Figure 6. The epithelial cells are often of the cuboid or flat type. The lateral intercellular spaces (LIS, arrows) have collapsed and the perisaccular tissue is no longer loose (asterisk).

pressure in the ES, so it is reasonable to suggest that the increase in HS in the ES lumen passed unobserved in the present investigation. Similar findings have been reported in the literature [3]. An increase in endolymph volume led to a decrease in stainable HS in the lumen of the sac; conversely, a reduced volume produced an increase in luminal HS. However, in contrast to this theory, Valk et al. [7] reported that no differences in luminal HS content were found between injected and non-injected ears immediately after termination. Furthermore, no distinct changes were observed in guinea pigs sacrificed at intervals up to 2 h. The reason for this discrepancy is still not known, although there are several explanations. It is known that there are physiological differences between guinea pigs of different strains, gender, size, and age [7]. There are even more differences between guinea pigs and mice. In mice, 30 and 60 min after the injection of glycerol, most of the sac lumens are filled with stainable substance. Two hours after, some specimens show clear endolymph, while others contain stainable substance. Most specimens show clear endolymph inside the lumen after 4 h [9,12]. These results may indicate that it takes 1 or 2 h for the disappearance or digestion of HS in mice. In contrast, in the guinea pig the disappearance of HS has been reported to occur within a matter of minutes [3]. The difference between these two studies may derive from the difference between the animals, i.e. guinea pig and mouse. The present study could not explain the discrepancy. Our results only suggest that the volume of the ES (and hence of the entire membranous labyrinth) may be actively regulated in the ES by active secretion and by dynamic regulation of the ES volume.

Concerning this endolymph volume regulation, one important question is: how does the inner ear sense a volume change? One possibility is transient receptor potential channels (TRPV4). Recently, TRPV4 were reported to be present in the inner ear, i.e. organ of Corti, stria vascularis, transitional and dark cells in the vestibular end organ, and epithelial cells of the ES. TRPV4 consists of osmotically activated channels and is suggested to participate in regulating osmotic hydrostatic pressure in the endolymph and to play an important role in the inner ear fluid homeostasis [15].

In conclusion, endolymph volume homeostasis is a complex mechanism, in which the ES may play an important role. Further investigation is necessary to elucidate the regulatory mechanisms of inner ear fluid volume, which will lead to an understanding of and treatment of an inner ear disease such as Meniere's disease and delayed EH.

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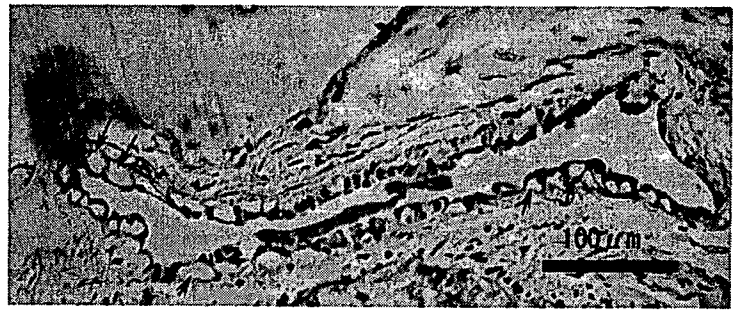

Figure 7. At 2 h after the injection, the endolymphatic sac (ES) lumen has normalized. The lateral intercellular spaces (LIS, arrows) are no longer collapsed and the perisaccular tissue is loose once more.

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ORIGINAL ARTICLE

A new animal model for Ménière's disease

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Abstract

Conclusion. A new murine model for the study of Ménière's disease has been developed by treatment with both lipopolysaccharide (LPS) and aldosterone. Induction of vestibular dysfunction in the hydropic animal model may entail additional stress such as reduced inner ear blood flow, and sudden acute changes in endolymph volume and/or pressure. *Objective.* The purpose of this study was to develop a more suitable animal model, showing closer resemblance to the pathophysiological process in Ménière's disease. *Materials and methods.* Adult CBA/J mice were treated by intratympanic injection of LPS, intraperitoneal injection of aldosterone, or injection of both LPS and aldosterone. Morphological analyses were performed in the cochlea and endolymphatic sac. *Results.* All experimental animals showed mild to moderate endolymphatic hydrops. Those treated with both LPS and aldosterone showed reversible vestibular dysfunction after the intratympanic injection of epinephrine.

Keywords: *Endolymphatic hydrops, lipopolysaccharide, aldosterone, mice, Ménière's disease*

Introduction

Since the discovery of endolymphatic hydrops (EH) in temporal bones of patients with Ménière's disease, EH has been accepted as the pathological origin of Ménière's disease [1,2]. Hydrops can result from the destabilization of natural regulation by excessive production of endolymph and/or reduced absorption of endolymph [3]. A number of methods have been used to develop an animal model for Ménière's disease. EH was observed after injecting distilled water, horse serum, tuberculin, or sodium chloride into the middle ear cavity, and following injection of pilocarpine or acetylcholine around the eighth nerve trunk in the internal auditory canal. When the functioning of the stria vascularis was modified by subcutaneous injection of arsenic or Atoxyl, or intraperitoneal injection of ethacrynic acid, hydrops developed in a few instances [4]. Injection of purified cholera toxin into scala media to stimulate endolymph secretion from the stria vascularis by stimulating adenyl cyclase resulted in a high incidence (81%) of hydrops [5].

Since its introduction by Kimura and Schuknecht in 1965 [6], surgical induction (by obliteration of the endolymphatic duct and sac) of EH in the guinea pig has become the standard model for the study of Ménière's disease. This procedure has been readily adopted by some investigators because it reliably produces both histological hydrops and hearing loss. However, this animal model does not result in anything resembling attacks of vertigo, even though a predictable low-tone hearing loss can ensue [7,8]. In this standard model produced by surgical obliteration, the hydrops is induced by destroying the endolymphatic sac (ES) and obliterating the endolymphatic duct (ED) with bone wax [6]. Recently, Dunnebier et al. [9] induced mild EH by total dissection of the extra-osseous part of the ES adjacent to the sigmoid sinus to obstruct the venous outflow to the sigmoid sinus and produce mild fibrosis of the most distal portion of the sac. Although several modifications of the standard animal model including Dunnebier's model [9] were developed, which produced hydrops in a

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varying degree, they were still too destructive or could not be standardized sufficiently to be superior to the standard model [4].

In contrast to the surgical obliteration model, 'overproduction' models have been developed that, like the surgical model, elicit both histological hydrops and hearing loss [4,7,8]. These models include the introduction of cholera toxin into the inner ear [5], long-term administration of vasopressin [10] or aldosterone [9], and various procedures to elicit inner ear autoimmune disease [8,11]. Because the mechanism for eliciting hydrops differs in overproduction models from that in the surgical model, it is possible that the pathophysiology of hearing loss is also different.

Recently, a two-phase hydrops guinea pig model has been developed [9]. This model is based on a combination of chronic ES dysfunction, induced by mild destruction of the most distal part of the ES, and acute stress-induced endolymph production by stimulating sodium-potassium activated adenosine triphosphatase (Na/K ATPase) in the stria vascularis with aldosterone.

At present it is still unclear whether one of these models better replicates the auditory and cochlear pathologic changes in Ménière's disease. It is therefore important to continue efforts to characterize all animal models as thoroughly as possible. It is not clear either that the characteristic vestibular symptoms of Ménière's disease are reproduced in the animal model, or even whether the basic physiology of hydrops production is the same as in the human condition [4,7,8].

The purpose of this study was to develop a more suitable animal model, which shows greater resemblance to the pathophysiological process in Ménière's disease. For this, we used a combination of both reduced absorption of endolymph and an enhanced production of endolymph fluid. A sudden reduction of absorption of endolymph is induced by the intratympanic injection of lipopolysaccharide (LPS) [12,13]. A mild increase in endolymph production was induced by administering aldosterone, to stimulate Na/K ATPase in the stria vascularis and the dark cells [9].

Materials and methods

Twelve healthy, otomicroscopically normal adult CBA/J mice of 20–25 g body weight and with a normal Preyer's reflex were used in this study. Care and use of the animals was approved by the Animal Experimentation Committee, Hiroshima University School of Medicine (permit no. A06-68) and 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.

The animals were randomly divided into three groups. Animals in group 1 ($n=5$) were inoculated once daily for 5 days with 1 mg LPS extracted from *Escherichia coli* (Sigma Chemical Co., St Louis, MO, USA) dissolved in 0.1 ml of sterile saline. The toxin was instilled into the left tympanic cavity through the tympanic membrane using a sterile 27-gauge needle. The right side (non-injected) ear served as a control. Animals in group 2 ($n=2$) received a daily intraperitoneal injection of aldosterone at a dose of 100 μg/100 g/day (Acros Organics, New Jersey, USA) for 5 days. Animals in group 3 ($n=5$) were inoculated with both LPS in the left ear and aldosterone intraperitoneally in the same way for 5 days. At 24 h after the last injection, the animals were deeply anesthetized with pentobarbital and fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The temporal bones were excised and immersed in the same fixative for another 2 h. They were decalcified with 0.1 M buffered Na-EDTA for 14 days. The specimens were then dehydrated with graded ethanols and embedded in a water-soluble resin (JB-4®). Sections were cut about 4 m thick and stained with toluidine blue for light microscopy.

