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Establishment of Cell Lines from the Human Middle and Inner Ear Epithelial Cells

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David J. Lim and Sung K. Moon

Abstract

The middle ear infection is the most common childhood infection. In order to elucidate the cell and molecular mechanisms involved in bacterial recognition and innate immune response, we have established a stable human middle ear cell line, which has contributed to the current knowledge concerning the molecular pathogenesis of the middle ear infection. The inner ear, a sensory organ responsible for hearing and balance, is filled with inner ear fluid, and disturbance of the fluid homeostasis results in dizziness and hearing impairment. It has been suggested that the endolymphatic sac (ES) may play a critical role in the fluid homeostasis of the inner ear. We have established a stable human ES cell line and are undertaking cell and molecular characterization of this cell line.

2.1 Introduction

The middle ear infection (otitis media) is the most common pediatric infectious disease and is the most common reason for physician's office visits and antibiotic prescriptions [1]. It was estimated to cost the US alone roughly five billion dollars per year for the management of otitis media (OM) in 1996 [2]. However, exact molecular

mechanisms involved in the pathogenesis of OM are poorly understood [3]. In order to elucidate a mechanism involved in bacterial recognition and innate and adaptive immune response, we have established a stable human middle ear cell line, which contributed to the current knowledge concerning the cell signaling involved in the pathogenesis of the middle ear infection.

The inner ear, which contains unique sensory organs responsible for hearing and balance, is filled with lymph fluids such as perilymph and endolymph [4]. The perilymph is characterized by high Na^+ ion, whereas the endolymph is characterized by high K^+ ion [5]. Disturbance of fluid homeostasis results in hearing impairment and vertigo. Particularly, Meniere's disease (MD), characterized by fluctuating hearing loss, tinnitus, and dizziness, is considered to be an example of

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inner ear fluid homeostasis disorders. While the underlying causes of MD are not yet known, it has been suggested that the endolymphatic sac (ES) may play a critical role in the pathogenesis of MD [6, 7]. It has been suggested that the ES may play an important physiologic function to regulate endolymph fluid homeostasis and also may function as an immune organ [8]. In order to elucidate the cell and molecular mechanisms involved in the physiology and pathophysiology of MD, it is critical to establish a stable human ES cell line. We report the progress thus far, which we have made in the characterization of this cell line.

2.2 Functional Morphology of the Ear

2.2.1 Middle Ear Epithelium

The middle ear cavity is connected to the oropharynx through the eustachian tube (E-tube), which provides pressure regulation and aeration of the tympanic cavity and the mastoid cavity (Fig. 2.1). The lining mucosa of the middle ear consists of ciliated cells, secretory cells, nonsecretory cells, and basal cells (Fig. 2.2) [9, 10]. The middle ear epithelial cells vary depending

Fig. 2.1 Schematic diagram of mastoid-E-tube-middle ear complex. Mastoid cavity is continuous to the middle ear cavity, which is connected to eustachian tube which is consisted of bony part and cartilaginous part, where mixed glands are well developed. The cartilaginous E-tube, which is open to the pharynx, is closed by tubal cartilage and opens intermittently to aerate and equalize pressure between middle ear cavity and ambient pressure of the

oropharynx. Poor tubal function is one of the major risk factors for otitis media susceptibility. The lining of the middle ear epithelium is covered by ciliated cells and secretory cells typical of respiratory epithelium. The mastoid cavity is largely covered by simple squamous mucosal epithelium. The eustachian tube is covered by tall columnar epithelial cells consists of ciliated and secretory cells. Adapted from Lim [13]

