Chapter 6

Sensory cell damage in two-phase endolymphatic hydrops; a morphologic evaluation of a new experimental model by low-voltage scanning techniques

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Introduction

Since Hallpike and Cairns and also Yamakawa in 1938 discovered hydrops of the endolymphatic system in the temporal bones of patients with Menière's disease, endolymphatic hydrops has been generally accepted as the basic histopathological substrate of Menière's disease[1,2].

The surgical destruction of the endolymphatic sac in guinea pigs and obstruction of the vestibular aqueduct with bone wax is a well-established model for inducing endolymphatic hydrops as observed in temporal bones of patients with Menière's disease[3]. This is, however, a non-physiological profound model for Menière's disease in humans. In our department we developed a new two-phase experimental model for endolymphatic hydrops to investigate the pathogenesis of Menière's disease[4]. In this model the absorption of endolymph has been chronically disturbed by surgical dissection of the distal portion of the endolymphatic sac without damaging the intermediate part. Periodic increase of endolymph production is induced by administration of aldosterone, to stimulate the Na/K ATP-ase activity in the stria vascularis and the dark cells of the inner ear. The acute endolymph production will disturb the endolymph homeostasis which is not capable to restore immediately due to the borderline capacity of the endolymphatic sac resulting in the development or increase of hydrops.

A crucial and complicated event in the hearing process is the transduction of mechanical stimuli into electrical signals by hair cells[5,6]. The hair cells have stereocilia of graded height on their apical surface which are interconnected by two different linkages; lateral links and tip links. Mechanical stimulus may deflect the stereocilia in several directions. In some theories, deflections of the bundle towards the tallest stereocilia open transduction channels by stretching the tip links. This is assumed to result in an inward flow of positive ions which depolarizes the hair cell.

Post-mortem scanning observations from the cochlea of a patient with Menière's disease revealed subtile pathological changes of the stereociliar complex[7]. Abnormal shortening of the short stereocilia followed by atrophy of short and middle stereocilia of the outer hair cells may be associated with early audiovestibular dysfunction in Menière's disease. Rydmarker and Horner[8] also reported about the structure of hair cell stereocilia in guinea pigs after surgical obliteration of the endolymphatic sac and duct according to the classical experimental hydrops model. They identified in SEM studies atrophy of the short and middle stereocilia on the outer hair cells while the inner hair cells did not have such pathology. The atrophy was merely restricted to the upper cochlear turns in remarkable correspondence with the low frequency sensitivity loss and was detected in the first stage of fluctuating thresholds. The middle and the short stereocilia appeared to be detached from the tall stereocilia, extensively shortened, and almost completely absent. However, the selective atrophy of short and middle stereocilia was not reported in another SEM study of experimental hydropic cochleas[9]. Furthermore, extrapolation of

the experimental results to the clinical situation of Menière's disease is complicated by the fact, that in all reported experiments the non-physiological experimental hydrops model was used.

In this experiment we used the Two-phase endolymphatic hydrops model, which may represent a more dynamic and physiological approach to Menière's disease. The sensory cells in the guinea pig cochlea were studied at low-voltage with field-emission scanning electron microscope (SEM) using special fixation methods. These new SEM techniques enable us to obtain a more detailed view of the stereocilia[10].

Materials and methods

Sixteen healthy female albino guinea pigs (Harlan, The Netherlands) with a mean weight of 250 g were used in this experiment. Animal care and use were approved by the experimental Animal Committee of the Groningen University, protocol number 0777-1193/1294, in accordance with the principles of the Declaration of Helsinki.

The animals were divided into two groups:

1 (n=6) dissection of the distal part of the endolymphatic sac of the left ear, followed by killing of the animals after four weeks. The right ear, which was unaffected, served as a control.
2 (n=10) dissection of the distal part of the endolymphatic sac of the left ear, followed by once-daily intraperitoneal injections of aldosterone during five days in the fourth week in a dose of 100 microgram aldosterone per 100 gram body weight per day. The animals were killed at the end of the fourth week. The right ear, which was influenced by the systemically injected aldosterone, served as a control.

The exact procedure of the distal endolymphatic sac dissection, the administration of aldosterone, and their light microscopic effects on the inner ear has been described in earlier papers[4,11].

Animal preparation and pre-fixation

All animals were killed by sublethal administration of sodium pentobarbital (60 mg/kg i.p.). The temporal bone was removed, the cochleas were dissected, and the top of the cochleas were opened as well as the round window. A fixation solution of 2.5% glutaraldehyde in 0.1M Na-cacodylate buffer [pH 7.4; 400 Mosm; 4°C] and calcium chloride (2 mM) was gently flushed through the cochlea from top to bottom for three minutes. Within 2-4 hours, this was followed by frequent flushing with a solution of 0.1M sodium-cacodylate buffer with 2mM calcium chloride and rinsing with distilled water.

Cochlea post-fixation

Post-fixation was performed according to one of the two following methods:

1 The OTOTO [OsO_4 - Thiocarbohydrazide - OsO_4 - Thiocarbohydrazide - OsO_4] non-coating technique involves the use of the ligand thiocarbohydrazide in combination with OsO_4 in 0.1 M Na-cacodylate buffer in the given sequence for various periods, as described by Jongebloed[12].
2 The TAO [tannic acid - arginine - OsO_4] non-coating technique involves the use of tannic acid in combination with arginine hydrochloride, glycine, sodium glutamate and sucrose for various periods [2%; 16 h; 20°C], rinsing with distilled water (3x), immersion in a mixture of tannic acid/ guanidine-HCL [2%; 8 h; 20°C], rinsing in distilled water (3x), immersion in OsO_4 solution [2%; 8 h; 20°C], rinsing with distilled water, and dehydration in an ethanol series as described by Kalicharan[13].

