selectively activating vanilloid receptors (VRs), the ligand-gated non-selective cation channels, leading to membrane depolarization and action potential generation. Furthermore, vanilloid-sensitive nerve endings are responsible for neurogenic inflammation, which is characterized by vasodilation and increased vascular permeability [4–6]. A subtype of VR, called VR1 (now renamed TRPV1) [7], has been cloned and shown to be expressed in a subset of sensory neurons in the dorsal root ganglia and trigeminal ganglia (TG) [3]. The expression of TRPV1 is not restricted to primary sensory neurons. Rather, TRPV1 and -2 (formerly called VRL-1; VR-like protein) receptors are reportedly expressed in the brain and certain non-neuronal tissues, which means that VRs have a much broader function than just pain perception [4,8–10]. Moreover, the expression of a VR-related osmotically activated channel (TRPV4) has been demonstrated in the mouse inner ear (i.e. the inner and outer hair cells and stria vascularis), as well as in the neurosensory cells in the CNS responsible for systemic osmotic pressure, in TG and in Merkel cells [11].

In the guinea pig inner ear, it has been reported [12,13] that capsaicin regulates cochlear blood flow via substance P and a nitric oxide-mediated mechanism. TRPV1 was identified in the organ of Corti and inner ear ganglion cells [14,15] and TRPV4 was found in both inner and outer hair cells of the organ of Corti, in auditory ganglia, marginal cells of the stria vascularis and the sensory cells of vestibular end organs [11,16]. However, the existence and distribution of TRPVs in the inner ear remain unclear, as is the question of whether they play any part in hearing and/or balance sensitivity.

The aim of this study was to determine the distribution of TRPV1 and -4 in the inner ear, in order to establish the functional significance of TRPVs in hearing and/or balance.

Material and methods

We used 5 healthy, otomicroscopically normal, adult albino guinea pigs with a body weight in the range 250–300 g and a normal Preyer's reflex. The care and use of the animals was approved by the Animal Experimentation Committee, Hiroshima University School of Medicine (permit no. F9606-028) and was carried out in accordance with the Guide to Animal Experimentation, Hiroshima University and the guidelines of the Committee on Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine. The animals were deeply anesthetized with pentobarbital and fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer solution, pH 7.4. The organ of Corti, vestibular end organs and endolymphatic sac were excised and immersed in the same fixative for a further 1 h for TRPV1 or -4 immunohistochemistry.

For antibody permeability, the specimens were immersed in 1.5% Triton X-100 in phosphate-buffered saline (PBS), pH 7.2, for 1 h, followed by immersion in 3% H_2O_2 for 5 min. After pretreatment with a normal goat serum for 30 min, the specimens were incubated with a rabbit polyclonal antibody to TRPV1 (Transgenic Inc., Kumamoto, Japan) at a dilution of 0.1 μg/ml, or with a rabbit polyclonal antibody to TRPV4 (Alomone Labs Ltd., Jerusalem, Israel) at a dilution of 1:200 in 0.3% Triton X-100 containing PBS at 4°C for 48 h. This primary antibody was raised against a peptide CDGHQQGYAPKWRAEDAPL corresponding to residues 853–871 of rat TRPV4 and was affinity-purified on immobilized antigen. The specimens were then washed in PBS and incubated with biotin-labeled anti-rabbit IgG for 1 h, followed by streptavidin-conjugated peroxidase for 1 h. Antibody binding was visualized with H_2O_2 and diaminobenzidine reaction. All specimens were then dehydrated through a graded ethanol series and embedded in a water-soluble resin (JB-4®). Sections were cut at a thickness of ≈4 μm. Methyl green was used for nuclear counterstaining. A Nikon standard photomicroscope was used for observation and photography of the sections. Control sections were incubated with PBS instead of the primary antibodies; the results in these sections were invariably negative.

Results

Distribution of TRPV1 (Figure 1)

In the guinea pig organ of Corti, TRPV1 labeling was evident chiefly in the outer hair cells (OHCs), with comparatively weaker labeling in the inner hair cells (IHCs). Positive labeling was also found in some supporting cells, especially in the inner and outer pillar cells and in Hensen's cells. The labeling in outer pillar cells was somewhat weaker than that in OHCs, and both cell types were significantly more intensely labeled than IHCs. In the stria vascularis, TRPV1 stained faintly. Evidence of TRPV1 expression was also found in the spiral ganglion cells.

In the vestibular epithelia of the crista ampullares, and in both the utricular and saccular maculae, immunoreactivity to TRPV1 was observed in both type I and type II sensory cells. Supporting cells did not display any significant labeling. The apical cytoplasm of dark and transitional cells showed weak immunoreactivity. In the subepithelial tissue, no immunoreactivity was observed in general. The vestibular ganglion cells also showed positive

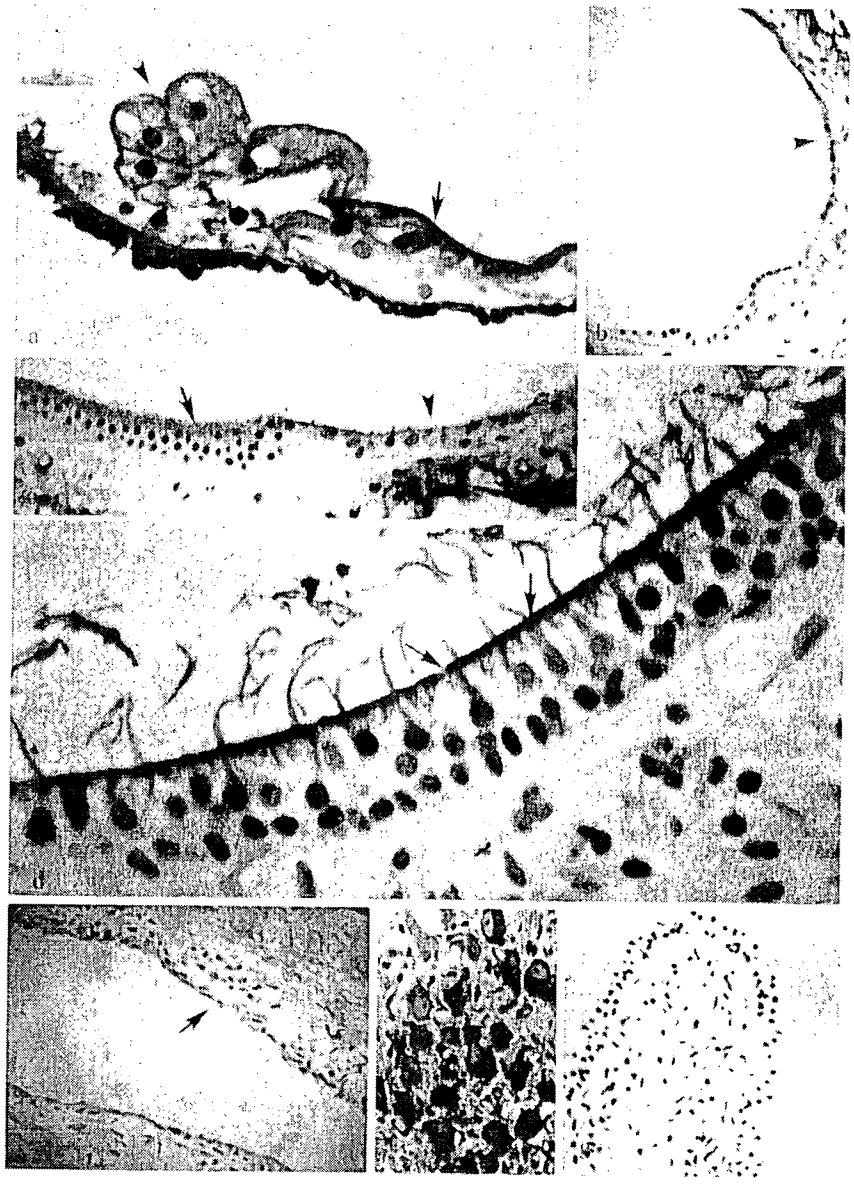

Figure 1. (a) TRPV1 labeling is evident in the OHCs (*arrow*). Positive labeling for TRPV1 is also seen in some supporting cells especially in the inner and outer pillar cells and in Hensen's cells (*arrowhead*). (b) In the stria vascularis (*arrowhead*), TRPV1 is faintly stained. (c) The apical cytoplasm of dark (*arrowhead*) and transitional cells (*arrow*) shows weak immunoreactivity. (d) In the vestibular epithelia of the crista ampullares, immunoreactivity to TRPV1 is observed in both type I and type II sensory cells (*arrows*). Supporting cells display no significant labeling. In the subepithelial tissue, no immunoreactivity is observed. (e) In the endolymphatic sac, epithelial cells (*arrow*) show only faint staining for TRPV1. (f) Vestibular ganglion cells show TRPV1-positive staining. (g) Control specimens show no significant staining.

staining. In the endolymphatic sac, epithelial cells showed barely detectable staining for TRPV1.

Distribution of TRPV4 (Figure 2)

In the guinea pig organ of Corti, TRPV4 labeling was evident in OHCs, with only comparatively weak labeling in IHCs. Positive labeling for TRPV4 was also found in some supporting cells, especially in the inner and outer pillar cells and in Hensen's cells. The region of the stria vascularis, especially the marginal cells, and also the spiral prominence revealed marked reactivity. Evidence of TRPV4 was also found in the spiral ganglion cells.

