Chapter 8

Ultrastructure of the endolymphatic sac in two-phase endolymphatic hydrops in the guinea pig

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Submitted for publication
Introduction

Endolymphatic hydrops is generally believed to be one of the main features of Menière’s disease, an inner ear disorder accompanied by fluctuating hearing loss, vertigo and tinnitus. Hydrops may arise as a result of an imbalance between production and absorption of endolymph. In the animal model of Kimura and Schuknecht, an experimental hydrops was obtained after surgical obliteration of the endolymphatic sac (ES), which is thought to play an important role in the regulation of the endolymph composition by way of absorption and, possibly, secretion. However, obliteration of the vestibular aqueduct is regarded as a nonphysiological model for Menière’s disease. In patients with Menière’s disease endolymphatic sac tissue still remains present, although the size of the ES is reduced, suggesting a reduction in resorptive capacity.

The production of endolymph is thought to be regulated by Na/K-ATPase in the marginal cells of the stria vascularis of the cochlea, as well as in the dark cells of the utricle and the cristae ampullares of the semicircular canals. In recent experiments, a relationship between circulating adrenal steroids and Na/K-ATPase activity in the inner ear was observed. Emotional stress leads to the activation of neuroendocrine effector systems, including the production of adrenal steroids such as aldosterone, and could thus increase the production of endolymph. A borderline capacity of the ES, in combination with a periodic increase of endolymph production caused by stressful situations may be responsible for the development of Menière’s disease. Indeed, manifestations of Menière’s disease frequently occur during stressful experiences in patients with physiological systems under challenge due to a neurasthenic psychological profile.

Our aim is to induce a mild decrease in absorption capacity, combined with a transient increased production of endolymph, to create a more physiological model for Menière’s disease. Hence, we developed the two-phase endolymphatic hydrops model, in which, on the one hand, the distal ES is dissected from the sigmoid sinus, leaving the intermediate part intact, and, on the other hand, the production of endolymph is temporary stimulated through systemic administration of aldosterone. Our model, which is discussed in more detail in an earlier paper, allows research on the entire inner ear, including the intermediate part of the ES, under experimental hydrops conditions. In this paper we describe the ultrastructure of the intermediate ES after dissection of the distal part and/or after administration of aldosterone, in order to obtain more insight into the precise effects of our model.
Materials and methods

In 5 female albino guinea pigs (Harlan, the Netherlands), weighing about 300 grams, the extraosseous part of the endolymphatic sac of the left ear was dissected from the sigmoid sinus. The right ear served as a control. After three weeks, three of the animals received a once-daily intraperitoneal injection of 1 ml aldosterone in a dose of 100 µg/100g (Sigma #A-6628) for five days. Four different groups of ears were created: ears without treatment, aldosterone-treated ears, dissected ears, and ears with a combination of dissection and aldosterone treatment.

Four weeks postoperatively, the guinea pigs were terminated, and both the endolymphatic sac and the cochlea were prepared for light and transmission electron microscopy. Animal care and use were approved by the Experimental Animal Committee of the University of Groningen, protocol number 0777-1193/1294, in accordance with the principles of the Declaration of Helsinki.

Surgical procedure

The operation was performed under halothane and O₂-N₂O anaesthesia, using a Zeiss stereo microscope. The body temperature was maintained using an electric heating pad. The most distal part of the endolymphatic sac was totally dissected from the sigmoid sinus through an extradural posterior fossa approach and a small sheet of latex was inserted in the thus-created space between the sigmoid sinus and the endolymphatic sac. The intermediate part of the ES was left intact.

Fixation and embedding procedure

The animals were terminated by decapitation. The bullae were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. The specimens were then decalcified for five days in 10% EDTA, pH 7.4, postfixed in 1% OsO₄ with 1% K₄Ru(CN)₆ for two to three hours, carefully rinsed in distilled water, dehydrated in a graded ethanol series followed by propylene oxide, and infiltrated using a mixture of 1:1 propylene oxide and Spurr’s low-viscosity resin for two hours and, finally, using pure resin overnight. Polymerization took place at 70°C after exsiccation in a vacuum.

The cochleas were cut in the midmodiolar plane and stained with toluidine blue. The extent of hydrops was classified as none, slight, moderate or severe, in accordance with Sperling et al.¹⁰. The specimens containing the endolymphatic duct and sac were cut from the proximal end towards the intermediate part, stained with toluidine blue, and evaluated by light microscopy.

