Ultrastructural Characteristics of the Round Window Membrane During Pneumococcal Otitis Media in Rat

To understand better the pathogenesis of inner ear (IE) damage caused by otitis media (OM), the round window membrane (RWM) structure was investigated in a rat model for pneumococcal otitis media (POM). The RWM of 25 rats were evaluated light and electron microscopically on 1 day, 3 days, 6 days, 10 days, and 20 days after the unilateral inoculation of type 3 pneumococcus suspension into their middle ear cavities. The thickness of the RWM increased in various stages of the pneumococcus-evoked otitis media, compared with that of the normal. The thickening was most pronounced on day 1, being about 4 to 5 times greater than that of the normal RWM. All layers of the RWM were affected by the pneumococcal infection, but the major changes were confined to the subepithelial space close to the basement membrane (BM). Together with alterations to the BM, the most distinct pathological features were characterized by an increase and hypertrophy of fibroblasts in association with abundant collagen fibers. Elastic fibers observed close to the inner mesothelial layer under a high power magnification also increased during the experiment. These results will be relevant to a better understanding of the histologic implication of RWM in stages of acute otitis media involving pneumococcus-evoked otitis media.

Key Words : Round Window Membrane; Pneumococcal Otitis Media

Yong-Joo Yoon, Sten Hellstrom*

Department of Otorhinolaryngology Chonbuk
National University Hospital, Chonju, Korea;
Department of Otorhinolaryngology*, University
Hospital, University of Umea, Umea, Sweden

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Address for correspondence

Yong Joo Yoon, M.D.
Department of Otorhinolaryngology Chonbuk
National University Hospital, 634-18 Knam-dong,
Chonju 560-180, Korea
Tel : +82.63-250-1988, Fax : +82.63-250-1986
E-mail : yjyoon@moak.chonbuk.ac.kr

INTRODUCTION

The round window membrane (RWM) located in the posterior inferior aspect of the medial tympanic wall deep inside the bony niche suggesting its unique function in the proximity of the inner ear (IE). This niche protects the RWM direct sound transmission and physical trauma, but also poses a threat to the IE, as noxious materials accumulated in the niche may traverse the soft tissue diaphragm, the RWM.

In sound transmission, the RWM acts as a relief organ for the waves of the cochlear fluid evoked by stapedial vibration. In addition, RWM might play a role in absorption and secretion between the middle ear (ME) and IE, and acts as a route for oxygenation of the IE tissue from the ME (1, 2). The RWM is readily permeable to many biological substances and may act as a port of entry for noxious substances from the ME into the IE, resulting in pathological changes in the IE (3, 4).

Histologically, the RWM consists of three basic layers: outer epithelial layer, middle connective tissue layer, and inner mesothelial layer (1, 5, 6). This triple-layered RWM, called secondary tympanic membrane (TM) is very similar to that of the TM. Interestingly, morphological characteristics of the pars flaccida in particular, markedly resemble those of the

RWM (7, 8), suggesting that they may have shared certain functions such as pressure equilibration. The epithelial layer contain metabolically active cells and the fibroblasts of the middle layer may play an important role in production of collagen fibers and of various active substances, which may be involved in the IE defense system (9).

In inflammatory conditions in ME, there are significant changes in the thickness and morphological characteristics of the RWM. Such alterations may affect the permeability of the RWM, which is an important barrier between the ME and the IE (10, 11). The ME in the rat and man share many similar structural characteristics. Recently, it was also shown that the reaction of the rat ME to *Streptococcus pneumoniae* closely resembles that in the human ME (12). This animal model for purulent otitis media has been utilized in studying the effects of pneumococci on the ME mucosa as well as the outcome of various therapeutic measures (13).

In the present study, we examined the fine structure of the RWM in the different stages of the pneumococcus-evoked otitis media model using rats. This will serve as a structural basis for a continuing studies on the role of the RWM for the protection of IE function in ME infections.

MATERIALS AND METHODS

Healthy male Sprague-Dawley rats (n=25) weighing 200–250 g were used for the study. All animals exhibited normal TM status. The rats were subjected to anesthesia with an intravenous injection of sodium methohexital (Brietal®), followed by an intraperitoneal injection of sodium pentobarbital (Mebumal®). Under an otomicroscope, a ventral vertical midline incision was made in the neck and the tympanic bullae were exposed bilaterally. Through the hole in the bulla, the left ME was inoculated with 0.05 mL suspension of type 3 *Streptococcus pneumoniae*. The right ear served as the control and was inoculated with culture medium only.