Fig. 2.2 Middle ear cavity is covered with respiratory epithelium consisting of ciliated cells (*arrowheads*), secretory cells, and nonsecretory cells. Viral upper respiratory

infection destroys these respiratory epithelial cells impairing mucociliary transport system essential for mucosal protection

on the location. Near the eustachian tube orifice, these cells are columnar or cuboidal, and they are becoming flat epithelial cells toward the mastoid cavity. The number of secretory cells is proportional to the number of ciliated cells. In the promontory region, the epithelial cells are largely cuboidal. The eustachian tube consists of two parts: bony parts closer to the tympanic cavity and the cartilaginous part connected to the oropharyngeal opening. This latter part has rich mixed glands. Tubal cartilage is closed most of time, but opens intermittently to equate pressure and aeration. The lining epithelium is largely ciliated and secretory cells and its major function is to transport unwanted particles trapped in the mucous blanket by mucociliary transport system. The tubal dysfunction is believed to be involved with the risk factor for OM in children during the winter months when upper respiratory infections (URI) are prevalent [11]. URI is known to impair or destroy mucociliary epithelium, leading to the

tubal dysfunction. The tubal dysfunction is believed to result in high negative pressure of the tympanic cavity. Therefore, the bolus of mucus containing pathogenic bacteria may enter into the tympanic cavity when the tube attempts to open.

The proportion of the ciliated and secretory cells reflects the history of inflammation in the middle ear, suggesting the middle ear epithelial cell type is in dynamic state [12]. The epithelial cells are also known to elaborate antimicrobial molecules, which constitute the innate immune defense mechanism [3].

2.2.2 Inner Ear

As to the functional morphology of the inner ear, the auditory sensory organ (organ of corti of the cochlea) is a unique sensory structure composed of one row of inner hair cells (IHC) and three (or four) rows of the outer hair cells in mammals

Fig. 2.3 A SEM photomicrograph of surface view of chinchilla organ of Corti showing complexity of cell structures including one row of inner hair cells (IHC) and three rows of outer hair cells (OH1–OH3), inner phalangeal

cells (IPx), outer phalangeal cells (OPx), and three rows of Deiter cells (D1–D3). The sensory cell hair bundles (stereocilia), arranged in “W” formation, are exposed to the endolymph (scala media). Adapted from Lim [14]

(Fig. 2.3) [13–16]. The IHC are considered the primary sensory cells responsible for transmitting electrical signals to the brain, whereas the outer hair cells are responsible for fine tuning (frequency resolution) of the hearing through motile activity of the sensory cells, by regulating their lengths. Electrical stimulation of the outer hair cells induces motor activity through their unique motor proteins (known as Prestin) embedded in the membrane structure [17].

In addition, the sensory organ is bathed in different fluid compartments. The endolymph compartment (scala media) is filled with K^+ ion-rich fluid, whereas the perilymph compartments, composed of scala vestibuli and scala tympani, contain Na^+ ion-rich fluid. The perilymph space is communicating with the CSF through the cochlea aqueduct.

2.2.3 Endolymphatic Sac

The endolymphatic compartment is connected to the blind sac known as ES through endolymphatic duct (ED). The ES is composed of intraosseous and intradural parts. In human, the ES is composed of multiple interconnecting tubules [18], occasionally filled with dense PAS-positive materials [19], which is believed to be the osmotic agent required for fluid volume regulation of the endolymph system. Experimental evidences suggest that ES epithelial cells may secrete these osmotic agents. In addition, the ion transport activities of the ES involve mainly Na^+ and Cl^- ions. The ES epithelial cells express ion channels and cotransporters, such as ENaC, Na^+ - K^+ -2Cl-type 2 (NKCC-2) cotransporter in addition

Fig. 2.4 Epithelial lining of the endolymphatic sac consists of mitochondria-rich light cell (M) and ribosome-rich dark cell (R). There are also considerable variations of luminal cell surface areas among different cells and extent of intercellular space (ICS) indicating various stages of fluid transport. This may represent dynamic states of the

fluid transport (absorption) at any given time. Loose subepithelial connective tissue contains capillaries (arrows) and nerve fibers (not shown), and has extensive network of fibrocyte processes (F) contacting to each other. Insert is a diagram of the endolymphatic sac and a line indicates the plane of section. Adapted from Lim [13]

to SLC26A4, carbonic anhydrase, and $\text{Cl}^-/\text{HCO}_3^-$ exchanger [5, 20, 21].