In the vestibular epithelia of the crista ampullares, and in both the utricular and saccular maculae, immunoreactivity to TRPV4 was observed in both type I and type II sensory cells. Supporting cells did not show any significant labeling. The apical cytoplasm of dark cells displayed marked immunoreactivity, while transitional cells showed moderately intense labeling. In the subepithelial tissue, no immunoreactivity was observed in

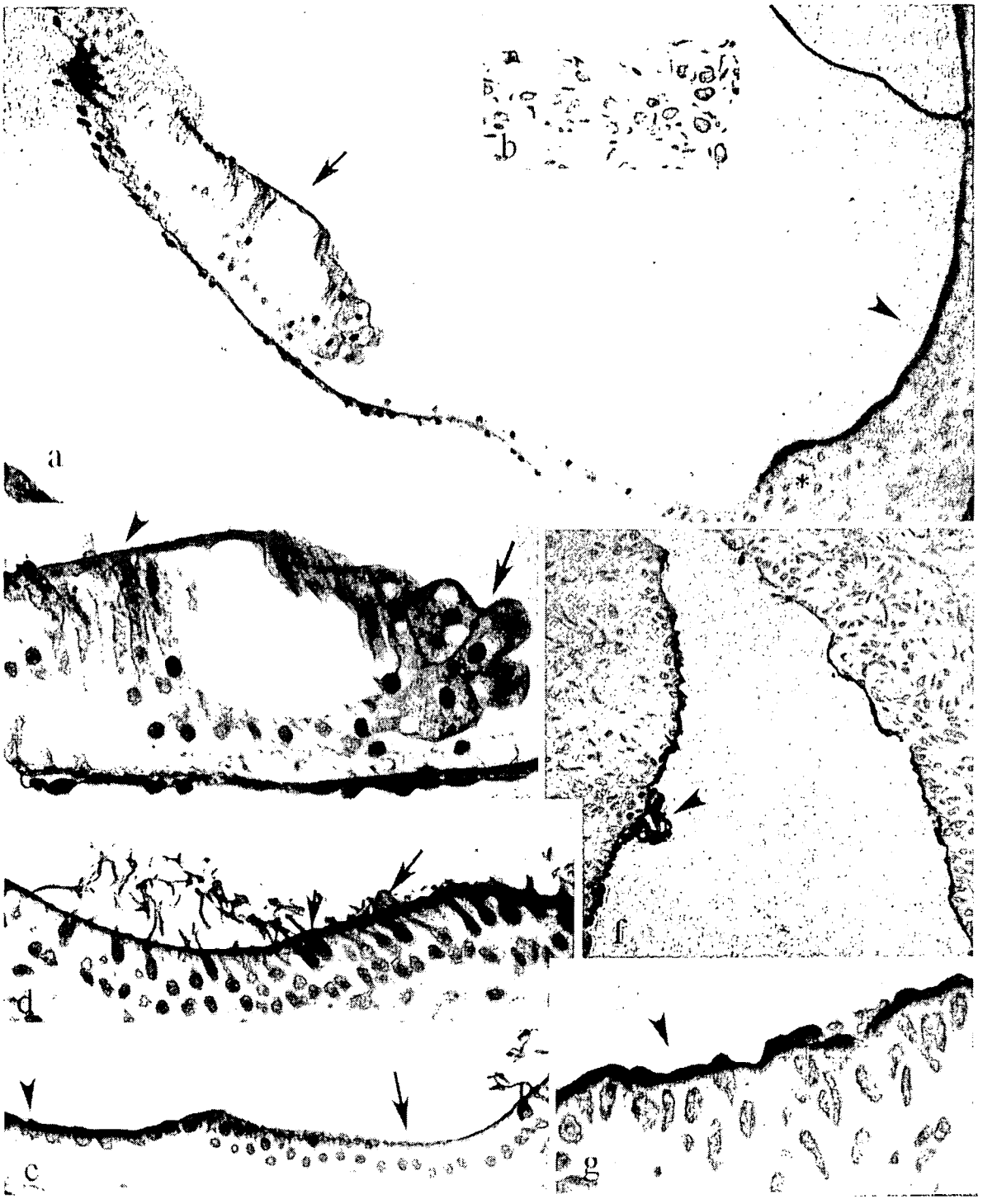

Figure 2. (a) TRPV4 labeling is evident in the organ of Corti (*arrow*). The region of the stria vascularis reveals marked reactivity (*arrowhead*). The spiral prominence also shows labeling (*asterisk*). (b) TRPV4 expression is seen in the spiral ganglion cells. (c) TRPV4 labeling is evident in the OHCs (*arrowhead*). Positive labeling for TRPV4 is seen in some supporting cells, especially in the inner and outer pillar cells and in Hensen's cells (*arrow*). (d) In the vestibular epithelia of the crista ampullares, TRPV4 is observed in both type I and type II sensory cells (*arrows*). Supporting cells do not show significant labeling. (e) The apical cytoplasm of dark cells (*arrowhead*) shows marked immunoreactivity, while transitional cells (*arrow*) show only moderate labeling. (f, g) In the endolymphatic sac, epithelial cells show intense immunoreactivity to TRPV4 (*arrowheads*).

general. The vestibular ganglion cells also showed positive staining. In the endolymphatic sac, epithelial cells displayed intense immunoreactivity to TRPV4.

Discussion

In primary sensory neurons, TRPVs are activated by heat, protons and mechanical pressure, transmitting

nociceptive information and being responsible for neurogenic inflammation. The expression and functional roles of TRPVs in other tissues remain unclear, although Birder and co-workers [17,18] found that bladder epithelial cells express TRPV1 and that TRPV1 knockout mice have altered bladder function, and Denda et al. [19] and Inoue et al. [20] found TRPV in epidermal keratinocytes. In the inner ear, TRPV1 was detected in the hair cells and supporting cells of the organ of Corti and in both the vestibular and spiral ganglion cells [14,15]. The present study revealed that TRPV1 is expressed in both the vestibular sensory cells and hair cells in the organ of Corti. It has been reported [14] that capsaicin raised the threshold of the auditory nerve compound action potential and reduced the magnitude of cochlear microphonic and electrically evoked otoacoustic emissions. These effects were reversible and could be blocked by a competitive antagonist, capsazepine. Based on the present findings and on previous physiological findings, it is suggested that TRPV1 may be involved in both cochlear and vestibular homeostasis, may have a suppressive effect on sensory cells and may participate in pathophysiological processes in the inner ear.

In contrast, the present study revealed that TRPV1 labeling was barely detectable in the fluid transporting cells, such as the stria vascularis, vestibular dark and transitional cells and endolymphatic sac. Despite alteration in the cochlear blood flow, the endocochlear potential was not affected by either capsaicin or resiniferatoxin, indicating normal functioning of the stria vascularis, which maintains the electrical and ionic environment of the endolymphatic compartment of the cochlea [14]. It is thus indicated that TRPV1 may not play an important role in the electrical, ionic or fluid homeostasis of the endolymph.

Recently [11,16], VR-related osmotically activated channels (TRPV4) were reported to be present in the inner ear. In the cochlea, the presence of TRPV4 was detected in marginal cells of the stria vascularis and in non-sensory cells that maintain the ionic composition of endolymph and the endocochlear potential. TRPV4 in these cells may help to regulate osmotic or hydrostatic pressure in the endolymph. The present study revealed that TRPV4 was present in cochlear and vestibular sensory cells as well as in fluid transporting cells, namely the stria vascularis, vestibular dark cells and epithelial cells of the endolymphatic sac. This suggests that TRPV4 could participate in regulating osmotic hydrostatic pressure in the endolymph and may play an important role in inner ear fluid homeostasis. TRPV4 was also detected in cochlear OHCs and IHCs and in vestibular hair cells. In fact, it has been reported [21,22] that hypo-osmotic challenge induces rapid contraction of vestibular and cochlear isolated hair cells. These changes were reversible upon returning cells to iso-osmotic conditions. The TRPV4 channel may therefore act as an osmotic sensor in the fluid homeostasis of these cells [11]. The function of TRPV4 in the inner ear sensory cells remains unclear, although it has been suggested that it may be closely involved in the functioning of sensory cells.

TRPV4 is also regulated by temperature in the normal physiological range [23]. It is possible that TRPVs are involved in inner ear nociception. Because they are sensitive to pH and heat, alterations in pH and temperature could activate the TRPVs on the sensory fibers innervating the cochlear blood vessels. This would result in a brief increase in cochlear blood flow and cause changes in vascular permeability [13]. What sensations might be perceived from activation of such sensory afferents are not known. It is noteworthy that the maximum sensitivity of TRPV4 at normal body temperature corresponds to peak thermal sensitivity for tactile and vibratory stimuli in mammals [11]. The molecular relationship of TRPVs makes it likely that this group of ion channels subserves diverse but related physiological functions.

