labyrinth was rinsed with 100% ethanol. Previous histological studies confirmed that the labyrinth was completely destroyed and that the lesion did not extend to the adjacent region such as ganglion cells [10]. The same procedure was repeated in the contralateral ear 14 days after the first operation. After a recovery period of 14 days, animals were exposed to 2 G hypergravity load for 4 h. Sham-operated rats received bilateral retroauricular incisions with an interval of 14 days and were then exposed to 2 G hypergravity load for 4 h.

Statistical analysis

Data are presented as mean ± SE. All p values were determined using one-way analysis of variance (ANOVA). Fisher's paired least significant difference test was used in the experiment for drug effects and Scheffe's F test in the experiment for H1 receptor mRNA expression. $p < 0.05$ was considered significant.

Results

Effects of histaminergic receptor blockers on kaolin intake induced by hypergravity load

Kaolin intake from day 1 to day 13 of each group is shown in Figure 1A. Rats did not take a significant amount of kaolin before hypergravity load (days 1–3, Figure 1A). However, kaolin intake in all of the groups increased after the first hypergravity load (day 4). The second hypergravity load was delivered 1 week after the first load and was preceded by the administration of the respective drugs. Accordingly, kaolin intake in the saline, terfenadine, and zolantidine groups on day 11 was increased compared with that of day 4, but it was decreased in the mepyramine group (Figure 1A). Indeed, kaolin intake of the saline group on day 11 was increased to 134.2% of the intake on day 4 in the same group. On the contrary, kaolin intake in the mepyramine group was decreased to 71.9% compared to that of day 4 in the same group. The ratio of kaolin intake (day 11/day 4) in this group was significantly lower than that of the saline group ($p < 0.05$, Figure 1B).

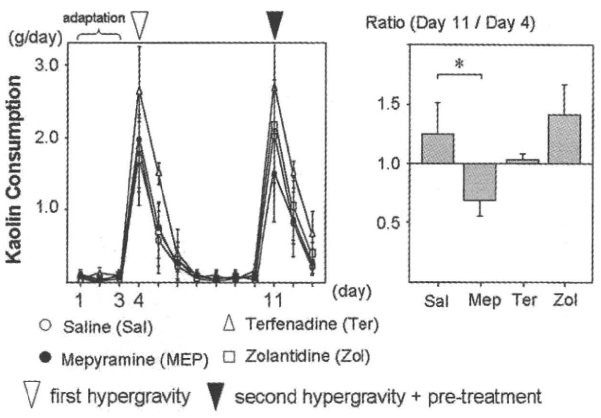

Figure 1. (A) Kaolin intake from day 1 to day 13 of each group. Kaolin intake in all of groups increased after the first hypergravity load (day 4). On day 11 after the second hypergravity load, which was preceded by drug administration, kaolin intake in the saline, terfenadine, and zolantidine groups was increased compared to that of day 4; however, it was decreased in the mepyramine group. (B) Ratio of kaolin intake on day 11 and day 4. Ratio of kaolin intake (day 11/day 4) in the mepyramine group was significantly lower compared with that of the saline group. *$p < 0.05$ vs saline group. Pretreatment with mepyramine suppressed the hypergravity-induced motion sickness.

H1 receptor mRNA expression in the hypothalamus, brainstem, and cerebral cortex after hypergravity load

Figure 2 shows the H1R mRNA expression in the hypothalamus of control rats (open column) and those exposed to hypergravity load for 0–12 h (closed column). Hypothalamic H1R mRNA expression remained unchanged in control animals, which were put into the centrifuge device but were not exposed to hypergravity load (open column). H1R mRNA expression in the hypothalamus gradually increased after hypergravity load (closed column). Hypothalamic H1R mRNA expression after 4 h load of hypergravity was 154.6% of the control animals. This difference reached a statistically significant level (4 h, open column vs 4 h, closed column, $p < 0.01$). It was also significantly higher compared with controls at 0 h (0 h, closed column vs 4 h, closed column, $p < 0.05$).

Figure 3 shows the H1R mRNA expression in the brainstem of animals exposed to hypergravity for 4 h

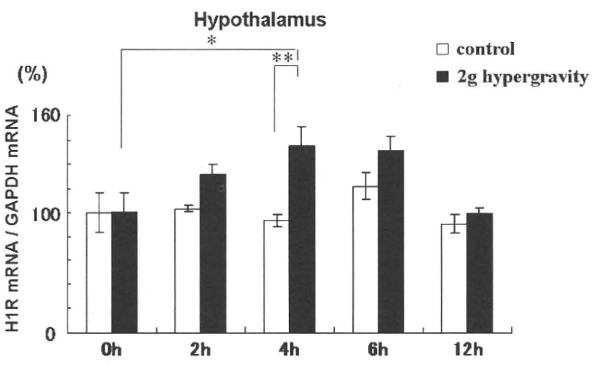

Figure 2. H1R mRNA expression in the hypothalamus of control rats (open column) and those exposed to hypergravity load for 0–12 h (closed column). Hypothalamic H1R mRNA expression after 4 h load of hypergravity was significantly higher than that of the control animals, which were in the animal cage placed beside the centrifuge device but not exposed to hypergravity for the same period (4 h, open column vs 4 h, closed column, **$p < 0.01$). It was also significantly higher compared with the controls at 0 h (0 h, closed column vs 4 h, closed column, *$p < 0.05$).

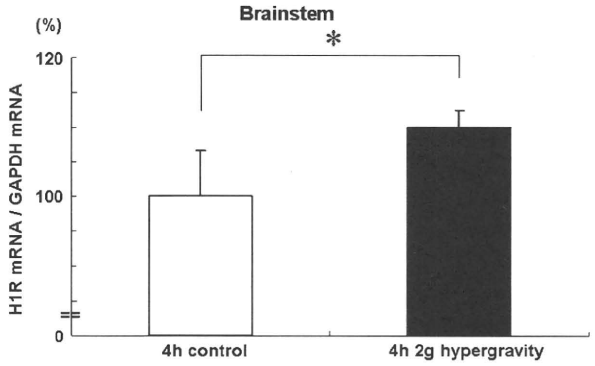

Figure 3. H1R mRNA expression in the brainstem. H1R mRNA expression in the brainstem of animals exposed to hypergravity for 4 h (closed column) was significantly higher than that of the control animals that were in the animal cage placed beside the centrifuge device but not exposed to hypergravity for the same period (open column). $*p<0.05$ vs controls.

(closed column) or control animals that were in the animal cage placed beside the centrifuge device but not exposed to hypergravity during the same period (open column). H1R mRNA expression in the brainstem of animals exposed to hypergravity was significantly increased to 110.0% of the control animals ($p<0.05$).

Figure 4 shows the H1R mRNA expression in the cerebral cortex of animals exposed to hypergravity for 4 h (closed column) or control animals that were in the animal cage placed beside the centrifuge device but not exposed to hypergravity during the same period (open column). No difference was noticed in H1R mRNA expression in the cerebral cortex between the controls and animals exposed to hypergravity.

Effects of labyrinthectomy on hypergravity-induced H1 receptor mRNA expression in the hypothalamus

H1R mRNA expression in the hypothalamus of the sham-operated rats exposed to hypergravity for 4 h

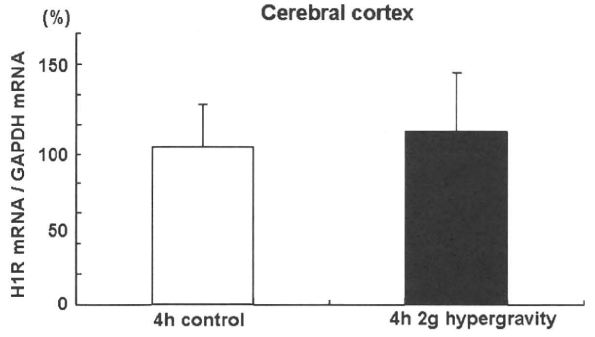

Figure 4. H1R mRNA expression in the cerebral cortex. There were no differences in H1R mRNA expression in the cerebral cortex between control animals and those exposed to hypergravity for 4 h.

was significantly increased to 118.2% of the bilaterally labyrinthectomized rats exposed to hypergravity for 4 h ($p<0.05$) (Figure 5).

Discussion

H1 and H2 receptors are the major post-synaptic histaminergic receptors in both peripheral and central tissues, although there are four subtypes of histamine receptors (H1–H4) [11]. H1 blockers are clinically effective in preventing motion sickness [2,3], suggesting that H1 receptors play an important role in the development of motion sickness. In our previous study, kaolin intake induced by double rotation was suppressed by diphenhydramine, which is less specific to H1 receptors with anticholinergic actions [12]. In the present study, an attempt was made to clarify which subtype of post-synaptic histaminergic receptors was responsible for the development of motion sickness. The hypergravity-induced kaolin intake was significantly suppressed by pretreatment with mepyramine but not with terfenadine or zolantidine. Both mepyramine and terfenadine are specific H1 receptor blockers; however, the latter does not penetrate the BBB, acting only on the peripheral tissues. On the other hand, zolantidine is an H2 receptor blocker that does cross the BBB [13]. Therefore, the present results indicate that the central H1 but not H2 or peripheral H1 receptors play a pivotal role in the development of motion sickness.

We reported in the previous study that histamine release from the hypothalamus in rats was increased by 2 G hypergravity load [14], suggesting that the

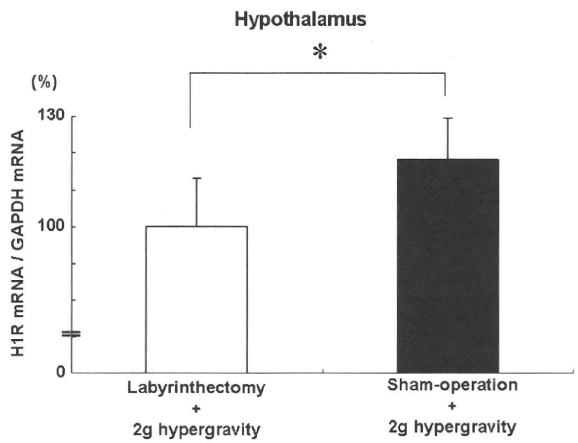

Figure 5. H1R mRNA expression in the hypothalamus of bilaterally labyrinthectomized rats (open column) and sham-operated rats (closed column) after hypergravity load for 4 h. H1R mRNA expression in the hypothalamus of the sham-operated rats was significantly increased in comparison with that of the bilaterally labyrinthectomized rats. $*p<0.05$ vs bilaterally labyrinthectomized rats.

histaminergic neuron system was activated by hypergravity. Histaminergic neurons were located in the tuberomamillary nucleus in the posterior hypothalamus and their axons project both rostrally and caudally [15]. Therefore, it is suggested that the histaminergic activation is transmitted to the brain region related to the physiological and behavioral responses of motion sickness. Recently, it was reported that the stimulation of H1 receptor up-regulates the level of H1 receptor protein expression through augmentation of H1 receptor mRNA expression in HeLa cells and rats nasal mucosa [8,9]. In the present study, the expression of H1 receptor mRNA in the hypothalamus was significantly increased after a 4 h load of 2 G hypergravity in rats. However, the up-regulation of H1 receptor mRNA after hypergravity was not seen in bilaterally labyrinthectomized rats. These findings suggest that hypergravity stimulation induced the release of histamine from the hypothalamus through vestibular end-organs and the released histamine stimulated H1 receptors, leading to its up-regulation in the hypothalamus. Moreover, 2 G hypergravity load also significantly increased the expression of H1 receptor mRNA in the brainstem, but not in the cerebral cortex in rats. The brainstem includes the nuclei complex related to nausea and vomiting such as area postrema and nucleus of solitary tract, and was reported to contain histaminergic axon terminals [15]. Therefore, these findings suggest that hypergravity stimulation also induced the release of histamine from the brainstem and released histamine-stimulated H1 receptors on the nuclei complex related to nausea and vomiting, resulting in the development of motion sickness. The cerebral cortex is suggested not to be involved in the histaminergic post-synaptic events in the processes of motion sickness.