Ultrathin sections of 100nm of the intermediate part were contrast-stained with 7% uranyl acetate in 70% methanol and lead citrate according to Reynolds, and examined using a Philips 201 transmission electron microscope (TEM) operating at 40 kV.
Results

Hydrops

No hydrops occurred in the control cochleas. The aldosterone-treated ears displayed a slight hydrops. A slight to severe hydrops occurred in all dissected ears, and the ears with a combination of dissection and aldosterone treatment were affected most. One cochlea was broken during preparation and could not be evaluated (*Table 1*).

**Table 1.** Degree of hydrops in the different treatment modalities.

<table>
<thead>
<tr>
<th>Treatment Modality</th>
<th>Degree of Hydrops</th>
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<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td>No treatment (n=2)</td>
<td>2</td>
</tr>
<tr>
<td>Aldosterone (n=2)</td>
<td>2</td>
</tr>
<tr>
<td>Dissection (n=2)</td>
<td>1</td>
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<tr>
<td>Dissection and Aldosterone (n=3)</td>
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Light microscopy

The control ears showed a normal endolymphatic duct and sac. Proceeding from the proximal end towards the intermediate part of the sac, the epithelium gradually changed from flat to cylindric in the intermediate part, and the surface became irregular and folded. The underlying connective tissue became more dense. Macrophages were found within the lumen (*Figure 1*).

Specimens treated with aldosterone showed no differences from the control ears. The epithelial type was the same, and the subepithelial tissue showed no differences in vascularity or cell density.

In the dissected ears, the endolymphatic duct seemed unaffected. More distally, the endolymphatic sac displayed an abnormal appearance. The epithelium did not change to cylindric but remained flat or cuboidal, and the underlying connective tissue became a very thin layer. In the intermediate part of the endolymphatic sac, the lumen of the endolymphatic space was very small and, further distally, the duct was completely blocked by newly formed bone tissue, demonstrating enhanced vascularization. In the subjacent connective tissue, at the boundary of the bone, many osteoblasts and some osteoclasts were found (*Figure 2*).

The ears with a combination of distal dissection and aldosterone application displayed the same features as the dissected ears without aldosterone.
Transmission electron microscopy

The intermediate endolymphatic sac of the control ears had a normal appearance. Light and dark cells could be distinguished, but many intermediate forms of these cell types were also found. The epithelial cells were filled with abundant endoplasmic reticulum and had a Golgi system close to the nucleus. They contained many mitochondria and some inclusion bodies of different types. The nucleus was either rounded and light, or it was elongated, irregular, and more dense. Many microvilli and pinocytotic vesicles were present at the apical surfaces. The cells were interconnected by tight junctions showing a non-leaky epithelium. Laterally, parts of adjacent cell membranes often interdigitated with each other, leaving some intercellular space. The basal membrane was in some places straight, and sometimes wrinkled parallel to the basal cell surface. The subepithelial tissue consisted of connective tissue containing capillaries and fibrocytes surrounded by collagen fibers (*Figures 3a and 3b*).

No difference could be detected between the normal ears and the aldosterone-treated ears. Microvilli, pinocytotic vesicles and lateral intercellular spaces were indistinguishable.
Dissected ears displayed marked differences compared with the control ears. The epithelial cells were flattened and displayed less microvilli, while the tight junctions still seemed intact. Within the cells, less intact mitochondria and a less prominent Golgi system were present. Numerous disruptions could be seen, many of which may have been degenerating mitochondria. Increased numbers of lysosomes, and some myeloid bodies and autophagosomes were found. The lateral intercellular spaces were dilated. The subepithelial tissue was more fibrous, and very active osteoblasts containing excessive amounts of RER were abundant at the bony wall, together with a few osteoclasts (Figures 4a and 4b).

No difference could be observed between the dissected ears and those that were both treated with aldosterone and dissected. The lateral intercellular spaces were not more dilated and the numbers of degenerating mitochondria, lysosomes, myeloid bodies and autophagosomes were comparable to those in the dissected ears without aldosterone treatment.