All specimens were critical point dried with liquid CO_2, and most specimen were sputter-coated with Au or Au-Pd (10 nm), and were studied in a JEOL Field Emission Gun Scanning Microscope type 6301F operated at 2 kV, with a spotsize 2 x 10^{-11} A, and a working distance (WD) of 6-11 mm.

Results

All *normal* control ears (n=6) showed normal structure of outer hair cells and inner hair cells, including the presence and configuration of their stereocilia. The tectorial membrane also appeared to be normal. No absence or disturbed stereociliar organization was observed. Artefacts due to preparation, such as the frequently damaged turn 4.0 to 4.5, were excluded for evaluation.

The organ of Corti of the cochleas in the dissected and/or aldosterone-treated groups were investigated on the following points of interest:

1 Sensory cells: the three rows of outer hair cells and inner hair cells with emphasis on *(a)* absence of sensory cells and the morphology of the cuticular plates, *(b)* the arrangement of the stereociliary bundle, *(c)* the morphology of the individual stereocilia, and *(d)* the structure of the interconnecting links.
2 The tectorial membrane and the supporting cells.
3 The tonotopic differences in the morphology between the apical (turn 3.0-4.0), middle (turn 2.0-3.0), and basal (turn 0.5-1.5) sites of the cochlea.

1. Sensory cells

Outer hair cells:

(a) Absence of the outer hair cells and morphology of the cuticular plates:
In severely affected cochleas, third and second row outer hair cells were frequently absent in the apical turns of the most damaged cochleas in 3 out of 6 dissected specimens and 7 out of 10 in the dissected/aldosterone-treated group, leaving gaps in the affected row of outer hair cells (*Figure 1*). In all cases of hair cell absence, the gaps between the remaining hair cells were filled by cells with an abundance of microvilli on their top which, because of their similarity, were most likely to be neighbouring supporting cells. In a few cases, the contours of cuticular plates of remaining hair cells were still visible between the abundance of microvilli of the surrounding cells. On these remaining cuticular plates short and thin microvillar structures, of another structure compared to the ones on the surrounding cells, seem to cover these plates (*Figure 2*). In less damaged

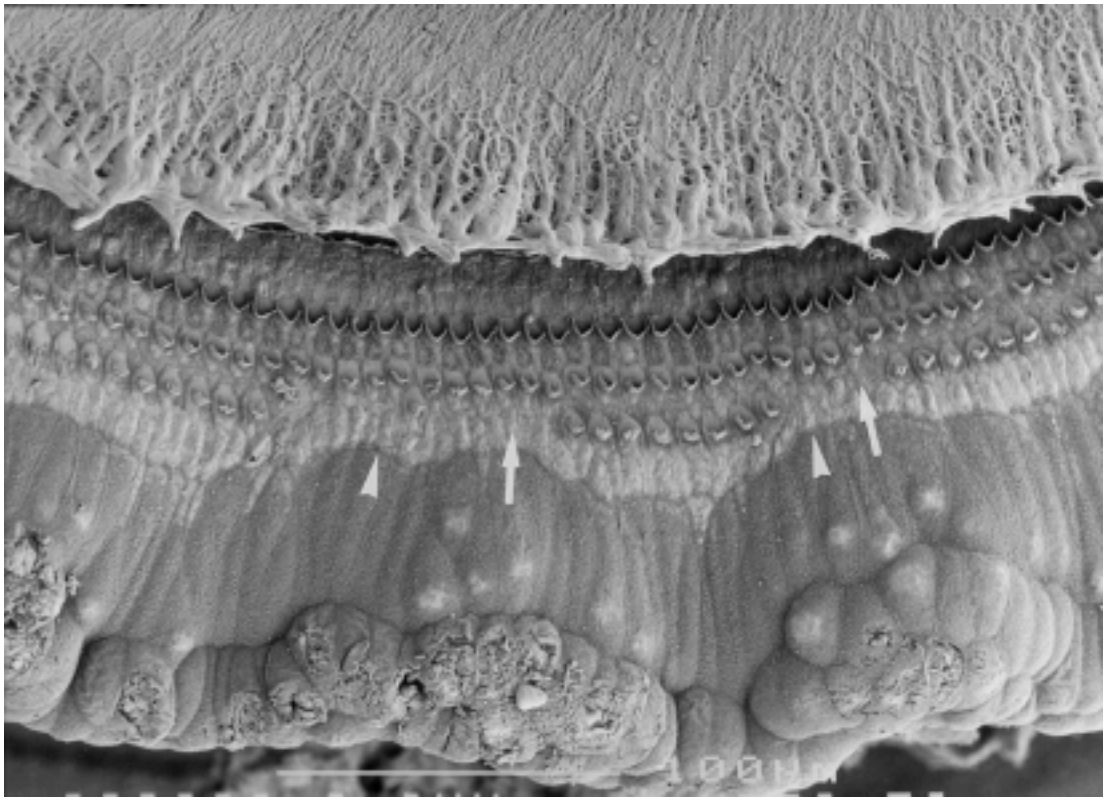

Figure 1. *Overview on the organ of Corti in an apical cochlear turn of a dissected and aldosterone-treated specimenn, in wich linear gaps in the first row of outer hair cells can be observed due to loss of these hair cells (arrows). Proliferation of supporting cells seem to fill these gaps as a compensation of missing hair cells, or perhaps may destruct these cells by oppression (arrowheads). Bar=100 µm.*

outer hair cells, the same microvillar structures seem to arise from their cuticular plates starting on the modiolar side of the hair cells near the location of the shortest stereocilia (*Figure 3*). Only one specimen in the dissected/aldosterone-treated group showed also hair cell loss in the middle turns.