In fact, the hypothalamus has a role in various autonomic reactions such as cold sweating, pallor, and oliguria that characterize the motion sickness syndrome. Also, the brainstem contains nuclei complexes responsible for vomiting, which is a major symptom of motion sickness, such as the medullary reticular formation, nucleus tractus solitarii, and nucleus originis vagi. On the contrary, the cerebral cortex is undoubtedly involved in motion sickness in the modification of conditioned responses. Nevertheless, it is not essential for the development of motion sickness, because motion sickness can be induced in the decorticate human [16].

The up-regulation of H1 receptors in the hypothalamus and brainstem also indicates a post-synaptic amplification of H1 transmission in the development of motion sickness. The up-regulation of H1 receptors may contribute to the characteristic time course of motion sickness symptoms that develop gradually at first and then progressively more rapidly.

Conclusion

In conclusion, hypergravity-induced motion sickness in rats was prevented by BBB-penetrating H1 receptor blockers but not by non-BBB-penetrating H1 receptor blockers or H2 receptor blockers, suggesting that activation of H1 receptors in the brain is important for the development of motion sickness. The expression of H1 receptor mRNA was increased in the hypothalamus and brainstem, but not in the cerebral cortex after the hypergravity load in rats. It is suggested that histamine was released from the axon terminals in the hypothalamus and brainstem and released histamine activated the post-synaptic H1 receptors there, resulting in the development of motion sickness. The up-regulation of post-synaptic H1 receptors would amplify the histamine-mediated responses in motion sickness that develop gradually at first and then progressively more rapidly.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Natural course of positional vertigo in patients with apogeotropic variant of horizontal canal benign paroxysmal positional vertigo

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Abstract

Objective: The purpose of this study was to assess the natural course of positional vertigo in patients with the apogeotropic variant of horizontal canal type of benign paroxysmal positional vertigo (AH-BPPV), which is reported to be more refractory to physiotherapy than the geotropic variant of horizontal canal type of BPPV (GH-BPPV).
Methods: 14 patients with AH-BPPV treated without physiotherapy were asked to visit the hospital every 2 weeks. At every follow-up visit, they were interviewed and positional nystagmus was assessed. After the disappearance of positional nystagmus, patients were asked about the time of cessation of the positional vertigo. Thus, the primary outcomes were evaluated by the self-reported onset and remission of positional vertigo. The time course of remission of positional vertigo was then calculated.
Results: The average and median period from the onset to natural remission of positional vertigo in patients with AH-BPPV was 13 and 7 days, respectively.
Conclusion: We have already reported that the average and median period from the onset to natural remission of positional vertigo in patients with GH-BPPV was 16 and 7 days, respectively (Imai et al., 2005 [8]). Thus, the natural course of AH-BPPV is not as refractory as that of GH-BPPV.

Keywords: Benign paroxysmal positional vertigo; Apogeotropic variant; Horizontal canal; Natural course; Cupulolithiasis

1. Introduction

In recent years, two types of horizontal canal benign paroxysmal positional vertigo (H-BPPV) have been recognized. One type shows geotropic positional nystagmus when the head is turned to the side while lying down (GH-BPPV) [1]. The other type shows apogeotropic positional nystagmus (AH-BPPV) [2,3]. The pathophysiology of GH-BPPV is thought to be canalolithiasis in the horizontal semicircular canal (HSCC) and physiotherapy such as canalith repositioning therapy was effective in patients with GH-BPPV [4–7]. Moreover, we reported that GH-BPPV disappeared naturally within about 2 weeks without physiotherapy [8]. On the other hand, the pathophysiology of AH-BPPV is still controversial, with cupulolithiasis in the HSCC being reported as the most plausible cause of AH-BPPV [2,3]. On the other hand, it was reported that outcomes of physiotherapy in patients with AH-BPPV were poorer as compared with those with GH-BPPV [6,7].

In the present study, in order to examine if AH-BPPV was more refractory than GH-BPPV without physiotherapy, we assessed the natural course of remission of positional vertigo in patients with AH-BPPV.

2. Methods

The present study included 14 consecutive patients who were diagnosed as AH-BPPV in the Department of

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Table 1
The data of all patients. m: male, f: female, CP: canal paresis, R-CP: canal paresis exist in right ear, L-CP: canal paresis exist in left ear, N.P.: not performed, SPEV (R): slow phase eye velocity of positional nystagmus when their head was right lateral position in supine, SPEV (L): slow phase eye velocity of positional nystagmus when their head was left lateral position in supine.

ID	Sex	Age (years)	Affected side	Visit (days)	Remit (days)	Caloric	SPEV (R) (°/s)	SPEV (L) (°/s)
A	m	59	Right	1	2	N.P	8.9	41.8
B	f	28	Left	0	3	R-CP	6.6	6.4
C	f	71	Right	1	3	No CP	3.5	53.5
D	f	85	Right	1	3	N.P.	25.5	28.4
E	m	26	Left	2	3	No CP	6.0	3.1
F	m	72	Left	1	4	N.P	83.6	65.5
G	f	57	Right	1	6	N.P.	9.7	50.1
H	f	61	Right	1	7	N.P.	1.9	2.7
I	m	61	Right	4	7	No CP	23.0	61.9
J	f	72	Left	7	23	N.P.	9.9	2.3
K	m	37	Left	10	26	L-CP	51.8	25.8
L	f	60	Right	12	27	No CP	4.6	15.4
M	m	73	Right	22	32	N.P.	1.1	7.6
N	m	70	Left	7	35	R-CP	1.7	1.3

Otolaryngology, Kansai-Rosai Hospital between April 2001 and November 2003 (7 males and 7 females; 26–85 years old: mean age, 59 years, Table 1). All patients who complained of dizziness and/or vertigo were tested by lateral head rotation in supine position. The positional nystagmus was recorded by an infrared CCD camera (*RealEyes*, Micromedical Technologies) in all subjects. Maximum slow phase eye velocity (max SPEV) of the positional nystagmus was analyzed using our own video-oculography system [9]. AH-BPPV was diagnosed on the following criteria; (i) a history of brief episodes of positional vertigo, (ii) observation of an apogeotropic direction-changing positional nystagmus mainly with a horizontal component triggered by lateral head rotations in supine position; the apogeotropic variant was indicated by an intense apogeotropic nystagmus beating away from the ground in lateral head position on both sides, reaching a maximum immediately after change in head position and lasting more than 1 min, and (iii) absence of an identifiable central nervous system disorder able to explain the positional vertigo following neurological and neurophysiological examinations. The affected ear was determined as the head turning side that created less intense nystagmus [3]. Patients with nystagmus, of which direction changed from apogeotropic to geotropic or from geotropic to apogeotropic, were excluded from this study.

The patients were asked about the onset time of positional vertigo through a detailed interview. After they were given the details of the physiotherapy [4], their informed consent to non-medicinal treatment was obtained. Thus, all patients received no drug and were advised to visit the hospital every 2 weeks after the initial visit. At every visit, they were interviewed and examined by lateral head rotation in supine position. After the disappearance of positional nystagmus, patients were required to indicate the time at which the positional vertigo disappeared through detailed interview. Thus, the primary outcomes were assessed by self-reported onset and remission of positional vertigo, while the time course of remission of positional vertigo after the onset was calculated by Kaplan–Meier method. Patients were then examined 4 weeks after the disappearance of positional nystagmus and remained free of positional vertigo and nystagmus.

Patients with AH-BPPV were divided into two groups: the early remission group including patients A–I whose remission period was within 7 days, and the delayed remission group made of patients J–N whose remission period was more than 3 weeks (Table 1). Differences in age, sex, affected side, result of caloric test and max SPEV of positional nystagmus at the first visit to the hospital between the two groups were analyzed using chi-square test.

3. Results

The residual rate of positional vertigo was 64% in the patients with AH-BPPV at 1 week after the onset and 14% at 1 month without physiotherapy (Fig. 1). The averaged period and median period from the onset to natural remission of positional vertigo was 13 ± 13 days and 7 days, respectively.

There were no significant differences in age, sex, affected side, result of caloric test and max SPEV of positional nystagmus at the first visit to the hospital between the early and delayed remission groups (Table 1).

4. Discussion

In the present study, we assessed the natural course of positional vertigo in patients with AH-BPPV (7 males and 7 females; mean age, 59 years) without physiotherapy and showed that the average and median period from the onset to natural remission of their positional vertigo was 13 and 7 days, respectively. We have already reported in patients with GH-BPPV (15 males and 21 females; mean age, 57 years)

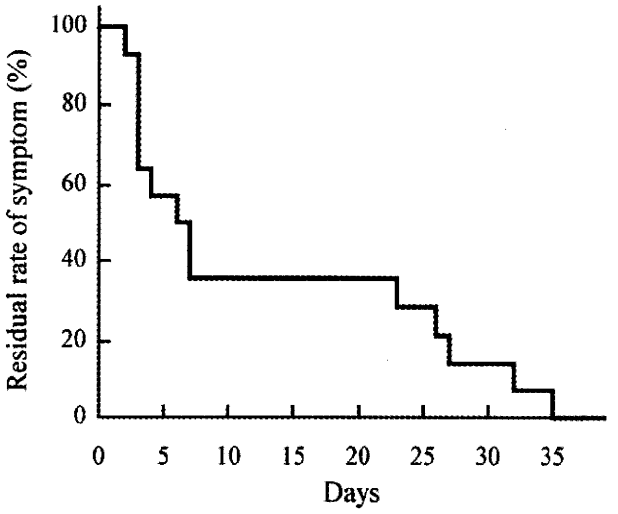

Fig. 1. Time course of positional vertigo after the onset without physiotherapy in patients with AH-BPPV was calculated using Kaplan–Meier method.

that the average and median period from the onset to natural remission of positional vertigo was 16 and 7 days, respectively [8]. Thus, the natural course of AH-BPPV is not as refractory as that of GH-BPPV.

GH-BPPV is thought to be induced by canalolithiasis made of the otoconial debris, which move under the influence of gravity within the distal long arm of HSCC when the head rotates in the supine position [1]. On the other hand, AH-BPPV is thought to be due to cupulolithiasis made of the otoconial debris attached to the cupula of HSCC and the gravity-sensitive cupula bends when the head rotates in the supine position [2,3,10]. Similarly to the posterior canal type of benign paroxysmal positional vertigo [11], the management of GH- and AH-BPPV is based on physiotherapy, having the same purpose of removing the debris from the location in the canal toward the utricle, even considering that the debris may be variously positioned in the lumen of the canal [6,7]. The physiotherapy of GH-BPPV is based on removing the debris from HSCC along its distal long arm into the utricle by various methods including 180° rotation [12], barbecue rotation [4], and forced prolonged position [5]; whereas the physiotherapy of AH-BPPV consists of detaching the debris from the cupula by various methods including therapeutic head shaking [13], modified Semont maneuver [14], Gufoni maneuver [6], and Vanucchi–Asprella maneuvers [15], and then removing the debris from HSCC along its distal long arm into the utricle by the same methods used for the physiotherapy of GH-BPPV. It was reported that outcomes of the physiotherapy in patients with AH-BPPV were poorer compared to those with GH-BPPV [6,7], probably because the physiotherapy of AH-BPPV contains two steps.

