Chapter 2

Morphology of the endolymphatic sac in the guinea pig after an acute endolymphatic hydrops

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Introduction

An endolymphatic hydrops (EH) is generally acknowledged as the fundamental histopathological substrate of Menière's disease (Hallpike and Cairns, 1938; Kimura and Schuknecht, 1965). An EH, which is an increased endolymph volume, may give rise to a disruption of inner ear function resulting in characteristic symptoms like vertigo, hearing loss, tinnitus and a subjective aural fullness. The mechanisms of endolymph volume regulation and pathophysiology of an EH remain enigmatic. Still, a tiny organ called the endolymphatic sac (ES) is generally believed to represent one of the primary loci for endolymph volume regulation (Kimura and Schuknecht, 1965; Rask-Andersen et al., 1999). The ES's anatomical and histological characteristics suggest an involvement in transport functions. Besides, different other functions have been ascribed to the ES: secretion of macromolecules, removal of waste products (Fukazawa et al., 1991), an immunological (Yan et al., 2003) and endocrine function (Qvortrup et al., 1999). Surgical ablation of the ES in the guinea pig results in a chronic EH (Kimura and Schuknecht, 1965), which has been widely investigated to gain insight into Menière's disease. In the present study, an acute EH was created by microinjection of artificial endolymph into scala media of the cochlea. In contrast to the chronic EH, the injection-produced hydrops develops rapidly and is not disturbed by complex cascades of secondary pathological changes.
In literature, there is only one report on the ultrastructural effects of microinjection of artificial endolymph on the ES. Rask-Andersen et al. (1999) provided a new perspective on endolymph volume homeostasis. They reported that directly after induction of an acute EH, there was an almost total absence of the normal intraluminal homogeneous substance (HS). On the other hand, when native endolymph was aspirated, the amount of HS increased substantially. It was therefore suggested that the volume of fluid in the ES, and hence the volume of the entire membranous labyrinth, might be regulated by a dynamic active secretion and degradation of the lumen-expanding HS.
Intentionally, the present study was performed to gain further insight into the most conspicuous finding of Rask-Andersen et al. (1999), which was a degradation of HS in the ES after an acute EH. An additional time-sequence study might identify specific ES time related mechanisms in the inner ear coping with an acute volume stress.

Materials and methods

Nineteen healthy female albino guinea pigs (Harlan, the Netherlands) with adequate Preyer's reflexes and a weight of 350-450 g were used in this study. Animal care and use were approved by the Experimental Animal Committee of Groningen University, protocol No. 2910.
General anesthesia was induced by intramuscular administration of ketamine/xylazine (60/3.5 mg/kg). For maintainance of an adequate anesthesial depth, additional injections were given every hour. Muscle relaxation was obtained with succinylcholine (2.5 mg/kg). The animals were artificially ventilated through a tracheostoma (Columbus Instruments, model 7950) with body temperature being maintained at 38°C. The heart rate was monitored with a pair of skin electrodes placed

on both sides of the thorax. The animal's head was kept in a stationary position by means of a steel bolt fixed to the skull with dental cement. Following a retroauricular incision, the bulla was opened and the round window was exposed. Through the round window membrane, the tip of a double-barreled micropipette was inserted into scala tympani. After perforation of the basilar membrane, the micropipette was advanced into scala media. An ~ 80 mV increase in DC potential at the pipette tip indicated measurement of the endocochlear potential and verified its correct position. The double-barreled micropipettes were drawn from borosilicate glass (1.5/0.84 mm diameter per barrel) with tips bevelled by a Narishige EG-40. The tip diameters were around 20 µm per barrel. One barrel of the pipette was used to measure inner ear pressure and DC potential (WPI 900A micropressure system). Through the other barrel, artificial endolymph (140 mM KCl + 25 mM $KHCO_3$) was injected. A constant flow rate was created by applying a controlable pneumatic pressure with a second WPI 900A micropressure system to the barrel end. Approximately 1.1 µl of artificial endolymph was injected with a rate of 91-109 nl/min. After injection, the animals were sacrificed by an intracardial injection of pentobarbital. In 6 animals microinjection was followed by immediate termination. Other animals were let to survive for a period of ½ h (n=3), 1 h (n=4) and 2 h (n=4).