(b) Disarrangement of the stereociliary bundle:
Disarrangement of the stereociliary bundle has been found as one of the main findings after endolymphatic sac dissection. All third row outer hair cells (in all 6 specimens) and a few of the second row (3 out of 6) in the upper cochlear turns were affected (*Figures 3 and 4*). Third row outer hair cells (5 out of 6) and second row outer hair cells (2 out of 6) were affected in the middle turns. Disarrangements due to artefacts were excluded.

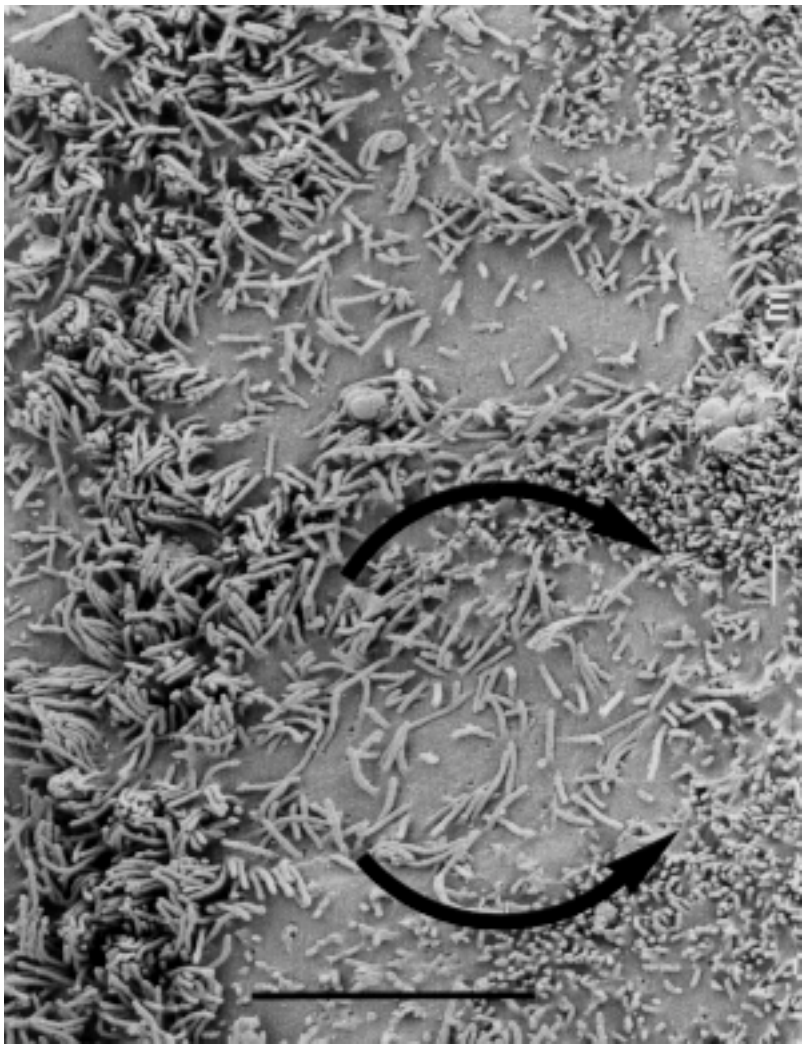

Figure 2. *Apical turn of a severely affected specimen (turn 3.0-4.0). The cuticular plates of these outer hair cells show total stereociliary atrophy without leaving stumps or holes, and overgrowth by microvillar structures (cell outlined by black arrows). Bar=5 µm.*

When aldosterone was injected three weeks after endolymphatic sac dissection, the most severe effects were observed.; stereociliary disarrangement was noticed in 10/10 of third row, 8/10 of second row, and even 2/10 first row outer hair cells in the apical turns. In the middle turns, 9/10 of third row and 3/10 of second row outer hair cells were affected.

(c) Morphology of the individual stereocilia:
The first signs of stereociliary damage started in the apical turns, and was in close connection to the findings of the stereociliary disarrangement (section b). In less affected specimens the shortest stereocilia of the third row outer hair cells become smaller in diameter, probably resulting in loss of side-to-side links. In some of these specimens a few of the shortest stereocilia were randomly missing in the third row outer hair cells (*Figure 3*).