But, as shown in the present study, the average period from the onset to natural remission of positional vertigo in patients with AH-BPPV was slightly shorter than that in patients with GH-BPPV. Indeed, in patients with GH-BPPV even without physiotherapy, the head movement of daily life moves canalolithiasis along the distal long arm of HSCC to the utricle. On the other hand, among patients with AH-BPPV even without physiotherapy, we hypothesized that the head movement of daily life makes the cupulolithiasis fall off from the cupula into the proximal short arm of HSCC in patients A–I and into its distal long arm in patients J–N. Thus, the short trajectory of the debris from the proximal short arm of HSCC into the utricle after its detachment from the cupula may be accomplished within 7 days in patients A–I, whereas the long trajectory of the debris from the distal long arm of HSCC into the utricle may take more than 3 weeks in patients J–N.

Because AH-BPPV in the early remission group disappeared within a week, it is possible that some other patients with AH-BPPV might have been cured naturally before the visit to the hospital behind the present study. This bias that cannot be estimated may slightly prolong the natural course of AH-BPPV.

In the present study, we showed that the average and median period from the onset to natural remission of positional vertigo in patients with AH-BPPV was 13 and 7 days, respectively. We concluded that despite the poorer physiotherapy outcomes in AH-BPPV patients, the natural remission of their disease was not as refractory in comparison with that of GH-BPPV patients.

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ORIGINAL ARTICLE

Meniere's Attacks Occur in the Inner Ear with Excessive Vasopressin Type-2 Receptors

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Meniere's disease is peculiar to humans and is characterised by episodic vertigo, fluctuating hearing loss and tinnitus, and attacks of the affliction occurring under conditions of stress. Its pathology was first revealed to be inner ear hydrops through temporal bone studies in 1938. Although subsequently proposed as a disorder of water metabolism in the inner ear, its pathogenesis remains unsolved. The present study aimed to assess the link between the inner ear pathology in Meniere's disease and vasopressin, an anti-diuretic stress hormone with a potential role in inner ear fluid homeostasis. Blood samples were obtained from Meniere's disease patients in the morning, before any surgical treatment, to examine plasma vasopressin (pAVP) levels, and then from inner ear tissue during surgical treatment, to examine vasopressin type-2 receptor (V2R) in the endolymphatic sac. pAVP and the relative V2R mRNA expression in the endolymphatic sac were examined using a real-time polymerase chain reaction. Relative cAMP activity in the endolymphatic sac was also examined using tissue culture and cAMP assay. Both pAVP (1.6-fold versus controls; $P = 0.048$) and inner ear V2R mRNA expression (41.5-fold versus controls; $P = 0.022$) were significantly higher in Meniere's patients. cAMP activity was basally up-regulated (2.1-fold versus controls) and cAMP sensitivity to vasopressin application was largely elevated (4.9-fold versus controls) in Meniere's patients. We conclude that, in the pathogenesis of inner ear hydrops, resulting in Meniere's attacks, elevation of pAVP levels (probably as a result of stress) may present as a matter of consequence, but susceptibility of the V2R-overexpressed and cAMP-hypersensitised inner ear to pAVP elevation might be essential as the basis of this disease. Further experimental and clinical studies are needed to better clarify the relationship between Meniere's disease and stress.

Key words: Meniere's disease, inner ear, stress hormone, vasopressin, V2 receptor, cAMP.

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Some sicknesses are well known to be provoked by inadequate adaptation to various kinds of stress experienced in daily life. Attacks of Meniere's disease characterised by episodic vertigo, fluctuating hearing loss and tinnitus are a common example. The otopathology of Meniere's disease was first revealed to be inner ear endolymphatic hydrops through temporal bone studies in 1938 (1, 2). It has been proposed that the pathogenesis in Meniere's disease could be inner ear endolymphatic hydrops due to a disorder of water metabolism.

Vasopressin acts upon water metabolism via vasopressin receptors and aquaporin channels in the inner ear as well as in the kidney (3). To assess the link between the inner ear pathology of Meniere's disease and the stress hormone vasopressin, we examined plasma vasopressin (pAVP) levels and vasopressin type-2 receptor (V2R) activity in the endolymphatic sac in Meniere's patients.

Materials and methods

Diagnosis and enrolment

Patients were eligible for enrolment if they had received a clinical diagnosis of Meniere's disease according to the 1995 AAO-HNS criteria (4). In brief, these criteria comprise: (i) repeated attacks of vertigo: a definitive spell was spontaneous vertigo lasting at least 20 min (a mixed type of spontaneous nystagmus was observed during attacks); (ii) fluctuating cochlear symptoms: the hearing test usually revealed a marked fluctuation of the threshold in

the low and middle tone range; and (iii) exclusion of other causes (to exclude other disorders, a thorough history, neurological, neurotological and magnetic resonance imaging examinations were carried out). Intractable Meniere's disease was designated in cases where various forms of medical and psychological managements failed for at least 6 months. Medical managements included diuretics, betahistine, diphenidol, dimenhydrinate and diazepam, which were thought to be effective for persistent symptoms in Meniere's disease (5).

Patients designated as intractable Meniere's disease underwent endolymphatic sac drainage, if there was no reason for declination of surgery. The technical details of this surgery have been described previously (6–8). The use of all the human materials in the present study was approved by the Ethics Committee of Osaka University, School of Medicine (certificate number: 0424).

Laboratory examination for plasma vasopressin

Patients and controls

Between 1998 and 2006, 105 patients were enrolled with Meniere's disease to examine plasma vasopressin level at Osaka University Hospital (unilateral Meniere's disease: 87 cases; bilateral Meniere's disease: 18 cases). Thirty patients with chronic otitis media without any direct inner ear damage were also prepared as controls. Before collecting blood samples, all the patients with Meniere's disease and chronic otitis media provided their informed consent. Blood samples in both groups were collected at 08.00–10.00 h on the day of surgery during remission of vertigo attacks. Endolymphatic sac drainage was performed as an inner ear surgery for Meniere's disease and tympanoplasty was performed as a middle ear surgery for chronic otitis media. There were no significant differences in patients' background (sex: $P = 0.131$, age: $P = 0.435$) between Meniere's disease (male : female = 50 : 55, 47.5 ± 1.3) and chronic otitis media (male : female = 19 : 11, 45.4 ± 2.5).

Procedures

The blood was transferred into an ethylenediaminetetracetic acid tube and centrifuged at 4 °C, and the separated plasma stored at −80 °C for plasma vasopressin assay. The plasma vasopressin level was determined by radioimmunoassay (arginine vasopressin radioimmunoassay kit; Mitsubishi, Tokyo, Japan). The normal plasma vasopressin level was in the range 0.3–4.2 pg/ml (mean 2.25 pg/ml) based on the data acquired by blood samples collected at 08.00–10.00 h from 105 healthy subjects with their informed consent (61 males and 44 females) who had no history of vestibular or cochlear disease (9).

Molecular examination for vasopressin receptor

Patients and controls

Before surgery, informed consent were obtained from 18 out of 105 Meniere's disease patients (unilateral Meniere's disease: 15 cases; bilateral Meniere's disease: three cases) for the collection of endolymphatic sac tissue during surgery. Six acoustic neurinoma patients without any direct endolymphatic sac damage were also prepared as controls. Tissue samples from both the groups were collected during surgery (endolymphatic sac drainage for Meniere's disease group and surgical removal of acoustic neurinoma for the other group). There were no significant differences in patients' background (sex: $P = 0.546$, age: $P = 0.551$) between Meniere's disease (male : female = 6 : 6, 47.9 ± 4.9) and acoustic neurinoma (male : female = 3 : 3, 53.0 ± 6.5).

Tissue preparation

Tissues were replaced immediately after collection in chilled phosphate-buffered saline (pH 7.3) and frozen with dry ice powder for real-time polymerase chain reaction (PCR) (Meniere's disease: 1–12; acoustic neurinoma: 1–6) and western blotting (other unilateral Meniere's disease: 13–15; acoustic neurinoma: 1–3) analysis.

Real-time PCR

Total RNA extraction. Total RNA was extracted from dissected frozen tissues using TRIzol reagents (Gibco BRL Gaithersburg, MD, USA). Briefly, samples were homogenised in 0.8 ml of TRIzol reagent. Chloroform was then added and the mixture was centrifuged in order to separate the RNA phase from the DNA phase. The RNA phase was used for RNA precipitation using isopropyl alcohol. The RNA samples were rinsed with ethanol and dissolved with RNase-free water. Finally, the RNA samples were treated with RNase-free Dnase I (Roche Diagnostics, Indianapolis, IN, USA) to remove contaminated genomic DNAs before reverse transcription.

Reverse transcription of RNA. The reverse transcription mixture included 10 μl of 10× PCR Taq Gold buffer II (Applied Biosystems, Foster City, CA, USA), 30 μl of 25 mM $MgCl_2$, 4 μl of 25 mM of each dNTP, 5 μl of 100 μM of random primers (Gibco BRL), 2 μl of RNasin (Applied Biosystems), 1.25 μl of Super-Script II (Applied Biosystems) and 5 μl (250 ng) of DNA-free total RNA in a final volume of 100 μl. The mixture was incubated at 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min in a 9600 Thermocycler (Applied Biosystems).

Reverse-transcriptase PCR. Samples with reverse transcriptase were forwarded for PCR (95 °C for 12 min and, 35 cycles at 95 °C for 15 s and 60 °C for 1 min) and electrophoresed on 1.5% agarose gel to check the results of reverse-transcriptase PCR. Samples without reverse transcription were also forwarded for PCR as negative controls to ensure that no genomic DNA contamination occurred.

PCR products were electrophoresed on 3% Seakem GTG agarose gel (FMC Bioproducts, Philadelphia, PA, USA) and purified using QIA quick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Sequencing was accomplished by means of ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit with ABI 310 DNA sequencer (Applied Biosystems).

Real-time quantitative PCR. PCR reactions were performed in the presence of the oligonucleotide primers for V2R (Takara Bio Inc., Otsu, Japan) and β-2 microglobrin (B2M) (Takara) shown in Table 1 and quantified by SYBR Green PCR reagents (Applied Biosystems). B2M, an endogenous housekeeping gene, was used as an internal control for this method. Each sample determination was performed in triplicate.

The PCR mixture included 5 μl of 10× SYBR PCR buffer, 6 μl of 25 mM $MgCl_2$, 4 μl of each dNTP (blended with 2.5 mM dATP, dGTP and dCTP, and 5 mM dUTP), 2.5 μl of each gene-specific primer (5 μM), 0.5 μl of AmpErase UNG (0.5 U), 0.25 μl of AmpliTaq Gold (1.25 U) and 5 μl of cDNA (250 ng) in a final volume of 50 μl. The conditions for the real-time PCR were: 50 °C for 2 min, 95 °C for 12 min, and 35 cycles at 95 °C for 15 s and 60 °C for 1 min in ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

Table 1. Gene-Specific Primers for Real-Time Polymerase Chain Reaction of Human V2 Receptor and β-2 Microglobulin.