There are also strong evidences that the ES is the immune organ of the inner ear, containing resident macrophages and lymphocytes [6], and it is suggested to play a critical role in the immune response of the inner ear [22]. The luminal fluid often contains dense glycoprotein substances as well as free floating macrophages. In addition to macrophages, there are reports to indicate the presence of intraepithelial lymphocytes [23].

The ES consists of epithelial cells and loose subepithelial connective tissue, which is continuous with the perilymphatic space of the ED. The ES epithelial cells are composed of relatively flat epithelial cells of two types (Fig. 2.4) [24]. Mitochondria-rich light cell is characterized by a large number of microvilli on its luminal surface,

whereas mitochondria-poor ribosome-rich dark cell has a relatively smooth luminal cell surface (Fig. 2.5). Mitochondria-rich cell is also believed to express Pendrin and carbonic anhydrase based on the studies with immunolabeling [25]. Foxi-1 gene is upstream for Pendrin expression, and Foxi-1-deficient mice develop endolymphatic hydrops and the ES of these animals lack Pendrin-positive (mitochondria-rich) cells [26].

The subepithelial connective tissue contains a large number of blood vessels and accompanying nerve supply. Sympathetic (superior cervical ganglion), parasympathetic (pterygopalatine ganglion), and somatosensory (trigeminal ganglion) innervation is known to be largely involved in regulation of the blood flow and possibly in the fluid absorption of the ES. They are either myelinated or unmyelinated [6].

Fig. 2.5 SEM photomicrograph of ES surface showing two types of epithelial cells: cell with extensive microvilli (M) and cell with smooth surface (S), and free-floating macrophages (Ma) in the luminal surface. The cell with

extensive microvilli is interpreted to be mitochondria-rich light cell and the cell with smooth surface is ribosome-rich dark cell. Adapted from Lim [13]

2.3 Human Middle Ear Cell Line

The middle ear epithelial cells are known to directly interact with the pathogens and pathogen-derived molecules. Since normal human middle ear epithelial cells are not easily obtainable, it is critical to establish a stable human middle ear epithelial cell line that express important genes/gene products involved in the bacteria–host interaction and resultant inflammatory response.

2.3.1 Immortalization

Small pieces of healthy human middle ear mucosa were harvested from the promontory area of the patient during translabyrinthine craniotomy. To induce proliferation of primary cells, explants of human middle ear mucosa were plated on 35-mm plastic culture dishes with a minimal volume of

media, allowing them adhere to the bottom [27]. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. The culture medium used was a 1:1 mixture of bronchial epithelial growth media (BEGM; Clonetics, Walkersville, MD) and Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Gaithersburg, MD), containing hydrocortisone (0.5 µg/mL), insulin (5 µg/mL), transferrin (10 µg/mL), epinephrine (0.5 µg/mL), triiodothyronine (6.5 µg/mL), gentamycin (50 µg/mL), and amphotericin B (50 ng/mL), all supplied by Clonetics (Walkersville, MD), and further supplemented with EGF (25 ng/mL; Collaborative Research, Bedford, MA), all-trans retinoic acid (5 × 10⁻⁸ M; Sigma, St. Louis, MO), bovine serum albumin (1.5 µg/mL; Sigma), and bovine pituitary extract (1% v/v; Pel Freez, Rogers, AR).

For immortalization, we infected cells using a retrovirus containing the E6/E7 genes of human papillomavirus type 16 [28]. Briefly, the PAS 17 amphotropic packaging cell line, stably transfected

with a replication-defective retrovirus construct (pLXSN16E6-E7), coding for HPV type 16 transforming oncoproteins E6 and E7, was grown to 70% confluence, and supernatants were collected after 24 h. Primary human middle ear epithelial cells (passage 3 at 50% confluence) were infected with 1 mL of virus stock diluted in 9 mL of fresh BEGM for 48 h. The medium was replaced with fresh BEGM and the cells were allowed to proliferate. For selection, cells were reseeded in fresh BEGM containing 0.4 mg/mL of G418 (Gibco BRL) and were kept in the selection media for 14 days. Multiple colonies were isolated using cloning rings and each clone was expanded for further characterization. One of clones appeared to be stably immortalized and was designated as HMEEC-1. The average doubling time of the HMEEC-1 was 23.8 h and appeared to preserve characteristics of epithelial cells such as expression of pan-cytokeratin, dome formation, and anchorage dependency. In addition, subcutaneous injection of HMEEC-1 cells to the nude mice did not result in tumor formation. Karyotypic analysis confirmed the immortalized cells are derived from male humans and have no major abnormality of chromosomes.