Finally, it is conceivable that TRPVs are involved in cochlear and vestibular pathophysiology. In the case of inflammation, a combination of endogenous vanilloids and inflammatory mediators may act on TRPVs, in turn initiating the inflammatory cascade [4]. It is possible that the TRPVs may also play a similar role in hearing loss during inflammatory processes in the inner ear or in pathological conditions that may affect inner ear lymph chemistry. Ménière's disease and autoimmune sensorineural hearing loss are examples of such conditions [14].

Acknowledgement

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ORIGINAL ARTICLE

Heat shock protein 70 delays gentamicin-induced vestibular hair cell death

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Abstract

In this study, geranylgeranylacetone (GGA) was shown to induce heat shock protein (HSP)70 in the vestibular end organs of the guinea pig and to alleviate gentamicin (GM) ototoxicity. This was accomplished without thermal preconditioning. In isolated guinea pig vestibular end organs we demonstrated possible prophylactic (preventive) effects of GGA on GM ototoxicity by actively inducing HSP70. When HSP70 was pre-incubated with GGA, its content in sensory cell cytoplasm and transitional dark cells was increased. Pre-incubation of vestibular end organs with GGA gave sensory cells partial protection from GM toxicity. These findings show that administration of GGA can protect vestibular sensory cells from GM ototoxicity and suggest that induction of HSP70 by GGA may be a useful adjunct for the treatment of vestibular disorders.

Keywords: *Gentamicin, heat shock protein, neuroprotection, vestibular hair cell*

Introduction

Heat shock proteins (HSPs) are highly conserved proteins that are formed in response to various types of stress and are known to protect cells in a variety of biological systems [1,2]. HSPs have been related to the acquired tolerance, and has been suggested that they may protect against various types of somatic condition. HSP70 forms transiently in response to stress, providing protection and aiding recovery and repair in several ways. For example, it binds to denatured proteins to prevent them from clumping and to promote their refolding [3] and also inhibits apoptosis [4].

HSP70 has been detected in the cochlea of rodents subjected to various forms of stress. In the cochlea of the unstressed guinea pig, a constitutive level of HSP72 is evident in Deiters cells and in interdental cells of the spiral limbus [5]. It has been found that this expression is increased by hyperthermic stress [6–8], transient hypoxia [9], noise [10,11] and administration of cisplatin [12]. The outer hair cells are a major target of HSP70 expression in the rat cochlea following exposure to noise [10], transient ischaemic stress [9] and cisplatin [12], whereas the stria vascularis is the target after hyperthermia [7]. How HSP70 arises in the vestibular end organs, however, remains unknown.

It has recently been shown [6] that the stress that upregulates HSP70 also provides protection (20–30 dB) from subsequent noise exposure (which would otherwise be harmful) during the peak period of HSP protection. In rodents, this heat-induced expression of HSP has been shown to attenuate noise-induced hearing loss [6]. Quantitative polymerase chain reaction experiments showed a 100–200-fold increase in HSP mRNA in treated mouse cochleae versus those of control mice.

The importance of HSP70 has also been demonstrated in another study using rats. Altschuler et al. [11] showed that when rats with profuse HSP expression (induced by noise) were exposed to noise a second time when HSP levels were high, they showed a greater recovery from noise trauma than a group without high levels of HSP. When rats were exposed to noise a second time when HSP levels were not so high, no enhanced recovery was found. These results are consistent with the concept that upregulation of HSP protects the inner ear from

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acoustic injury. Precisely how HSPs function in the auditory system is unclear, but they may play an important role in protecting the inner ear from further damage and thus may also lie behind the conditioning phenomenon.

Geranylgeranylacetone (GGA; teprenone), an acyclic poly-isoprenoid developed and used clinically in Japan, is a unique anti-ulcer drug that protects the gastric mucosa without affecting either gastric acid or pepsin secretion [13]. Its cytoprotective effects have been found to be correlated with the expression of HSPs in gastric mucosal cells, when induced by systemic administration [14]. GGA induces the expression of proteins in the HSP60, −70 and −90 families in gastric mucosal cells, both in vivo and in vitro, by activating heat shock factor 1 (HSF1), the transcription factor for HSPs [14]. GGA induces HSPs in numerous tissues in the rat: the small intestine [15], liver [15,16], lung [15], kidney [15], heart [15,17] and retinal ganglion cells [18]. In addition, GGA has been suggested to have potential therapeutic attributes for the prevention or treatment of ischaemia–reperfusion injury, trauma, inflammation, infection, stress ulcers and organ transplantation [19]. The effects of GGA in the inner ear have not yet been investigated.

The aim of this study was to ascertain whether GGA administration could induce HSP70 in the vestibular end organs of the guinea pig and attenuate gentamicin (GM) ototoxicity. Using isolated guinea pig vestibular end organs, putative prophylactic effects of GGA on GM ototoxicity by the active induction of HSP70 in the vestibular end organs were investigated.

Material and methods

Animal dissection

Ten healthy, otomicroscopically normal Hartley guinea pigs with body weights in the range 200–250 g and a normal Preyer's reflex were used. The care and use of the animals, as approved by the Animal Experimentation Committee, Hiroshima University School of Medicine (permit No. F9606–028), was in accordance with the Guide to Animal Experimentation, Hiroshima University and the guidelines of the Committee on Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine.

All animals were anaesthetized deeply with pentobarbital and immediately decapitated. The temporal bones were quickly removed and the individual vestibular end organs were dissected in ice-cold Hanks balanced salt solution (HBSS; Gibco BRL, LideTechnologies, Oriental Inc., Tokyo, Japan) buffered at pH 7.2 and 300 mOsm with 1 mM 4-(2-hydroxyethyl)-1-piperazinethansulfonic acid (Sigma-Aldrich Co., St. Louis, MO).

Induction of HSP

In order to induce HSP in the inner ear, the individual vestibular end organs were incubated in 3 μM GGA (a generous gift from Eisai Co., Ltd., Tokyo, Japan) for 4 h [16] and prepared for further experimentation.

Immunocytochemistry

After incubation with 3 μM GGA, the specimens were fixed for 30 min in 4% paraformaldehyde/PBS, and the membranes were rendered permeable by incubation in 0.2% Triton X-100 in PBS. After pretreatment for 30 min with a blocking serum (Dako), the specimens were incubated with a mouse monoclonal antibody for HSP70 (Stressgen Biotechnologies, Victoria, B.C., Canada) diluted 1:200 in 0.3% Triton X-100 in PBS at 4°C for 48 h. The specimens were then washed in PBS and incubated for 1 h with Alexa Fluor 488 rabbit anti-mouse IgG (Molecular Probes, Eugene, OR). After dehydration with cold methacrylate monomer, the specimens were embedded in water-soluble resin (JB-4®) in the cold (4°C) [20]. Sections were cut at a thickness of ≈3 μm. Immunofluorescent images were obtained using a Nikon fluorescence microscope (Eclipse E600) equipped with appropriate filter sets (excitation 465–495 nm; emission 515–555 nm). Control sections were incubated with PBS instead of the first antibodies; the results were invariably negative.

Effect of GGA on GM-induced ototoxicity

After pre-incubation in GGA, the utricular macula and crista ampullares were dissected in order to expose the surface of the sensory epithelium. The otoconia and otoconial membrane, or the cupula, were then removed from the surface of the sensory epithelium. In order to obtain isolated vestibular sensory cells, the crista ampullares and utricular macula were then incubated for 20 min in HBSS containing 0.25 mg/ml collagenase. Further dissociation was effected mechanically and the isolated sensory cells were collected for subsequent experiments.

In order to evaluate the effect of GGA on GM-induced cell damage to the neurosensory epithelium, specimens with or without pre-incubation in GGA were incubated for up to 8 h with GM (2 mg/ml; Sigma-Aldrich).

To assess cell viability, a two-colour fluorescence cell viability assay (LIVE/DEAD Viability/Cytotoxicity Assay Kit®; Molecular Probes) was used. Live cells were distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence (excitation/emission ≈495 nm/≈515 nm). Ethidium homodimer (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (excitation/emission ≈495 nm/≈635 nm). EthD-1 is excluded by the intact plasma membrane of live cells. The determination of cell viability depends on these physical and biochemical properties of cells. Cytotoxic events not affecting these cell properties may be inaccurately assessed with this method.

Specimens were stained after different periods of time (1, 2, 4 and 8 h of incubation). The specimens were incubated for 40 min with 2 μM calcein AM and 4 μM EthD-1 in PBS, after which they were rinsed with HBSS and placed in an 8-well perfusion chamber (depth 0.5 mm, diameter 9 mm, volume 3.5 μl; PC8R-0.5; Grace Bio-Labs Inc., Bend, OR) filled with HBSS for imaging.

The specimens were viewed in a Nikon fluorescence microscope (Eclipse E600) equipped with an appropriate filter set (Nikon dual-band filter block F-R). Fluorescence analogue images were obtained with an intensified digital colour charge-coupled device camera (C4742–95; Hamamatsu Photonics) and stored as digital images, using IP Lab Spectrum software (version 3.0; Signal Analytics Corporation). After completion of each experiment, the numbers of live and dead cells were counted. Cell survival was calculated as a percentage [20].

Statistics

For the statistical analysis, 50 isolated sensory cells were randomly selected from each specimen, and the cell survival was calculated for each time point. The mean value was obtained by averaging the cell survival of six to eight specimens generated at each time point. A standard error was calculated from the means for each individual specimen. These data were analysed using two-way ANOVA.