The animals were inspected daily under the otomicroscope throughout the experiment. They were divided into 5 rats in each. The groups of animals were killed on 1, 3, 6, 10, and 20 days after inoculation, respectively. Before sacrifice, the TM status was examined and carefully recorded. After an intraperitoneal overdose of Mebumal®, and their animals were decapitated, the temporal bones were roughly trimmed and then fixed by immersion in a solution containing 3% glutaraldehyde in a 75 mM sodium cacodylate buffer with 4% polyvinylpyrrolidone and 2 mM CaCl_2 was added. The temporal bones were then carefully dissected and further fixed for 48 hr in the same fixative solution. After several rinses in buffer, the specimens were transferred to a 10% EDTA solution and decalcified in a microwave oven (Miele M696), at a setting of 150 W, 35°C, until softened (14).

The round window niche with the RWM was now cut out in a single block, which then was dehydrated in a graded series of ethanol to xylene and embedded in an epoxy resin. The tissues were then cut with an ultramicrotome in 1 μm sections for light microscopic examination. The tissue sections were stained with toluidine blue, mounted, and examined under the light microscope. Ultrathin sections (70 nm) were cut in an ultramicrotome and stained with uranyl ace-

Fig. 1. The surface is curved as a cone with its apex directed towards the scala tympani. $\times 50$. MEC, middle ear cavity; ST, scala tympani.

tate and lead citrate for transmission electron microscopy. The specimens were studied under JEOL 1200 electron microscope.

RESULTS

The thickness of the RWM

The control RWM had a convex shape facing the scala tympani (Fig. 1). It was thickened at the edge, due to a sudden increase in the middle connective tissue layer. In the central portion of control RWM, the mean thickness was $11.5 \pm 2.1 \mu\text{m}$ (n=25).

In the pneumococcus-inoculated ears, the RWM was thickened considerably. The average thicknesses of each group are summarized in Fig. 2. The change in the thickness of the RWM in pneumococcal otitis media (POM) was most pronounced on day 1 when the thickness was 4 to 5-fold greater than that of the control. The alterations were confined mainly to the central portion of the RWM (Fig. 2, 3).

RWM in Pneumococcal Otitis Media

Day 1: All layers of the RWM were markedly thickened, especially in the central part of the RWM. The most prominent thickening was confined to the subepithelial space immediately below the outer epithelial layer. Abundant inflammatory cells, mainly macrophages but also polymorphonuclear neutrophils (PMNs) were observed in this area (Fig. 4). Macrophages were also observed within the outer epithelial layer (Fig. 4). The surface of the outer epithelial cells exhibited an increased number of microvilli, especially at the periphery of the cells. The cytoplasm of the epithelial cells showed an

Fig. 2. The kinetics of average thickness (μm SD) of the rat RWM in controls (n=25) and on days 1, 3, 6, 10, and 20 in pneumococcus-induced otitis media (n=20).

extensive vacuolization. Most of the nuclei of the outer epithelial cells appeared pale and swollen, with a dark outline. Mitochondria, Golgi complex, and endoplasmic reticulum were increased in the dark granulated cells (6). The outer epithelium exhibited widened intercellular spaces, and the epithelial junctions were often widened to some extent. The enlarged intercellular space was most pronounced towards the basement membrane (BM). The BM showed distinct changes compared with controls and was widened and partly interrupted. When in contact with inflammatory cells, the BM was folded (Fig. 4). The fibroblasts were increased in numbers and accumulated close to leukocytes in the connective tissue layer. The inner epithelium was not so thick as the outer epithelium (Fig. 3).

Day 3: The RWM showed severe changes, characteristic of inflammation in the deep connective tissue layer with edema, invasion of inflammatory cells and an increased vascularization. The thickness of the RWM was slightly decreased compared with that of the day 1, in spite of the pronounced in-

flammatory changes (Fig. 5). Inflammatory cells were observed among the outer epithelial cells, as well as in the subepithelial space. Many of the epithelial cells were increased in size. The cytoplasm of outer epithelial cells showed an increased electron density of the ground substance. Swollen mitochondria were observed. Adjacent to the BM, the epithelial cells exhibited small round clear vacuoles scattered in the cytoplasm. Towards the BM, the epithelial cells showed complicated infoldings and cytoplasmic processes. The BM seemed thickened but the uninterrupted BM was noticeable in this group. The connective tissue layer contained abundant hypertrophic fibroblasts with a marked increase in the cytoplasm, whereas fibrocytes seemed to be slightly decreased in number. The purulent effusion in the tympanic cavity was diminished and had fewer inflammatory cells than on the day 1 (Fig. 5).

