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Morphological changes in the round window membrane associated with *Haemophilus influenzae*-induced acute otitis media in the chinchilla

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ABSTRACT

Objective: The round window membrane (RWM) encloses the round window, the opening into the scala tympani (ST) from the middle ear. During the course of acute otitis media (AOM), structural changes of the RWM can occur that potentially affect sound transmission into and out of the cochlea. The relationship between such structural changes and conductive hearing loss during AOM has remained unclear. The focus of the current study was to compare the thickness distribution across the RWM surface between normal ears and those with AOM in the chinchilla. We assessed the occurrence of AOM-associated histological changes in this membrane compared to uninfected control animals after AOM of two relatively short durations.

Material and methods: AOM was induced by transbullar injection of the nontypeable *Haemophilus influenzae* strain 86-028NP into two groups of adult chinchillas (n = 3 each). Bullae were obtained from the two infected groups, at 4 days or 8 days post challenge. Structures and thickness of these RWMs were compared between the two infected treatment groups and to RWMs from uninfected control animals (n = 3) at seven different RWM locations.

Results: RWM thickness in infected chinchillas increased significantly at locations along the central line on the 4th day post bacterial challenge compared to values found for uninfected control animals. Lymphocyte infiltration and edema were the primary contributors to these thickness increases. No significant further increases in RWM thickness were observed when RWMs from chinchillas ears infected for 4 and 8 days were compared. Thickness and structural changes at the RWM lateral and medial areas were less visually obvious and not statistically significant among the three treatment groups. These latter RWM regions clearly were less affected during AOM than the central areas.

Conclusions: This histological study establishes that *H. influenzae*-induced AOM causes significant acute changes in chinchilla RWM structure that are characterized by region-specific increases in thickness. Our new morphological findings comparing normal and diseased chinchilla RWMs identify yet another biomechanical mechanism by which nontypeable *H. influenzae* may contribute to hearing loss in AOM.

1. Introduction

The round and oval windows are the two openings from the middle ear that directly connect the middle ear and cochlea. The round window membrane (RWM) covers the round window and vibrates with a phase opposite to the vibration of the stapes at the oval window. The structure of the RWM consists of three layers: an outer epithelial layer facing the middle ear, a core comprised of connective tissue, and an inner epithelial layer facing the scala tympani (ST) [1–5]. Compliance of the RWM affects its ability to be displaced by fluid inside the cochlea, which has a major impact on the transmission of sound into the cochlea [1,4,6,7]. Previous studies have revealed that the RWM also functions as a channel for the transmission of specific substances between the middle ear and the cochlea [1–5,8]. The mechanical properties and permeability of the RWM are closely associated with its morphology, especially the

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thickness of the membrane [6,7].

Acute otitis media (AOM), the most commonly diagnosed disease among young children, is typically caused by bacterial invasion of the middle ear and can result in temporary or permanent hearing loss. Structural changes in the soft tissues of the middle ear, e.g. the tympanic membrane (TM), the stapedial annular ligament and the RWM, contribute significantly to conductive hearing loss induced by AOM [9–13]. A number of published studies have described the morphology of both the healthy and diseased RWMs. These studies include ones involving humans [14,15] and various clinically relevant animal models such as cats [16,17], chinchillas [18–23] and guinea pigs [11,24,25]. However, the orientation of most of these studies is largely histopathological. There are no published data describing the quantitative measurement of the thickness distribution across RWM surfaces in either normal or AOM ears. The occurrence of thickness changes in the RWM during the course of AOM remains undescribed. Since the stiffness and permeability of the RWM are likely to be dependent on its thickness, a systematically obtained data set describing the thickness distribution of both normal and diseased RWMs is crucial for detailed biomechanical analyses of RWM structure-function relationships [6,7].

The broad aim of the present study was to begin to fill this important gap in knowledge relevant to hearing loss during acute otitis media caused by the bacterium, *Haemophilus influenzae*. Nontypeable (acapsular) *H. influenzae* is now the most frequent cause of acute otitis media. Previously our research team has investigated the mechanism by which this bacterium alters the microstructure and functions of middle ear soft tissues in the chinchilla model of AOM [12]. Recently we described the occurrence of significant *H. influenzae*-induced microstructural changes in the chinchilla TM. Such changes included inflammatory cell infiltration into the membrane, cell hyperplasia and edema [13]. As an extension of the previous research, the focus of the present study was to describe how *H. influenzae*-induced infection of the middle ear alters the structure of the RWM.