Results

Immunohistochemistry

The vestibular end organs incubated with HBSS alone (controls) showed weak immunoreactivity for HSP70. In surface preparation specimens, some of the sensory cells showed weak immunoreactivity. The fluorescence was observed chiefly in the cytoplasm of the sensory cells (Figure 1). In the thin section specimens, fluorescence was observed only faintly (Figure 2). Immunoreactivity for HSP70 was significantly increased in the specimens pre-incubated with GGA (Figure 1). The number of positively immunoreactive sensory cells increased and almost all sensory cells displayed intense fluorescence (Figure 1). In the thin section specimens, the fluorescence was visible mainly in the cytoplasm of the sensory cells (Figure 2). It was also stronger in the transitional and dark cells. In the subepithelial space, blood vessels also showed fluorescence (Figure 3).

Protection against GM ototoxicity

Using the two-colour fluorescence cell viability assay, live cells show green fluorescence and dead cells show red fluorescence. Pre-incubation with GGA did not affect the survival rate of sensory cells. As previously reported [20], the sensory cells incubated in HBSS maintained almost complete viability for up to 8 h. Subsequently they began to die but after 10 h of incubation their survival rate was still 73%±13.5%. Those incubated in the presence of 2 mg/ml GM were significantly damaged ($p < 0.01$) after 1 h (cell survival: 54%±11.6%). Viability declined further after 2 h to 39%±15.4%. After 4 and 8 h of incubation with GM, viability had decreased to 17%±16.9% and 2%±10.5%, respectively.

Pre-incubation of vestibular end organs with GGA provided sensory cells with partial protection from GM toxicity. Cell survival was 78%±17.0% after 1 h and was thus significantly ($p < 0.01$) better than that of the cells treated with GM alone. Viability decreased to 70%±14.1% after 2 h, 56%±11.1% after 4 h and 43%±13.6% after 8 h, but was still significantly better than that of the cells treated with GM alone (Figure 4).

Discussion

In this study, HSP70 expression was induced in guinea pig vestibular end organs by in vitro incubation with GGA, an HSP inducer. We further demonstrated the protective effects of GGA on vestibular hair cell survival against GM ototoxicity. This is the first report to demonstrate that GGA induces HSP70 in the inner ear.

GGA was developed in Japan [13] and is used clinically for the treatment of gastric ulcers and gastritis. In gastric mucosal cells, GGA promotes

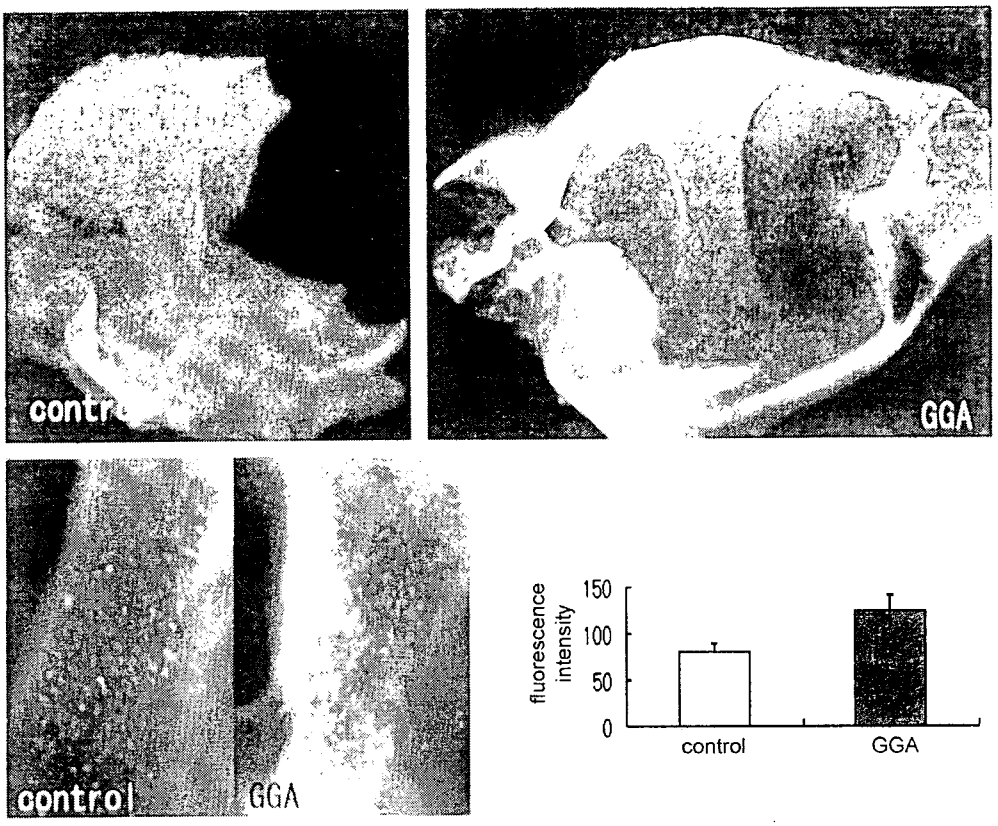

Figure 1. Vestibular end organs incubated with HBSS alone (upper left panel: *control*) showed weak immunoreactivity to HSP70 (lower left panel: *control*). Some of the sensory cells also showed weak immunoreactivity. Lower right graph: immunoreactivity to HSP70 was significantly increased in the specimens pre-incubated with GGA. Upper right and lower left (GGA) panel: the number of positive immunoreactive sensory cells increased after pre-incubation with GGA and almost all sensory cells showed intense fluorescence.

biosynthesis of glycolipid intermediates in microsomes and improves the metabolism of mucous glycoprotein. Its major pharmacological effects include promotion of biosynthesis of gastric mucus, protection of the gastric mucosa and increased gastric blood flow. A number of studies [14] have shown, both in vivo and in vitro, that administration of GGA protects rat and guinea pig gastric mucosa from various stressors. It has also been suggested [17] that GGA may exert its cytoprotective action by increasing prostaglandin E2, maintaining nitric oxide synthase activity or inducing HSPs.

Consistent with the results in other tissues, e.g. small intestine, liver, lung, kidney and heart [15–17], we observed that the administration of GGA induces expression of HSP72 in the sensory, transitional and dark cells of the vestibular end organs of the guinea pig. The mechanism by which GGA induces HSP is not clearly understood, but it is likely that GGA activates HSF1, a transcription factor that

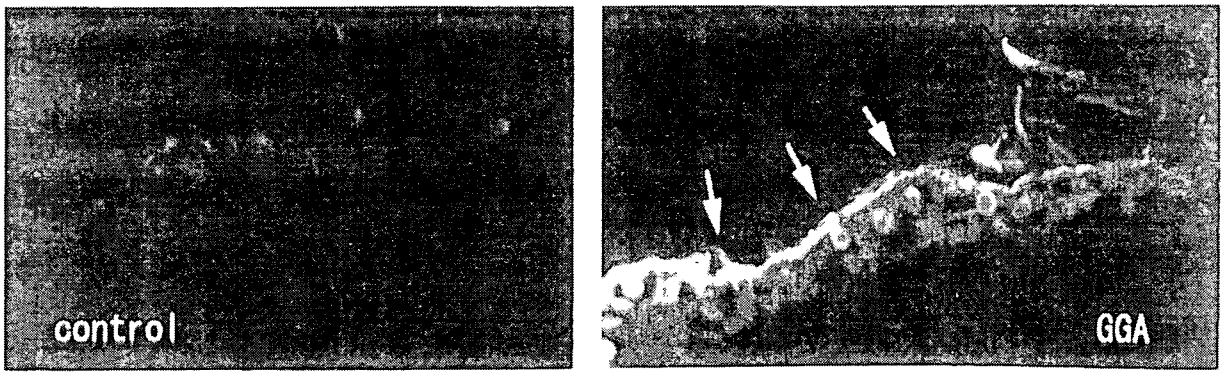

Figure 2. Control: faint fluorescence is seen. GGA: increased fluorescence is observed mainly in the cytoplasm of the sensory cells (*arrows*).