After decapitation, bullae were rapidly dissected, opened and immersed in formalin 10%. The time lag between termination and fixation was approximately 5 min. Subsequently, the specimens were decalcified in 0.1 M Na-EDTA for at least 14 days. After rinsing in distilled water and dehydration in a graded ethanol series (30-100%), the specimens were placed in ethanol:HPMA (1:1) for 8 h. In the following 48 h the specimens were embedded in HPMA with addition of a catalyst (N,N-dimethylaniline, PEG 400; 15:1). For LM, 4 µm-sections were stained with toluidine blue and contrast-stained with basic fuchsin. The ES was sectioned longitudinally to obtain optimal data on luminal filling and staining characteristics. The contralateral, non-injected ear served as a histological control.
For transmission electron microscopy, the endolymphatic sacs (n=5) were fixed in a solution of 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4; 4 °C; 400 mOsm) and 2 mM $CaCl_2$. After fixation, the specimens were postfixed in 1% OsO_4 with 1% $K_4Ru(CN)_6$ for 3-4 h, carefully rinsed in distilled water, and then dehydrated in a graded ethanol series followed by propylene oxide. They were then infiltrated using a mixture of 1:1 propylene oxide and Spurr's low viscosity resin for 1 h and pure resin overnight. Polymerisation took place at 70 °C after exsiccation in a vacuum. Ultrathin sections (100 nm) of the ES were contrast-stained with 7% uranyl acetate in 70% methanol and lead citrate according to Reynolds, and examined using a Philips 201 operating at 40 kV.

Additional experiments involved microinjections of suspended polystyrene microspheres (molecular probes ®) into scala media of the cochlea (n=2). The microspheres, with a diameter of 2 µm, can not penetrate inner ear membranes. After histological processing, the polystyrene microspheres in the endolymphatic compartment were visualised by using a similar method as described by Voie (2002); the orthogonal-plane fluorescence optical sectioning (OPFOS) microscopy.

Results

The microinjections of 1.1 µl of artificial endolymph into scala media of the cochlea were successfully performed in all guinea pigs (n=17). The mean rate of injection was 98 nl/min (91-109). In 2 additional experiments 2.2 µl of suspended polystyrene microspheres (molecular probes ®) were injected.

The mean endocochlear potential (EP) prior to manipulation of endolymph volume was 77.2 mV (± 5.1). During the period of injection the EP remained stable. The animals which were let to survive for 2 h demonstrated an EP of 64.5 mV on average prior to termination.

After histological processing, polystyrene microspheres were visualised by OPFOS microscopy. The microspheres could be detected in the endolymphatic compartment of the cochlea (Fig. 1). In this figure, the endolymphatic scala media is filled with microspheres. There is no presence of microspheres in the perilymphatic scala vestibuli and scala tympani. A distension of Reissner's membrane in the different coils of the cochlea demonstrates an acute endolymphatic hydrops.

The ES was analyzed by light microscopy with emphasis on luminal filling (Fig. 2). The level of intraluminal HS was categorized as low, medium or high. Category "low" equals no HS or only traces of HS, whereas the category "high" equals an ES full of HS. To surpass the possible visual bias of overlooking a HS-filled lumen with a very low density, the scoring was objectively analysed by checking the RGB-values in the pixels of the LM micrographs with ImageJ (http://rsb.info.nih.gov/ij). All injected and non-injected contralateral ears were scored (table 1).

The ES of the guinea pigs that were terminated directly after injection showed no differences between the injected and non-injected contralateral ear. The ES's luminal homogeneous substance (HS) was always present and often to a large extent. Furthermore, no obvious differences in luminal filling with regard to the amount of HS or presence of macrophages, were found between animals which were let to survive for different periods after induction of an acute EH (Fig. 2).

HS was found primarily in the proximal and intermediate part of the ES, with the distal part being the least HS-filled. Typically, in 12 out of 17 specimens HS was also present in the endolymphatic duct.