V2 receptor (NAA: 000054)
Forward: ACTGTGAGGATGACGCTAGTGATTG
Reverse: GGACACGCTGCTGCTGAAAG
p-2 microglobrin (NAA: 004048)
Forward: CGGGCATTCCTGAAGCTGA
Reverse: GGATGGATGAAACCCAGACACATAG

7700 Sequence Detection software was used for instrument control, automated data collection and data analysis.

Data analysis. The number of PCR cycles was recorded until the fluorescence intensity exceeded the pre-determined threshold. The quantification of the initial amounts of template molecules relied on this number of PCR cycles, which is termed the cycle threshold (CT). The dCT represents the CT of the target gene normalised to the human endogenous B2M (dCT = $CT_{target} - CT_{B2M}$). Relative quantification of the mRNA expression levels of target genes (= fold range) was calculated using the 2^{-ddCT} method, where ddCT = $(CT_{target} - CT_{B2M})_A - (CT_{target} - CT_{B2M})_B$ (10). For example, changes in the gene expression of V2R in endolymphatic sac in Meniere's disease (MD) compared with acoustic neurinoma (AN) were quantified as the fold range: 2^{-ddCT}[ddCT = $(CT_{V2R} - CT_{B2M})_{MD} - (CT_{V2R} - CT_{B2M})_{AN}$].

Western blotting

Samples from endolymphatic sac were homogenised on ice with polytron homogeniser (PCU-11; Kinematica, Bohemia, NY, USA) in 20 mM HEPES (pH 7.2), 25 mM NaCl, 2 mM ethylennebis(oxonnitrilo)tetraacetic acid, 50 mM NaF, 1 mM Na3VO4, 25 mM β-glycerophosphate, 0.2 mM dithithreitol, 1 mM phenylmethanesulfonyl fluoride, 60 μg/ml aprotinin, 2 μg/ml leupeptin and 0.1% Triton X-100). After incubation at 4 °C for 30 min, homogenates were sonicated (Sonifier 250; Branson Ultrasonics, Danbury, CT, USA) on ice for 1 min and centrifuged at 10 000 *g* at 4 °C for 30 min. The supernatant was collected. Protein concentrations of these supernatants were measured with a protein assay kit (Pierce, Rockford, IL, USA). Gel samples were prepared by adding sample buffer, containing final concentrations of 50 mM Tris (pH 6.7), 2% SDS and 2% mercaptoethanol. Protein extracts (20 μg) were boiled for 10 min, cooled to room temperature and loaded on 10% SDS-polyacrylamide gels. Equal amounts of protein in each sample were further checked by immunoblotting with β-actin monoclonal antibody (diluted 1 : 500) (Oncogene Research Products, Calbiochem, San Diego, CA, USA).

Proteins were transferred to Hybond-PVDF membranes (Amersham Pharmacia, Piscataway, NJ, USA) by using standard electroblotting procedures. Membranes were incubated sequentially in the following solutions at 4 °C: 2% nonfat dry milk, 1% bovine serum albumin and normal goat serum (NGS) in 0.3% Triton-X 100 in phosphate-buffered saline (PBS) for 3 h; antisera against V2R (diluted 1 : 500) (sc-18100-R; Santa Cruz Inc., Santa Cruz, CA, USA) in 1% BSA and NGS in 0.3% Triton-X 100 in PBS for 24 h; 0.1 M PBS for 30 min; horseradish peroxidase-conjugated secondary antibody (Dako, Carpinteria, CA, USA) in 1% BSA and NGS in 0.3% Triton-X 100 in PBS for 3 h; 0.1 M PBS for 30 min. Protein bands were visualised using an ECL detection kit and Hyperfilm MP (Amersham Pharmacia) and analysed using Scion Image software (Scion Corp., Frederick,. MD, USA).

Organotypic culture of endolymphatic sac tissues

Tissue culture

According to a previous study (11), parts of the endolymphatic sac tissues from patients with Meniere's disease (cases 16–18) and acoustic neurinoma (cases 4–6) were rapidly removed and placed in cold HEPES-buffered saline with Hank's balanced salt solution (HHBSS: 4 °C, pH 7.3). The tissues were mounted flat on culture slides coated with 20 μl of a 1 : 5 dilution of Cell Tek (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and covered with 300 μl of minimum essential medium containing D-valine (MEM D-Val), to suppress fibroblast growth, as well as 10% fetal calf serum, 10 mM HEPES, 100 IU/ml penicillin and 2 mM glutamine. Cultures were maintained under a 5% CO_2 atmosphere at 37 °C for up to 12 h. The morphology of the cultured tissues was monitored by differential interference contrast infra-red light microscopy.

cAMP assay

After culture for up to 12 h, the endolymphatic sac tissues were incubated with HHBSS (37 °C, 5% CO_2, pH 7.3) for 30 min. [Arg8]-vasopressin alone (Sigma Aldrich, St Louis, MO, USA) at 5–10 nM (AVP5-10) or vasopressin/V2R specific antagonist OPC31260 (Otsuka Pharmaceutical Inc., Tokushima, Japan) at 10 nM/10–50 nM (AVP10/OPC10-50) was added to the HHBSS at the incubation. Next, the supernatants from the tissues were placed in 96-well microplates and incubated with a cAMP assay solution (cAMP Fluorescence Assay Kit; Nippon Genetics, Tokyo, Japan) at 37 °C for 30 min. Fluorescence images were captured and measured three times for each sample by using an AX-70 fluorescence microscope (Olympus, Munster, Germany). The cAMP activity was calculated as the relative fluorescence intensity on the basis of the fluorescence intensity in acoustic neurinoma samples in a control solution (AN in control = 1).

Statistical analysis

Statistical differences of patients' backgrounds (sex, age) between Meniere's disease and controls were examined by the Mann–Whitney U-test. Statistical differences of the data between two groups in Figs 1 and 2 were determined by an unpaired t-test. Co-relationships between two parameters in Fig. 3 were analysed by Pearson's test. In Fig. 4, the Bonferroni/Dunn test was adopted to examine statistical changes among multiple factors in each group and then an unpaired t-test was complementarily used to check a trend of differences (strictly no significant differences) between two factors in two groups. $P < 0.05$ was considered statistically significant (or to demonstrate a trend of differences for the unpaired t-test in Fig. 4).

All the statistical analyses in the present study were carried out using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) according to the suggestions of a registered statistician (Dr Y. Yamagiwa, certificate number: 0540072).

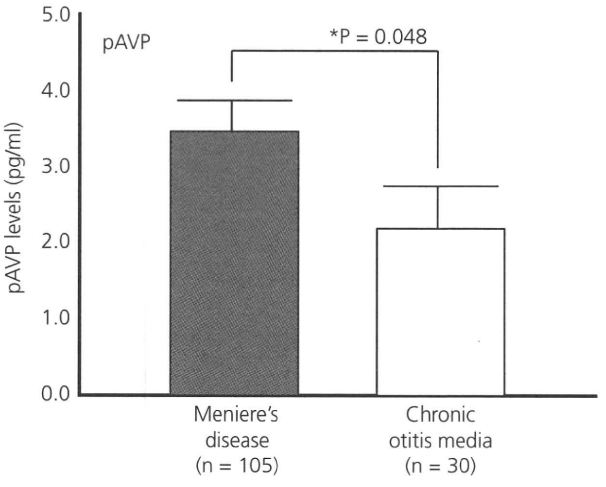

Fig. 1. Plasma vasopressin levels in Meniere's disease patients compared with control patients. Plasma vasopressin (pAVP) was significantly higher in Meniere's disease (MD) patients (n = 105; 3.30 ± 0.30 pg/ml) compared to control chronic otitis media (OM) patients (n = 30; 2.11 ± 0.38 pg/ml) during the early morning of the day of surgery (unpaired t-test: *P = 0.048). There were no significant differences between the pAVP levels in unilateral (n = 87; 3.47 ± 0.35 pg/ml) and bilateral (n = 18; 2.49 ± 0.40 pg/ml) MD patients (unpaired t-test: P = 0.221).

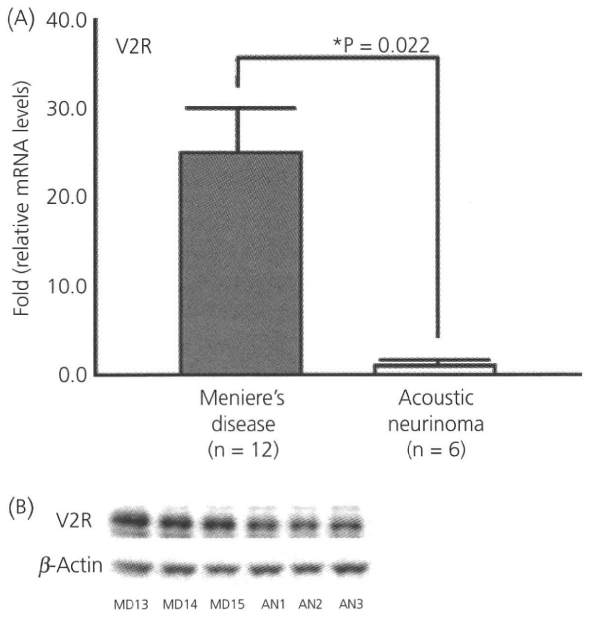

Fig. 2. V2 receptor mRNA and protein expression levels in the endolymphatic sac in Meniere's disease patients compared with control patients. (A) The relative V2 receptor (V2R) mRNA expression in the endolymphatic sac was significantly higher in Meniere's disease (MD) patients (n = 12; 24.89 ± 6.65 fold) compared to control acoustic neurinoma (AN) patients (n = 6; 0.62 ± 0.10 fold) as evaluated by real-time PCR (unpaired t-test: *P = 0.022). There were no significant differences between the V2R mRNA expression levels in unilateral (n = 9; 27.86 ± 8.19 fold) and bilateral (n = 3; 15.96 ± 10.72 fold) MD patients (unpaired t-test: P = 0.465). (B) V2R protein expression in the endolymphatic sac was also higher in MD patients compared to control AN patients as evaluated by western blotting.

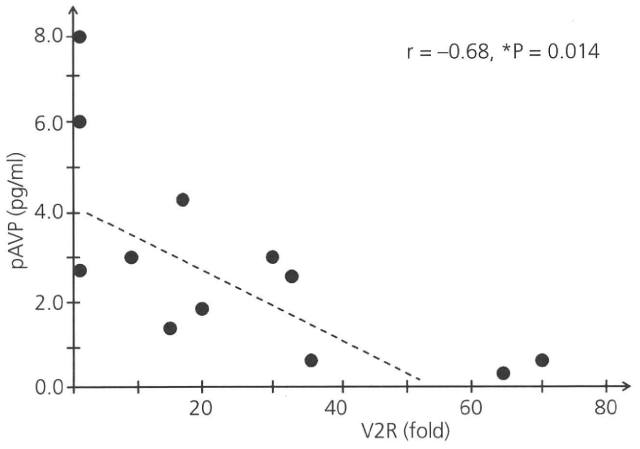

Fig. 3. Co-relationship between plasma vasopressin and V2 receptor mRNA expression in the endolymphatic sac in 12 cases of Meniere's disease. There was a significant negative co-relationship between plasma vasopressin (pAVP) and V2 receptor (V2R) mRNA expression in the endolymphatic sac in 12 cases of Meniere's disease (Pearson's test: r = −0.68, *P = 0.014).

Results

The raw data for 12 cases with Meniere's disease, including their plasma vasopressin levels, V2R mRNA expression level in the endolymphatic sac, vertigo frequency, hearing level and duration of disease before surgery, are shown in Table 2. We judged these patients' data of vertigo frequency, hearing level and duration of disease by questionnaire and/or review of the clinical notes.

The plasma vasopressin level was 1.6-fold higher in Meniere's disease patients (n = 105; 3.30 ± 0.30 pg/ml) compared to control chronic otitis media patients (n = 30; 2.11 ± 0.38 pg/ml) and this difference was significant (unpaired t-test: P = 0.048; Fig. 1). There were no significant differences between the plasma vasopressin levels in unilateral (n = 87; 3.47 ± 0.35 pg/ml) and bilateral (n = 18; 2.49 ± 0.40 pg/ml) Meniere's disease patients (unpaired t-test: P = 0.221).