**Figure 2.** LM picture of the intermediate ES one month after dissection of the distal part, showing flat epithelium (thin arrow) and new bone formation (thick arrow). An osteoclast (OC) can be observed. A few macrophages (M) are present in the lumen. Bar=20 µm.
Discussion

No artefacts due to fixation or preparation techniques were observed. Mitochondria and other cell organelles were well preserved. The morphology of the control sacs was comparable to findings of other authors\textsuperscript{11,12}, although frequently no sharp distinction could be made between light and dark cells. Typical light and dark cells as described by Lundquist\textsuperscript{11} were observed, but many cells combined properties of both light and dark cells. Cells with different types of nuclei contained cytoplasm with the same density and a comparable amount of mitochondria and other cell organelles. This discrepancy could be explained by differences in fixation techniques or by the use of different species and strains of animals\textsuperscript{13}, but perhaps light and dark cells are two extremes of a gradual variation in cell morphology, possibly representing differences in activity.

The daily systemic aldosterone caused a slight hydrops in the cochlea, indicating an enhanced endolymph production. In earlier experiments, we observed a possible effect of aldosterone in the ES at the light microscopical level\textsuperscript{9}, but this could not be confirmed by transmission electron microscopy. No signs of enhanced activity or other changes were observed in the epithelium. However, small fluctuations in fluid transport might not be detectable with TEM. The administration period of five days might be too short to cause visible alterations in the ES.

Dissection of the distal endolymphatic sac had strong effects: the intermediate epithelium showed dilated lateral intercellular spaces, suggesting an increase of fluid transport through the epithelial membrane\textsuperscript{13}. However, the epithelium also displayed clear signs of degeneration: the cells were flattened and contained disrupted mitochondria, and increased numbers of lysosomes and autophagosomes were found.

The endolymphatic duct seemed to become completely blocked after one month due to the generation of new bone tissue. The locally increased fluid transport in the intermediate part of the ES is possibly a compensating mechanism of the distal dysfunction due to the bone barrier. Studies in the mouse after labyrinth destruction show that the ES epithelial integrity might be dependent on the existence of the longitudinal fluid transport into the ES\textsuperscript{13}. These observations suggest that degeneration of the intermediate ES epithelium could also have been a result of the arrested fluid transport, possibly causing problems in osmolarity, ionic composition, or pH, instead of a direct effect of the surgical process. However, the excessive bone formation could well be a consequence of damage at the time of surgery. Ibrahim and Linthicum\textsuperscript{15} reviewed the various proposed mecha-
nisms by which osteogenesis occurs within the cochlea. New bone may arise from the osteal lining cells of the labyrinth, as a result of metaplasia of the connective tissue in the membraneous labyrinth or as a result of disruption of the endosteum. In general, the early phase of a sterile labyrinthitis consists of activation of inflammatory cells that secrete mediators that may stimulate osteoprogenitor cells in the inner ear to proliferate and secrete matrix which ultimately becomes ossified. It is proposed that the inner ear does not have a strong immunosuppressive mechanism and is unable to withstand inflammatory insults. This could explain that our small disruption of the distal ES, evoking a wound-healing reaction, may lead to the excessive bone formation we found.

Additional administration of aldosterone may have caused a slight augmentation of hydrops in the cochlea, in accordance with earlier findings, but had no visible effects in the ES. Indications of enhanced activity or degeneration were similar to the dissected ears without aldosterone treatment. Aldosterone may act directly on the stria vascularis, where receptors for adrenal steroids were detected, rather than on the ES, where more acid-base transport systems are located. For any visible changes, perhaps a stronger stimulation of endolymph production is required.

In this study the dissection had a more severe impact on the ES than we found in our earlier studies. This might have been induced by a more accurate surgical technique due to our increased experience. In the earlier studies the distal ES might not have been completely dissected, which could be a more satisfactory method, because this may avoid excessive bone formation leading to partial obstruction of the endolymphatic duct. The integrity of the distal ES is apparently a significant factor in maintaining the structure and functioning of the intermediate ES.

Refinements of our model will concentrate on a more subtle dissection of the distal ES and preventing damage to the intermediate ES, in combination with a more prolonged stimulation of endolymph production.

**Figure 4. TEM pictures from the intermediate ES after dissection.**
* (top left) Dissected ear without aldosterone treatment, showing flat epithelium (arrows) with less intact mitochondria, less microvilli and increased numbers of dark-staining lysosomes. Underneath, bone remodelling and fibrous connective tissue can be observed. *Bar*=2 µm.
* (bottom left) Dissected and aldosterone-treated animal, with features comparable to dissection alone. Note also the dilated lateral intercellular spaces (arrows). *Bar*=2 µm.
References