The light microscopic specimens were viewed in a Nikon photomicroscope (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).

Quantitative assessment of volumetric changes of the scala media

For the quantitative assessment of volumetric changes in the scala media (S), the change ratios of the cross-sectional area of S were measured from the mid-modiolar sections of the cochlea [10]. For this analysis, the following two parameters were measured in the lower and upper turns, not including the hook portion: 1) the cross-sectional area of S, enclosed by the distended Reissner's membrane, and 2) the cross-sectional area of the original scala media (S*), enclosed by a straight line segment, which represents the normal position of the idealized Reissner's membrane connecting the normal lateral attachment of Reissner's membrane 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 the IP Lab Spectrum software.

From these parameters, the increase ratio (%) of the cross-sectional area of S (IR-S) of an upper or lower turn was calculated according to the equation described below.

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

Quantitative assessment of luminal size change of the ES

The luminal sizes of the ES in the intra-osseous rugose portion as well as the extra-osseous distal portion were calculated in the following way. One light micrograph from the stored digital images was prepared at the mid-portion of the intra-osseous rugose portion of each ES. On these photographs, the lumen of the sac and the bony lining of the vestibular aqueduct were outlined. 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. In the distal portion of the ES, one light micrograph from the stored digital images was prepared at the distal portion of each ES. On these photographs, the short diameter of the lumen of the sac and that of the outer lining of whole ES were measured. The relative diameter was calculated again using the IP Lab Spectrum software [14].

Induction of vestibular dysfunction

In order to create the vestibular dysfunction, some animals in each group were inoculated with 1:10 000 epinephrine into the middle ear cavity through the tympanic membrane (TM). Their behavior was recorded with VTR. The nystagmus was recorded in darkness using a Frenzel's glass with an infrared CCD camera with a video monitor.

Results

Endolymphatic hydrops

In the control ears, which were not inoculated LPS nor treated with aldosterone, no hydrops was detected in any of the turns of these cochleas (Figure 1a).

In the ears treated with aldosterone only, all cochleas demonstrated a mild to moderate degree of EH. Intracochlear variation in the severity of hydrops was observed in this group. Three of nine cochleas showed more severe hydrops in the lower turn and six revealed more severe hydrops in the upper turn. Elongation and folding of Reissner's membrane was noted in four of nine cochleas, especially in the lower turn (Figure 1b).

In the ears inoculated with LPS, but not treated with aldosterone, a slight or moderate severity of hydrops was observed in the cochlea. All cochleas showed more severe hydrops in the upper turn. Folding of Reissner's membrane was noted in one of five cochleas (Figure 1c).

In the ears in which LPS was inoculated and aldosterone was administered, all cochleas showed mild to moderate EH. Intracochlear variation in the severity of hydrops was also demonstrated in this group. The combined treatment with both LPS and aldosterone did not clearly demonstrate a shift to a more severe degree of hydrops when compared with only LPS or aldosterone treatment. However, aldosterone did increase the severity of hydrops in the lower turn. The severity of hydrops was more distinct in the upper turn of the cochlea in general. Elongation and folding of Reissner's membrane was noted in two of five cochleas, especially in the lower turn (Figure 1d).

The increase ratios (IR) of the cross-sectional area of the SM of upper and lower turns were calculated. In the control ears, IR was $97 \pm 6.1\%$ (mean $\pm$ SD, $n = 5$) in the upper turn and $100 \pm 5.2\%$ in the lower turn. In the aldosterone-treated ears, IR was significantly increased both in the upper turn ($122 \pm 8.2\%$, $n = 9$, $p < 0.01$; Student's t test) and in the lower turn ($117 \pm 10.4\%$, $p < 0.01$). IR was also significantly increased in the LPS-treated ears in the upper turn ($126 \pm 6.7\%$, $n = 5$, $p < 0.01$) and in the lower turn ($115 \pm 6.5\%$, $p < 0.01$), and in the LPS+aldosterone-treated ears in the upper turn ($131 \pm 8.4\%$, $n = 5$, $p < 0.01$) and in the lower turn ($115.4 \pm 10.0\%$, $p < 0.05$) (Figure 2). The significant difference in IR was noted between the upper and lower turns in the LPS-treated ears ($p < 0.05$) and aldosterone+LPS-treated ears ($p < 0.01$), whereas no significant difference was noted in the aldosterone-treated ears. No significant difference was observed between the experimental groups.

Endolymphatic sac

Light microscopic examination of the normal and experimental animals was performed to assess the effect of LPS inoculation, administration of aldosterone, or both LPS and aldosterone on the ES.

In the control ears, the intra-osseous portion of the ES was easily identified by its cylindrical cells, which protrude into the lumen as irregular papillae. The epithelial lining of the distal portion of the ES was cuboid, except at the extreme end, where it was squamous. In the intra-osseous portion of the ES, numerous distended lateral intercellular spaces (LIS) were seen in the epithelial cell lining of the ES. The epithelial cells were columnar in general. In

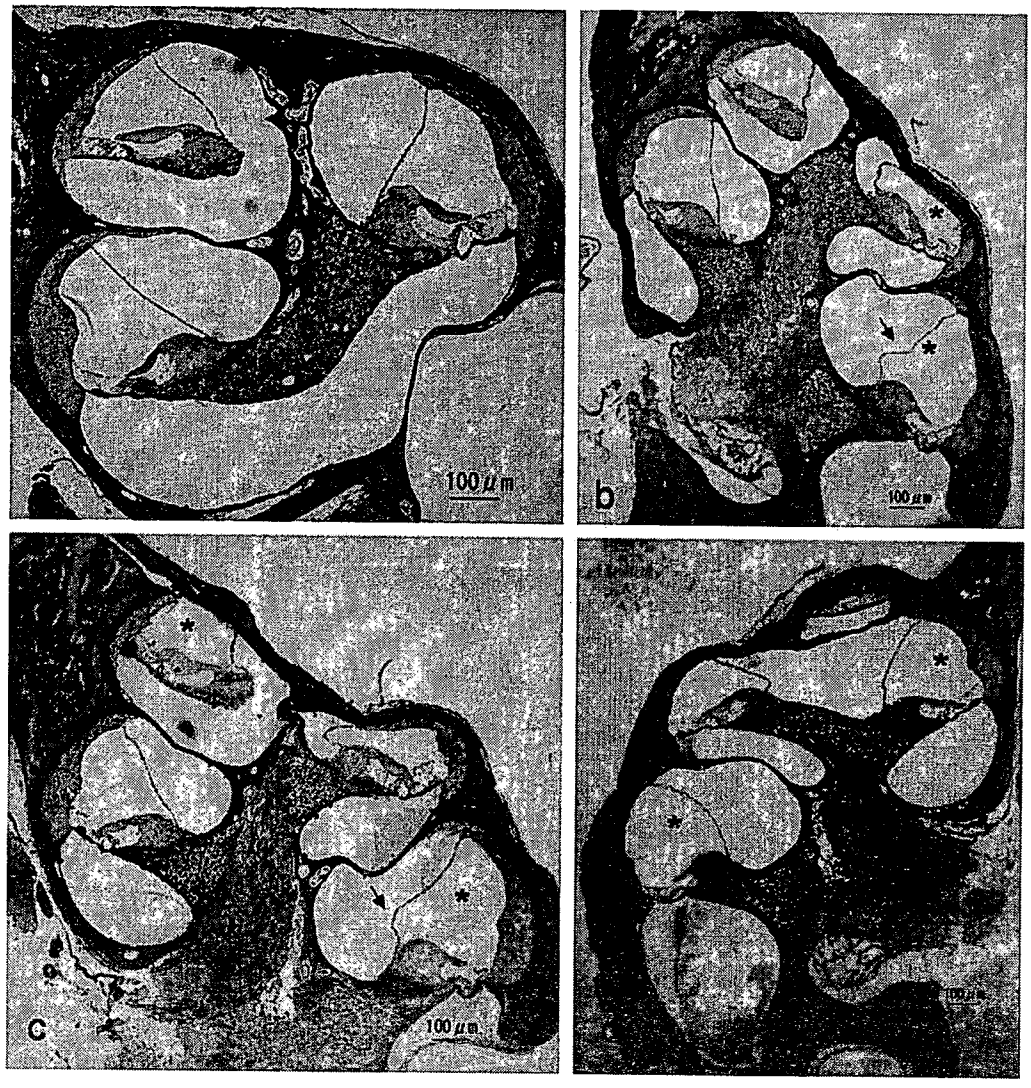

Figure 1. (a) Control ears: no hydrops in any of the turns of the cochlea. (b) Aldosterone-treated ears: mild endolymphatic hydrops (asterisks); elongation and folding of Reissner's membrane in the lower turn (arrow). (c) LPS-inoculated ears: slight or moderate degree of hydrops (asterisks); hydrops is more severe in the upper turn; folding of Reissner's membrane (arrow). (d) LPS + aldosterone-treated ears: moderate endolymphatic hydrops (asterisks); the endolymphatic hydrops is more uniform in this group.

the distal portion of the ES, LIS were not so distended as in the intra-osseous portion, but were nevertheless evident (Figure 3a,b).