2.3.2 Major Research Findings Resulting from the Human Middle Ear Cell Line

2.3.2.1 Secretion of Mucins

The sterility of the eustachian tube and the middle ear is maintained not only by the adaptive immune system, but also by the innate immune system such as mucociliary system and the antimicrobial molecules [3]. Mucins are high molecular weight glycoproteins that constitute the major component of mucus secretions in the eustachian tube and middle ear. The core proteins are encoded by different mucin genes (MUC genes); and we showed the expression of MUC genes such as MUC1, MUC2, MUC5AC, and MUC5B in the primary human middle ear epithelial cells [29]. Moreover, we demonstrated that HMEEC-1 cells up-regulate MUC5AC expression in response to cytoplasmic proteins of nontypeable *Haemophilus influenzae*

via a p38 MAP kinase signaling pathway [30], which is negatively regulated via the transforming growth factor beta-Smad signaling pathway [31]. In addition, we showed that HMEEC-1 cells up-regulate MUC2 expression through the cooperation of transforming growth factor-beta-Smad signaling pathway and NF- κ B activation [32].

2.3.2.2 Induction of Antimicrobial Agents

To defend against invading pathogens, the tubotympanum is known to secrete antimicrobial molecules including lysozyme, lactoferrin, and beta defensins [3]. The beta defensins are mainly produced by epithelial cells of the skin, kidneys, and respiratory lining of nearly all vertebrates [33]. The beta defensins are released upon microbial invasion and are located at the host–environment interfaces, such as mucosal surfaces and skin. Beta defensin 2, which is released from the epithelial cells in response to microorganisms or cytokines, exhibits potent antimicrobial activity against gram-negative bacteria and candida. HMEEC-1 cells were found to up-regulate beta defensin 2 in response to interleukin 1 alpha (IL-1 α) via a Src-dependent Raf-MEK1/2-ERK signaling pathway [34]. We also found that nontypeable *H. influenzae*-induced up-regulation of beta defensin 2 requires a TLR2/MyD88-dependent p38 MAP kinase pathway [35]. As shown in Fig. 2.6, we demonstrated that IL-1 α is secreted by middle ear epithelial cells upon exposure and that it can synergistically act with nontypeable *H. influenzae* molecules to up-regulate beta defensin 2 via the p38 MAP kinase pathway [36].

2.3.2.3 Expression of Toll-Like Receptors

Toll-like receptors (TLRs) have been suggested to play a critical role in the recognition of various bacterial components such as lipoprotein, peptidoglycan, lipoteichoic acid, and lipopolysaccharide [37]. We found that HMEEC-1 cells regulate TLR2 expression and that glucocorticoids synergistically enhance nontypeable *H. influenzae*-induced TLR2 expression via a negative cross-talk with p38 MAP kinase [38]. Moreover, we demonstrated that TLR2 is involved in nontypeable

Fig. 2.6 Schematic illustration summarizes signaling pathways involved in the individual and synergistic effects of IL-1 alpha and nontypeable *H. influenzae* (NTHi) on beta defensin 2 (DEFB4) transcription in the middle ear epithelial cells

H. influenzae-induced NF- κ B activation through the TAK1-dependent NIK-IKK-I κ B and MKK3/6-p38 MAP kinase signaling pathways [39]. In addition, we found the involvement of TLR2 in the recognition of nontypeable *H. influenzae* molecules, resulting in induction of beta defensin 2 [35].

2.4 Human Endolymphatic Sac Cell Line

Because of the location of the ES deep inside the petrosal bone and the difficulty of obtaining epithelial cells, experiments using the human ES epithelial cells have been difficult to perform. Recently, we have successfully developed a human ES cell line preserving the characteristics of normal ES epithelial cells such as the expression of Pendrin.