The relative V2R mRNA expression level in the endolymphatic sac was 41.5-fold higher in Meniere's disease patients (n = 12; 24.89 ± 6.65-fold) compared to control acoustic neurinoma patients (n = 6; 0.62 ± 0.10-fold) and this difference was significant (unpaired t-test: P = 0.022; Fig 2A). These results were confirmed at the protein expression level by western blotting (Fig. 2B). There were no significant differences between the V2R mRNA expression levels in unilateral (n = 9; 27.86 ± 8.19 fold) and bilateral (n = 3; 15.96 ± 10.72 fold) Meniere's disease patients (unpaired t-test: P = 0.465).

In Meniere's disease patients, there was a significant negative co-relationship between plasma vasopressin and V2R mRNA expression in the endolymphatic sac (Pearson's test: r = −0.68, P = 0.014; Fig. 3).

According to the results for endolymphatic sac tissue cultures (Fig. 4), the relative cAMP activity in the endolymphatic sac was 2.1-fold higher in Meniere's disease patients (n = 3: 2.59 ± 0.39 fold) compared to control acoustic neurinoma patients (n = 3: 1.24 ± 0.21 fold) and this difference had a tendency for significance. (Meniere's disease in control versus acoustic neurinoma in control: unpaired t-test: P = 0.039). Furthermore, the vasopressin sensitivity of cAMP in the endolymphatic sac was 4.9 times higher in Meniere's disease patients (n = 3: 12.25 ± 1.30 fold) compared to control acoustic neurinoma patients (n = 3: 2.49 ± 0.29 fold) and this difference also had a tendency for significance. (Meniere's disease in AVP10 versus acoustic neurinoma in AVP10: unpaired t-test: P = 0.002). These elevations were significantly suppressed by V2R specific antagonist OPC31260 in dose-dependent manners in both groups (Meniere's disease: Bonferroni/Dunn test: P = 4.07E-06; acoustic neurinoma: Bonferroni/Dunn test: P = 0.005).

All the values in presented are expressed as the mean ± SEM. All the error bars in Fig. 4 were normalised to the acoustic neurinoma control value.

Discussion

For more than a decade, it has been known that plasma vasopressin levels are significantly higher in patients with inner ear endolymphatic hydrops, including Meniere's disease, during remission (12) or attacks (9), than those in patients with vertigo due to non-endolymphatic hydrops, such as benign paroxysmal positional vertigo and vestibular neuronitis. Systemic injection of vasopressin was also found to induce bilateral endolymphatic hydrops and hearing

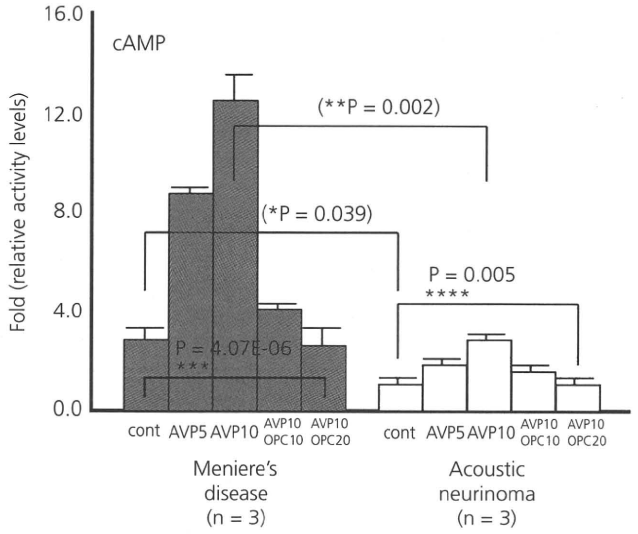

Fig. 4. cAMP activity in the endolymphatic sac in Meniere's disease patients compared with control patients. The relative cAMP activity in the endolymphatic sac was basally up-regulated in Meniere's disease (MD) patients (n = 3; 2.59 ± 0.39 fold) compared with control acoustic neurinoma (AN) patients (n = 3; 1.24 ± 0.21 fold), and this difference had a tendency MD in control versus AN in control: unpaired t-test: *P = 0.039). Furthermore, the vasopressin sensitivity of cAMP in the endolymphatic sac was largely elevated in MD patients (n = 3; 12.25 ± 1.30 fold) compared with control AN patients (n = 3; 2.49 ± 0.29 fold) and this difference also had a tendency (MD in AVP10 versus AN in AVP10: unpaired t-test: **P = 0.002). These elevations were significantly suppressed by V2R specific antagonist OPC31260, in dose-dependent manners in both groups (MD: Bonferroni/Dunn test: ***P = 4.07E-06; AN: Bonferroni/Dunn test: ****P = 0.005). Control, control solution only; AVP5-10, with 5–10 nM vasopressin; AVP10/OPC10-50, with 10 nM vasopressin and 10–50 nM V2R antagonist OPC31260.

deterioration in guinea pigs (13). These findings led to the hypothesis that a high level of plasma vasopressin is one of the causes of inner ear endolymphatic hydrops in Meniere's patients. However, it has recently been reported that plasma vasopressin levels in patients with unilateral Meniere's disease do not differ significantly from those in healthy volunteers (14). Furthermore, the hypothesis of a high level of plasma vasopressin, which should have equal effects on the bilateral ears, contradicts the fact that 70–80% of Meniere's disease patients are unilaterally affected (15). Therefore, the above hypothesis appears to be insufficient to explain the pathogenesis of endolymphatic hydrops, which has remained unexplained ever since Yamakawa (1) and Hallpike and Cairns (2) first demonstrated endolymphatic hydrops in Meniere's temporal bone in 1938. In the present study, to resolve this controversial relationship between vasopressin and Meniere's disease, we focused on plasma ligands and also on receptors in the inner ear in Meniere's disease.

Regarding vasopressin receptors, V2R molecules have been detected in rat (11, 16) and human (11, 17) inner ear endo-organ tissues. V2R is clearly distributed together with a V2R-linked water channel molecule, aquaporin-2 (AQP2), in the luminal epithelium of the human endolymphatic sac (17). Interestingly, the physiological interactions between vasopressin and V2R in the endolymphatic sac attenuate membranous turnover via cAMP-dependent signalling in

Table 2. Raw Data for 12 Cases of Meniere's Disease.

	pAVP (pg/ml)	V2R mRNA (fold)	Vertigo frequency (per month)	Hearing level (dB)	Duration (per month)
MD 1	0.5	64.78	1.0	30.0	34
MD 2 (Bil)	0.8	36.90	3.3	42.5	12
MD3	0.8	69.28	1.0	45.0	60
MD4	1.3	13.72	1.3	66.3	84
MD5	2.0	20.18	3.3	60.8	98
MD6	2.7	1.90	1.7	70.0	48
MD7	2.7	32.38	7.3	57.5	18
MD 8 (Bil)	3.1	9.45	2.0	30.7	45
MD9	3.5	29.52	8.0	66.5	30
MD10	4.2	17.32	4.0	60.0	48
MD11	6.0	1.70	2.0	58.5	60
MD12 (Bil)	8.0	1.52	2.5	75.6	96

The raw data for 12 cases of Meniere's disease (MD) include the plasma vasopressin level (pAVP), V2 receptor mRNA expression level in the endolymphatic sac (V2R), vertigo frequency, hearing level and duration of disease (Duration) before surgery. Bil, bilateral Meniere's disease.

a manner that contrasts to that in the kidney in rats (11). These interactions also translocated AQP2 from the luminal to the basolateral side in a manner that contrasts to that in the human kidney (Maekawa C, Kitahara T, Doi K, Horii A, Kizawa K, Kiyama H; unpublished data). These findings indicate that V2R and cAMP-linked signalling could suppress endolymphatic fluid absorption in the endolymphatic sac. First, in the present study, V2R mRNA expression in the endolymphatic sac was much higher in Meniere's patients compared to controls (41.5-fold); whereas plasma vasopressin level was only elevated 1.6-fold. cAMP activity was basally up-regulated 2.1-fold and cAMP sensitivity to vasopressin was significantly elevated 4.9-fold in the endolymphatic sac in Meniere's patients compared to controls. All these findings suggest that V2R overexpression and cAMP hypersensitivity in the endolymphatic sac could attenuate membranous turnover and cause endolymphatic fluid overflow into the endolymphatic space, even after a small increase in plasma vasopressin. In other words, patients with V2R overexpression and cAMP hypersensitivity in the unilateral endolymphatic sac could develop unilateral inner ear susceptibility to plasma vasopressin elevation, resulting in unilateral endolymphatic hydrops and subsequent attacks of vertigo, with hearing loss and tinnitus in the unilateral ear. Second, we detected a significant negative co-relationship between plasma vasopressin and V2R expression in the endolymphatic sac in Meniere's patients, consistent with an earlier study on the application of vasopressin in normal rats (16). Taken together with the results of high levels of both plasma vasopressin and V2R in the endolymphatic sac in Meniere's patients, it is suggested that a negative-feedback system between plasma vasopressin and its receptor in the endolymphatic sac in normal subjects could function for inner ear fluid homeostasis and avoid generating endolymphatic hydrops after plasma vasopressin elevation. Although the mechanisms of this negative-feedback system remain to be clarified, it may explain why previous studies

of plasma vasopressin in Meniere's disease have produced a large variety of controversial results (9, 12, 14).

Finally, we would like to speculate about the possible causes of attacks associated with inner ear pathology in Meniere's disease. It has been reported that Meniere's disease is usually triggered by immune, infectious, traumatic or other insults to the inner ear in association with a small misplaced malfunctioning endolymphatic sac (18, 19). Among these insults, immune-mediated responses in the inner ear endo-organs, such as the endolymphatic sac, stria vascularis and spiral ligament, are thought to be the main causes of fluid homeostatic disorder in Meniere's disease (20, 21). Certain virus infections of the endolymphatic sac in early childhood, such as varicella-zoster, Epstein–Barr and adenovirus infections, represent other possible causes for the dysfunction of fluid homeostasis (22, 23). Taken together with the present data, it is suggested that autoimmune responses and/or virus infections could modulate V2R regulatory genes in the endolymphatic sac for V2R overexpression and hypersensitivity of cAMP-linked signalling there. After such insults to the inner ear, endolymphatic hydrops could gradually be generated because of the susceptibility of the V2R-overexpressed and cAMP-hypersensitised inner ear to plasma vasopressin elevation, and perhaps the rupture of Reissner's membranes (24), thereby resulting in attacks of Meniere's disease.

In conclusion, we hypothesised that Meniere's patients with overexpression and hyperactivity of V2R in the endolymphatic sac develop endolymphatic hydrops and vertigo attacks after plasma vasopressin elevation. However, it remains unclear when or what kinds of insults could make the inner ear susceptible to plasma vasopressin elevation, probably as a result of stress, and what kinds of or how long a period of stress could lead to a high level of plasma vasopressin during the development of endolymphatic hydrops in Meniere's disease. Experimental and clinical studies are required to better clarify the relationship between stress and Meniere's disease.