In the aldosterone-treated ears, the lumen of the distal portion of the ES was markedly dilated. The epithelial cells became thinner and the LIS had collapsed. Inside the lumen, the ES contained clear endolymph without macrophages. The intra-osseous portion of the ES was also dilated. The LIS had also collapsed and the looseness of the perisaccular tissue was no longer observed. The epithelial cells were often of the cuboid or flat type (Figure 3c,d).

In the LPS-treated ears, the lumen of the distal portion of the ES was narrower and had collapsed. Distended LIS were observed between the cuboidal epithelial cells. Inside the lumen, a number of macrophages were observed. In the intra-osseous portion of the ES, the lumen of the ES had collapsed and contained a stainable substance with a number of macrophages. The LIS appeared distended, showing a general increase in size (Figure 4a,b).

In the LPS + aldosterone-treated ears, intrasaccular variation in the morphologic features was observed. The distal portion of the ES showed wide variations, from near collapse to marked dilation. The LIS appeared distended in general. The intra-osseous portion of the ES showed a wide variation too, while the lumen of the ES was slightly dilated in general. The LIS were generally distended (Figure 4c,d).

The relative luminal size of the ES was judged using the ratio between the size of the ES lumen and that of the bony vestibular aqueduct in the intermediate,

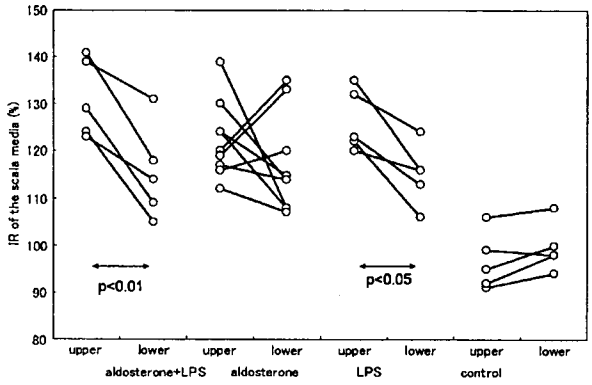

Figure 2. The increase ratios (IR) of the cross-sectional area of the scala media of upper and lower turns. In the control ears, IR is 97±6.1% (upper turn) and 100±5.2% (lower turn). In the aldosterone-treated ears, IR is significantly increased both in the upper turn (122±8.2%, $p<0.01$) and in the lower turn (117±10.4%, $p<0.01$). IR is also significantly increased in the LPS-treated ears in the upper turn (126±6.7%, $p<0.01$) and in the lower turn (115±6.5%, $p<0.01$), and in the LPS+aldosterone-treated ears in the upper turn (131±8.4%, $p<0.01$) and in the lower turn (115.4±10.0%, $p<0.05$). The significant difference in IR is noted between the upper and lower turns in the LPS-treated ears ($p<0.05$) and aldosterone+LPS-treated ears ($p<0.01$).

rugose portion as well as the ratio between diameter of the ES lumen and that of the whole ES in the distal portion. In the normal condition the relative size of the lumen of the ES was 47±7.1% (mean±SD, $n=5$) in the intra-osseous portion and 68±3.6% in the distal portion. In the aldosterone-treated ears, the luminal size of the ES was significantly increased, to 61±2.8% (mean±SD, $n=9$) in the intra-osseous portion ($p<0.01$) and 82±1.7% in the distal portion ($p<0.01$), while it was significantly decreased to 35±1.4% ($n=5$) (intra-osseous portion) ($p<0.01$) and 58±3.4% (distal portion) ($p<0.01$) in the LPS-treated ears. In the LPS+aldosterone-treated ears, the luminal size of the ES in the intra-osseous portion (43±2.8%) was similar to that of the control, while the distal portion (71±5.1%) showed wide variations in size, from normal to marked dilation (Figure 5). In the aldosterone-treated ears, the relative size of the ES lumen was significantly greater than that of the LPS- or aldosterone+LPS-treated ears ($p<0.01$).

Behavioral data

Treatment with epinephrine did not induce any behavioral changes in the animals treated with aldosterone or LPS alone, whereas treatment with both aldosterone and LPS had drastic effects. Four of five animals in this group showed signs of severe vestibular dysfunction. Following epinephrine injection, the vestibular effects started to manifest within 5 min. In the most affected stage, the animals could not maintain a stable posture but leaned to the left (injected) side (Figure 6). They were unable to swim and when walking showed a tendency to turn to left. At the same time faint but distinct nystagmus toward the right (non-injected) side was noted with infrared Frenzel's glass. This behavior reached its maximum after 10 min and continued for about 30 min. The

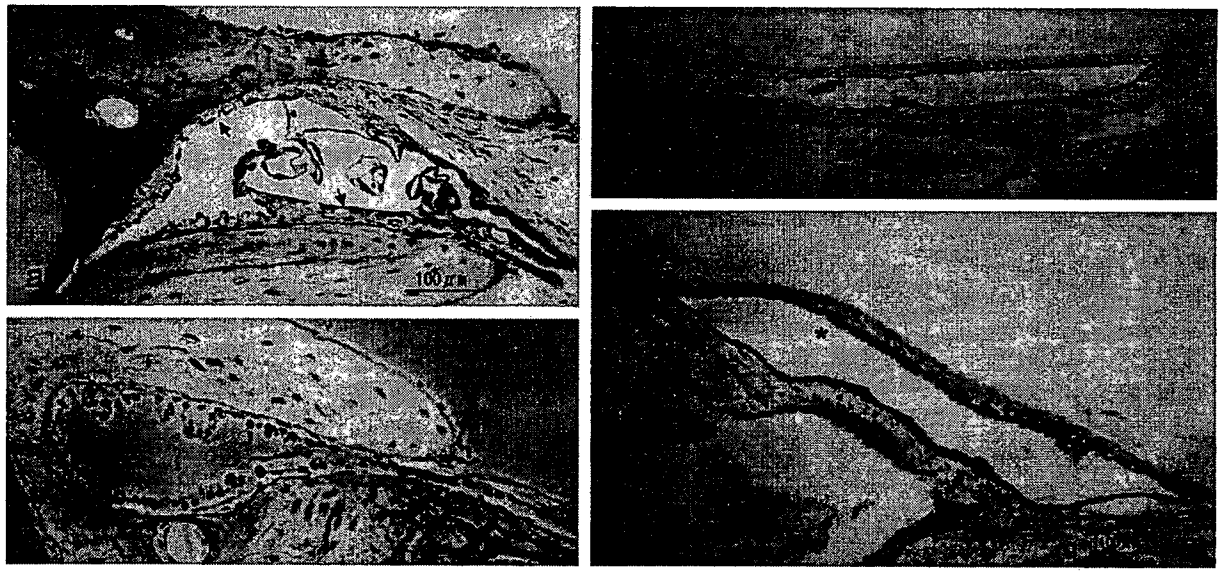

Figure 3. (a) In the control ears, the intra-osseous portion of the endolymphatic sac (ES) is identified by its cylindrical cells, which protrude into the lumen. Numerous, distended lateral intercellular spaces (LIS) are seen in the epithelial cell lining of the ES (arrows). (b) In the distal portion of the ES, the epithelial lining is cuboid. LIS is not evident compared with the intra-osseous portion. (c) In the aldosterone-treated ears, the intra-osseous portion of the ES is dilated. The LIS have collapsed and the looseness of the perisaccular tissue is no longer observed. The epithelial cells are of the flat type. (d) The lumen of the distal portion of the ES is markedly dilated. The epithelial cells have thinned and the LIS have collapsed. Inside the lumen, the ES contains clear endolymph without macrophages (asterisk).