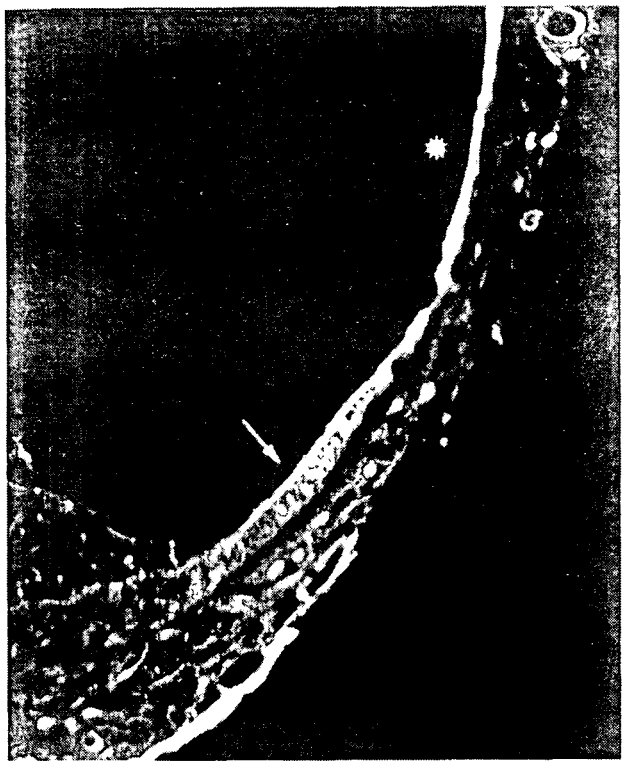

Figure 3. Fluorescence is also increased in the transitional cells (*arrow*) and dark cells (*asterisk*). In the subepithelial space, blood vessels also display fluorescence.

stimulates synthesis of mRNA for HSP72 [14,16]. HSF1 is present in the cytoplasm as an inactive monomer but, when exposed to stressors, it undergoes oligomerization to a DNA-binding trimer followed by phosphorylation, translocates into the nucleus and binds to heat shock element (HSE). HSE is located upstream of the HSP72 gene and the binding causes synthesis of HSP72 [21]. Cellular expression and activation of HSF1 in the central nervous system has been demonstrated previously [22]. HSP72 is believed to protect cerebral neurons against ischaemia, seizure, stroke and hyperthermia. The molecular mechanism of HSP72 expression induced by GGA and the involvement of HSF1 in neurons are topics for further investigation.

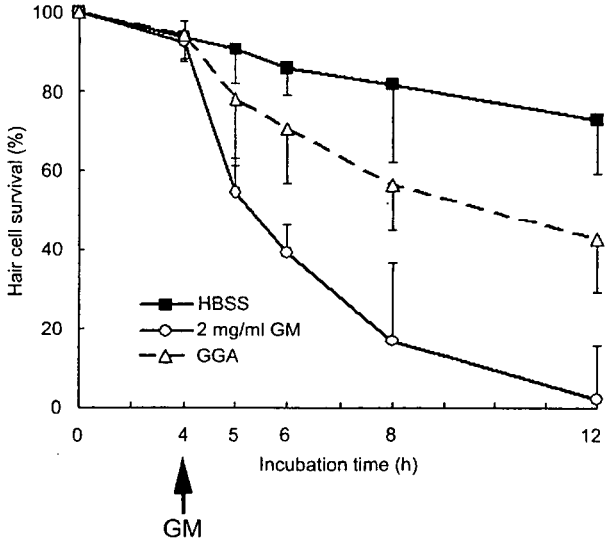

Figure 4. Sensory cells incubated in HBSS maintain almost complete viability for up to 8 h. Subsequently they begin to die but after 10 h of incubation their survival rate is still 73% ± 13.5%. Sensory cells incubated in the presence of 2 mg/ml GM are significantly damaged ($p < 0.01$). Pre-incubation of the vestibular end organs with GGA gives partial protection of sensory cells against GM toxicity. Cell survival is significantly ($p < 0.01$) better than in cells treated with GM alone.

The GM ototoxicity model used here demonstrated that pretreatment with GGA moderated GM ototoxicity. The role of HSPs in the inner ear is poorly understood; consequently, it is difficult to offer a compelling hypothetical mechanism whereby HSPs might protect the vestibular end organs from GM ototoxicity. Nevertheless, recent work has suggested a possible line of investigation [23,24]. The exact mechanism of HSP70 synthesis in vestibular sensory cells remains unclear. However, the ameliorative effect of GGA on GM ototoxicity in this model supports the notion that the use of GGA could be beneficial to patients not only with aminoglycoside ototoxicity but also several vestibular disorders, such as Ménière's disease, by enhancing endogenous self-defence mechanisms. Beere et al. [4] have proposed that HSP72 is an anti-apoptotic chaperone protein that may interfere with several stages of the apoptotic process. These stages include suppression of c-Jun N-terminal kinase (JNK) activation, prevention of cytochrome c release, disruption of apoptosome formation, inhibition of apoptotic protease-activating factor (Apaf) − 1 oligomerization and suppression of procaspase recruitment. This mechanism has also been proposed for the inner ear [25]. Aminoglycoside exposure leads to stress in the inner ear hair cells, possibly by generating reactive oxygen species and activating the JNK pathway and apoptosis. It has been suggested that JNK activation could be one of the major intracellular processes by which hair cells respond to changes in the homeostasis of their environment following exposure to ototoxic drugs or noise. A similar response may also occur in conjunction with presbyacusis.

There is also interaction between pathways, whereby the HSF1/HSP pathway can interact with nuclear factor-kappa B pathways or with the oxidative stress pathways involving caspase and activator protein − 1, with alternating fates of protection and cell death. More recently, Ikeyama et al. [16] demonstrated that GGA induces rapid accumulation of HSP72 mRNA and HSP72, suppresses hydrogen peroxide- and ethanol-induced phosphorylation of

JNK and interferes with caspase −9 and subsequent activation of caspase-3-like proteases in rat hepatocytes. Whether GGA inhibits apoptosis in this GM ototoxicity model on several levels, or whether there are preferential sites of action of HSP72, needs further evaluation [23–25].

In conclusion, administration of GGA induces an increase in HSP70 in the vestibular end organs and may protect vestibular sensory cells from GM ototoxicity. It has been suggested that induction of HSP70 by GGA may be useful for the treatment of vestibular disorders.

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ORIGINAL ARTICLE

Ménière's disease: A long-term follow-up study of bilateral hearing levels

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Abstract

Conclusion. It is suggested that a holistic factor – such as psychological stress – is involved in Menière's disease (MD) and that the pathological changes in MD may be a result not only of endolymphatic hydrops but also of disorders affecting the entire cochlea. *Patients and methods.* Changes in the hearing of 51 patients with unilateral MD were investigated to ascertain the correlation between changes in hearing loss (a) in the affected ear vs the contralateral ear and (b) at low frequencies vs high frequencies. *Results.* About half of the MD patients showed a significant positive correlation between the hearing level in the affected ear and that in the contralateral ear and also between the average hearing level at lower frequencies and that at 8 kHz. These tendencies were more pronounced in patients with severe fluctuation of hearing and/or severe hearing loss.

Keywords: *Ménière's disease, hearing loss, contralateral ear, follow-up*

Introduction

In Ménière's disease (MD), a syndrome of peripheral cochleo-vestibular origin, vertigo coincides with (initially fluctuating) hearing loss, tinnitus and aural fullness. Although most MD cases are unilateral, the incidence of bilateral involvement tends to increase as the duration of MD extends [1–5]. Concerning changes in hearing, long-term follow-up studies have found that in bilateral MD, there is an alternating 'see-saw' fluctuation and/or simultaneous fluctuation of bilateral hearing loss [6–8]. However, a few studies have been made into the changes in hearing that occur in unilateral MD, especially the correlation between the affected ear and the contralateral ear [9–11]. In the present study, we focused attention on the hearing loss of patients with unilateral MD, especially the correlation of changes in hearing loss (a) in the affected ear vs the contralateral ear, and (b) at low vs high frequencies.

Patients and methods

The study subjects were recruited from a group of registered unilateral MD patients at the Department of Otolaryngology at Hiroshima University Hospital, and at Kochi Medical School Hospital, Japan. Based on history and audiometric data, each patient was classified as having confirmed MD in accordance with the 1995 guidelines of the Committee on Hearing and Equilibrium of the American Academy of Otolaryngology – Head and Neck Surgery (CHE AAO-HNS) [12]. Patients participating in the study were carefully followed up for at least 1 year. Their average age (22 males, 29 females) was 51.5 years (range 17–71 years). The right ear was affected in 22 patients and the left ear in 29 patients. The average follow-up period was 76.5 months (range 16–161).

Evaluation of hearing

Only pure-tone thresholds were used in this study. Determination of hearing change was based on

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average hearing levels in the lower frequencies (125, 250, 500 Hz) and at all frequencies (125 Hz, 250 Hz, 500 Hz, 1 kHz, 2 kHz, 4 kHz, 8 kHz) in the affected ear, on the hearing level at 8 kHz in the affected ear, and on average hearing levels in the lower and all frequencies in the contralateral ear.

Results

The present study found that in most cases of unilateral MD, hearing fluctuated not only in the affected ear but also in the contralateral ear (Figure 1). We calculated the correlation coefficient between the average hearing level in the affected ear and that in the contralateral ear. In order to elucidate the characteristics of MD, comparison was made between a group of MD patients and a control group with conditions such as sudden deafness, idiopathic fluctuating hearing loss, and acute low-tone sensorineural hearing loss.

In the unilateral MD group, 28 cases showed a significant positive correlation (Figure 2), while in 23 cases there was no such correlation at lower frequencies between the affected and the contralateral ear. In the control group there was a significant correlation in 1/7. Thus there was a significant difference ($p < 0.05$) between the MD group and the controls. At all frequencies, 32 cases showed a significant positive correlation, whereas 19 showed no significant correlation in the MD group. In the control group, a significant difference was observed in one of seven, showing a significant difference ($p < 0.05$).

We also calculated the correlation coefficient between the average hearing level at lower frequencies and that at 8 kHz in the affected ear. In the MD group, 28 cases showed a significant positive correlation, whereas 23 showed none (Figure 2). In the control group, a significant correlation was observed in seven of eight, showing a significant difference ($p < 0.01$) (Table I).