Day 6: Most specimens showed a general thickening of the outer epithelial layer and an increased vascularization of the subepithelial tissue. PMNs were rarely observed. Macro-

Fig. 3. Light microscopic changes in the thickness of the RWM stained for toluidine blue on normal control (A) and day 1 in pneumococcus-induced otitis media (B). The change was most prominent on day 1, which was most evident in the outer epithelium and subepithelial space of the RWM. $\times 200$. MEC, middle ear cavity; ST, scala tympani.

Fig. 4. Electron micrographs of RWM on day 1 of pneumococcus-induced otitis media. (A) The most prominent thickening is confined to the subepithelial space immediately below the outer epithelium. $\times 1,000$. (B) Abundant inflammatory cells, mainly of macrophages (M) but also PMNs (P) are present in the outer epithelial layer and subepithelial space. The BM (small arrow heads) shows distinct changes including widening, and is deeply folded and partly interrupted (short thick arrow), $\times 2,500$.

phages, evident in the outer epithelial and connective tissue layer on day 3, now appeared enlarged and filled with phagocytosed material. The epithelial cells, varying in size, exhibited a cytoplasm rich in dense granules. The fibroblasts seemed larger with an increased volume of cytoplasm. Collagen fibers had a less irregular distribution in the subepithelial layer and occurred at a higher density close to fibroblasts, but not in the vicinity of fibrocytes (Fig. 5). Although the outer and middle layers were less thick than on the days 1, 3, and 6, the thickening of the inner mesothelial layer still remained. Discontinuity in the mesothelial cell layer was remarkable, suggesting that the perilymph may communicate directly with the middle connective tissue. There was no longer purulent effusion in the tympanic cavity (Fig. 5).

Day 10: The structure of the RWM now started to regain its normal characteristics (Fig. 5). However, in the outer epithelium, ciliated epithelial cells appeared in the peripheral part of the RWM close to the junction with the ME mucosa. The mitochondria of epithelial cells showed a marked swelling. This layer still showed an increased number of intracellular vacuoles and enlarged intercellular spaces.

In the deep part of the connective tissue layer, the inflammatory cell infiltration was still observed. In contrast to the earlier observations, the number of macrophages was reduced.

PMNs were occasionally found in the subepithelial space. The inner mesothelial cells had a large nucleus and contained many dark granules in their cytoplasm.

Day 20: On day 20, the structure of the RWM appeared almost normalized except for a minor thickening of the subepithelial tissue and the deep connective tissue layer. The thickened BM still remained. The connective tissue layer was less rich in cells but had denser bundles of collagen fibrils. The elastic fibres in the connective tissue layer were more pronounced at this stage of the experiment than any other earlier observations. In particular, close to the inner layer, accumulations of elastin fibrils were observed (Fig. 6).

DISCUSSION

Inoculation of *Streptococcus pneumoniae* into the ME had an immediate effect on the epithelial lining facing the ME cavity, but also caused more slowly developing changes in the connective tissue layer. In fact the interface area between the outer epithelium and the connective tissue layer showed striking changes including an interrupted BM, which could significantly affect the permeability of the RWM. Various animal experiments have led to the assumption that RWM response

Fig. 5. Electron micrographs of RWM in the pneumococcus-induced otitis media. (A) third, (B) sixth, (C) tenth days. $\times 1,500$. MEC, middle ear cavity; ST, scala tympani.

Fig. 6. Electron micrographs of the RWM on day 20 pneumococcus-induced otitis media. The elastic fibers (E) in the connective tissue layer become most prominent especially close to inner mesothelial cells (white asteriks) under high power view on day 20. The connective tissue layer is less rich in cells but had dense bundles of collagen fibers (C). $\times 12,000$.

and permeability to ME inflammatory conditions can vary according to the different stages of acute otitis media (AOM) (15). Researchers have tried to understand the functional significance of the RWM as a port of entry from the ME to the IE. Various kinds of materials instilled into the ME cavity. ME inflammatory conditions, and the presence of bacterial toxin have all been shown to induce morphological and functional alterations in the RWM (15, 16).