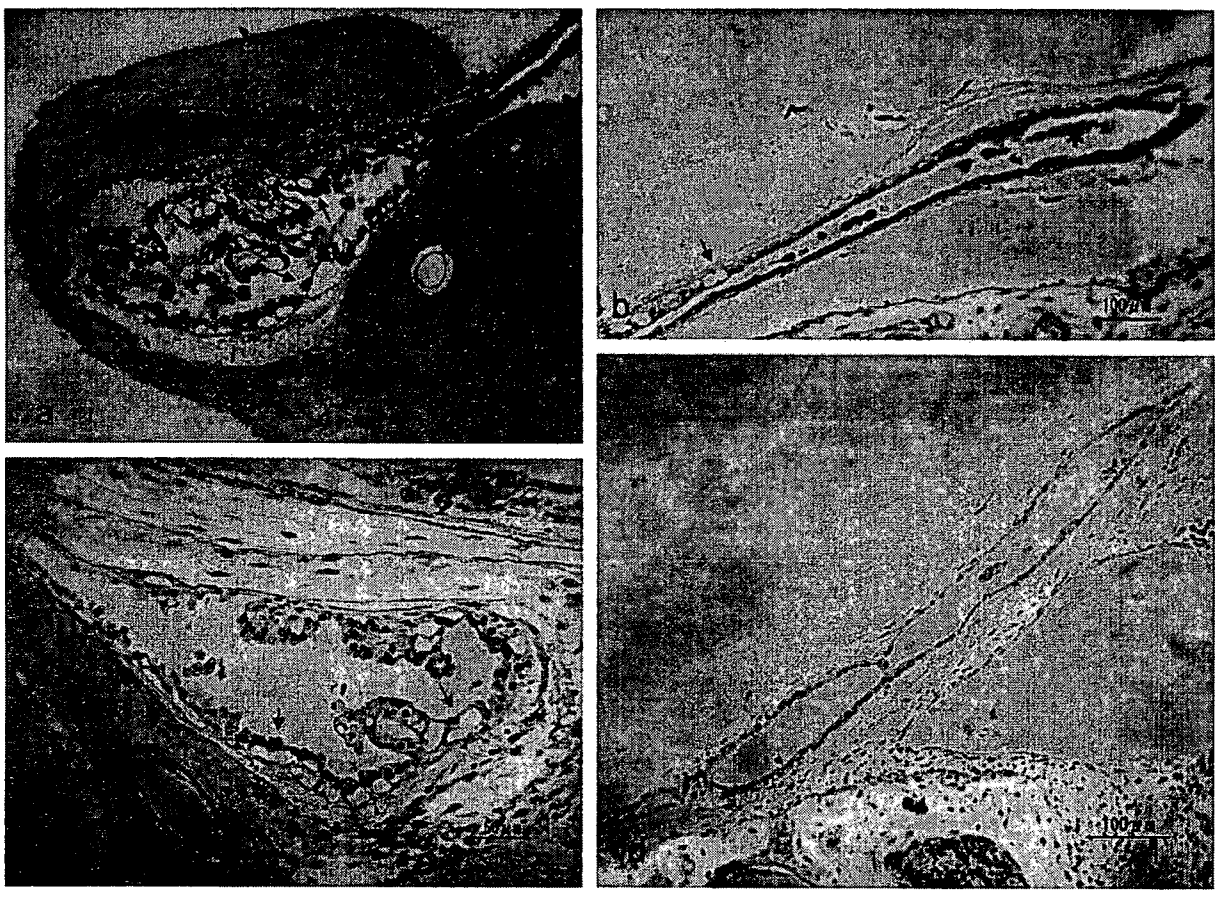

Figure 4. (a) In the LPS-treated ears, the lumen of the intra-osseous portion of the endolymphatic sac (ES) has collapsed and contains a stainable substance with a number of macrophages (asterisk). The LIS appear distended, showing a general increase in size (arrows). (b) The lumen of the distal portion of the ES is narrower and collapsed. Distended LIS are observed; inside the lumen, a number of macrophages. (c) In the LPS+aldosterone-treated ears, the intra-osseous portion of the ES shows a wide variation as well, while the lumen of the ES is slightly dilated in general. The LIS are generally distended (arrows). (d) The distal portion of the ES shows wide variations from near-collapse to marked dilation.

animals then gradually recovered and after 1 h all animals displayed normal vestibular function. Signs of ataxia and weakness of muscle tonus were no longer observed at any given time.

Discussion

Even though obliteration of the ED and ES in the guinea pig is most the ideally available procedure for producing hydrops, this model is not perfect for Ménière's disease, because after such procedures these animals rarely show episodic vestibular symptoms [4,7,8]. Furthermore, this model requires the surgical obstruction of ED and ES. Several modifications of this animal model, which produced hydrops to a varying degree, were still too destructive and were not physiologically accurate models for Meniere's disease. In the present study, we succeeded in producing hydrops without any surgical procedures on the ED and ES. Another interesting point was that we were able to induce EH in the mice. By surgical obliteration of the ES, EH has been produced in the guinea pig with 100% accuracy. Another reliable species for production of EH by this procedure is the rabbit. In the cat, EH can be produced in 80% of animals. The sac blockage procedure was not successful in monkeys and the success rate was poor in chinchillas [4]. In mice, surgical obliteration of the ES is difficult because of its size and location. Actually, mice have several advantages as an animal model, compared with the guinea pig. Mice have now been widely used for inner ear research, have a number of antibodies available, and also have more technical advantages for the investigation of genetic problems.

In the present investigation, we used a combination of overproduction of endolymph and reduced absorption of endolymph (or ES dysfunction) to induce EH.

Endolymph overproduction was induced by stimulating Na/K ATPase in the stria vascularis with aldosterone [9]. The specific chemical composition of endolymph and the generation of the transepithelial

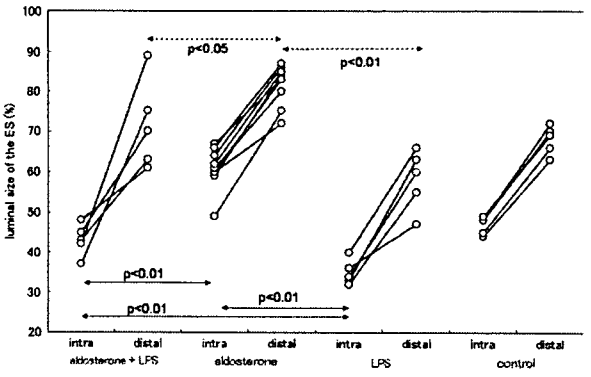

Figure 5. The relative luminal size of the endolymphatic sac (ES) was judged using the ratio between the size of the ES lumen and that of the bony vestibular aqueduct in the intermediate portion as well as the ratio between diameter of the ES lumen and that of the whole ES in the distal portion. In the normal condition the relative size of the lumen of the ES is $47 \pm 7.1\%$ in the intra-osseous portion and $68 \pm 3.6\%$ in the distal portion. In the aldosterone-treated ears, the luminal size of the ES is significantly increased, to $61 \pm 2.8\%$ in the intra-osseous portion ($p < 0.01$) and $82 \pm 1.7\%$ in the distal portion ($p < 0.01$), but significantly decreased to $35 \pm 1.4\%$ (intra-osseous portion) ($p < 0.01$) and $58 \pm 3.4\%$ (distal portion) ($p < 0.01$) in the LPS-treated ears. In the LPS + aldosterone-treated ears, the luminal size of the ES in the intra-osseous portion ($43 \pm 2.8\%$) is similar to that of the control, while the size of the distal portion ($71 \pm 5.1\%$) varies widely. In the aldosterone-treated ears, the relative size of the ES lumen is significantly greater than that of the LPS- or aldosterone + LPS-treated ears ($p < 0.01$).

positive potential are considered to be regulated by membrane-bound Na/K ATPase in the marginal cells of stria vascularis and the dark cells of the vestibular labyrinth [15]. In the guinea pig, large amounts of Na/K ATPase were detected in several inner ear structures [16,17] and a relationship between circulating adrenal steroid and Na/K ATPase activity in the inner ear has been demonstrated [18–20]. Depletion of aldosterone after bilateral adrenalectomy of the rat reduces ATPase activity in the stria vascularis, which induced morphological changes within the cellular structure of the cochlea have also been observed [18]. Re-establishment of an endogenous level of aldosterone restores the cellular morphology and increases the Na/K ATPase activity [19]. The Na/K ATPase level in the stria vascularis increases as a result of enhanced aldosterone levels induced by low-sodium high-potassium diets [20]. Aldosterone levels may also be increased by emotional stress, which has been suggested as a precipitating factor in Ménière's disease [21]. The strial Na/K ATPase activation by aldosterone may cause an increased secretion of potassium ions in the endolymphatic compartment and excess production of endolymph. This may contribute to the development of EH as seen in Ménière's disease [9].

Figure 6. After intratympanic injection of epinephrine, the animals treated with both aldosterone and LPS could not maintain a stable posture, but leaned to the left (injected) side.

In the present investigation, all aldosterone-treated cochleas demonstrated mild to moderate EH. This aldosterone-induced EH has already been reported in the guinea pig [9]. In mice, as in guinea pigs [9], some aldosterone-treated cochleas showed more severe hydrops in the lower turn. This is a characteristic finding for aldosterone-induced hydrops, as in the standard surgical model, the hydrops is concentrated in the apical turns of the cochlea [6]. Concerning the mechanisms of the aldosterone-induced EH, the localization and properties of ATPase in the inner ear have been put forward [15]. It has been reported that there was a marked decrease in strial enzyme activity, from the base to the apex of the cochlea. Stimulation of this enzyme activity by aldosterone stimulates the basal turn more to develop hydrops, probably because of the relatively intense enzyme activity in this part of the stria.

The present study also revealed that aldosterone-treated ES showed a marked dilation of the lumen of the ES, in both the intra-osseous and the extra-osseous portion. A similar finding was also noted in the acute EH induced by injecting distilled water into the middle ear cavity in mice [22]. The dilation of the ES lumen may reflect a sudden volume increase in the endolymph, which could thus be an additional support for the overproduction of endolymph by aldosterone.