In order to illustrate the pathophysiology of MD, comparison was made between a severe fluctuating group and a mild fluctuating group, depending on the average annual numbers of hearing fluctuation. The average number was calculated as the annual number of changes in average hearing threshold exceeding 10 dB at lower or all frequencies. Severe fluctuation was defined as more than 1.19 fluctuations/year and mild fluctuation as fewer than 1.19 fluctuations. At lower frequencies, 11 cases in the severe group showed a significant positive correlation between the affected and the contralateral ear, while 6 cases showed no significant correlation; in the mild fluctuation group, 17 showed a significant positive correlation and 17 showed none. There was a tendency ($p < 0.1$) for the severe group to have a closer correlation between the affected and the contralateral ear. At all frequencies, 14 cases in the severe group showed a significant positive correlation between the affected and the contralateral ear and 3 cases showed none, while 18 in the mild group showed a significant positive correlation and 16 showed none. Thus the severe group had a significantly closer correlation ($p < 0.05$) between the affected and the contralateral ear. According to the correlation between the average hearing level at the lower frequencies and that at 8 kHz, 12 cases in the severe group showed a significant positive correlation and 5 showed no significant correlation,

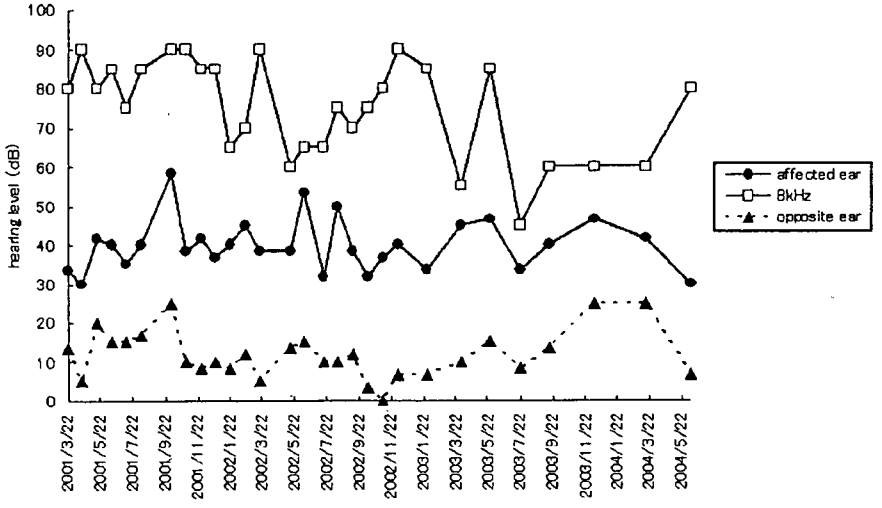

Figure 1. Time course of average hearing loss for lower frequencies in unilateral Ménière's disease. Mild fluctuations are shown for the affected ear. The contralateral ear remains in almost normal condition, although mild fluctuations were noticed, linked to those in the affected ear. Hearing loss at 8 kHz is also fluctuating, linked to that at lower frequencies.

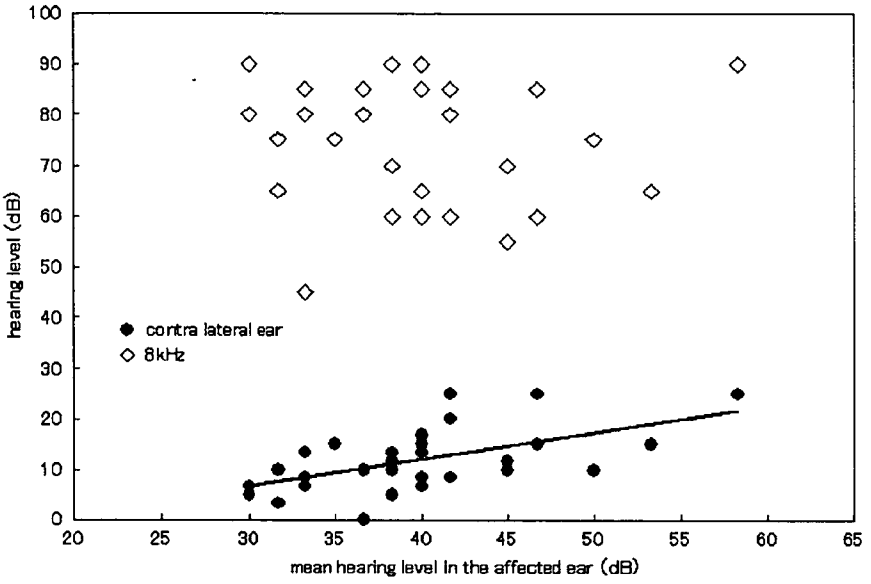

Figure 2. Correlation coefficient between average hearing level at lower frequencies in the affected ear and those in the contralateral ear as well as between average hearing level at lower frequencies and that at 8 kHz. A significant positive correlation is observed between average hearing level at lower frequencies in the affected ear and those in the contralateral ear, while no significant correlation is evident between average hearing level at the lower frequencies and that at 8 kHz.

while 16 cases in the mild group showed a significant positive correlation and 18 showed no significant correlation. There were no significant differences between these groups (Table II). In addition, we measured the average plasma ADH level and calculated the correlation coefficient between that level and the average annual number of fluctuations in hearing, showing a significant positive correlation ($p < 0.05$) (Figure 3).

Analysis was made between severe hearing loss (exceeding 40 dB in the average of all frequencies) and mild hearing loss (< 40 dB). At lower frequencies, 16 cases in the severe group showed a significant positive correlation and 11 showed no significant correlation, while 12 cases in the mild group showed a significant positive correlation and 12 showed none. There were no significant differences between the two groups. At all frequencies, 19 cases in the severe group showed a significant positive correlation and 8 showed no significant correlation, while 13 cases in the mild group showed a significant positive correlation and 11 showed none. There were no significant differences between the two groups. According to the correlation between the average hearing level at lower frequencies and that at 8 kHz, 18 cases in the severe group showed a significant positive correlation and 9 showed no significant correlation, while 10 cases in the mild group showed a significant positive correlation and 14 showed no significant correlation. There was a tendency ($p < 0.1$) for the severe group to have a closer correlation between the average hearing level at the lower frequencies and that at 8 kHz (Table III).

Discussion

The present study has established that the hearing changes found in the contralateral ear were reflected by those in the affected ear, as has been previously reported in bilateral MD [6,7,9–11]. In about half of the unilateral MD patients there was a significant

Table I. Numbers of positive correlation of hearing level.

	Lower hearing level (affected vs contralateral ear)		All hearing levels (affected vs contralateral ear)		Lower hearing level vs 8kHz	
Group	Positive	Negative	Positive	Negative	Positive	Negative
Ménière's disease	28	23	32	19	28	23
Control disease	1	6	1	6	7	1
p value	< 0.05		< 0.05		< 0.01	

Table II. Numbers of positive correlation of hearing level.

Average number of fluctuations	Lower hearing level (affected vs contralateral ear)		All hearing levels (affected vs contralateral ear)		Lower hearing level vs 8 kHz	
	Positive	Negative	Positive	Negative	Positive	Negative
> 1.19	11	6	14	3	12	5
< 1.19	17	17	18	16	16	18
p value	< 0.1		< 0.05		NS	

positive correlation between the hearing level in the affected ear and that in the contralateral ear. This tendency was more pronounced in cases of severe fluctuation of hearing, whereas such fluctuation was inconsiderable in control diseases such as sudden deafness, idiopathic fluctuating hearing loss, and acute low-tone sensorineural hearing loss. In unilateral MD, hearing change in the contralateral ear has not been examined in a similar way, possibly because of the numerous reports of hearing normality. Consequently, even when there was a deterioration, it attracted little attention. Actually, hearing loss in the contralateral ear was scarcely acknowledged and in the present investigation too, the fluctuation was inconsiderable. In a number of cases, however, there was an obvious correlation between the hearing level in the affected ear and that in the contralateral ear when hearing on both sides was compared. It has therefore been suggested that there is some correlation between the two.

It has previously been suggested that a large proportion of patients with what appears to be MD show signs of endolymphatic hydrops in the contralateral asymptomatic ear [13,14]. Concerning the

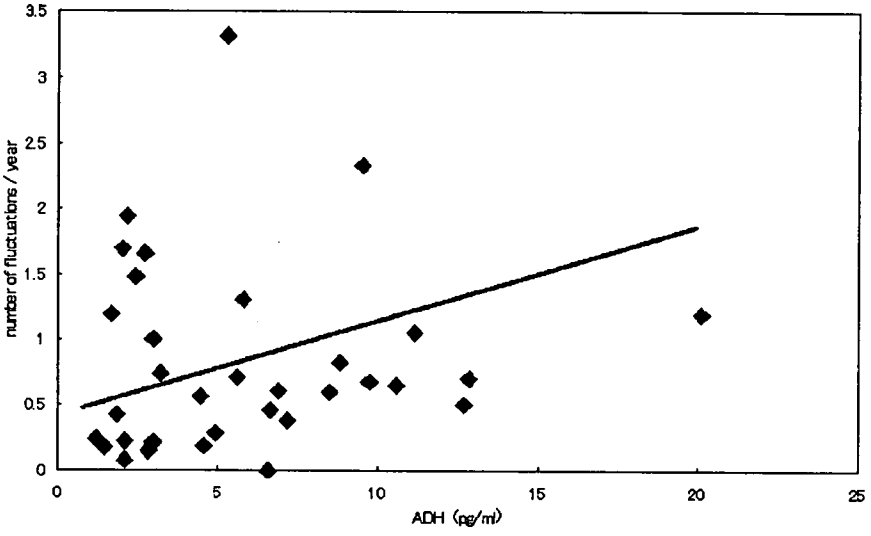

Figure 3. Significant positive correlation coefficient ($p < 0.05$) observed between plasma ADH level and average annual number of fluctuations of the hearing.