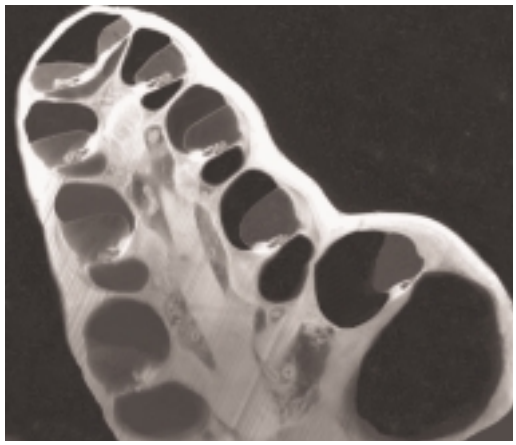

Figure 1: Cochlear image acquired by OPFOS. The grey coloured areas beneath Reissner's membrane depict the endolymphatic scala media of the cochlea which is filled with microspheres. A bulging Reissner's membrane in the different coils of the guinea pig's cochlea indicates an endolymphatic hydrops. The photograph is blurring towards the lower left corner because of overprojection of cochlear structures.

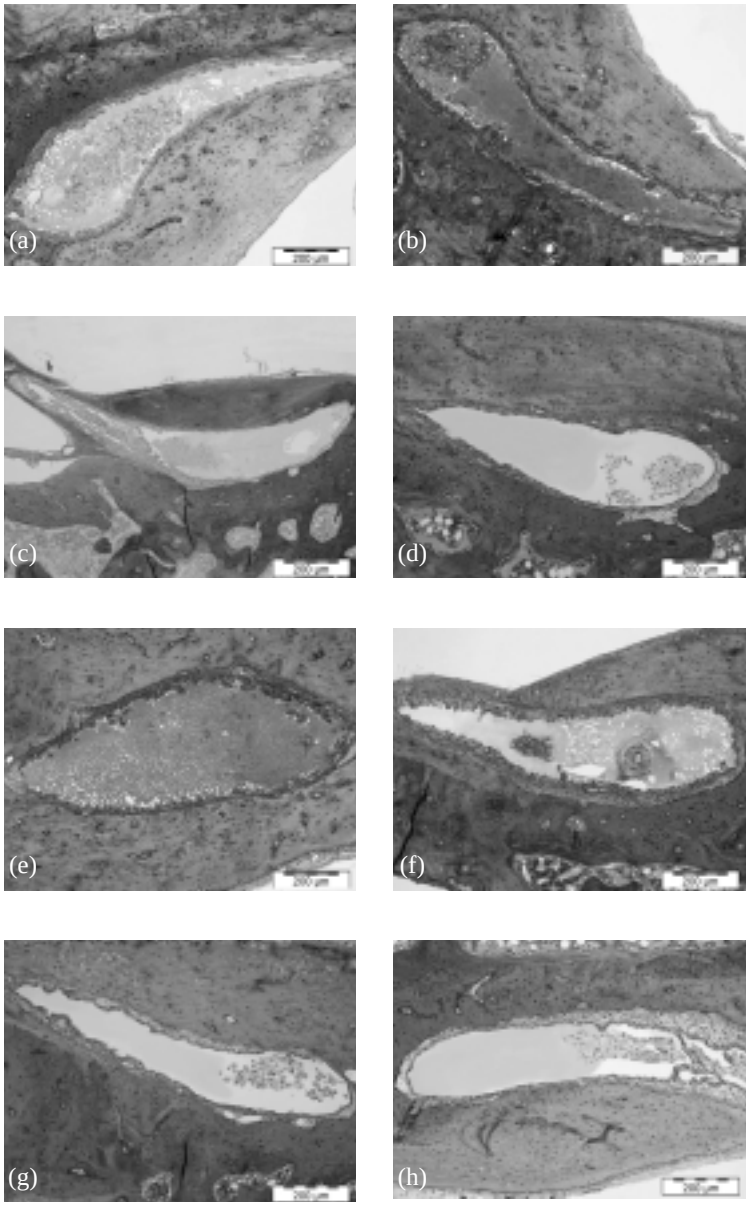

Figure 2: Light microscopic photographs of the intermediate part of the ES of guinea pigs terminated after different time intervals.
A: injected ear, directly terminated after endolymph injection. B: contralateral non-injected ear, direct. C: injected ear after ½ h. D: contralateral non-injected ear, ½ h.
E: injected ear after 1 h. F: contralateral non-injected ear, 1 h.
G: injected ear after 2 h. H: contralateral non-injected ear, 2 h.
HS is always present to a large extent. Also note the difference in HS-densities between the specimens.