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ORIGINAL ARTICLE

Expression and Translocation of Aquaporin-2 in the Endolymphatic Sac in Patients with Meniere's Disease

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Meniere's disease, characterised by episodic vertigo, fluctuating hearing loss and tinnitus, can occur under conditions of stress. Its pathology was first revealed to be inner ear hydrops through temporal bone studies in 1938. Although its pathogenesis has been proposed to be a disorder of water transport in the inner ear, subsequently, it remains unsolved, until now. A recent study revealed that both plasma stress hormone, vasopressin (pAVP) and its receptor, V2 (V2R) expression in the inner ear endolymphatic sac were significantly higher in Meniere's patients. In the present study, to link V2R-related molecules and inner ear hydrops, we examined V2R-linked water channel molecule, aquaporin-2 (AQP2) expression and translocation in human endolymphatic sac. AQP2 mRNA expression in the endolymphatic sac was significantly higher in Meniere's patients by using real-time polymerase chain reaction, as further confirmed by western blotting. AQP2-like immunoreactivity (-LIR) was translocated from luminal to basolateral side with endosomal trapping in the endolymphatic sac at the time of AVP exposure in human endolymphatic sac tissue culture. The similar AQP2-LIR translocation was also demonstrated by forskolin and blocked by vasopressin/V2R specific antagonist, OPC31260 and protein kinase A (PKA) specific antagonists, H-89 and KT-5720. We concluded that in the pathogenesis of inner ear hydrops resulting in Meniere's attacks, pAVP elevation as a result of stress and subsequent V2R-cAMP-PKA-AQP2 activation and endosomal trapping of AQP2 in the endolymphatic sac, might be important as a basis of this disease. Further experimental and clinical studies are needed to better clarify the neuroscientific relationship between stress and Meniere's disease.

Key words: Meniere's disease, stress, vasopressin, endolymphatic sac, V2 receptor, cAMP, protein kinase A, aquaporin-2.

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The otopathology in Meniere's disease was first revealed to be inner ear endolymphatic hydrops through temporal bone studies in 1938 (1,2), so that it has been gradually understood the fluid homeostatic system in the endolymphatic sac via water transport-related molecules such as vasopressin and aquaporin (3). Subsequently, it was proposed that the pathogenesis in Meniere's disease could be inner ear endolymphatic hydrops as a result of a disorder of water transport-related molecules.

A previous study revealed that both plasma stress hormone, vasopressin (pAVP) and its receptor, V2 (V2R) expression in the inner ear endolymphatic sac were significantly higher in Meniere's patients (4,5). In the present study, to link V2R-related molecules and inner ear hydrops, we examined V2R-linked water channel molecule, aquaporin-2 (AQP2) expression and translocation in human endolymphatic sac.

Materials and methods

The use of all the human materials in the present study was approved by the Ethics Committee of Osaka University, School of Medicine (certificate number: 0424).

Diagnosis and enrolment

Patients were eligible for enrolment if they had received a clinical diagnosis of Meniere's disease according to the 1995 AAO-HNS criteria (6). These criteria comprise: (i) repeated attacks of vertigo: a definitive spell is spontane-

ous vertigo lasting at least 20 min (a mixed type of spontaneous nystagmus is observed during attacks); (ii) fluctuating cochlear symptoms: the hearing test usually reveals a marked fluctuation of the threshold in the low and middle tone range; and (iii) exclusion of other causes: to exclude other disorders, a thorough history, neurological, neurotological and magnetic resonance imaging examinations were carried out. Intractable Meniere's disease was designated in cases where various forms of medical and psychological managements failed for at least 6 months. Medical managements included diuretics, betahistine, diphenidol, dimenhydrinate and diazepam, which were considered to be effective for persistent symptoms in Meniere's disease (7).

Patients diagnosed with intractable Meniere's disease were treated with endolymphatic sac drainage if there were no contraindications to surgery. The technical details of this surgery have been described previously (8–10).

Molecular examination for vasopressin receptor

Patients and controls

Before surgery, we obtained permission for collection of endolymphatic sac tissue during surgery from 15 Meniere's disease (MD) patients (unilateral MD: 12 cases; bilateral MD: three cases). We also prepared nine vestibular schwannoma (VS) patients without any direct endolymphatic sac damage as controls. Tissue samples from a part of the endolymphatic sac in both groups, MD and VS, were collected during surgery (endolymphatic sac drainage for MD and acoustic neurinoma removal surgery for VS). There were no significant differences in patients' background (sex and age) between MD (male : female = 7 : 8, 48.6 ± 5.8) and VS (male : female = 4 : 5, 52.0 ± 7.5). A part of samples used in the present study have been described previously (4).

Tissue preparation

For real-time polymerase chain reaction (PCR) (MDs: 1–12; VSs: 1–6) and western blotting (MDs: 13–15; VSs: 1–3), tissues were obtained from the endolymphatic sac during endolymphatic sac drainage for MD or vestibular schwannoma removal surgery for VS, replaced immediately in chilled phosphate-buffered saline (PBS) (pH 7.3) and frozen with dry ice powder.

Real-time PCR

Total RNA extraction

Total RNA was extracted from dissected frozen tissues using TRIzol reagents (Gibco BRL, Gaithersburg, MD, USA). Briefly, samples were homogenised in 0.8 ml of TRIzol reagent. Chloroform was then added and the mixture was centrifuged to separate the RNA phase from the DNA phase. The RNA phase was used for RNA precipitation using isopropyl alcohol. The RNA samples were rinsed with ethanol and dissolved with RNase-free water. Finally, the RNA samples were treated with RNase-free Dnase I (Roche Diagnostics, Indianapolis, IN, USA) to remove contaminated genomic DNAs before reverse transcription.

Reverse transcription of RNA

The reverse transcription mixture included 10 μl of 10× PCR Taq Gold buffer II (Applied Biosystems, Foster City, CA, USA), 30 μl of 25 mM $MgCl_2$, 4 μl of 25 mM of each dNTP, 5 μl of 100 μM of random primers (Gibco BRL), 2 μl of RNasin (Applied Biosystems), 1.25 μl of Super-Script II (Applied Biosystems) and 5 μl (250 ng) of DNA-free total RNA in a final volume of 100 μl. The mixture was incubated at 25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min in a 9600 Thermocycler (Applied Biosystems).

Reverse-transcriptase PCR

Samples with reverse transcriptase were forwarded for PCR (95 °C for 12 min and 35 cycles at 95 °C for 15 s and 60 °C for 1 min) and electrophoresed on 1.5% agarose gel to check the results of reverse-transcriptase PCR. Samples without reverse transcription were also forwarded for PCR as negative controls to ensure that there was no genomic DNA contamination.

PCR products were electrophoresed on 3% Seakem GTG agarose gel (FMC Bioproducts, Philadelphia, PA, USA) and purified using QIA quick Gel Extraction kit (Qiagen, Valencia, CA, USA). Sequencing was accomplished by means of ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction kit with ABI 310 DNA sequencer (Applied Biosystems).

Real-time quantitative PCR

PCR reactions were performed in the presence of the oligonucleotide primers for AQP2 (forward: 5′-CCACCTCCTTGGGATCCATT-3′; reverse: 5′-GTGACGACAGCTGGAGCCA-3′) (NM: 000486) (Takara Bio Inc., Otsu, Japan) and β-2 microglobrin (B2M) (forward: 5′-CGGGCATTCCTGAAGCTGA-3′; reverse: 5′-GGATGGATGAAACCCAGACACATAG-3′) (NM: 004048) (Takara Bio Inc.) and quantified by SYBR Green PCR reagents (Applied Biosystems). B2M, an endogenous housekeeping gene, was used as an internal control for this method. Each sample determination was performed in triplicate.

The PCR mixture included 5 μl of 10× SYBR PCR buffer, 6 μl of 25 mM $MgCl_2$, 4 μl of each dNTP (blended with 2.5 mM dATP, dGTP and dCTP, and 5 mM dUTP), 2.5 μl of each gene-specific primer (5 μM), 0.5 μl of AmpErase UNG (0.5 U), 0.25 μl of AmpliTaq Gold (1.25 U) and 5 μl of cDNA (250 ng) in a final volume of 50 μl. The conditions for the real-time PCR were: 50 °C for 2 min, 95 °C for 12 min and, 35 cycles at 95 °C for 15 s and 60 °C for 1 min in ABI PRISM 7700 Sequence Detection System (Applied Biosystems). 7700 Sequence Detection software was used for instrument control, automated data collection and data analysis.

Data analysis

The number of PCR cycles was recorded until the fluorescence intensity exceeded the pre-determined threshold. The quantification of the initial amounts of template molecules relied on this number of PCR cycles, which is termed the cycle threshold (CT). The dCT represents the CT of the target gene normalised to the human endogenous B2M ($dCT = CT_{target} - CT_{B2M}$). Relative quantification of the mRNA expression levels of target genes (= fold range) was calculated using the 2^{-ddCT} method, where $ddCT = (CT_{target} - CT_{B2M})_A - (CT_{target} - CT_{B2M})_B$ (11). For example, changes in the gene expression of AQP2 in endolymphatic sac in MD compared with VS were quantified as the fold range: 2^{-ddCT} ($ddCT = (CT_{AQP2} - CT_{B2M})_{MD} - (CT_{AQP2} - CT_{B2M})_{VS}$).

Western blotting

Samples from endolymphatic sac were homogenised on ice with polytron homogeniser (PCU-11; Kinematica, Bohemia, NY, USA) in 20 mM HEPES (pH 7.2), 25 mM NaCl, 2 mM EGTA, 50 mM NaF, 1 mM Na_3VO_4, 25 mM β-glycerophosphate, 0.2 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 60 μg/ml aprotinin, 2 μg/ml leupeptin and 0.1% Triton X-100. After incubation at 4 °for 30 min, homogenates were sonicated (Sonifier 250; Branson Ultrasonics, Danbury, CT, USA) on ice for 1 min and centrifuged at 10 000 *g* at 4 °C for 30 min. The supernatant was collected. Protein concentrations of these supernatants were measured with a protein assay kit (Pierce, Rockford,

IL, USA). Gel samples were prepared by adding sample buffer, containing final concentrations of 50 mM Tris (pH 6.7), 2% sodium dodecyl sulphate (SDS) and 2% mercaptoethanol. Twenty microgrammes of protein extracts were boiled for 10 min, cooled to room temperature and loaded on 10% SDS-polyacrylamide gels. Equal amounts of protein in each sample were further checked by immunoblotting with β-actin monoclonal antibody (diluted 1 : 500) (Oncogene Research Products, Calbiochem, San Diego, CA, USA).

Proteins were transferred to Hybond-PVDF membranes (Amersham Pharmacia, Piscataway, NJ, USA) by using standard electroblotting procedures. Membranes were incubated sequentially in solutions (at 4 °C) of: 2% nonfat dry milk, 1% bovine serum albumin (BSA) and normal goat serum (NGS) in 0.3% Triton-X 100 in PBS for 3 h; antisera against AQP2c for C-terminal intracellular domain (sc-28629; Santa Cruz Inc., Santa Cruz, CA, USA) (diluted 1 : 500) in 1% BSA and NGS in 0.3% Triton-X 100 in PBS for 24 h; 0.1 M PBS for 30 min; horseradish peroxidase-conjugated secondary antibody (Dako, Carpinteria, CA, USA) in 1% BSA and NGS in 0.3% Triton-X 100 in PBS for 3 h; 0.1 M PBS for 30 min. Protein bands were visualised using an ECL detection kit and Hyperfilm MP (Amersham Pharmacia) and analysed using Scion Image software (Scion Corp., Frederick, MD, USA).

