

Potential contaminants in AT I cell preparations included AT II cells, macrophages, and alveolar fibroblasts. To quantify contamination in freshly isolated AT I cell preparations, cells from each AT I cell preparation were grown on gelatin-coated 8-well glass chamber slides for 48 h in RPMI 1640 with 10% FBS. Then the modified papanicolaou staining kit [9] and alkaline phosphatase staining kit [10] were used to detect AT II cells and a leukocyte esterase staining kit was used to detect macrophages [10]. Cell preparations were stained to detect vimentin, a marker for mesenchymal cells; human dermal foreskin fibroblasts were used as a positive control. Digital images of stained cells were captured and used to quantify AT II cells, macrophages, and fibroblasts.

2.1.6. AT I cell characterization

To characterize rat primary AT I cells we first evaluated AT I cell morphology. Four randomly selected microscopic fields (1 mm^2) of