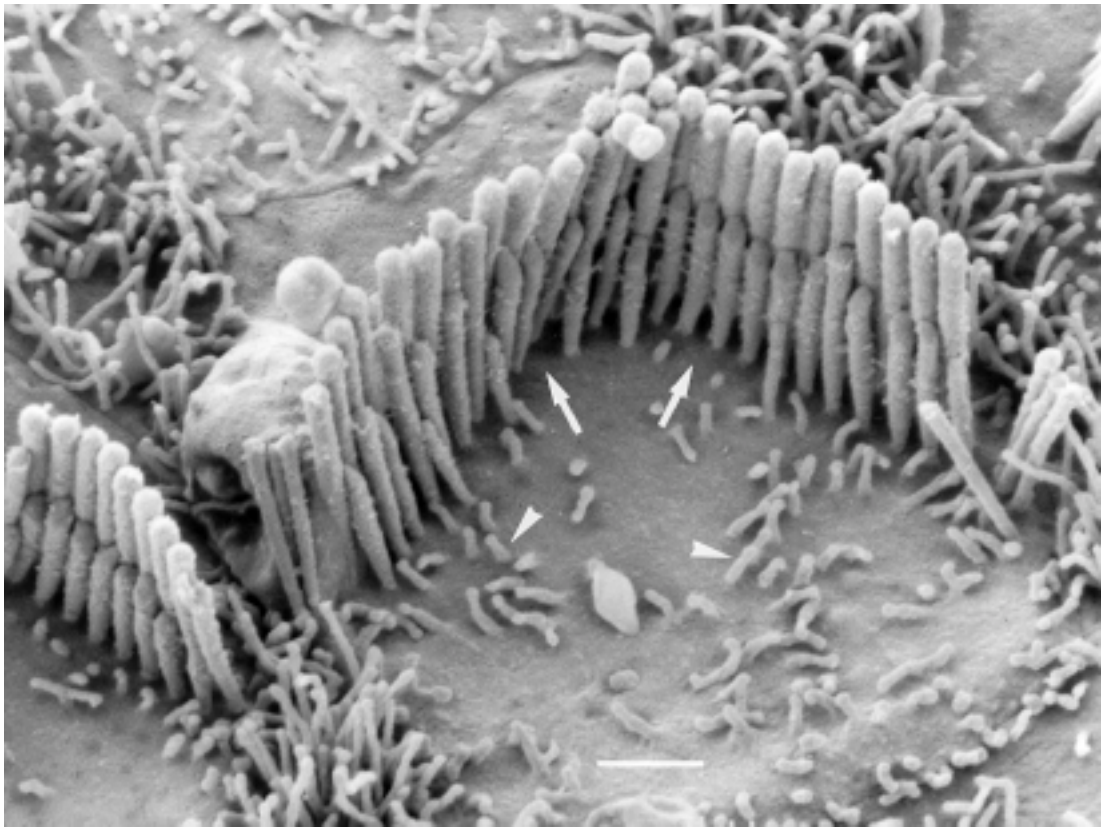

Figure 3. *Second row outer hair cell of the apical turn (turn 3.0-4.0) after treatment by distal dissection of the endolymphatic sac as well as aldosterone injections. Most of the shortest stereocilia are missing (arrows). The middle and longest stereocilia and their W-configuration seems to remain intact. Some microvillar structures arise from the cuticular plate (arrowheads). Bar=1 µm.*

In more affected specimens, the above mentioned observations were most frequently observed in the second row outer hair cells of the apical turns as well in the third row outer hair cells of the middle turns, as will be discussed in the section of tonotopical changes. The third row outer hair cells of the apical turns in these severely affected specimens showed complete loss of the shortest stereocilia, smaller diameters of middle and longer stereocilia including loss of these remaining stereocilia starting from the lateral sides, and disarrangement of the stereociliary bundle (*Figure 4*). In some cases, all stereocilia were disappeared and the remaining cuticular plates of these cells were overgrown by an abundance of the above mentioned microvillar structures (*Figure 2*, see also section a).