Organotypic culture of endolymphatic sac tissues

Tissue culture

According to the previous study (12), parts of the endolymphatic sac tissues from patients with VSs (cases 4–9) were rapidly removed and placed in cold HEPES-buffered saline with Hank's balanced salt solution (HHBSS: 4 °C, pH 7.3). The tissues were mounted flat on culture slides coated with 20 μl of a 1 : 5 dilution of Cell Tek (Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and covered with 300 μl of minimum essential medium containing D-valine to suppress fibroblast growth as well as 10% foetal calf serum, 10 mM HEPES, 100 IU/ml penicillin and 2 mM glutamine. Cultures were maintained under a 5% CO_2 atmosphere at 37 °C for up to 12 h. The morphology of the cultured tissues was monitored by differential interference contrast infrared light microscopy.

Immunohistochemistry

Intracellular translocation of AQP2-like immunoreactivity

After culture at 37 °C for up to 12 h, the endolymphatic sac tissues were incubated with HHBSS (37 °C, pH 7.3) for 30 min and then divided into three pieces in each VS case 4–9. [Arg8]-vasopressin alone (Sigma Aldrich, St Louis, MO, USA) at 10 nM (AVP10) or [Arg8]-vasopressin with vasopressin/V2R specific antagonist, OPC31260 (Otsuka Pharmaceutical Inc., Tokushima, Japan) at 5–10 nM/10–20 nM (AVP5–10/OPC10–20) was added to the HHBSS at the incubation. Forskolin alone (Sigma Aldrich) at 10–50 μM (forskln10–50) was also added to the HHBSS at the incubation. Furthermore, [Arg8]-vasopressin with protein kinase A (PKA) specific antagonist, H-89 (Sigma Aldrich) at 10 nM/0.5–1 μM (AVP10/H0.5–1) or KT-5720 (Sigma Aldrich) at 10 nM/5–10 μM (AVP10/KT5–10) was added to the HHBSS at the incubation. The doses of reagents were determined according to previous studies (4,12,13). Whole amounts of the pieces were post-fixed in 4% paraformaldehyde for 24 h, incubated in 30% sucrose for 24 h and washed in 0.1 M PBS for 3 h at room temperature.

Whole amounts of the pieces were incubated sequentially in the following solutions without Triton X-100 at RT: 5% normal donkey serum in 0.1 M PBS for 2 h; antisera against AQP2c for C-terminal intracellular domain (sc-28629: Santa Cruz Inc.) and AQP2n for N-terminal extracellular domain (sc-9880: Santa Cruz Inc.) (diluted 1 : 1000) in 0.1 M PBS for 72 h; 0.1 M PBS for 15 min; fluorescein isothiocyanate (FITC)-conjugated anti-goat immunoglobulin G secondary antibody (Jackson ImmunoResearch, Bar Harbor, ME, USA) (diluted 1 : 1000) in 0.1 M PBS for 24 h; 0.1 M PBS for 15 min, and then examined under a fluorescence microscope. All images in Fig. 2(A,B) were viewed from the luminal side (see the schema in Fig. 2c). For negative controls, primary antibodies were either preabsorbed with each control peptide (diluted 1 : 50) or the primary antibody was omitted.

Fluorescence images were captured four times in each (n = 4) from the luminal side by using an AX-70 fluorescence microscope (Olympus, Hamburg, Germany). AQP2-like immunoreactivity (-LIR) was calculated as the rel-

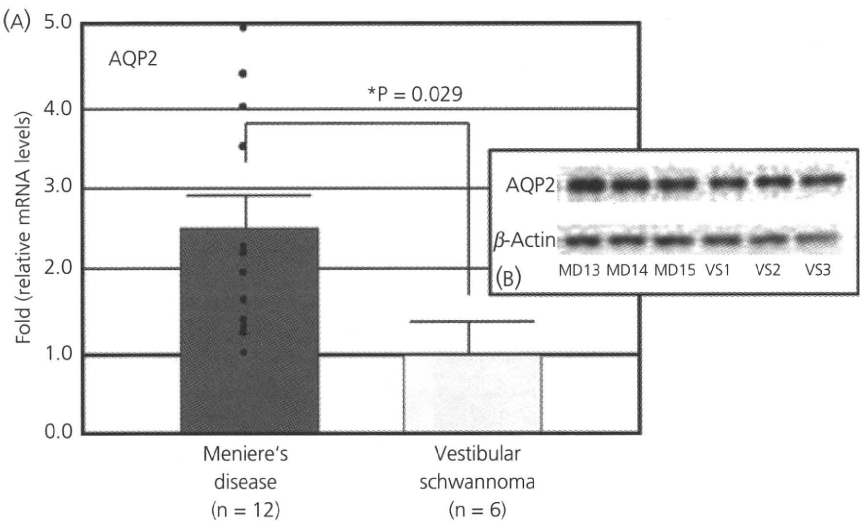

Fig. 1. Aquaporin-2 (AQP2) mRNA and protein expression levels in the endolymphatic sac in Meniere's disease patients compared with control patients. (A) The relative AQP2 mRNA expression in the endolymphatic sac was significantly higher in Meniere's disease (MD) patients (n = 12; 2.52 ± 0.40-fold) than in control vestibular schwannoma (VS) patients (n = 6; 1.02 ± 0.36-fold) as evaluated by real-time polymerase chain reaction (unpaired t-test: *P = 0.029). (B) AQP2 protein expression in the endolymphatic sac was also higher in MD patients than in control VS patients as evaluated by western blotting.

Table 1. Raw data for 12 cases of Meniere's disease.

	V2R mRNA (fold)*	AQP2 mRNA (fold)*	Vertigo frequency (/months)	Hearing level (dB)	Duration (months)
MDI	64.78	4.05	1.0	30.0	34
MD2 (Bil)	36.90	1.92	3.3	42.5	12
MD3	69.28	3.62	1.0	45.0	60
MD4	13.72	2.10	1.3	66.3	84
MD5	20.18	1.26	3.3	60.8	98
MD6	1.90	1.06	1.7	70.0	48
MD7	32.38	5.02	7.3	57.5	18
MD8 (Bil)	9.45	1.70	2.0	30.7	45
MD9	29.52	4.45	8.0	66.5	30
MD10	17.32	2.21	4.0	60.0	48
MD11	1.70	1.35	2.0	58.5	60
MD12 (Bil)	1.52	1.48	2.5	75.6	96

Showing V2 receptor (V2R) and aquaporin-2 (AQP2) mRNA expression levels in the endolymphatic sac, vertigo frequency, hearing level and duration of disease before surgery. There was a significant positive co-relationship between V2R and AQP2 mRNA expression levels in the endolymphatic sac (Pearson's test: *r = +0.69, P = 0.013). Bil, bilateral Meniere's disease.

ative fluorescence intensity on the basis of the fluorescence intensity in each VS case 4–9 in a control solution (CONT = 1 in Fig. 3).

Co-localisation of AQP2 and EEA1-like immunoreactivity

To determine whether the AQP2 molecule was stored intracellularly after AVP stimulation, a co-localisation study of AQP2 and an endosomal marker was performed. Using both kinds of antibodies, AQP2c for aquaporin-2 C-terminal domain (sc-28629: Santa Cruz Inc.) (FITC-labelled: green colour) and EEA1 for early endosome antigen-1 (sc-6415: Santa Cruz Inc.) (texas-red labeled: red colour), co-localisation was detected at the 5-μm thick cryostat sections by means of confocal laser scanning microscopy as merged cells in the saline- (control, n = 3) and AVP-treated endolymphatic sac (AVP10, n = 3) of control patients (yellow coloir).

Statistical analysis

Statistical differences of patients' backgrounds (sex, age) between Meniere's disease and controls were examined by the Mann–Whitney U-test. Statistical differences of the data between two groups in Fig. 1(A) were determined by unpaired t-test. In Fig. 3, a Bonferroni/Dunn test was adopted to examine statistical changes among multiple factors in each group and then an unpaired t-test was used complementarily to check a trend of differences (strictly no significant differences) between two factors in two groups. In Table 1, statistical relationships between two factors were evaluated by Pearson's correlation coefficient.

$P < 0.05$ (Fig. 1A and Table 1) were considered statistically significant. $P < 0.05$ (Fig. 3) were considered to show a trend of differences. All the statistical analyses in the present study were carried out using SPSS, version 14.0 (SPSS Inc., Chicago, IL, USA). All values are expressed as the mean ± SEM.

Results

The raw data for 12 cases with Meniere's disease, including their V2R and AQP2 mRNA expression levels in the endolymphatic sac, vertigo frequency, hearing level and duration of disease before surgery, are shown in Table 1. We judged these patients' data of ver-

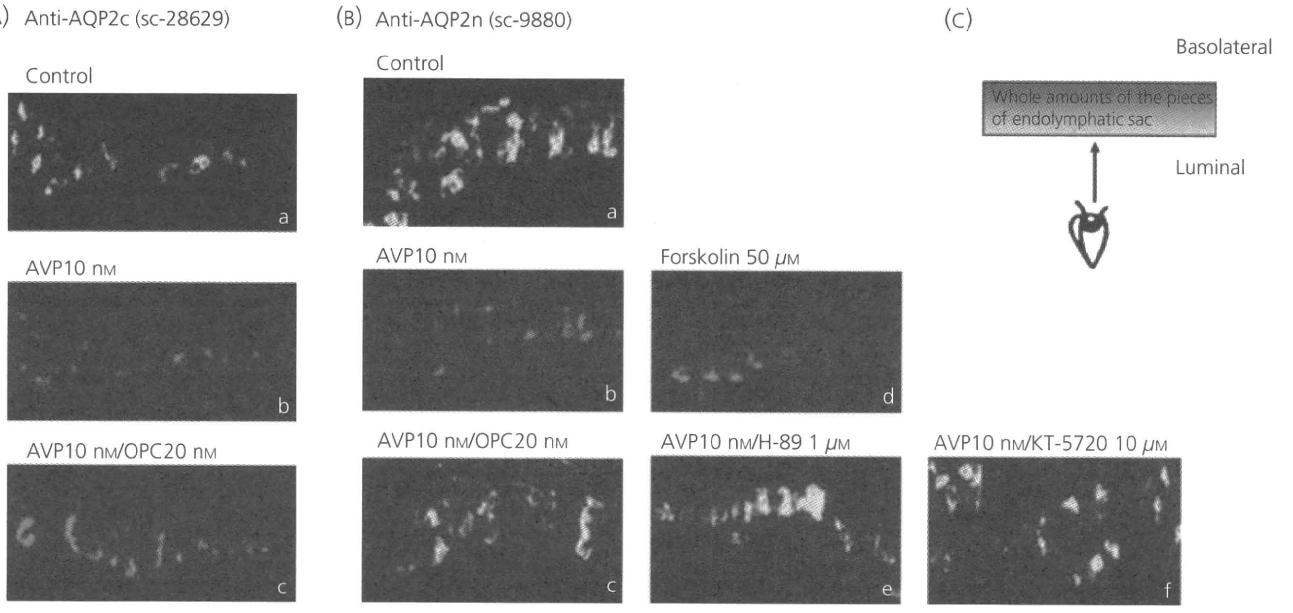

Fig. 2. Intracellular translocation of aquaporin-2 (AQP2)-like immunoreactivity (-LIR) via V2 receptor (V2R)-cAMP-protein kinase A (PKA) system in the human endolymphatic sac. Using both kinds of antibodies, AQP2c for C-terminal (A) and AQP2n for N-terminal (B), AQP2-LIR was detected in the luminal side of endolymphatic sac tissue (Aa, Ba). Furthermore, the AQP2-LIR was translocated from luminal to basolateral of the endolymphatic sac at the time of vasopressin (AVP) exposure (Ab, Bb) and reversed by AVP/V2R specific antagonist, OPC31260 (OPC) (Ac, Bc). Using AQP2n antibody only, the similar AQP2-LIR translocation was also demonstrated by forskolin (forskln) (Bd) and blocked by PKA specific antagonists, H-89 (Be) and KT-5720 (Bf). All images were viewed from the luminal side of whole amount of pieces of endolymphatic sac (see the schema in C).