Table III. Numbers of positive correlation of hearing level.

Hearing level	Lower hearing level (affected vs contralateral ear)		All hearing levels (affected vs contralateral ear)		Lower hearing level vs 8 kHz	
	Positive	Negative	Positive	Negative	Positive	Negative
> 40 dB	16	11	19	8	18	9
< 40 dB	12	12	13	11	10	14
p value	NS		NS		< 0.1	

correlation with the hearing change on the affected side, causing a change on the contralateral side, the participation of a holistic factor such as psychological stress is regarded as a cause of such change. It is well known that stress is a considerable causative factor in MD [15,16].

The present study using the ADH level as a stress marker revealed a significant correlation with the severity of the hearing fluctuation. In the severe fluctuation group, there was a closer correlation between the hearing level in the affected ear and that in the contralateral ear.

These results may mean that one of the causes of MD is a holistic factor, and one that may affect both ears [13,14,16]. It seemed that this was related to the fact that the number of bilateral MD cases increased with the duration of the disease. It has been claimed that MD is a bilateral disease in 'all patients'. Theoretically this might be true if patients were to 'live long enough', beyond their average lifespan [9].

In addition, when examining the relationship between the average hearing level at the lower frequency and the 8 kHz hearing level in the affected ear, it was found that almost all cases of control disease had a positive significant correlation, while only half of the MD patients showed a significant correlation. This tendency was more prominent in the cases with severe hearing fluctuation and/or severe hearing loss. Generally speaking, endolymphatic hydrops is known to be more pronounced in the upper turns of the cochlea, while the degeneration of the sensory cells is most prominent in the basal turn [17]. It has therefore been suggested that the hearing loss at the lower frequencies may be caused by the endolymphatic hydrops, while that at 8 kHz is caused by sensory cell disorders. Based on these facts, the significant correlation between the hearing level at lower frequencies and at 8 kHz may indicate that pathological changes in MD may be caused not only by the endolymphatic hydrops but also by some disorder affecting the entire cochlea, probably the degeneration of cochlear hair cells, which impairs hearing including high frequencies in the long term.

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ORIGINAL ARTICLE

Effect of acute endolymphatic hydrops overload on the endolymphatic sac

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Abstract

Conclusions. Homeostasis of endolymph volume is a complex mechanism, in which the endolymphatic sac (ES) may play an important role. *Objectives.* To elucidate the effect of acute endolymphatic hydrops (EH) on the ES and to gain further information about the volume and pressure regulative function of the ES. *Materials and methods.* Distilled water was injected into the middle ear cavity of adult CBA/J mice. The ESs were studied morphologically by light and transmission electron microscopy. *Results.* Mild EH was found, particularly in the upper turn of the cochlea. Acute EH led to an increase in the size of the ES lumen, accompanied by collapse of the lateral intercellular spaces and dense perisaccular tissue, changes which had reversed 2 h after the injection.

Keywords: *Endolymphatic sac, hydrops, morphology, endolymph, homeostasis*

Introduction

The volume and composition of endolymph are of great importance for the normal functioning of the inner ear. Changes in the endolymph are believed to give rise to serious derangement of inner ear function and may be involved in certain diseases, such as Meniere's disease and delayed endolymphatic hydrops (EH). The mechanism of endolymph volume regulation and the pathophysiology of EH still remain obscure. Far from being a passive reservoir for endolymph, the endolymphatic sac (ES) probably acts as a site of endolymph volume regulation [1–4]. The anatomical and histological characteristics of the ES suggest its involvement in transport functions, and other functions have been suggested, such as secretion of macromolecules, removal of waste products and immunological, volumetric and pressure regulative functions [4,5].

Surgical ablation of the ES in the guinea pig results in chronic EH, which has been widely investigated to gain insight into Meniere's disease [1,6]. Acute EH has been induced by microinjecting artificial endolymph into the scala media of the cochlea [3,7] or by intratympanic injection of distilled water into the middle ear cavity [6,8]. In contrast to chronic EH, hydrops caused by the injection develops immediately and is not disturbed by the complex cascades of secondary pathological changes [7].

The literature contains two contradictory reports on the effects of acute EH on the ES. Rask-Andersen et al. [3] reported that, directly after the microinjection of artificial endolymph, there was almost total absence of the normal intraluminal, stainable, homogeneous substance (HS) on the injected side. 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-distending HS. In contrast to this theory, Valk et al. [7] reported that no differences were found in the ES between injected and non-injected ears and no distinct changes were observed in guinea pigs terminated after different time intervals. HS was invariably

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present in the lumen of the ES and was often copious.

The present study was designed to elucidate the effect of acute EH on the ES and to gain further information about the volume and pressure regulative functions of the ES. Mice were used for this purpose, as the HS in the ES and its related secretory function of the ES has already been fully investigated in mice [4,5,9,10].

Materials and methods

Twenty adult CBA/J mice, of 20–25 g body weight and with a normal Preyer's reflex, were used in this study. The care and use of the animals was approved by the Animal Experimentation Committee, Hiroshima University School of Medicine (permit no. A06-68) and was in accordance with the Guide to Animal Experimentation, Hiroshima University and the Committee on Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine.

Distilled water was injected into the middle ear cavity through a tiny puncture in the posterior-inferior quadrant of the tympanic membrane. In order to study any morphological changes in the ES, the mice were sacrificed under deep anesthesia with pentobarbital and decapitated at the following times: 0, 15 and 30 min, and 1 and 2 h after the instillation. The temporal bones were excised and fixed by immersion in 3% glutaraldehyde and decalcified with 0.1 M buffered Na-EDTA for 10 days. The specimens were then stained with 1% OsO_4 for 2 h, dehydrated with graded ethanols and embedded in Agar 100 resin. Sections were cut in routine fashion. Thick sections, about 3 μm, were stained with toluidine blue and thin sections were counterstained with uranyl acetate and lead citrate.

The light microscopic specimens were viewed in a Nikon photo microscope (Eclipse E600). Analog images were obtained using an intensified digital color charge-coupled device camera (C4742-95; Hamamatsu Photonics) and stored as digital images using IP Lab Spectrum software (version 3.0; Signal Analytics Corporation). A JEOL 100CXII transmission electron microscope was used to observe and photograph the electron microscopic specimens.

Quantitative assessment of volumetric changes in the scala media

For the quantitative assessment of volumetric changes in scala media, the change ratios of the cross-sectional area of the scala media were measured in mid-modiolar sections of the cochlea [11]. For this analysis, the following two parameters were measured in the lower and upper turns, not including the hook portion: 1) cross-sectional area of scala media (S), enclosed by the distended Reissner's membrane; 2) cross-sectional area of the original scala media (S*), enclosed by a straight line segment, representing the normal position of the idealized Reissner's membrane connecting its normal lateral attachment at the upper margin of stria vascularis to its normal medial attachment at the spiral limbus. The anatomical measurements were carried out in 'blind' fashion using IP Lab Spectrum software.

From these parameters, the increased ratios (%) of the cross-sectional area of scala media (IR-S) of an upper turn were calculated according to the following equation:

$$\text{Increase ratio (\%) of the cross-sectional area of the scala media (IR-S)} = 100 \times \sum(Sx\text{-}S^*x)/\sum S^*x \text{ (x: upper turn)}$$

Quantitative assessment of luminal size change of the ES

The size of the ES lumen in the intra-osseous rugose portion was calculated as follows. One light micrograph (×200) from the stored digital images was prepared from the mid-portion of the intra-osseous rugose part of each ES. The lumen of the sac and the bony lining of the vestibular aqueduct were outlined on these photographs. The relative size of the lumen, as compared with the bony lining of the vestibular aqueduct, was calculated again using the IP Lab Spectrum software [9,12].

Results

After the injection of distilled water into the middle ear cavity, mild but definite EH was observed, especially in the upper turn. The endolymphatic space appeared significantly larger than that of non-injected control ears, at both 15 and 30 min after the injection (Figure 1), but decreased gradually. Two hours after the injection, the endolymphatic space was normal again.

The increase ratio (IR) of the cross-sectional area of scala media of the upper turn was calculated. On the injected side, IR tended to increase after 15 min (IR = 134 ± 27.2%; mean ± SD, $n = 4$) and 30 min (123 ± 4.1%, $n = 4$). IR became normal again after 1 h (105 ± 4.5%, $n = 4$) and 2 h (105 ± 2.6%, $n = 4$). On the control side, IR was 93 ± 3.0% ($n = 4$) after 15 min, 93 ± 4.3% ($n = 4$) after 30 min, 94 ± 5.2% ($n = 4$) after 1 h and 93 ± 9.8% ($n = 4$) after 2 h (Figure 2).