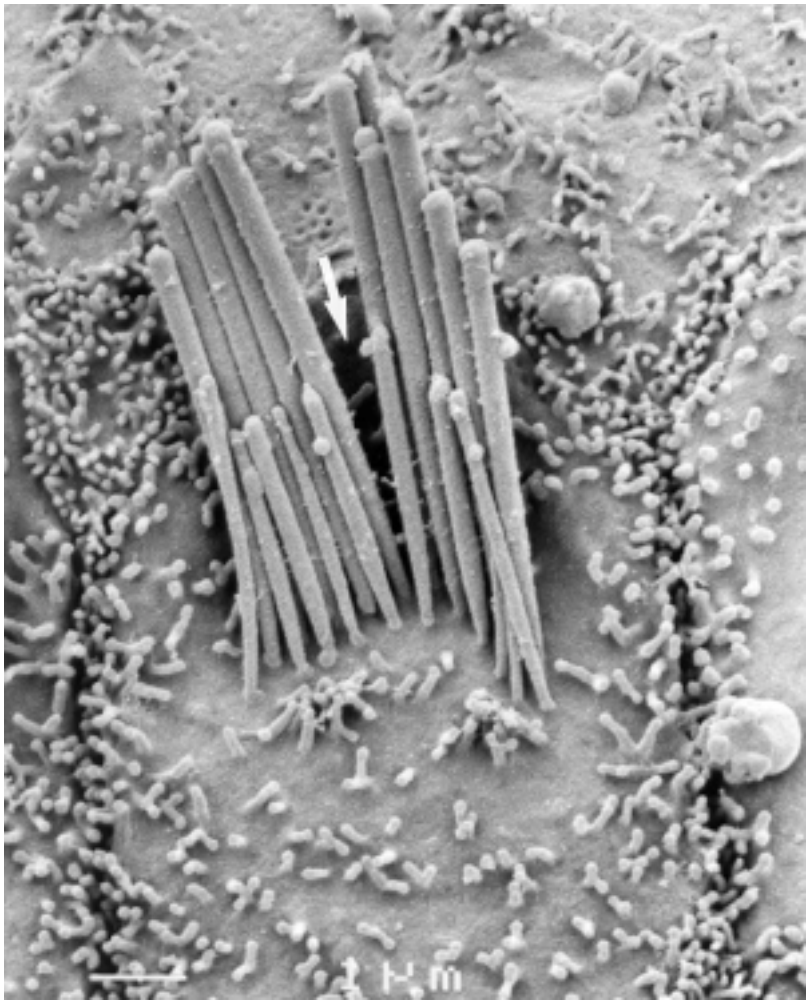

Figure 4. *Third row outer hair cell of the apical turn (turn 3.0-4.0) after treatment by distal dissection of the endolymphatic sac as well as aldosterone injections. All shortest stereocilia are missing. Some of the middle and longest stereocilia are missing from the lateral side and their W-configuration is disorganized, probably due to loss of side-to-side links (arrow). Many microvillar structures arise from the cuticular plate. Bar=1 µm.*

Disruption of the stereocilia from the cuticular plates, leaving cuticular stumps or holes were regarded as artefacts and were excluded.

In an earlier study, a network of long filamentous structures has been identified mainly at the top of the longest outer hair cell stereocilia and the undersurface of the tectorial membrane[10]. This structure may represent a glycocalyx layer. In this study, a few specimen demonstrated indication of the presence of these filaments, although their visibility had to be sacrified by a very thin metal sputtercoating to prevent conduction of some cochlear sections. Conductivity disorders may result in incorrect interpretations in comparing these structures in different sections of our cochleas. It was therefore not possible to separate the effects of our model on the glycocalyx in our different subgroups.

(d) Structure of the interconnecting links:
Short and middle stereocilia seemed to be connected to adjacent longer stereocilia by normal appearing tip links. The presence and structure of row-to-row links could not be visualized well enough in all groups. The side-to-side links between the middle and long stereocilia seemed to be normal in specimens in which only a few of the shortest stereocilia were missing (*Figure 3*).

However, in more affected specimen in which no short stereocilia could be observed anymore, side-to-side links seemed to be affected resulting in a disarrangement of the W-configuration of the remaining middle and long stereociliary bundles (*Figure 4*).

Inner hair cells
No inner hair cells seemed to be missing. Although the strial side of the inner hair cells appeared to be normal, in a few of these inner hair cells the modiolar side showed some of the same morphological changes as observed in the slightly affected outer hair cells. Some of the shortest stereocilia were missing, while the side-to-side links of the remaining shortest stereocilia seemed to be disrupted and their diameter seemed to be reduced (*Figure 5*), mostly in the aldosterone-treated group.

Most middle and longest stereocilia were normal in length and diameter. Side-to-side links between these stereocilia were also rare, resulting in disarrangement of these stereociliary rows. On the cuticular plates of these affected inner hair cells, microvillar structures seem to arise as described in the section of the outer hair cells.

2. The tectorial membrane and the supporting cells

The undersurface of the *tectorial membrane* was observed for the imprints of the tips of the longest stereocilia of the three rows of outer hair cells. No stereocilia were caught inside these imprints in the tectorial membrane. All imprints were empty with a normal W-configuration and linear arranged according to three rows of outer hair cells, also in severely affected groups.

At low magnifications of the *organ of Corti*, an overview of the three rows of outer hair cells has been obtained. In severely affected cochleas, the linear arrangement of the first row of outer hair cells remained intact in all turns. The second and in particular the third row of outer hair cells showed the earlier mentioned stereociliary disarrangement and atrophy, and loss of outer hair cells. A remarkable finding is loss of several outer hair cells in a row resulting in linear gaps which seem to be the result of oppression by surrounding outer phalangeal cells (*Figure 1*). In some cases, it is unclear whether the second or third row of outer hair cells is affected (*Figure 1*).

3. Tonotopic organization

As mentioned in the earlier described sections, the sensory cells in the apical turns were severely affected according to a radial gradient in which the third row outer hair cells were most damaged.

A longitudinal gradient was also obvious between the apical, middle, and basal turns of the cochlea. Stereociliary disorganization and atrophy was observed in the middle