Albumin, which normally does not pass the RWM, will traverse the RWM under inflamed conditions (10). In experiments testing the RWM permeability, these large molecules had difficulty in traversing the connective tissue layer, thus suggesting the involvement of fibroblasts in the permeability control mechanisms of the RWM.

Streptococcus pneumoniae is the most common bacterial species isolated from patients with AOM. The condition resolves spontaneously within 10–14 days. In the present study, a rat model was used to elucidate the morphological events of the RWM in pneumococcal AOM. The rat AOM model developed by Hermansson et al. (12) is well suited for studies of AOM because of the close resemblance of the clinical course of the pneumococcal infection between man and rat. A significant difference in the thickness of the RWM was observed between five groups. In the early stage of infection, there was a marked widening of the subepithelial space and hypertrophy of the outer epithelial cells. The thickened RWM may act as an effective barrier protecting the IE from harmful agents, such as bacterial products and inflammatory mediators. Ikeda et al. (15) reported a reduced permeability reaction of the RWM to tetraethylammonium ions in the convalescent stages of AOM. It is assumed that in convalescent stages of AOM, the RWM permeability is reduced, but that the permeability could be increased in the acute stage of inflammation. Proliferation of the secretory epithelium and production of dense granules in the proximity of the apical surface in the cytoplasm in the pneumococcus-induced otitis

media may suggest an increased activity of epithelial cells against inflammation. Numerous PMNs and macrophages suggest that the increased RWM permeability in the acute stage may be attributed to bacterial toxins and/or such as cytokines, fibronectin, elastase, and peptides derived from these cells (17, 18).

The BM may be of physiological importance with regard to the permeability as it may act as a filter between the epithelial layer and the connective tissue layer (19). The widening and multiple foldings of the BM during the infectious condition may indicate strengthened barrier mechanism against bacteria and its byproducts.

Fibroblasts and collagen fibers, the principal elements of the connective tissue layer, may constitute a second line of defence. The elongated cells, arranged in a radial fashion, are in contact with one another and form a network. In inflammatory conditions and following physical trauma, the fibroblasts expand. We consider it of utmost importance to clarify the role of the fibroblasts in the permeability mechanisms of the RWM.

In contrast to the collagen fibers, elastic fibers are sparse in the normal rat RWM. The elastic fibers could not be distinguished under the light microscope, but were detected by the electron microscope. The elastic fibers were shown to be located predominantly in the region close to the inner mesothelial layer. It may be assumed that the area of connective tissue layer close to the inner mesothelial layer could be associated with the perilymph wave and thus involve elasticity. The high density of elastic fibers toward the mesothelial layer in the convalescent stage of POM suggested that they may participate in the barrier function of the RWM in the later stage of inflammation. With the progression of inflammation, the elastic fibers became progressively easier to visualize in the connective tissue layer. The connective tissue layer of the RWM could be considered the most important layer in aural physiology, as it permits movements of IE fluid, yet maintaining the constant shape and tension. Elastic fibers stretch easily, and when released after stretching, they return almost completely to their original length. The strength and elasticity of the RWM are preserved by combination of these fibers. On the other hand, elastic fibers may lose their innate characteristics by elastase (18) secreted from PMLs in the AOM resulting in a sensori-neural hearing loss. However, there is little information so far in this area.

The inner layer of the RWM is composed of mesothelial cells and lacks both microvilli and BM. Lim described that there are numerous underlying micropores and holes for free communication of perilymph between surrounding perilymphatic tissue and the scala tympani (spiral ligament, basilar membrane, and osseous spiral lamina), but could not point them out in the RWM (2). This indicates an interchange between the RWM and perilymph regarding absorption or secretion, and suggests the possibility of presence of similar micropores and holes on the inner epithelial surface of the

RWM.

In summary, alterations in the RWM structure were shown to occur in pneumococcus-induced ME inflammation. Increased knowledge of these dynamic changes in the RWM structure will improve our understanding of how substances pass from the ME to the IE. Further investigations are necessary to clarify the functional significance of these structural changes using more advanced methods in the various pathological conditions.

The thickening was most pronounced on day 1, being about 4 to 5 times greater than that of the normal RWM. All layers of the RWM were affected by the pneumococcal infection, but the major changes were confined to the subepithelial space close to the BM. Together with alterations in the BM, the most distinct pathological features were characterized by an increase and hypertrophy of fibroblasts in association with abundant collagen fibers. Elastic fibers observed close to the inner mesothelial layer also increased during the experiment.

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