The reduced absorption of endolymph or ES dysfunction was induced by the intratympanic injection of LPS. Intratympanic injection of LPS induces otitis media. EH has been observed in animal studies of otitis media [4,12,13]. Fluctuating sensorineural hearing loss in chronic otitis media has led to the hypothesis that the latter can cause hydrops [23]. Histopathologic studies on human temporal bones have found EH to be a common occurrence in human cases of otogenic or meningogenically induced suppurative or serous labyrinthitis. Paparella and Djalilian [23] reported that in a study of 560 temporal bones, 75 of 194 bones with otitis media

were affected by EH. The frequency of the presence of both disease processes supports the hypothesis that the two diseases may be related. In the guinea pig [13] and chinchilla [12], mild EH has been observed after injecting LPS. The most common pathologic findings in the cochleas after LPS inoculation were inflammatory cell infiltration and bleeding in the perilymphatic and endolymphatic spaces, distension of the intercellular space of stria vascularis, hair cell damage, vacuolation of supporting cells, i.e. Hensen's cells and Deiter's cells, and mild EH [12,13]. In the ES, inflammatory cell infiltration was frequently seen in the lumen but also to a small extent in the subcellular connective tissue. The ES lumen was generally filled with stainable substance [13].

The present study also demonstrated mild EH in the cochlea. In the ES, the lumen contained stainable substances and inflammatory cell infiltration. The luminal size of the ES was diminished in both the intra-osseous and the distal portion. Similar findings, i.e. an increase in stainable substance and decrease in luminal size of the ES, have also been reported following administration of glycerol in mice [14]. These changes were suggested to reflect a reduced absorption of endolymph, thus possibly indicating that inoculation of LPS causes the reduced absorption of endolymph as well.

By using the combination of these two methods, we hoped that a new experimental mouse model could be developed, showing closer resemblance to the pathophysiologic process in Ménière's disease. The present study revealed that treatment with aldosterone, LPS, or both aldosterone and LPS, induced EH in mice. Our findings may mean that EH is the result of either insufficient resorption of or excess production of endolymph. The combined treatment with both LPS and aldosterone did not clearly demonstrate a shift to a more severe degree of hydrops, when compared with only LPS or aldosterone treatment. However, aldosterone did increase the severity of hydrops in the lower turn. Elongation and folding of Reissner's membrane, as observed in the present study, may indicate a previous occurrence of EH. As a result, combined treatment induced a uniform pattern of hydrops.

Despite the successful induction of EH, none of the present experimental animals showed any signs of vestibular dysfunction as did previous animal models [4,7,8]. Although it is being extensively studied, the standard guinea pig model does not reliably reproduce vestibular symptoms. Importantly, hydrops is produced by under-resorption of the endolymph, a mechanism that might not directly imitate the production of hydrops in Ménière's disease. Other induced models, i.e. by delayed perilymphatic infusion of keyhole limpet hemocyanin [11], anti-CB11 monoclonal antibodies, cholera toxin [5], chronic administration of vasopressin [10], and the two-phase hydrops guinea pig model [9], all appear to have the same limitations inherent in the standard guinea pig model [4,7,8]. Based on the present findings and previous investigations, the vestibular dysfunction might not result from the existence of EH alone. Actually in the human temporal bone studies, EH was observed in the temporal bones of persons showing symptoms and signs of Ménière's disease [1,2,23]. Indeed, hydrops is both the histological hallmark of Ménière's disease and the working concept of its pathogenesis. Paradoxically, not all people with symptoms of Ménière's disease have hydrops, and not all people with hydrops discovered at autopsy had symptoms during life [23].

Numerous investigations have suggested that the symptoms of Ménière's disease derive from a disturbance in the volume/pressure relationship of the endolymph. It is not yet known whether hydrops is the cause of the symptoms, or simply a side effect of the disorder. In fact, Ménière's disease patients never suffer from incessant vertigo. Typically, the vertigo begins fairly quickly and builds in intensity over minutes or hours. When severe, nausea and vomiting may occur, movement during a vertigo attack exacerbates the nausea, so patients quickly learn to stay motionless during an attack. The vertigo usually lasts more than 20 min but rarely more than 24 h. Vertigo attacks occurred episodically, elicited by additional factors such as emotional stress [8]. In another way, all animal models – including the present one – might be a model of an asymptomatic period in Ménière's disease. Based on this hypothesis, we injected epinephrine into the middle ear cavity as an additional stressor. As a result, we succeeded in inducing reversible vestibular dysfunction in our animal model treated with both aldosterone and LPS. It has been reported that after topical epinephrine application, cochlear blood flow immediately decreased to 20% of baseline and maintained this level for about 5 min before returning toward baseline [24]. It is therefore suggested that the vestibular dysfunction observed in the present investigation may have been induced by the reduced inner ear blood flow in the hydropic ear. Similar reversible vestibular dysfunction was noted after injecting glycerol in the guinea pig surgical obliteration model [25]. These results may indicate that the occurrence of vestibular dysfunction in hydropic animal models may require additional stress such as reduced inner ear blood flow, sudden changes in endolymph volume and/or pressure. The present study also revealed that the animals treated with LPS or aldosterone alone did not show any vestibular dysfunction after the intratympanic injection with

epinephrine. This may indicate that the overproduction of endolymph or its reduced absorption (ES dysfunction) alone is not enough to produce vestibular dysfunction after additional stress.

Based on the previous and present investigation, we consider the pathogenesis of Ménière's disease as follows. EH is caused by many extrinsic and intrinsic factors [23]. These include hypopresia of the vestibular aqueduct and sac, racial genetic factors, autoimmunity, otitis media, trauma, otosclerosis, vasopressin, allergy, and viral infection. Patients with EH alone are generally free from vertigo. However, additional stress such as sudden changes in endolymphatic pressure, restricted inner ear blood flow, rupture of the endolymphatic membrane (Reissner's or the saccular wall), may cause a vertigo attack. It seems logical that both physical and chemical mechanisms can concomitantly cause the symptoms. Chemical factors characteristically include osmotic and hydrostatic pressure alterations and, most likely, changes in membrane permeability, causing egress of inappropriate ions across the membrane barrier so as to incite sensory dendrites, leading to cochlear and vestibular symptoms.

In conclusion, the new murine model explains some of the shortcomings of the classical model, and may relate to many observations in Ménière's disease. Although many more morphological and pathophysiological investigations with this new model need to be performed, the present results indicate that it may contribute to a better understanding of the pathogenesis. Consequently, this study may contribute to a more sophisticated therapeutic approach to Ménière's disease, in particular by restricting endolymph production.

Acknowledgements

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ORIGINAL ARTICLE

Protective effect of edaravone against endolymphatic hydrops

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Abstract

Conclusion. Our findings suggest that edaravone prevented the production of reactive oxygen species (ROS). Edaravone also delayed the formation of endolymphatic hydrops in guinea pigs, but had no effect on endolymphatic hydrops. *Objective.* To analyse the protective effect of a free radical scavenger, edaravone, on endolymphatic hydrops. *Materials and methods.* Guinea pigs were subjected to surgical obliteration of the endolymphatic duct (ED). For the detection of ROS, group 1 received intraperitoneal injections of edaravone (3 mg/kg/day) for 2 days, group 2 received edaravone for 2 weeks, group 3 saline for 2 days, and group 4 saline for 2 weeks. ROS production by the organ of Corti and stria vascularis was examined by using dihydrotetramethylrosamine. For the morphological analysis, guinea pigs were divided into five groups, i.e. 2 or 4 weeks after ED obliteration, 2 weeks with edaravone, first or last 2 weeks with edaravone and sacrificed 4 weeks after ED obliteration. Increases in the ratios of the cross-sectional area of scala media were analysed quantitatively to assess the degree of endolymphatic hydrops among the above-mentioned five groups of the hydropic cochlea. *Results.* ROS was detected both in the organ of Corti and in the lateral wall of cochleae 2 days after ED obliteration. Edaravone prevented the production of ROS and also attenuated the formation of endolymphatic hydrops in the acute hydrops group.

Keywords: *Edaravone, hydroxyl radical scavenger, endolymphatic hydrops, reactive oxygen species*

Introduction

Meniere's disease is characterized histologically by endolymphatic hydrops (ELH), which is known to cause degenerative damage to various tissues of the inner ear. Not only were degenerative changes found in Reissner's membrane, but also atrophic changes in the lateral cochlear wall and the organ of Corti in Meniere's disease [1,2]. Similar findings were confirmed in experimental animals with chronic ELH [3,4]. Although the mechanism underlying the degenerative process in hydropic ears remains to be settled, it was suggested earlier that oxidative insult probably contributed to the pathology associated with ELH [5].