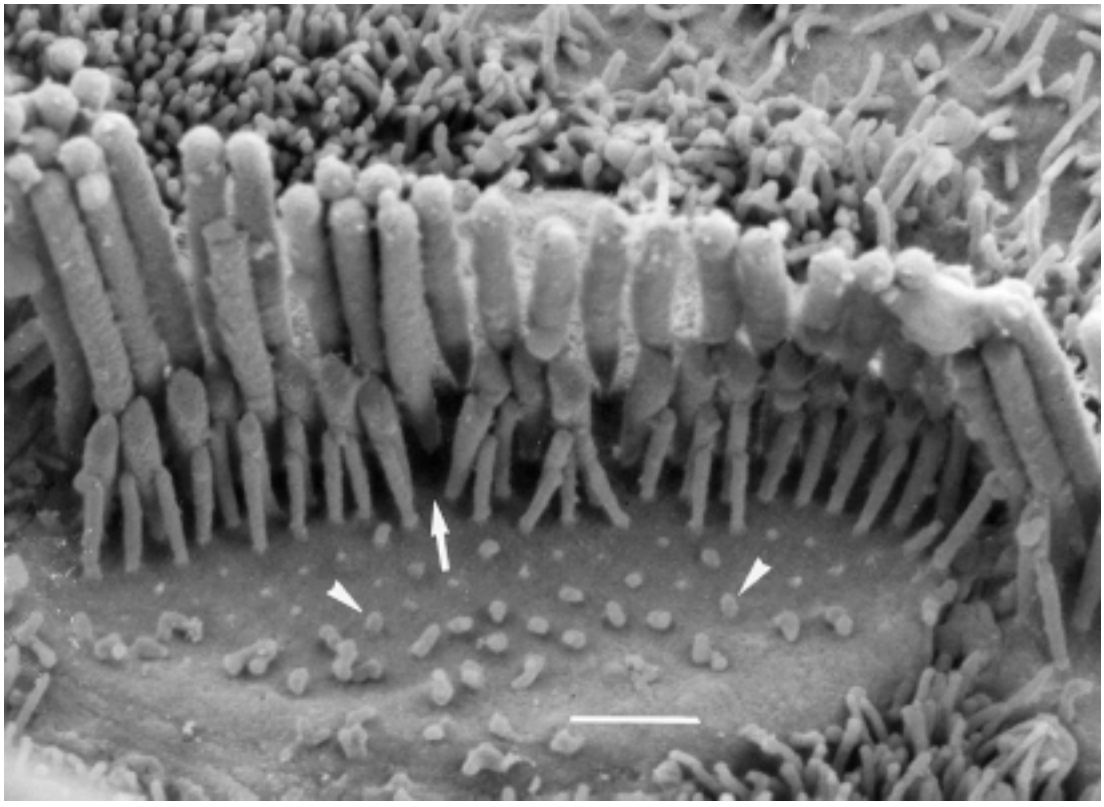

Figure 5. *Inner hair cell of a severely affected apical turn (turn 3.0-4.0). On the modiolar side of these hair cells, loss of some shortest stereocilia was observed (arrow). The configuration of the different rows of stereocilia seemed to be disorganized by loss of side-to-side linkes. Some microvillar stuctures also seem to arise on these cells (arrowheads). Bar=1 µm.*

turns of severely affected cochleas (*Figure 6a*) in both groups, however, only a few specimen showed loss of outer hair cells in the middle turns (section 1a). The basal turns showed three normal linear arranged rows of outer hair cells with W-configurated stereocilia (*Figure 6b*). No additional effects in aldosterone treated cochleas were observed in the basal turns.

No abnormal inner hair cell structure or abnormalities of the tectorial membrane were noted in the middle and basal turns.

Discussion

In this study the Two-phase Endolymphatic Hydrops model was used as a dynamic and physiological model for Menière's disease. Most severe effects were observed in the group treated according to the two-phase endolymphatic hydrops theory in which guinea pigs were treated with unilateral distal endolymphatic sac dissection followed by systemic administration of aldosterone. However, moderate effects were also noted in our subgroups treated by endolymphatic sac dissection or aldosterone alone. The exacerbation of morphological damage as demonstrated in our two-phase endolymphatic hydrops model may indicate that this model represents a more physiological approach to Menière's disease compared to the classical experimental model.

A specific sequence of morphopathology of various structures in the organ of Corti could be observed, which was predominantly located in the outer hair cells: first of all, some of the shortest stereocilia were absent in the affected third row outer hair cells in the apical turns. The stereociliary bundles become smaller starting from the lateral side of the shortest and middle stereocilia. Also, the stereociliary interconnections seemed to be absent in moderate to severe degrees wich resulted in separated or collapsed stereocilia with loss of the W-configuration. This process has been defined as stereociliary atrophy (*Figures 3 and 4*). This atrophy confirms earlier observations by Rydmarker and Horner[8] who demonstrated selective atrophy of the short and middle stereocilia of the apical outer hair cells, while the longest stereocilia appeared to remain intact. In these studies, endolymphatic hydrops was induced by classical (total) dissection of the endolymphatic sac.

After initial stereociliary disarrangement and stereociliary atrophy, the sequence of damaging effects proceeded to total absence of the shortest and, in later stages, the middle stereocilia. In these cases, the cuticular plates may still be recognizable but were frequently possessed or overgrown by microvillar structures (*Figure 2*). To our knowledge, these structures were not described before. Whether these structures were degenerative changes of the cuticular plates and the stereocilia or whether they may indicate some kind of regeneration still remains unclear.