The ES and duct were both analysed histologically. In the non-injected control ears, numerous distended lateral intercellular spaces (LIS) were seen between

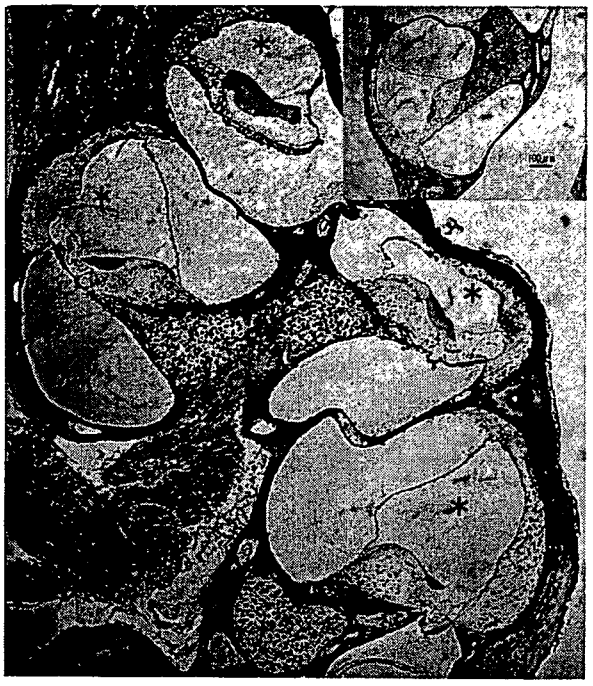

Figure 1. At 15 min after injection of distilled water into the middle ear cavity, mild endolymphatic hydrops (EH) is evident in the cochlea (asterisks). Inset: non-injected control ear showing absence of EH.

the dark and light cells of the epithelial lining of the ES (Figures 3 and 4). The epithelial cells were usually columnar, particularly in the intra-osseous portion of the ES (Figure 3). Ultrastructurally, different epithelial cells could be clearly distinguished. The light cells showed light cytoplasm and were outnumbered by dark cells. The more columnar light cells displayed abundant supuranuclear mitochondria and apical microvilli (Figure 4). Other common morphological features included a basally situated round nucleus with both smooth and rough endoplasmic reticulae, Golgi apparatus and lysosomal structures. At some points, light cells were covered over by dark cells. The dark cells showed a dark cytoplasm without apical microvilli and few mitochondria. They showed more variety in cell and nucleus shape. In general, they had an indented nucleus. The subepithelial tissue appeared loose and contained a network of blood vessels surrounding the ES (Figure 4). Inside the ES lumen, little if any intraluminal homogeneous stainable substance was observed (Figure 3).

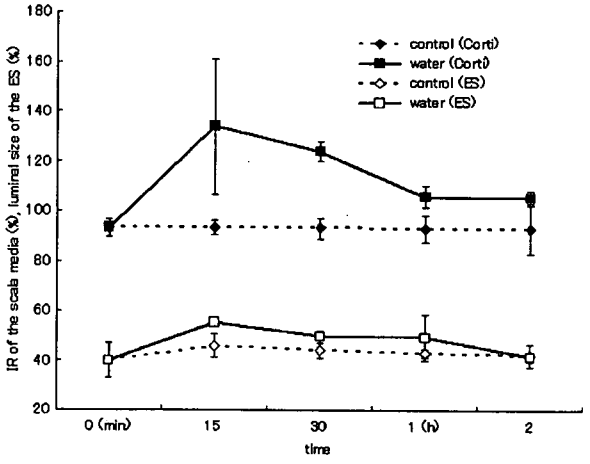

Figure 2. In the injected ear (water (Corti)), the increase ratios (IRs) of the cross-sectional area of scala media of the upper turn tended to increase after 15 and 30 min, but became normal again after 1 and 2 h. On the control side (control (Corti)), IRs show no change. In the injected ears, the relative size of the lumen of the endolymphatic sac (ES) increases significantly after 15 and 30 min, then decreases after 1 h and returns to normal again after 2 h (water (ES)). On the control side (control (ES)), the luminal side of the ES shows no change.

Although there were no obvious changes in the endolymphatic duct, the ES exhibited distinct changes compared with the non-injected side. On the injected side, there was very little intraluminal HS. The luminal size of the ES appeared significantly larger than normal at 15 and 30 min after the injection. The LIS had collapsed and the looseness of the perisaccular tissue was no longer apparent (Figure 5). The epithelial cells were often of the cuboidal or flat type. The so-called granular cells frequently observed after the injection of glycerol [9,12] were not observed. The dark cells generally did not cover the apical surface of light cells (Figure 6). Two hours after injection, the ES lumen had normalized. The LIS were no longer collapsed and looseness of perisaccular tissue was observed again. The epithelial cells were generally columnar (Figure 7).

The relative luminal size of the ES was judged according to the ratio between the size of the ES lumen and that of the bony vestibular aqueduct in the intermediate, rugose portion. In normal conditions the relative size of the lumen of the ES was $40 \pm 7.1\%$ (mean $\pm$ SD, $n = 4$), but it increased significantly to $55 \pm 1.3\%$ ($n = 4$) after 15 min, and to $49 \pm 2.5\%$ ($n = 4$) after 30 min. The luminal size then diminished after 1 h ($48 \pm 9.3\%$, $n = 4$) and returned to normal again after 2 h ($41 \pm 2.0\%$, $n = 4$) (Figure 2).

Discussion

We found that acute EH results in pronounced morphological changes in the ES. Acute EH led to increase in the luminal size of the ES, accompanied by the collapse of the LIS and dense perisaccular tissue. These changes were not observed in the non-injected control ears, suggesting that they resulted directly from the induced change in acute EH. This pattern of change in the ES, i.e. increased luminal size, was in contrast to that previously reported

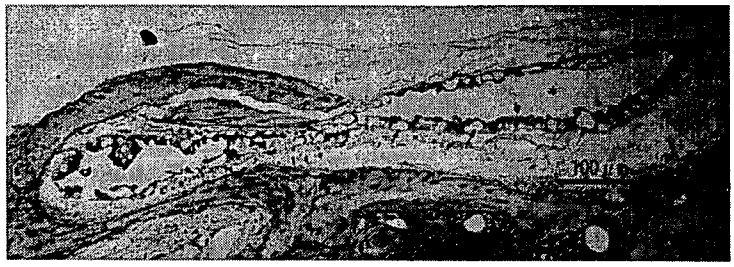

Figure 3. In the non-injected (control) ears, numerous, distended lateral intercellular spaces (LIS) are visible between the dark and light cells of the epithelial lining of the endolymphatic sac (ES, arrows). The lumen of the ES (asterisk) is not dilated.

Figure 4. The epithelial cells are generally columnar, particularly in the intra-osseous part of the endolymphatic sac (ES). The light cells display abundant supranuclear mitochondria and apical microvilli with a basally situated round nucleus (arrow). The dark cells have a dark cytoplasm without apical microvilli and few mitochondria. They show more variety in cell and nucleus shape (arrowheads). At some points, light cells are covered by dark cells (double arrow). The lateral intercellular spaces (LIS, asterisks) are distended.

following systemic treatment with glycerol [9,12]. Some 15–60 min after systemic glycerol administration, the ES volume had decreased. During this time, the ES lumen often collapsed or became obliterated. The LIS were distended, which may be a sign of increased transepithelial movement of fluid [13]. This finding, together with signs of increased looseness of the perisaccular tissue, may indicate that fluid may also be withdrawn from the interior through the epithelial barrier due to local osmotic forces [12]. Subsequently, the lumen dilated. The initial volume increase of the ES in this study may reflect the endolymphatic volume and pressure increase induced by acute EH. This is a conceivable reaction, since the increase in endolymph volume and pressure could thus be compensated by the ES volume increase. It may therefore be suggested that the ES controls the endolymph volume and pressure by actively regulating its size [4].

Concerning the stainable HS in the ES lumen, the present study revealed that most of the ES showed clear endolymph inside the lumen, in both injected and non-injected (control) ears. This is not surprising. Around 80% of normal mice contain clear endolymph, while the remainder have only a small amount of HS (around 10–40% of the lumen) [10,12]. This is in contrast to normal guinea pigs, which generally have a large amount of HS in the ES. This increase in HS, with consequent reduction in endolymph volume, has been reported previously following systemic treatment with glycerol [5,9,12]. The HS is a composite of complex macromolecules, of which the principal glycosaminoglycan is hyaluronan [14], and could have the property of drawing water osmotically from outside of the ES, thus increasing the volume and pressure within the ES and thereby compensating for the withdrawal of fluid from the rest of the inner ear [9,12]. In acute EH, there is no need to increase the volume and

Figure 5. On the injected side, the lumen of the endolymphatic sac (ES, asterisk) appears significantly larger than normal 15 min after the injection. The lateral intercellular spaces (LIS) have collapsed and the perisaccular tissue is no longer loose (arrow).