2.4.1 Immortalization

Primary cell culture preparation of ES epithelial cell has been described [40]. Briefly, human ES

were excised during translabyrinthine acoustic neuroma surgery at Uppsala University Hospital with an institutional approval. The intraosseous portion of the human ES was drilled out, leaving a thin, movable eggshell layer of bone on its anterior surface. The human ES was separated from the posterior bony surface with a mucosal knife and was cut with a pair of scissors at the external aperture of the vestibular aqueduct. Thus, only the intraosseous portion of the human ES was retained. The sample was then cut into small pieces ($<1 \text{ mm}^3$) and incubated at 4°C in 0.17% trypsin in PBS for 15–18 h. The resulting suspension of single cells and small aggregates were transferred to a test tube, and an equal volume of growth medium was added. The cells were centrifuged for 5 min at 1,000 rpm, resuspended in growth medium, and seeded into a 35-mm diameter fibronectin and collagen coated tissue culture dish. The cells were grown in a 3:1 mixture of (DMEM) and Ham's F12 medium (Gibco BRL) supplemented with 10% fetal calf serum (FCS) (Hyclone), $0.4 \mu\text{g/mL}$ hydrocortisone (Sigma), $10 \mu\text{g/mL}$ human epidermal growth factor (EGF) (Austral Biologicals), 10^{-10} M cholera toxin

(Sigma), 5 µg/mL Zn-free insulin (Lilly Research Laboratories), 24 µg/mL adenine (Sigma), and 2×10^{-9} M 3,3,5-triiodo-L-thyronine (Sigma).

For immortalization, primary ES epithelial cells were infected using a retrovirus containing the E6/E7 genes of human papillomavirus type 16 as described [28]. Infected cells were selected using the G418-containing medium and multiple colonies were isolated. Each clone was expanded for the further characterization, and one clone showing stable immortalization was designated as HESC-1. HESC-1 cells appeared to preserve the characteristics of epithelial cells such as expression of ZO-1 and cytokeratin. Karyotypic analysis demonstrated that the immortalized cells have no major abnormality in chromosomes. Although HESC-1 cells were originated from a single cell, they appeared to differentiate into two distinct subtypes, resembling ribosome-rich dark cells and mitochondria-rich light cells of the ES epithelial cells. Immunolabeling and FACS analysis showed that there are two subpopulations according to the expression of Pendrin and richness of mitochondria.

2.4.2 Research Findings Resulting from the Human Endolymphatic Sac Cell Line

Secretory capacity of the ES has been postulated by some authors [41–43]. In about 30% of the cross-sections of the mouse ES, a small amount of homogeneous substance was observed. This homogeneous substance is believed to contain acidic protein-bound carbohydrates [44], proteoglycan [45], sulfur compounds [46], hyaluronan [47], and soluble megalin [48]. Although the function of this homogeneous substance is unclear, it is suggested to be involved in the attraction of water and small water soluble cations, which could influence the regulation of fluid volume of the inner ear endolymphatic compartment [45].

Unexpectedly, we found that our HESC-1 cells secrete viscous substance in response to EGF. Dot blot analysis showed that the supernatant of HESC-1 cells contains mucin core proteins and hyaluronan. qRT-PCR analysis demonstrated that HESC-1 cells up-regulate mucin core proteins and

hyaluronic acid synthases in response to EGF. Further studies are needed to determine the regulatory mechanisms involved in secretion and biological functions of these molecules. We anticipate that our HESC-1 cells will provide an in vitro model for the studies of secretory function of the ES.

2.5 Conclusions

We have successfully immortalized epithelial cells from human middle ear and ES. The human middle epithelial cells have been used for elucidating cell signaling pathways involved in inflammatory responses including induction of beta defensins and mucin genes. The human ES cell line is in the process of further characterization. Preliminary results show the expression of genes involved in water and ion transportation, such as Pendrin and aquaporins. Particularly, the human ES cell line was found to secrete osmotically active substances, and further studies are necessary to identify these molecules and determine their function in the regulation of endolymphatic fluid volume.

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