The epithelial architecture changed from cubic in the proximal to cylindrical in the intermediate and again to cubic in the distal part of the ES. Again, no differences could be observed between injected and non-injected ears.

Ultrastructurally, different epithelial ES cells could be clearly distinguished. The light cells or mitochondrium rich cells (MRC) were markedly outnumbered by dark cells, also called ribosome rich cells (RRC) or chief cells (Fig. 3). The more columnar MRC displayed abundant supranuclear mitochondria and apical microvilli. Other common morphological features included a basally placed, round nucleus with smooth and rough endoplasmic reticulae, Golgi-apparatus and lysosymal structures (Fig. 3). At some points MRC's were covered over by RRC's (Fig. 4). The RRC showed less mitochondria and showed more variety in cell and nucleus shape. In general they had a more calyx-like structure with often an indented nucleus (Figs. 3, 4).

Typically, a clear luminal zone was present adjacent to the epithelial cells (Fig. 4). This was predominantly seen on the luminal side of the RRC's (Figs. 4,5). In figure 5, a detailed example of such a clear zone is demonstrated. The clear zone possesses some remnants of HS, which forms a matrix in which the microvilli stand out. This clear zone was not continuous and exhibited several interruptions, which gave them a vesicle-like structure. Further, in the majority of specimens a completely HS filled lumen of the ES showed several vacuole-like spheres (Fig. 6). The spheres of dissolved HS at the epithelial surface outnumbered the spheres in the ES's lumen. Apparently, the spheres originate from the epithelium with subsequent drifting into the dynamic HS-filled lumen.

Intraluminal macrophages were full of activity showing abundant lysosomes involved in trapping and digesting HS (Fig. 7). The macrophages typically contained several HS filled vesicles with different densities. Aggregates of macrophages were often found in the intermediate part of the ES. No differences in macrophage activity between injected and non-injected ears could be found.

Discussion

The present results do not confirm the findings of Rask-Andersen et al. (1999), who observed dramatic changes in ES's luminal filling immediately following endolymph volume manipulation. It was suggested that endolymph volume might be regulated by a dynamic secretion and degrada-

Table 1

Time after induction of an acute EH	Number of specimens	Level of intraluminal HS		
		Low	Intermediate	High
Direct	6		++++	++
control		+	+++	++
½ h	3	+	+	+
control		+	+	+
1 h	4		++	++
control			+++	+
2 h	4		++	++
control		+	+	++

tion of the lumen-expanding HS. In contrast with this theory, no differences in luminal HS content or ES's epithelia were found between injected and non-injected ears after immediate termination. Additionally, no distinct changes were observed in guinea pigs terminated after time-intervals up to 2 h.

There are several possible differences with the study performed by Rask-Andersen et al. (1999). They do not state the details of the animals they used. It is known that there are physiological differences between guinea pigs of different strain, gender, size or age. In addition, other anesthetics may have been used. A technical explanation for the contrasting findings remains obscure, for almost exactly the same techniques were used. The only difference was the approach for microinjection. Whereas they microinjected directly into the second turn of scala media in the cochlea, we reached scala media by puncturing the round window and basilar membrane respectively. The transition from the perilymphatic to the endolymphatic compartment was confirmed by measuring the endocochlear potential (± 80 mV). To further corroborate that the microinjection was actually performed in the endolymphatic scala media, additional experiments in which polystyrene microspheres were injected, were carried out. With afterwards tracing of the microspheres in the endolymphatic compartment, a correct injection site was ensured (Fig. 1).