2. Material and methods

2.1. The chinchilla AOM model

Nine adult chinchillas (*Chinchilla laniger*) of mixed gender weighing 600–800 g were utilized in this study. These animals were purchased from a USDA approved vendor, Moulton Chinchilla Ranch, Rochester, MN. The protocol for the experiments was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma and met the guidelines of the National Institutes of Health. All animals were examined by otostoscopy before infection to assure that their ears were free from disease prior to administration of nontypeable *H. influenzae*.

Animals were divided into three groups (3 animals for each): untreated control, AOM of 4 days post bacterial challenge, and AOM of 8 days post challenge. AOM was established by bilateral transbullar injection of nontypeable *H. influenzae* strain 86-028NP (a low passage number human clinical isolate) suspended in sterile phosphate buffered saline. The bacterial culture protocol followed the procedure described by Morton et al. [26]. Under general anesthesia (10 mg/kg ketamine and 2 mg/kg xylazine), 0.3 ml of a bacterial suspension containing 3000 CFU of *H. influenzae* was injected into the superior bulla with a 1 ml syringe equipped with a 26 gauge needle. After the bacteria challenge dose was administered, daily otoscopic examination and animal body temperature measurements were conducted until the animals were euthanized (by Euthasol injection). The uninfected control group was euthanized in the same manner.

2.2. Histological preparation

On either the 4th or 8th day post bacterial challenge, animals were deeply anesthetized by an overdose of ketamine (100 mg/kg) and xylazine (20 mg/kg). Then a multiple-frequency tympanometer (MAICO MI24, MAICO Diagnostics, Eden Prairie, MN) and an otoscope were used to confirm the presence of AOM in the two infected groups of animals and that the ears of control animals remained uninfected. All animals, including the untreated controls, were then perfused intracardially with fixative (4.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2) [27]. The bullae were harvested immediately and then were opened widely. The cochlear apex and the middle ear superior and inferior bony walls were removed to fully expose the RWM to fixative. The bullae then were immersed in fixative overnight at room temperature. On the following day, the bullae were removed from the fixative, placed into phosphate buffered saline solution, and subsequently washed in phosphate buffered saline. These specimens then were decalcified with 10% ethylene diamine tetra acetic acid (EDTA) for 8–10 days. The EDTA solution was replaced daily with fresh solution. When calcium was no longer discernable in the EDTA wash solution, the decalcification process was terminated. Then the ossicular chain of the bulla was separated at the incudostapedial joint with a #11 surgical blade. The cochlea, with its intact RWM, was dissected from the bulla. Thereafter, specimens were dehydrated in a graded series of ethanol solution and subsequently were decolored in xylene.

2.3. Specimen embedding and sectioning

Upon completion of dehydration step, the RWM specimens were removed from xylene, placed into molten paraffin at a temperature of 54 °C, and incubated at this temperature overnight. On the following day, these specimens were immersed in fresh molten paraffin to remove any remaining xylene. The RWMs then were embedded in new paraffin at an orientation such that the plane of sectioning was approximately perpendicular to the short axis of the RWM and parallel to the long axis. After the paraffin solidified, each specimen was sectioned (8 μm in thickness) from the lateral end to the medial end. These sections were mounted sequentially on glass slides, stained with hematoxylin-eosin, and coverslips were applied.

2.4. Image selection, thickness measurement, and statistical analysis

A Nikon E-800 optical microscope was used to examine and photograph the histologic sections. The microscope was calibrated with a standard calibration slide before measurements were made. Particular attention was given to the thickness of the RWM. Initially, an overview of RWM was captured at lower magnification. The orientation and position of seven target sites used for our membrane thickness measurements are indicated in Fig. 1A. A central line of the RWM from the anterior to posterior (Fig. 1A) was determined by identifying the histologic section that had the longest membrane cross section. Five of the seven locations (A2, A1, O, P1, and P2 shown in Fig. 1A), starting from the anterior region and proceeding to the posterior region of the round window, were evenly distributed along the central line. Locations L and M were selected 0.2 mm laterally and medially, respectively to the center point O. Membrane thickness at each location was determined by averaging the measured values of 4 nearby points within an approximate distance of 150 μm as shown in Fig. 1B. At each of the noted sites or locations, one-way AONVA and Tukey's Honest Significance Tests were used to determine whether the thickness