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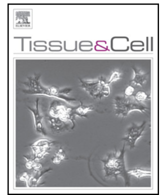

Structurally and functionally characterized *in vitro* model of rabbit vocal fold epithelium

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ABSTRACT

In this paper, we describe a method for primary culture of a well differentiated electrically tight rabbit vocal fold epithelial cell multilayer and the measurement of transepithelial electrical resistance (TEER) for the evaluation of epithelial barrier function *in vitro*. Rabbit larynges were harvested and enzymatically treated to isolate vocal fold epithelial cells and to establish primary culture. Vocal fold epithelial cells were co-cultured with mitomycin C-treated feeder cells on collagen-coated plates. After 10–14 days in primary culture, cells were passaged and cultured until they achieved 70–90% confluence on collagen-coated plates. Epithelial cells were then passaged onto collagen-coated cell culture inserts using 4.5 cm^2 membrane filters (1.0 μm pore size) with 10% fetal bovine serum or 30 μg/mL bovine pituitary extract to investigate the effects of growth-promoting additives on TEER. Additional experiments were performed to investigate optimal seeding density (1.1, 2.2, 4.4, or 8.9×10^5 $cells/cm^2$), the effect of co-culture with feeder cells, and the effect of passage number on epithelial barrier function. Characterization of *in vitro* cultures was performed using hematoxylin and eosin staining and immunostaining for vocal fold epithelial cell markers and tight junctions. Results revealed higher TEER in cells supplemented with fetal bovine serum compared to bovine pituitary extract. TEER was highest in cells passaged at a seeding density of 2.2×10^4 $cells/cm^2$, and TEER was higher in cells at passage two than passage three. Ultrastructural experiments revealed a well-differentiated epithelial cell multilayer, expressing the epithelial cell markers CK13, CK14 and the tight junction proteins occludin and ZO-1.

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1. Introduction

Voiced speech is the result of acoustic energy created by airflow modulation of the vocal folds. Located in the larynx, the vocal folds are a pair of unique tissue structures that play a role in the biological and non-biological functions of the larynx and consist of epithelial, muscle, and connective tissue (Hirano, 1974). Phonation is the physiological process that results in vibration of the vocal folds and the production of acoustic sound energy (Van Den Berg, 1958). During vocal fold vibration, the epithelium and superficial layer of the lamina propria (referred to as the vocal fold cover) vibrate freely over the deeper connective tissue layers and muscle (Hirano and Kakita, 1985). The epithelium and superficial connective tissue layer are altered after disease and injury, leading to abnormal sound production (Benninger et al., 1996; Colton et al., 2011).

The vocal fold epithelium acts chiefly as a functional barrier to protect the underlying connective tissue layers against various external threats. Disrupted epithelial function after disease and injury interrupts the integrity of the epithelial barrier and may exacerbate disease risk (Levendoski et al., 2014). The structural integrity of this stratified, non-keratinized squamous vocal fold epithelium is maintained by the junctional complex. The junctional complex consists of tight junctions, adherens junctions, and desmosomes (Tsukita et al., 2001). Tight junctions are located at the apical portion of the lateral membrane and are responsible for intercellular signaling, whereas adherens junctions and desmosomes mechanically link adjacent cells to one another (Suzuki, 2013; Tsukita et al., 2001).

The investigation of epithelial barrier function provides an important approach for evaluation of the integrity of the vocal fold

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barrier. Transepithelial electrical resistance (TEER) has been shown to be a valid and reliable method for measurement of the functional integrity of the epithelial barrier (Hasegawa et al., 1999; Hashimoto et al., 2008; Meyer et al., 2001; Reichl et al., 2011). TEER in whole-mount tissue specimen of ovine, porcine, and rabbit vocal fold has been studied *ex vivo* in Ussing chambers (Alper et al., 2011; Kojima et al., 2014a; Sivasankar and Fisher, 2008, 2007). What remains is a need for the development of experimental protocols and methods for the *in vitro* measurement of vocal fold barrier function in epithelial cell culture. The measurement of transepithelial resistance *in vitro* allows for the investigation of epithelial barrier function *over time* and may be useful for the preclinical testing of novel treatments for restoration of barrier function after injury.

It is important to create such an *in vitro* model of epithelial cells in order to provide a robust system in which to test novel treatments of vocal fold injury. In determining changes in epithelial cell characteristics, such as epithelial inflammatory responses, apoptosis, or changes in barrier function, we can better understand the mechanism of each drug or therapy.

The purpose of the current study was to describe a method for primary culture and passaging of functionally characterized *in vitro* vocal fold epithelial cells from New Zealand white breeder rabbits. We investigated the effects of growth-promoting additives, seeding density, cell passaging, and co-culture with and without 3T3 feeder cells on epithelial barrier function. Additionally, we characterized our *in vitro* cultures through the detection of the vocal fold epithelial cell markers pan-CK, CK13, and CK14, and the tight junctions occludin and ZO-1 to verify the nature of the cells cultured. Throughout the development of this preliminary culture method, and across culture conditions, we measured TEER to quantify barrier integrity of the cell layer.