Despite our apparent negative findings concerning the role of HS in endolymph volume homeostasis some interesting clinical data are well documented about HS. In patients with Menière's disease a higher incidence of eosinophilic intraluminal HS is found in the ES compared to non-Menière's controls (Ikeda and Sando, 1984). Besides, a feature of Menière's disease is a positive glycerol test. In this somewhat controversial test a hearing improvement occurs after ingestion of

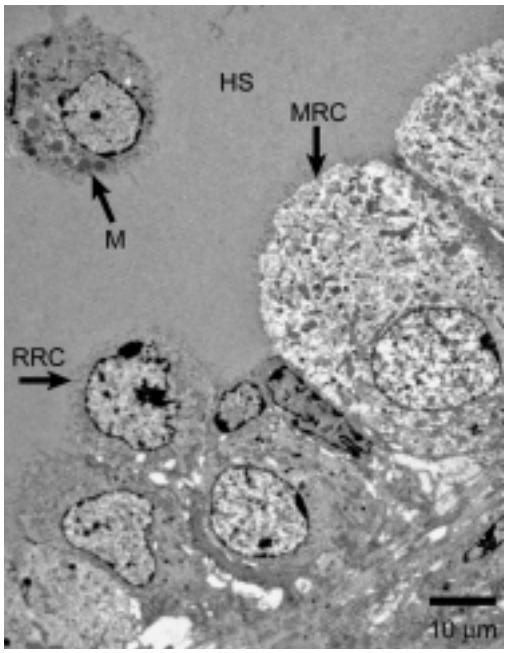

Figure 3: TEM image showing different epithelial cells in the intermediate part of the ES. MRC, mitochondrium rich cell. RRC, ribosome rich cell. The lumen of the ES is completely filled with HS. There is also a macrophage (M) in the ES's lumen.

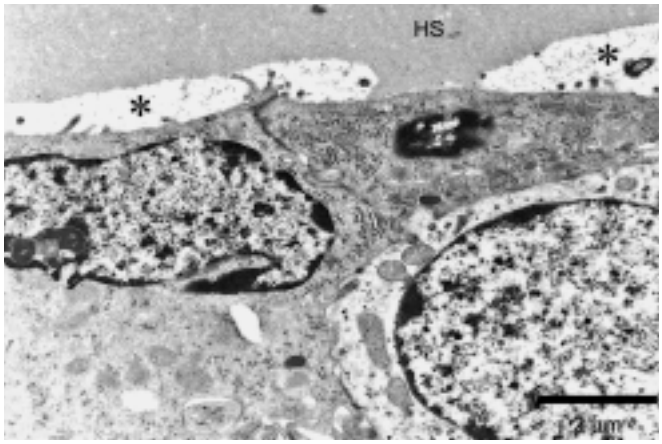

Figure 4: TEM image of a ribosome rich cell (darker cell) covering a mitochondrium rich cell (light cell). There is a HS clear zone (asteriks) adjacent to the luminal side of the darker RRC's. The lumen of the ES is again filled with HS.