Recently, a considerable volume of evidence has accumulated showing that free radicals are generated in the developmental process of ELH [6–8]. ELH elicits inducible nitric oxide synthase (iNOS) in structures of the cochleo-vestibular system [6,7]. Expression of iNOS is thought to lead to excessive nitric oxide (NO) production. High concentrations of NO and NO-related reactive oxygen species (ROS) can have cytotoxic and neurotoxic effects, resulting in loss of hair cells and ganglion cells and also strial atrophy. Excessive NO and ROS, when detected in the early stages of ELH before the development of inner ear dysfunction, could therefore be involved in the pathophysiology of vestibular and cochlear dysfunction late in Meniere's disease [6–8].

It has been reported that allopurinol, a xanthine oxidase inhibitor and free radical scavenger, reduced the incidence of hydrops-associated atrophy in sensorineural structures [9]. Allopurinol also delayed the development of ELH after obliteration of the endolymphatic sac. Clinically, a recent trial

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of treatment for Meniere's disease using radical scavengers suppported the experimental findings [10]. On the basis of these results, it is plausible that free radical reactions may play some part in the development of cochleo-vestibular disorders associated with ELH. The aim of the present study was to elucidate the effects of edaravone in normal and hydropic ears.

Edaravone is an extremely potent scavenger of hydroxyl radicals, not only inhibiting them but also healing iron-induced peroxidative injuries. Edaverone has therefore been used clinically in Japan to treat cerebral infarction in the acute phase [11,12]. If the development of ELH promotes the generation of ROS, edaverone is expected to vigorously inhibit its production, resulting in the delay of ELH development.

Materials and methods

Two experiments were performed in this study. Experiment 1 was designed to elucidate the effects of edaravone on ROS in hydropic ears and experiment 2 the effects of edaravone on volumetric changes in scala media in hydropic ears (Figure 1).

These experiments were approved by Animal Care and Use Committee of the Kochi Medical School, conforming to the Animal Welfare Act and the guiding principles for animal care promulgated by the Ministry of Education, Culture, Sports and Technology, Japan.

Experiment 1

Six female guinea pigs with an intact Preyer's reflex and weighing about 300 g were used. They were immobilized in prone position with a head holder and operated on under sterile conditions. A dorsal midline scalp incision was made under local anesthesia with xylocaine. Using an epidural occipital approach the left occipital bone was removed to expose the aperture of the endolymphatic duct. In four animals the operculum of the endolymphatic duct was removed using a 0.5 mm diamond burr (Sac-op). The scalp incision was then closed. In the remaining two animals, closure of the scalp incision was made without removing the operculum (Sham-op). Two of the four sac-obliterated animals were sacrificed 2 days postoperatively (Sac-op (2d)); one was given intraperitoneal (i.p.) injection of edaravone (3 mg/kg/day) in the meantime (Sac-op $(2d)_E$). Edaravone was supplied by Mitsubishi Pharma Corporation (Osaka Japan). The remaining two sac-obliterated animals were sacrificed 2 weeks after the operation (Sac-op (2w)); one was given edaravone i.p. (3 mg/kg/day) during the 2-week period (Sac-op $(2w)_E$). Sham-operated animals served as controls, being sacrificed 2 days after the surgical procedure (Sham-op (2d)).

Detection of ROS

Animals were anesthetized deeply with pentobarbital and immediately decapitated. The temporal bones

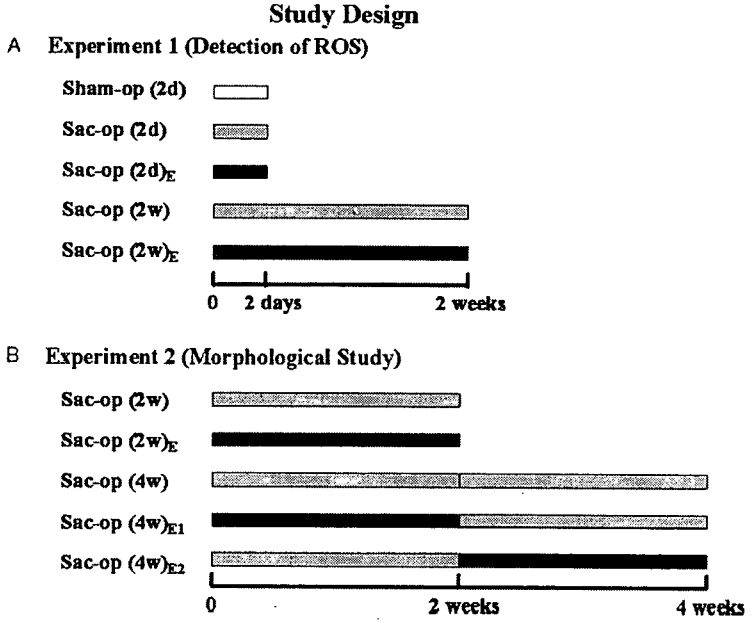


Figure 1. Summarized study designs of experiments 1 and 2. Edaravone injections were made according to the schedule described in the study design.

were quickly removed and the cochleae were excised in artificial perilymph (AP) containing NaCl 130 mM, KCl 5.4 mM, $CaCl_2/2H_2O$ 1.25 mM, buffered to pH 7.2 with 5.0 mM HEPES and adjusted to 300 mosm with glucose and equilibrated with 95% O_2/5% CO_2. The bony capsule of each cochlea was dissected meticulously to expose the cochlear duct. The organ of Corti and stria vascularis were then removed under a stereoscopic microscope.

The specimens collected were loaded with 20 μM dihydrotetramethylrosamine (DHTMRos) (Molecular Probes, Eugene, OR, USA) in AP for 20 min at 37°C in the dark. The specimens were then rinsed with AP and placed in an eight-well perfusion chamber (depth 0.5 mm, diameter 9 mm, volume 3.5 μl; PC8R-0.5, Grace Bio-Labs, Bend, OR, USA) filled with AP for imaging.

The specimens were viewed in a Nikon fluorescence microscope (Eclipse E600) equipped with appropriate filter sets, i.e. an excitation (510–560 nm) and emission (590 nm LP). Fluorescence analog images obtained using an intensified digital color CCD camera (C4742-95, Hamamatsu Photonics) were stored as digital images (IP Lab Spectrum software, version 3.0; Signal Analytics). After completion of the fluorescence data sampling, fluorescence intensities were measured and analyzed from the stored digital image sequence, using the IP Lab Spectrum software. For the statistical analysis, 30 spots were randomly selected from each specimen, and the fluorescence intensity of stria vascularis and the organ of Corti was measured. Means and standard errors were calculated from the intensity values of these 30 points. Data were analyzed by a one-way analysis of variance (ANOVA) [13].

Experiment 2

Fifty female pigmented guinea pigs with an intact Preyer's reflex and weighing about 300 g were used. All experimental animals underwent surgical obliteration of the endolymphatic sac of the left ear, as described above. The animals were divided into five groups. Twenty animals, not injected with edaravone, served as controls. Ten of these 20 animals were fed for 2 weeks after the surgical obliteration (Sac-op (2w)), the remaining 10 for 4 weeks postoperatively (Sac-op (4w)).

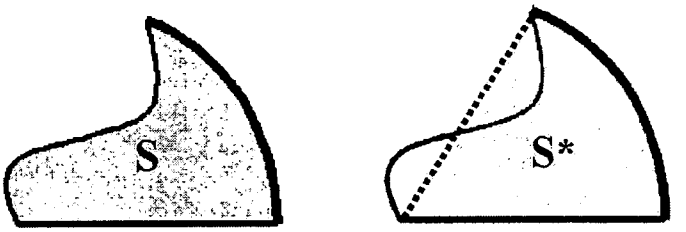
The other 30 animals served as experimental animals. Ten of them received edaravone i.p. (3 mg/kg) daily for 2 weeks starting immediately after the surgical obliteration and were sacrificed 24 h after the last injection (Sac-op $(2w)_E$). Ten of the experimental animals also received i.p. injections of edaravone, at the same dosage, for 2 weeks starting immediately after the surgical obliteration, and were fed for 2 weeks thereafter without any injection (Sac-op $(4w)_{E1}$). The remaining 10 animals were fed for 2 weeks without injection, and then given edaravone injections, at the same dosage, for 2 weeks (Sac-op $(4w)_{E2}$) (Figure 1). All animals were left undisturbed with unrestricted mobility in individual cages in a quiet room throughout the period, except during experimental procedures. All animals were sacrificed for quantitative assessment of the volumetric changes in scala media.

Quantitative assessment of volumetric changes of the scala media

Animals were perfused transcardially with physiological saline solution under deep anesthesia (a peritoneal injection of pentobarbital), and fixation was performed with 10% formalin. The temporal bones were removed and post-fixed in 10% formalin solutions for 10 days or more. Thereafter, they were decalcified with 5% trichloroacetic acid for 12 days and dehydrated in a graded ethanol series, before embedding in paraffin and celloidin. The prepared blocks were cut horizontally into 6 μm sections, stained with hematoxylin and eosin and observed under a light microscope.