2. Materials and methods

2.1. Isolation of vocal fold epithelial cells

The procedures used in this study were approved by the Vanderbilt University Institutional Animal Care and Use Committee. The larynges of 4 New Zealand white breeder rabbits were harvested following sedation and euthanasia. Excised larynges were treated with 66 U/mL Dispase II (Rosche Life Science, Indianapolis, IN) in culture medium at 37 °C for 4 h to digest the collagenous extracellular matrix of the lamina propria. Following incubation, the epithelial layer of the true vocal fold was removed and treated with 0.05% trypsin-0.02% EDTA solution (Sigma-Aldrich, St. Louis, MO) at 37 °C for 20 min. Additional medium was then added to counteract trypsin activity, and the cells were suspended using gentle pipetting. The suspension of dissociated cells was then centrifuged and the obtained pellet was re-suspended in culture medium. Cell were counted using a hemocytometer and then co-cultured with feeder cells (3T3-Swiss Albino, ATCC CCL®-92™, ATCC, Manassas, VA) on collagen-coated 6 well plates. To coat the plates with collagen, they were incubated at 37 °C for 2.5 hours with 2 ml of 0.6 mL of a 37.5 μg/mL collagen solution (Advanced Biomatrix PureCol) in each well. The excess liquid was aspirated and the plates were rinsed with PBS. The plates were then re-sterilized under UV light for 30 minutes. Feeder cells were treated with 10 μg/mL mitomycin-C (Sigma-Aldrich) at 37 °C for 3 h to halt proliferation, and seeded with the epithelial cells at a density of 2.0×10^4 cells/cm^2.

2.2. Cultivation of vocal fold epithelial cells

Unless otherwise indicated, culture medium was composed of DMEM/F12 (1:1 with 1-glutamine, 15 mM HEPES, 1 mM $CaCl_2$, GIBCO, Grand Island, NY), 10% fetal bovine serum (FBS, HyClone, South Logan, UT), penicillin (100 U/mL), streptomycin (100 μg/mL, HyClone, South Logan, UT), epidermal growth factor (10 ng/mL, Peprotech, Rocky Hill, NJ), insulin (5 μg/mL, Sigma-Aldrich), adenine (24 μg/mL, Sigma-Aldrich), hydrocortisone (0.4 μg/mL, Sigma-Aldrich), cholera toxin (0.1 nM, Sigma-Aldrich), and triiodo-L-thyronine (2 nM, Sigma-Aldrich) (Spurr-Michaud and Gipson, 2013). The cells were cultured on collagen-coated plates. In primary culture, medium was exchanged on day 4. On day 7, feeder cells were exchanged and medium was changed. Because the epithelial cells adhere more tightly to the collagen coated plate than the feeder cells, treatment with 0.05% trypsin-0.02% EDTA for 2 minutes allowed detachment of the feeder cells (and any contaminating fibroblasts) while the epithelial cells remained adherent. Following removal of the medium containing the suspended feeder cells and fibroblasts and a rinse with phosphate buffered saline, new feeder cells were seeded into the plate containing the epithelial cells. After 10–14 days of primary culture, epithelial cells were locally confluent and ready for passaging. Using the same adherence principle described above, feeder cells and any contaminating vocal fold fibroblasts were removed from the plates using 0.05% trypsin-0.02% EDTA solution. Detachment of these cells was confirmed using microscopic observation. Immediately after the feeder cells and vocal fold fibroblasts were detached from the surface of the culture plates, the trypsin cell suspension was aspirated and wells containing the epithelial cells were washed with phosphate buffered saline. Epithelial cells were then harvested *via* plate detachment using a longer (<10 minute) treatment with 0.05% trypsin-0.02% EDTA solution, confirmed *via* microscopic observation. These cells were then spun down, resuspended in medium, and counted using a hemocytometer. In passage 1 (P1), cells were passaged at a density of 2.0×10^4 cells/cm^2, again co-cultured with mitomycin-C treated feeder cells (at a density of 2.0×10^4 cells/cm^2) on collagen-coated 6-well plates.

In subsequent passages (P2+), after achieving 70–90% confluence, cells were passaged at a density of 2.0×10^4 cells/cm^2. Within P2, two cell culture methods were used. One method was consistent with the method described for P1 (Section 2.2), with epithelial cells and feeder cells co-cultured on a collagen-coated 6-well plate. The second method involved the use of a collagen-coated cell culture insert, where epithelial cells were seeded in the insert, and feeder cells were seeded in the plate below each insert, described in Section 2.4.1 (Fig. 1A).

2.3. Characterization of cultured cells

The following antibodies were used for immunohistochemistry and immunofluorescence: mouse monoclonal anti-pan-Cytokeratin (CK) AE1/AE3 (1:100, Abcam, Cambridge, MA), mouse monoclonal anti-CK 14 (1:100, Abcam), mouse monoclonal anti-CK 13 (1:10, Abcam), mouse monoclonal anti-occludin (1:100, Invitrogen, Camarillo, CA), and mouse monoclonal anti-ZO-1 (1:100, Invitrogen).

2.3.1. Hematoxylin and eosin (H&E) and immunohistochemical staining

P2 cells were cultured on inserts for 14 days and fixed using 10% neutral buffered formalin and embedded in paraffin for H&E and immunohistochemical staining. Five-micron-thick serial sections were prepared in the coronal plane. H&E staining was performed using routine methods. For immunohistochemistry, slides were deparaffinized and antigen retrieval was performed by microwave irradiation using Target Retrieval Solution (Dako North America, Carpinteria, CA) for 20 min. Sections were treated with peroxidase blocking solution (Dako North America) for 20 minutes and blocked in 1% bovine serum albumin for 1.5 h. Sections were incubated with primary antibodies at 4 °C overnight. Secondary antibody incu-

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