the hyperosmolar diuretic glycerol. A possible explanation is a raised blood osmolarity which removes fluid from the inner ear. Further, it is known that after intravenous injection of urea or glycerol in the guinea pig, the HS in the ES's lumen is substantially increased (Erwall et al., 1988).
When we take a closer look at this intriguing substance, it is known that HS is a composite of complex macromolecules (Thalmann, 2001), of which the principal glycosaminoglycan is hyaluronan (Parker et al., 1992). Furthermore, HS is the major contributor to the different protein profile and 50 fold higher protein content in the ES in respect to cochlear endolymph. The presence of HS in the ES seems to be linked to the epithelial cells. In the ES of the guinea pig the ultrastructural morphological appearance of both MRC and RRC suggests an active involvement in secretion and degradation of HS respectively. Peters et al. (2003) studied ES cells in detail in the rat. The different subtypes of ES cells were suggested to be dynamically active in endolymph homeostasis. MRC's could be involved in proton secretion and Cl^-/HCO_3^- exchange. This is supported by Stankovic et al. (1997), who located proton secreting cells in the ES of the guinea pig. Peters et al. (2003) also suggested that RRC's are actively involved in secretion and absorption of complex proteins. RRC's appeared to synthesize and secrete glycoproteins into the ES lumen. In our study, in both injected and non-injected ears, the RRC's appeared to secrete a lytic substance that was digesting the HS. For there were no enzyme filled granules in the apical cytoplasm of the RRC, the lytic enzymes might be secreted by diffusion. It seemed plausible that intraluminal macrophages had similar enzymes involved in digesting HS. Several deglycosylating ES enzymes are known to be able to degrade intraluminal HS (Thallman, 2001).
In our study HS was present in all hydropic and non-hydropic specimens. The role of HS in endolymph volume homeostasis therefore remains obscure. In order to elucidate the mechanisms in which endolymph volume is regulated, these results prompt us to find an answer in other directions.
In the acute EH model, Salt and DeMott (1997) demonstrated that small volume disturbances are corrected locally in the cochlea, whereas larger disturbances produced a longitudinal flow of endolymph out of the cochlea. Through the ductus reuniens, saccule and endolymphatic duct, the endolymph would eventually reach the ES. This overflow system could represent a mechanism

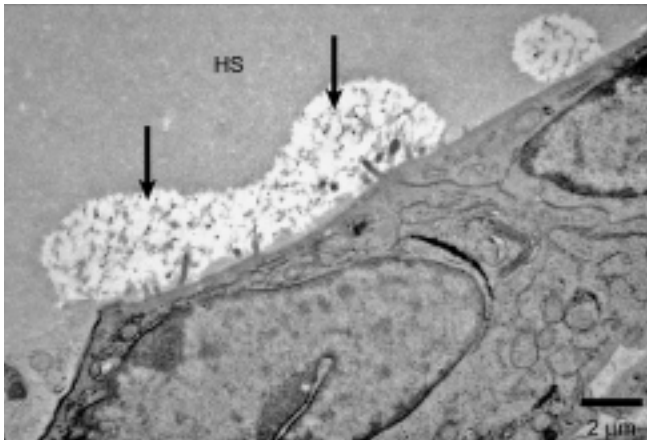

Figure 5: Detailed TEM image of a HS clear zone demonstrating a matrix (arrows) in which the microvilli extend.

contributing to inner ear volume correction. As the total endolymph volume in the inner ear of the guinea pig is around 4.7 µl (Shinomori et al., 2001), a volume increase of 1.1 µl corresponds with an EH of ~23%. Perhaps this degree of an EH is within the coping limits of the inner ear. This is in accordance with a previous study (Valk et al., 2004), where the same degree of hydrops only had a minor reversible influence on cochlear function, as measured by distortion product otoacoustic emissions (DPOAE). However, it remains doubtful, if the relatively small ES with its volume of ~0,1 µl is able to correct an endolymph excess of this magnitude. The excess of endolymph could be taken up by the perisaccular tissue and surrounding vascular network. An important

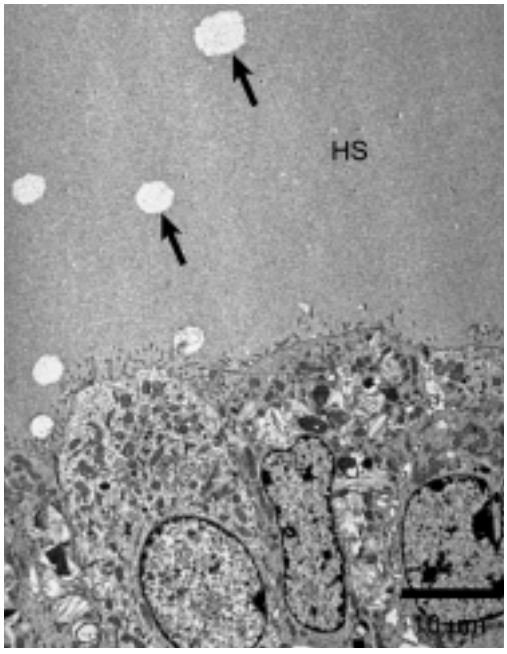

Figure 6: TEM image showing several vacuole-like HS free spheres (arrows) in an otherwise completely HS filled lumen of the ES.