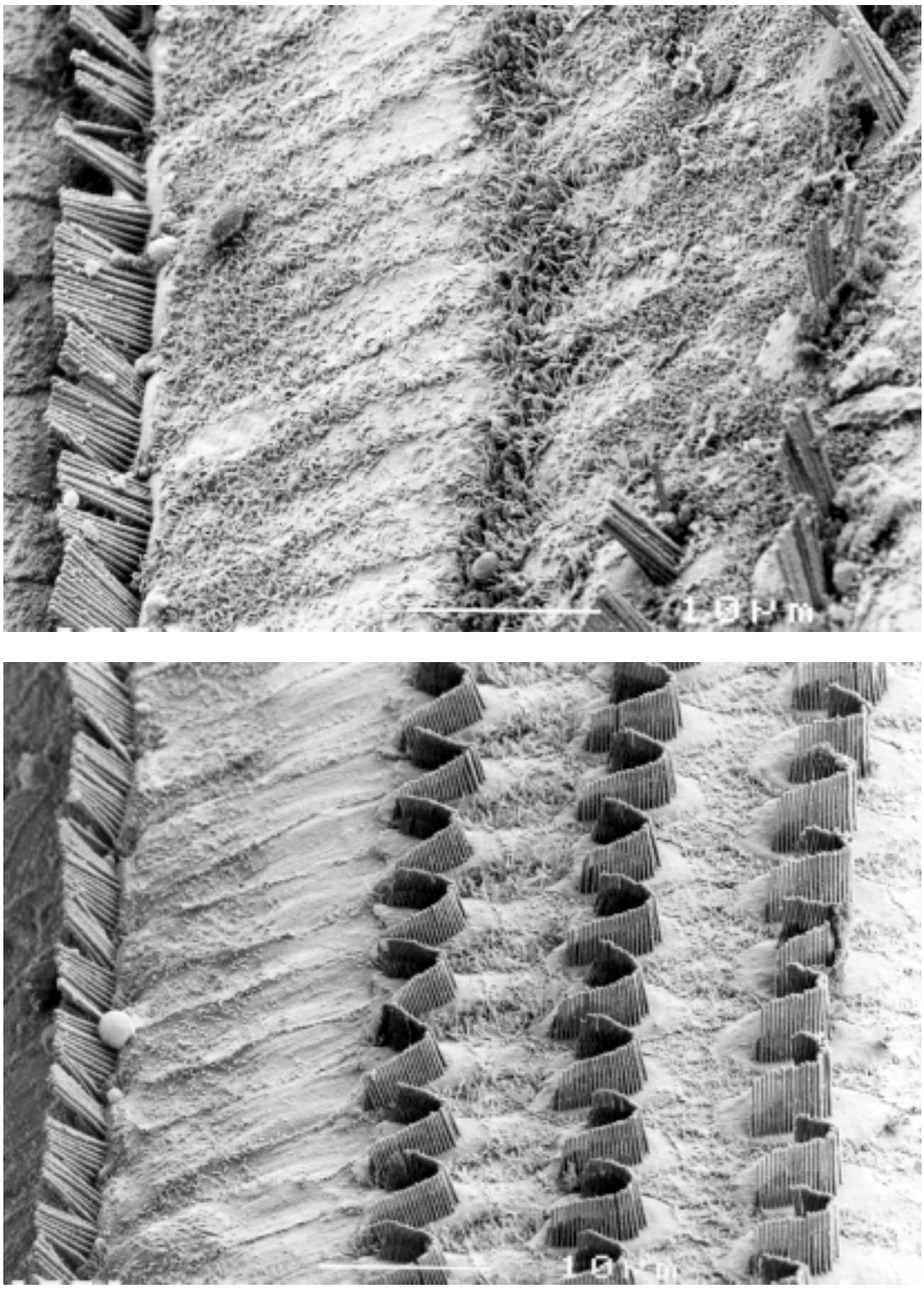

Figure 6. *Tonotopic organization of damaging effects in the different turns of a cochlea treated by distal sac dissection followed by aldosterone injections. This has been clearly demonstrated by the contrast between the severe damage in the apical turn (top) and the absence of damage in the basal turn of the same cochlea (bottom). Bar=10 µm.*

In later stages the cuticular plates could not be recognized at all, and the outer hair cells seemed to be replaced by adjacent supporting cells. As described in our results, gaps in the second or third row of outer hair cells seem to be in relation to expansion of supporting cells from the region of the outer phalangeal cells (*Figure 1*). It is uncertain whether the outer hair cells are degenerated at first and then compensated by proliferation of supporting cells, or that the loss of outer hair cells is the result of oppression by surrounding cells.

Evidence of hair cell regeneration has been identified in noise-damaged or aminoglycoside-treated cochleas[14]. Damaged hair cells and their stereociliary budles may be repaired by a reactivated differentiation process. In several studies however, damaged or lost hair cells seemed to be replaced by expansion of supporting cells. These supporting cells may reenter the cell cycle and divide and then go on to differentiate into either hair cells or supporting cells. Once new hair cells differentiate, the microvillar structures found on these cells mature to form stereociliary bundles which will be reconnected to the tectorial membrane[14].

In our specimens, the microvillar structures on the damaged hair cell may represent a regenerative effort of these cells to repair the stereociliary damage by compensation of making new ones. Expansion and proliferation of supporting cells was observed in some of our specimens without signs of transition of these supporting cells to new hair cells. In our section on the tonotopic organization, we described a radial gradient of morphological changes between the different rows of outer hair cells as well as a longitudinal gradient along the cochlea. The above described sequence of damaging effects seems to proceed in a radial gradient in which the third row of outer hair cells is affected the first and in later stages the most severe. However, a sequence of damaging effects can also be recognized along the several cochlear turns starting with the first signs, and in later stages the most severe effects in the apical turns (*Figure 6a*) proceeding to the lower turns.

Although a radial gradient along the upper cochlear turns has been described before by Rydmarker and Horner[8], in their study morphological changes due to hydrops seemed to be focused to these upper cochlear turns. A detailled scanning image using new techniques and observation of several obvious co-existing effects in the same cochlea in such a relatively short hydrops was never described before.