For the quantitative assessment of volumetric changes in scala media, the change ratios of the cross-sectional area of the scala were measured from the mid-modiolar sections of the cochleae. Basically, the measurement was performed as described previously [14]. In principle, one specimen was used for analysis of one cochlea. When the plane of a section deviated slightly from the mid-modiolar axis, the section that was cut closest to the mid-modiolar plane in individual turns was used. For this analysis, the following two parameters were measured in the basal, second, third, and apical turns, excluding the hook portion: 1) the cross-sectional area of scala media (S), enclosed by the distended Reissner's membrane (RM), and 2) the cross-sectional area of the original scala media (S*), enclosed by a straight line segment, representing the normal position of the idealized RM connecting the normal lateral attachment of RM at the upper margin of stria vascularis to its normal medial attachment at spiral limbus (Figure 2). The measuring system comprised a video camera, a computer, and a digitizer (Video Micro Meter VM-30, Olympus Co., Tokyo). The anatomical measurements were carried out in a blinded fashion.

From these parameters, the increased ratios (%) of the cross-sectional area of scala media (IR-S) of a total of four turns were calculated according to the equation as described below.



Increase ratio (%) of the cross-sectional area of the scala media (IR-S) = 100 × Σ (Sx − S*x)/Σ S*x (x: base, second, third, apex)

Figure 2. Parameters for the quantitative measurement of scala media dilatation. *S* (dark gray area): the cross-sectional area of scala media, enclosed by the distended Reissner's membrane. *S** (light gray area): the cross-sectional area of the original scala media, enclosed by a straight line segment (broken line). The broken line segment represents the normal position of the idealized Reissner's membrane, which connected the normal lateral attachment of Reissner's membrane at the upper margin of stria vascularis to its normal medial attachment at the spiral limbus. These parameters were measured using a computerized digitizer (Video Micro Meter VM-30, Olympus Co., Tokyo).

Increased ratio (%) of the cross-sectional area of scala media (IR-S) =

$$100 \times \sum(\text{Sx-S*x})/\sum \text{S*x}$$

(x: base, second, third, apex)

Results

Detection of ROS

Production of ROS was represented by a red-fluorescing signal. This fluorescence was evident both in the stria vascularis and in the organ of Corti sampled from the cochlea in the sac-obliterated animal not injected with edaravone (Sac-op(2d)), which was sacrificed 2 days after obliteration of the endolymphatic sac, but was much weaker in the sac-obliterated animal given an i.p. injection of edaravone (Sac-op $(2d)_E$) (Figure 3). No fluorescent signal was evident in the cochlear specimens from the sac-obliterated animal that was sacrificed 2 weeks later (Sac-op (2w)), or from sham-operated animals (Sham (2d)). There was no significant difference in fluorescence intensity of the organ of Corti and stria vascularis between Sac-op (2d) non-operated side, Sac-op $(2d)_E$, Sac-op (2w), Sac-op $(2w)_E$ and Sham (2d) (one-way ANOVA). However, fluorescence intensities of both the organ of Corti and stria vascularis were significantly increased only in the operated ear of Sac-op (2d) (one-way ANOVA, $p < 0.001$) (Table I).

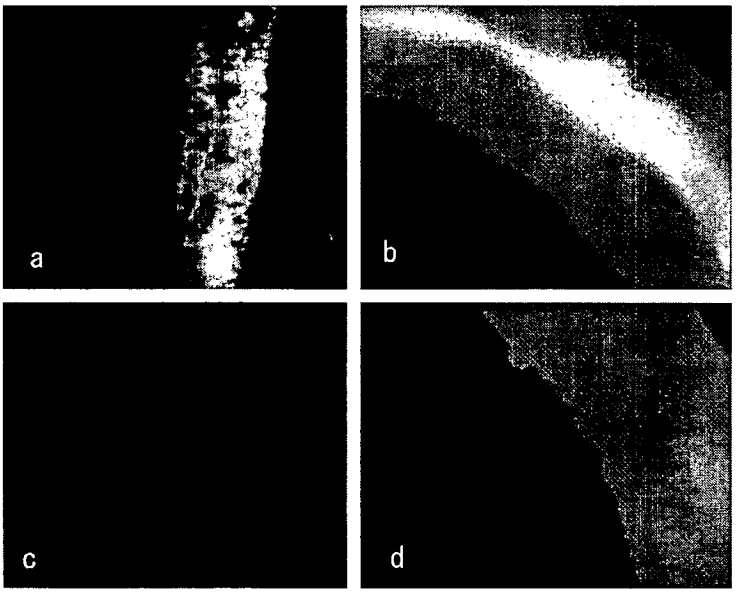


Figure 3. Fluorescence images of the dye-loaded stria vascularis and organ of Corti. The fluorescence is evident both in the organ of Corti (a) and in stria vascularis (b) sampled from the cochlea in the sac-obliterated animal without injection of edaravone (Sac-op(2d)), but markedly reduced in the sac-obliteraed animal with an intraperitoneal injection of edaravone (Sac-op $(2d)_E$). (c) Organ of Corti; (d) stria vascularis.

Table I. Means and standard deviations of fluorescence intensities in the organ of Corti and stria vascularis.

Procedure	Mean±SD	
	Stria vascularis	Organ of Corti
Sham-op (2d)	20.0±10.4	28.9±7.5
Sac-op (2d)		
Non-operated side	20.1±12.5	30.5±3.5
Operated side	67.0±14.4*	101.0±13.3*
Sac-op $(2d)_E$	19.9±6.8	31.5±5.5
Sac-op (2w)	28.2±15.7	42.5±14.5
Sac-op $(2w)_E$	21.1±11.9	38.9±10.5

Volumetric changes in scala media

In Sac-op (2w) animals, Reissner's membranes (RM) were distended and ELH was noticeably developed. In Sac-op $(2w)_E$ animals, however, the RM bulged only slightly and ELH was not so evident. The endolymphatic ducts were completely obliterated in both animals. Therefore, the mild development of ELH in Sac-op $(2w)_E$ animals was not thought to be due to the incomplete obliteration of the endolymphatic ducts (Figure 4). Mean IR-Ss of non-operated and operated ears in Sac-op (2w) and Sac-op $(2w)_E$ groups were 1.07, 1.65, 1,09, and 1.20, respectively. IR-Ss in the operated ears were significantly higher in both Sac-op (2w) and Sac-op $(2w)_E$ groups (paired t test, $p<0.001$ and $p<0.05$, respectively). However, an increase in IR-S was not so marked in the Sac-op $(2w)_E$ group. The difference in IR-Ss of operated ears was statistically significant between Sac-op (2w) and Sac-op $(2w)_E$ groups (Cochran-cox test, $p<0.01$) (Table II).

In the 4-week observation groups, marked ELH was noted in Sac-op (4w) and Sac-op $(4w)_{E2}$ animals, but not in Sac-op $(4w)_{E1}$ animals (Figure 5). The means of Sac-op (4w) and Sac-op $(4w)_{E2}$ groups were significantly higher than that of Sac-op $(4w)_{E1}$ (Dunnet multiple comparison test, $p<0.05$, $p<0.01$). The mean value of Sac-op $(4w)_{E1}$ did not differ significantly from that of Sac-op $(2w)_E$ (one-way ANOVA) (Table III). The endolymphatic sac and duct were histologically confirmed to be completely obliterated in all animals (not shown).

Discussion

The present study has shown that edaravone remarkably prevented the development of ELH in the early stage of ELH formation, but could not prevent it in the late stage. Edaravone inhibited the generation of ROS, which suggests that the generation of ROS in the cochlea may be an important factor in the formation of ELH in the early stage, but not in the late stage. As the endolymphatic sac and duct were completely obliterated in Sac-op $(2w)_E$ and Sac-op $(4w)_{E1}$ groups, the present results lead to the assumption that the inhibition of ROS production may be closely linked to the prevention of ELH development.

ROS forms under physiological conditions in the human body and is removed by the cellular antioxidant defense system. However, this delicately balanced process is easily triggered off in the presence of oxidative stress, or due to external

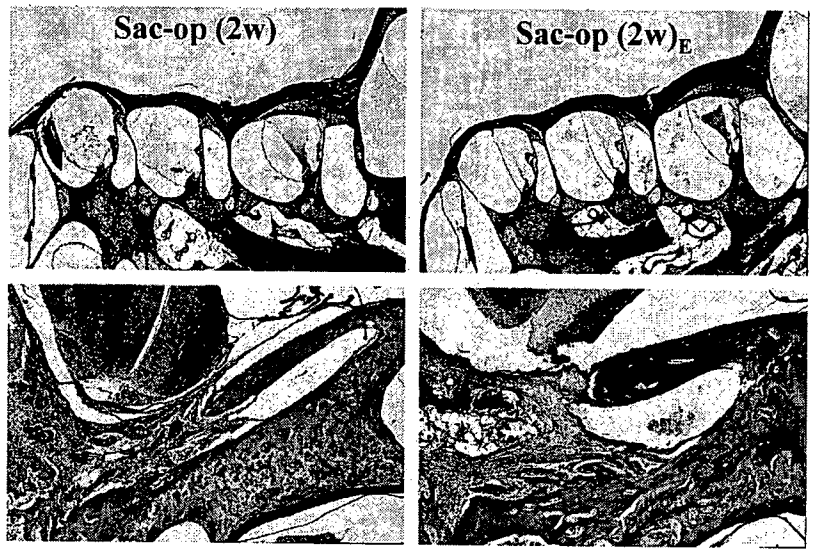


Figure 4. Representative pictures of the cochlea and the endolymphatic sac and duct in Sac-op (2w) and Sac-op $(2w)_E$ animals. The distension of Reissner's membrane and endolymphatic hydrops is noticeable in a Sac-op (2w) animal, but not so evident in an animal injected with edaravone. Note that the endolymphatic duct was completely obliterated in both animals.