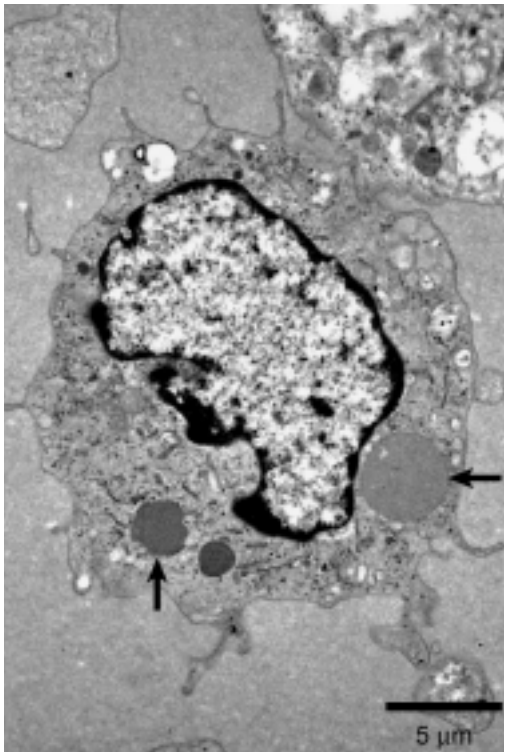

Figure 7: Highly detailed image of a macrophage harbouring several membrane bound vacuoles with different electron densities in which there appears to be endocytosis of a substance similar to HS (arrows); the smaller the size of the vacuole, the denser the contents.

blood vessel would than be the sigmoid sinus which is anatomically closely related to the ES (Rask-Andersen, 1979). This might seem plausible, for an EH results after removal of the vascular network from the ES (Lee and Kimura, 1992).

Endolymph volume might also be regulated by an inhibitor of sodium reabsorption. Qvortrup et al. (1996) found a natriuretic factor in extracts of the ES. Cytoplasmatic granules in the RRC or the HS might contain this factor and thus control fluid transport in the ES. In similar respect, pressure changes of the inner ear have been suggested to control antidiuretic hormone release (Bartoli et al., 1989).

Another intriguing theory is that of specific water channels which are found in several organs including the inner ear. These specific water channels are composed of aquaporin (AQP) proteins. Recently, Takeda et al. (2003) found that water homeostasis in the inner ear of the guinea pig is regulated via the vasopressin-AQP2 system. As AQP2 could be involved in endolymph volume regulation, a “leaky boat” model was proposed by Beitz et al. (2003). In this model endolymph volume is regulated by osmotic water influx into the endolymph through AQP2 (the leaks in the boat) and removal of endolymph by isoosmotic pinocytosis (scooping out buckets of water). The whole process would be balanced by vasopressin, which depends on the systemic hydration status. In this light the choice of xylazine as an anesthetic turns out to be less fortunate, because this anesthetic is known to have diuretic properties which might interfere with systemic hydration status.

Another important question in endolymph volume regulation is: how does the inner ear sense a volume change? To answer this question, it might be of interest to look at cell volume regulation. Different mechanisms are proposed for cells actively regulating their volume. Possibly, specific cell mechanisms could be similar for the inner ear as a whole. One of the proposed cell mechanisms involves mechanosensitive ion channels which would respond to mechanical stretching or compression of the membrane lipid bilayer. Another volume sensor might be a high concentration of macromolecules in the cytoplasm which would affect molecular and cellular functions (Mongin and Orlov, 2001). Further, Grunnet et al. (2003) found that so called KCNQ4 channels are tightly regulated by small cell volume changes, when expressed with aquaporin water channels. Remarkably, these channels are also expressed in hair cells and the auditory tract.

In conclusion, endolymph volume homeostasis is a complex mechanism, in which the role of HS in the ES remains obscure. Further investigation is necessary to elucidate regulatory mechanisms of inner ear fluid volume. This will hopefully lead to understanding and treatment of inner ear diseases, like Menière's.

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