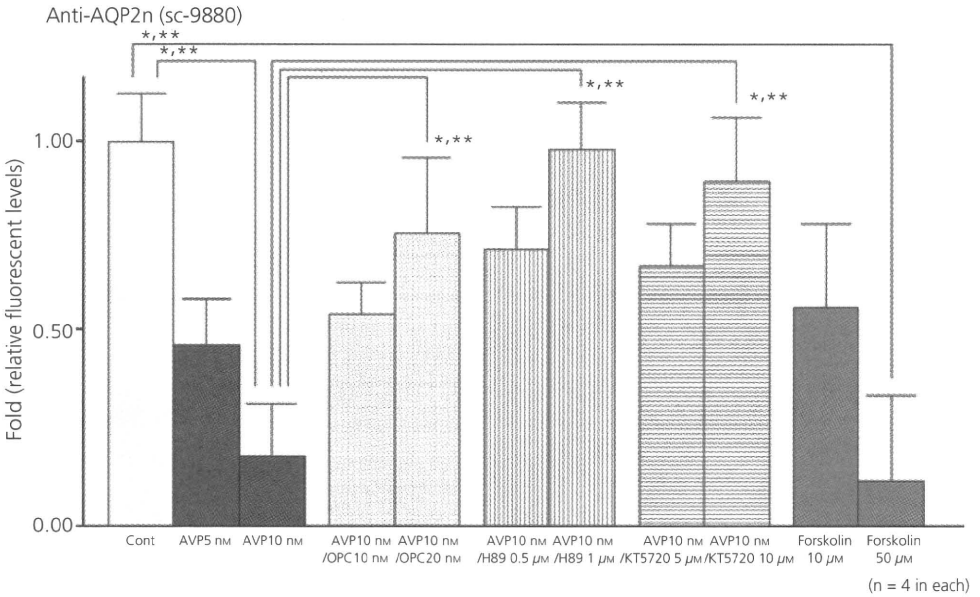

Fig. 3. Statistical analysis of aquaporin-2 (AQP2)-like immunoreactivity (-LIR) intensity in the endolymphatic sac. Changes in all the relative fluorescence intensity of AQP2n-LIR in Fig. 2B were confirmed statistically. The blockade of vasopressin (AVP) effects by OPC31260, H-89 or KT-5720 was demonstrated in a dose-dependent manner (Bonferroni/Dunn test: **P < 0.05; unpaired t-test: *P < 0.05). The relative fluorescence intensity was calculated on the basis of the fluorescence intensity in each case in a control solution (Cont = 1). OPC, AVP/V2R specific antagonist, OPC31260; forskln, forskolin; H, protein kinase A (PKA) specific antagonist, H-89; KT, PKA specific antagonist, KT-5270.

tigo frequency, hearing level and duration of disease by questionnaire and/or review of the clinical notes.

The relative AQP2 mRNA expression level in the endolymphatic sac was 2.47-fold higher in Meniere's disease patients (n = 12; 2.52 ± 0.40-fold) than in control vestibular schwannoma patients (n = 6; 1.02 ± 0.36-fold), and this difference was significant (unpaired t-test: P = 0.029) (Fig. 1A). These results were confirmed at the protein expression level by western blotting (Fig. 1B). In Meniere's disease patients, there was a significant positive co-relationship between V2R and AQP2 mRNA expression levels in the endolymphatic sac (Pearson's test: r = +0.69, P = 0.013) (Table 1).

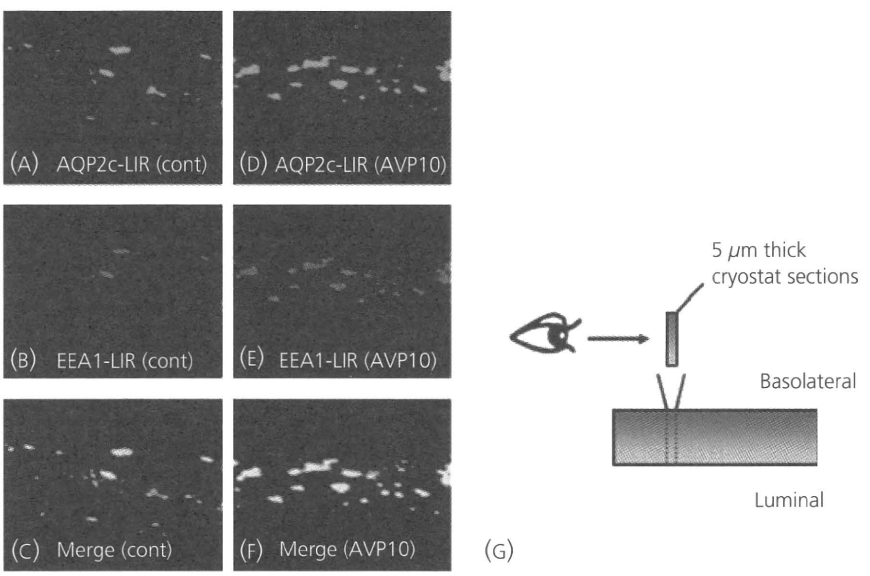

Fig. 4. Co-localisation of aquaporin-2 (AQP2)- and early endosome antigen (EEA)1-like immunoreactivity (-LIR) in the human endolymphatic sac. Using both kinds of antibodies, AQP2c for aquaporin-2 C-terminal domain (A, D: green) and EEA1 for early endosome antigen-1 (B, E: red), co-localisation was shown as merged cells in the endolymphatic sac of control (cont) patients (C: yellow). Furthermore, the number of merged cells was increased in the endolymphatic sac at the time of vasopressin (AVP) exposure (F: yellow). All images were viewed from the lateral side of 5 μm thick cryostat sections: upward basolateral; downward luminal (see schema in G).

Using both kinds of antibodies, AQP2c and AQP2n, AQP2-LIR was detected in the luminal side of endolymphatic sac tissue (Fig. 2Aa,Ba). Furthermore, the AQP2-LIR was translocated from luminal to basolateral side of the endolymphatic sac at the time of AVP exposure (Fig. 2Ab,Bb) and reversed by vasopressin/V2R specific antagonist, OPC31260 (Fig. 2Ac,Bc). Using AQP2n antibody only, the similar AQP2-LIR translocation was also demonstrated by forskolin (Fig. 2Bd) and blocked by PKA specific antagonists, H-89 (Fig. 2Be) and KT-5720 (Fig. 2Bf). Changes in all the relative fluorescence intensity of AQP2-LIR in a dose-dependent manner were confirmed statistically, as shown in Fig. 3 (Bonferroni/Dunn test: $^{**}P < 0.05$; unpaired t-test: $^{*}P < 0.05$).

Co-localisation of AQP2c and EEA1-LIRs was demonstrated in Fig. 4. In the control state, some merged cells (yellow) were seen in the human endolymphatic sac (Fig. 4c). In the AVP-treated state (AVP10), the number of merged cells (yellow) was increased (Fig. 4f).

Discussion

Subsequent to the end of the last century, it has been discussed that the plasma vasopressin levels in patients with Meniere's disease (endolymphatic hydrops), during remission (14) as well as during attacks (15), were significantly higher than those in patients with vertigo as a result of benign paroxysmal positional vertigo and vestibular neuronitis (non-endolymphatic hydrops). It was also revealed that systemic application of vasopressin induced bilateral endolymphatic hydrops and hearing loss in guinea pigs (16). These findings suggest that a high level of plasma vasopressin is one of the causes of inner ear endolymphatic hydrops in Meniere's patients. By contrast, it was reported that the plasma vasopressin levels in patients with unilateral Meniere's disease did not change significantly compared with those in healthy volunteers (17). Furthermore, the hypothesis of a high level of plasma vasopressin, which should have equal effects on the bilateral ears, cannot explain the fact that 70–80% of patients with Meniere's disease are unilateral (18).

Disregarding the idea of vasopressin ligands, V2 receptor and its related- molecules have been detected in rat (12,19) and human (12,20) inner ear endo-organ tissues. V2R was clearly distributed together with a V2R-linked water channel molecule, aquaporin-2, in the luminal epithelium of the human endolymphatic sac (20). Interestingly, the physiological interactions between vasopressin and V2R in the rat endolymphatic sac attenuated the membranous turnover via cAMP-dependent signalling in a contrasting manner with the kidney (12). Indeed, our recent study revealed that V2R and subsequent cAMP activation in the endolymphatic sac in patients with Meniere's disease increased significantly for its pathogenesis (4). In the present study, the AQP2 mRNA and protein expression level in the endolymphatic sac was much higher in Meniere's patients than in control patients. There was a significant positive co-relationship between V2R and AQP2 mRNA expression levels in the endolymphatic sac. Furthermore, both the cAMP and PKA were activated and then AQP2 was translocated from luminal to basolateral side and trapped within the cytoplasmic endosome in cultured human endolymphatic sac tissues. All these findings suggest that V2R-cAMP-PKA-AQP2 activation and endosomal trapping of AQP2 in the endolymphatic sac could attenuate the membranous turnover and cause the endolymphatic fluid overflow into the endolymphatic space after even

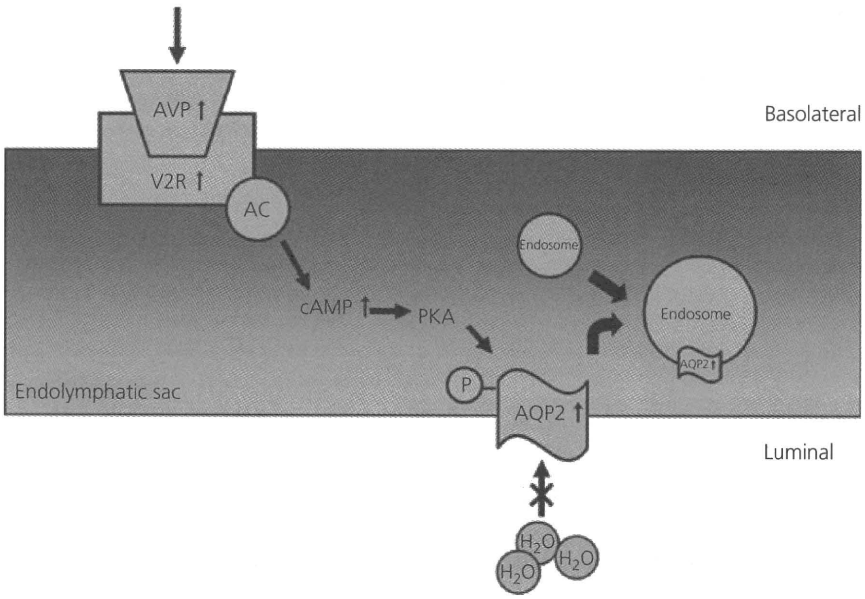

Fig. 5. Schematic representation of a hypothetical mechanism for endolymphatic hydrops generation in Meniere's disease via stress hormone vasopressin (AVP) and water channel aquaporin-2 (AQP2). Plasma AVP elevation and subsequent V2 receptor (V2R)-cAMP-protein kinase A (PKA) activation in the endolymphatic sac might lead the intracellular translocation of AQP2 from luminal side to basolateral side with endosomal trapping, resulting in the pathogenesis of inner ear hydrops (i.e. Meniere's disease). Upward side, basolateral side; downward side, luminal side. AC, adenylate cyclase; P, phosphorylated.