Table II. A comparison of mean IR-S values between non-operated and operated ears in Sac-op (2w) and Sac-op $(2w)_E$ groups.

	Sac-op (2w)		Sac-op $(2w)_E$	
	Non-opside	Opside	Non-opside	Opside
Mean±S.D.	1.07±0.02	1.65±0.32	1.09±0.02	1.20±0.11
	***		*	
			**	

*P <0.05, **P <0.01, ***P <0.001.

factors such as radiation, environmental chemicals, pollutants, carcinogens, mutagens, or due to endogenous factors, e.g. a faulty defense mechanism (owing to genetic factors), leading to increased concentrations of free radicals. In the inner ear, increased ROS production is well known to be involved in noise trauma [15], cisplatin ototoxicity [16], and aminoglycoside ototoxicity [17,18]. The present study revealed that increased ROS production also occurred in the cochlea in the early stage of ELH development.

Possible mechanisms underlying an increase in ROS production may be enumerated as follows: 1) increased hydrostatic labyrinthine pressure, 2) changes in the cochlear ion environment, and/or 3) distension of the labyrinthine membrane in the process of ELH development. Endolymphatic hypertension is unlikely to be significant in the early stages of hydrops [19,20]. Although a rapid extension of the endolymphatic compartment was reported to result in a transient pressure imbalance between endolymphatic and perilymphatic compartments, the pressure changes are within physiological fluctuation limits of intralabyrinthine pressure during pulsation, breathing, and postural change [21]. Changes in the ionic environment of the cochlea also can be ruled out. No alteration in ionic composition in the cochlea was detected in the early stage until 2 weeks after the obliteration of the endolymphatic sac and duct [22,23]. In contrast, mechanical stress of the labyrinthine membrane is thought to occur during the process of ELH development, leading to ROS generation. Actually, the RM was distending during the development of ELH. The same mechanical stress could occur in the organ of Corti and stria vascularis. These mechanical stresses probably impair the endogenous antioxidant defense mechanisms, allowing an excessive production of ROS. In the present study, generation of ROS was detected in the organ of Corti and stria vascularis. Unfortunately, detection of ROS in the RM could not be performed in the present work due to technical difficulties. However, ROS is also expected to be present in the RM, where the most prominent morphological changes appear. If this is the case, it can be concluded that the RM was would be impaired by the noxious ROS.

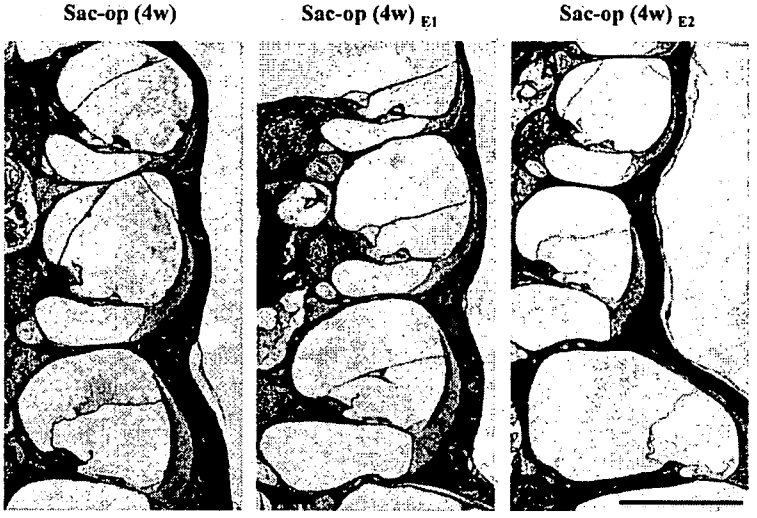


Figure 5. Representative pictures of the cochlea of Sac-op (4w), Sac-op $(4w)_{E1}$ and Sac-op $(4w)_{E2}$ animals. Marked endolymphatic hydrops is evident in a Sac-op $(4w)_{E2}$ animal with late application of edaravone, but not in a Sac-op $(4w)_{E1}$ animal. Early application of edaravone noticeably prevented the development of endolymphatic hydrops.

Table III. A comparison of means and standard deviations of IR-S among Sac-op (4w), Sac-op $(4w)_{E1}$ and Sac-op $(4w)_{E2}$ groups.

	Sac-op(4w)	Sac-op$(4w)_{E1}$	Sac-op$(4w)_{E2}$
Mean ± SD	1.53 ± 0.40	1.20 ± 0.08	1.62 ± 0.34
	$P < 0.05$	$P < 0.01$	
	NS		

Recently, much interest has focused on the RM. Three types of ion channel were identified on the apical membrane of epithelial cells of the RM: namely, a stretch-activated nonselective cation, a chloride and a potassium channel [24,25]. These stretch-activated ion channels might be involved in regulating the endolymphatic volume, in balancing the hydrostatic pressure across RM, and in maintaining the electrochemical composition of the endolymph [24]. If these channels are damaged by an excessive production of ROS in the process of developing ELH, a regulating system of the endolymphatic volume of RM will be disturbed, resulting in failure to prevent ELH formation caused by the obliteration of the endolymphatic sac and duct. However, the development of ELH could be prevented if the generation of ROS was inhibited by a radical scavenger.

In the present study, generation of ROS was detected in the organ of Corti and in stria vascularis during the early stage of ELH development, but not in the late stage. The difference in ROS formation between the two stages is thought to be due to the presence or absence of rapid mechanical stress. ROS generation would produce some tissue damage in the organ of Corti and stria vascularis [10]. In particular, damage to stria vascularis, which is the site of endolymph homeostasis, is thought to be crucial for endolymph regulation. The RM is also vulnerable to ROS. It is likely that the damage to RM plays an essential part in the faulty regulation of endolymph, as speculated earlier. Therefore, early application of the free radical scavenger, edaravone, is believed to prevent ROS generation, resulting in a delay in or alleviation of ELH development.

Finally, a question arose: is the attenuation of ELH caused by the effects of edaravone, inhibiting the production of endolymph? The assumption was discarded, however, as edaravone had no effect on the endolymphatic volume of the non-operated (right) ears. Nor did edaravone decrease ELH in the late stage. Consequently, a free radical scavenger (including edaravone) might be useful for the treatment of the early stage of diseases associated with ELH.

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Microarray Analysis of Stress-Related Gene Expression in Patients with Ménière's Disease

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Key Words
Microarray analysis · Stress response · Vertigo attack · Ménière's disease

Abstract
We developed an original microarray carrying 1,467 cDNAs of stress-related genes for the assessment of stress responses. In this study, we used microarray analysis to assess the stress-related gene expression profiles in peripheral leukocytes in 2 patients with definite Ménière's disease. In the attack and active phases, mRNA expression levels of 57 genes and 163 genes were either up-regulated more than twofold or down-regulated by less than half in patient 1 and patient 2, respectively. Patient 1 had sporadic episodes of vertigo attack, while patient 2 had an intractable course with frequent vertigo attacks, suggesting that the magnitude of changes in gene expression is correlated with the severity of the disorder in Ménière's disease. The expression of a total of 26 genes commonly changed in both patients in the attack and active phases and returned to the baseline levels in the remission phase, suggesting the involvement of the distinct group of stress-related genes in the development of vertigo attacks in Ménière's disease. We then examined the effects of caloric stimulation on the stress-related gene expression profiles in peripheral leukocytes in 5 healthy volunteers. Although unilateral caloric stimulation with cold water caused acute vertigo with nystagmus, the expression profiles of stress-related genes did not significantly change after this experiment. This finding indicated that the up- or down-regulated genes in the attack and active phases in patients with Ménière's disease are not secondary to vertigo or vertigo-associated anxiety. All these findings suggested that the distinct group of stress-related genes contributed to the development of vertigo attacks of Ménière's disease and that stress-related gene expression profiles in peripheral leukocytes can be a predictive and therapeutic tool for episodic vertigo attacks in patients with Ménière's disease.

Introduction

Ménière's disease is characterized by spontaneous episodic vertigo, hearing loss and tinnitus [1]. It has a pathologic correlate in endolymphatic hydrops of the inner ear, but the exact cause of the increased volume of endolymph is still unknown. The causal relationship between stress and vertigo attacks of Ménière's disease has been discussed in the literature [2, 3]. Indeed, patients with Ménière's disease often describe a possible association of

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