In one of our earlier guinea pig SEM studies[10] using low voltages and non-coating techniques, an abundance of filamentous structures were found on the tips of the longest stereocilia of the outer hair cells. These structures were also found in the same radial and longitudinal gradients as the sequential damaging effects described in this study. Hypothetically, these structures may represent some kind of glycocalyx layer and may render the stereocilia more sensitive or, in contrary, may have a protective function by supporting the shape and configuration of the (longest) stereocilia and thus delaying the damaging effects.

In several studies this glycocalyx layer, which consists of glycocojugates, has been demonstrated on the endolymphatic surfaces of both sensory cells and supporting cells. Albers et al. demonstrated a less prominent glycocalyx contrast-staining of the stereociliary membranes together with disappearance of interconnecting material between the stereocilia in two and three-month hydropic cochleas after obliteration of the endolymphatic duct[15]. Changes in the endolymphatic fluid balance might influence the biochemical composition of the glycocalyx which may affect the molecular and ion exchange between the intra- and extracellular compartments[16].

In our study most specimen were sputtercoated with a very thin layer of gold in order to prevent conductivity errors and thus enabling us to study all cochlear structures and sections adequately in a radial as well as in a longitudinal gradient. Although this sputtercoating may prevent incorrect interpretation due to conductivity disorders, delicate structures such as the cross-links or filamentous structures on the tips of the longest stereocilia[10] may become merely invisible. In this study indications of the presence of these filaments has been found in all groups with a slight tendency of absence in the two-phase treated group. However, due to clotting under the metal coating as well as their invariable presence in all groups, no conclusion could be drawn from these findings.
This study describes the scanning findings in a newly developed experimental model of endolymphatic hydrops. In this model, aldosterone not only demonstrated to aggravate hydrops[4], but also resulted in extensive stereociliary atrophy and outer hair cell loss in the apical and, to some extent, in the middle cochlear turns.

In a few of our control ears which were influenced by systemically administrated aldosterone only, some remarkable changes such as stereociliary disarrangement and atrophy in some of the third and second row outer hair cells were observed. Although these effects seem to be of minor damaging value, their importance must not be underestimated. Aldosterone proved to induce changes in cochlear structures which supports our hypothesis that the inner ear homeostasis may be influenced by aldosterone and probably other endocrine hormones[4].

However, our scanning findings after aldosterone are in contrast with the presence of hydrops in our LM study. The damage observed in this study is mainly located in the apical regions, while in our LM study, slight endolymphatic hydrops has been observed in the basal turns due to aldosterone alone. This may indicate that although hydrops may be present in the basal turns, as indicated by the raised endolymphatic fluid volume in these specimen, the basal sensory cells may be more resistant to compromising influences than the apical sensory cells.

Whether the damaging effects in the apical turns are directly related to the degree of hydrops, or undirectly related by intermediate factors, remains uncertain. One of these intermediate factors may be the mechanical influence of the filamentous structures which were found mainly on the tips of the longest outer hair cell stereocilia of the third and second row outer hair cells[10]. These filaments may play a role in altering the sensitivity

to compromising factors as discussed before in this section. This may also be demonstrated by the fact that first row outer hair cells and inner hair cells as well as basal second and third row outer hair cells, which do not possess these filamentous structures, appear to remain intact for the longest time. In contrary, the shortest stereocilia which are also deficient of these filamentous structures were first affected in this study. Also, in earlier studies, mechanical trauma merely seems to affect the longest stereocilia of the inner hair cells[17].

As the morphological changes described in this study does not seem to be directly related to endolymphatic fluid volume, or indirectly by mechanical influences on the cochlear structures, other intermediate influences such as biochemical disturbances of the endolymphatic fluid composition must be considered. Alteration of this biochemical composition may be regulated by a disturbed resorptive function of the endolymphatic sac, as well as a disturbed production of endolymph regulated by endocrine factors. Biochemical changes may interfere with sensory cell function. This may result in an ineffective sensory transduction process and perhaps even in degeneration of these sensory cells.

It would be very interesting and relevant to determine the functional implications of our scanning observations. In patients with Menière's disease audiometry often shows initial fluctuating low frequency hearing losses, followed by high and middle frequency losses resulting in an irreversible flat hearing loss in the last stage of Menière's disease.

In several models of experimental endolymphatic hydrops, this pattern of hearing loss seems to correspond to the clinical findings in patients as well as to the morphological findings earlier found in scanning studies[8]. The observations of our study will be evaluated in a future electrophysiological study for several reasons; to study the tonotopic distribution of hearing loss in relation to the severity of the damage we observed in this study, as well as to study the additional influences of aldosterone and its probable fluctuating influences on the sensory cells, resulting in reversible or irreversible electrophysiological damage.

In conclusion, the two-phase endolymphatic hydrops results in a wide spectrum of slight to severe specific morphological effects on the organ of Corti in radial as well as longitudinal gradients.

This two-phase concept demonstrates the influence of endocrine factors on endolymph production, resulting in exacerbation of endolymphatic hydrops, and its damaging effects on surface morphlogy of the sensory structures. The process of degeneration of stereociliary structures, or the possible regeneration of these structures as may be demonstrated by the microvillar strucures as found on the cuticular plates, may be a consequence of biochemical imbalance of the endolymphatic fluid composition.

The specific sequence of damage in this study, and the combination of multiple factors in exacerbating endolymphatic hydrops, may indicate a complex underlying etiology of

Menière's disease. However, this study may attribute to a better understanding of the sequential and fluctuant clinical findings as found in patients with Meniere's disease.

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