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ORIGINAL ARTICLE

Tinnitus as a prognostic factor of sudden deafness

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Abstract

Conclusions. The 'tinnitus-rare' group had a poorer prognosis for hearing than the 'tinnitus-often' group in all sudden sensorineural hearing loss (SSNHL), although the 'shorter duration' group had better prognosis than the 'longer duration' when restricted to SSNHL accompanied by tinnitus. This indicates that tinnitus itself may not be a sign for poor hearing prognosis but might be an essential sound for the initiation of repair of a damaged auditory system. *Objectives.* We examined the hearing improvement rate (HIR) and tinnitus at the onset of SSNHL to elucidate the prognostic value of tinnitus accompanying SSNHL. *Patients and methods.* Fifty patients with SSNHL were treated with systemic administration of steroids. Hearing recovery was determined by comparing the hearing levels before and after treatment. Tinnitus was subjectively evaluated by the tinnitus scoring questionnaire. The score for the five-step evaluation of the subjective tinnitus feelings 'loudness', 'duration' and 'annoyance' was obtained at the onset. *Results.* In terms of 'duration', when we divided all the cases into 'tinnitus-rare' group and 'tinnitus-often' group, HIR in the 'tinnitus-rare' group was significantly lower than that in 'tinnitus-often' group. When restricted to the 'tinnitus-often' group, HIR for 'shorter duration' was significantly higher than that for 'longer duration'.

Keywords: *Sudden deafness, vertigo, tinnitus, hearing improvement rate, prognostic factor*

Introduction

Sudden sensorineural hearing loss (SSNHL) is defined as a sensorineural hearing loss of 30 dB or worse in three consecutive speech frequencies that has occurred with sudden onset [1,2]. The incidence of SSNHL is estimated to range from 5 to 20 per 100 000 population [2]. Various kinds of causes of SSNHL have been suggested as follows: viral infection of labyrinth or cochlear nerve, vascular insult, intralabyrinthine membrane rupture and perilymphatic fistula [1].

To date, some prognostic factors for SSNHL have often been reported. Vertigo, particularly severe vertigo, has been considered as a negative prognostic factor [1–3]. Delayed start of treatments after the onset has also been considered as a negative prognostic factor [2,4]. Byl Jr also reported that age over 60 years or below 15 years has been considered a negative prognostic factor [2]. Additionally, the severity of the initial hearing level has been considered a negative prognostic factor [1,2,5,6]. As regards tinnitus, although several papers on this topic have already been published, the prognostic value has been controversial. Wilson et al. and Moskowitz et al. reported that tinnitus was a negative prognostic factor for hearing after SSNHL [7,8]. Cadoni et al. also described that hearing recovery was poorer in SSNHL patients with both tinnitus and vertigo than those with only vertigo [5]. While Byl Jr reported that tinnitus had little prognostic value [2], Danino et al. reported that tinnitus was a favourable prognostic manifestation in an analysis of symptoms and recovery rates in 60 patients with SSNHL [9]. Ben-David et al. found tinnitus to be strongly associated with hearing improvement in 67 patients with SSNHL and indicated that tinnitus was a positive prognostic factor for hearing after SSNHL [10].

In the present study, to elucidate the relationship between hearing prognosis and tinnitus at the onset

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of SSNHL, we examined changes in hearing and tinnitus of patients with SSNHL using pure-tone audiometry (PTA) and the tinnitus scoring questionnaire [11].

Patients and methods

Charts of 50 consecutive patients with the diagnosis of SSNHL accompanying vertigo (25 females and 25 males, mean 48.6 years, range 11–80 years) attending the Dizziness & Vertigo Section of the Department of Otolaryngology in three hospitals (Osaka Rosai Hospital, Osaka University Hospital and Tondabayashi Hospital) from 1998 to 2007 were reviewed in this retrospective study. Patients with Meniere's disease were carefully excluded.

All the patients underwent PTA, electronystagmography (ENG) and MRI for the purpose of excluding possible retrocochlear lesions, including demyelinating diseases, at the first visit. All the treatments started within 7 days after the onset (2.8±1.7 days), including bed rest and intravenous applications of hydrocortisone sodium succinate (from 500 mg/day with dose reductions of 200 mg every 3 days to zero) and lasted for 1–2 weeks at most.

Hearing recovery was determined by comparing the audiometric results at the first visit (2.8±1.7 days: pretreatment) and the last visit approximately 6 months later, when hearing function was assumed to be fixed completely (6.6±1.3 months: post-treatment). The hearing improvement rate (HIR) was used as a credible parameter for hearing recovery after SSNHL [12]. Hearing gain was an absolute value of changes in averaged hearing levels of 250, 500, 1000, 2000 and 4000 Hz from pretreatment to post-treatment. HIR was defined as a result of hearing gain divided by differences between averaged initial hearing levels in the affected and unaffected ear, multiplied by 100.

Tinnitus was subjectively evaluated by the tinnitus scoring questionnaire according to the Tinnitus Research Group of Japan Audiological Society (TRGJ) in 1993 (Table I) [11]. The score of five-step evaluation from 1 to 5 in the three items of subjective tinnitus feelings – 'loudness', 'duration' and 'annoyance' – was obtained from patients at the onset of SSNHL.

For neuro-otologists, it is very important to tell patients with SSNHL their hearing prognosis at the first visit. Therefore, we examined the relationship between tinnitus score at the onset and HIR.

Statistical analysis was performed based on the Mann-Whitney test and Spearman correlation test. All reported p values were two-sided and those under 0.05 were considered to be significant.

Results

The number of patients for each item of the pretreatment tinnitus score, 'loudness', 'duration' and 'annoyance', is summarized in Figure 1.

(i) Tinnitus score pretreatment and HIR

None of three items in the pretreatment tinnitus score, 'loudness', 'duration' and 'annoyance', was significantly related to HIR according to the Spearman correlation test. As no one selected score 3 for 'duration' (Figure 1), we divided the pretreatment tinnitus scores for 'duration' into two groups. We defined scores 1 and 2 as 'tinnitus-rare' and scores 4 and 5 as 'tinnitus-often'. HIR for the 'tinnitus-rare' group ($n=8$; 16.3±34.0%, range −5.0 to 100.0%) was significantly lower than that for the 'tinnitus-often' group ($n=42$: 53.2±35.4%, range −19.0 to 119.0%) (Mann-Whitney: U=72.0, $p=0.011<0.05$) (Figure 2A). When restricted to the 'tinnitus-often' group ($n=42$), we compared the HIR for score 4, 'shorter duration' and score 5, 'longer duration'. HIR with 'shorter duration' of tinnitus ($n=9$: 99.5±9.5%, range 88.0–119.0%) was significantly higher than that for 'longer duration' of tinnitus ($n=33$: 40.6±29.3%, range −19.0 to 100.0%) (Mann-Whitney: U=5.0, $p=1.17\text{E-}05<0.001$) (Figure 2B).

(ii) Tinnitus score pretreatment and hearing level pretreatment

None of three items in the pretreatment tinnitus score was significantly related to pretreatment hearing level

Table I. Tinnitus scoring questionnaire: Tinnitus Research Group of Japan Audiological Society, 1993.

	Score				
Parameter	1	2	3	4	5
Loudness	Very quiet	Quiet	Medium	Loud	Very loud
Annoyance	Not at all	Slightly	Frequent	Always	Very much
Duration	Rare	Less often	Often	Very often	Constant

The nature and severity of tinnitus were evaluated in 5 steps from 1 to 5 in the three different categories of subjective tinnitus feelings – 'loudness', 'duration' and 'annoyance'.

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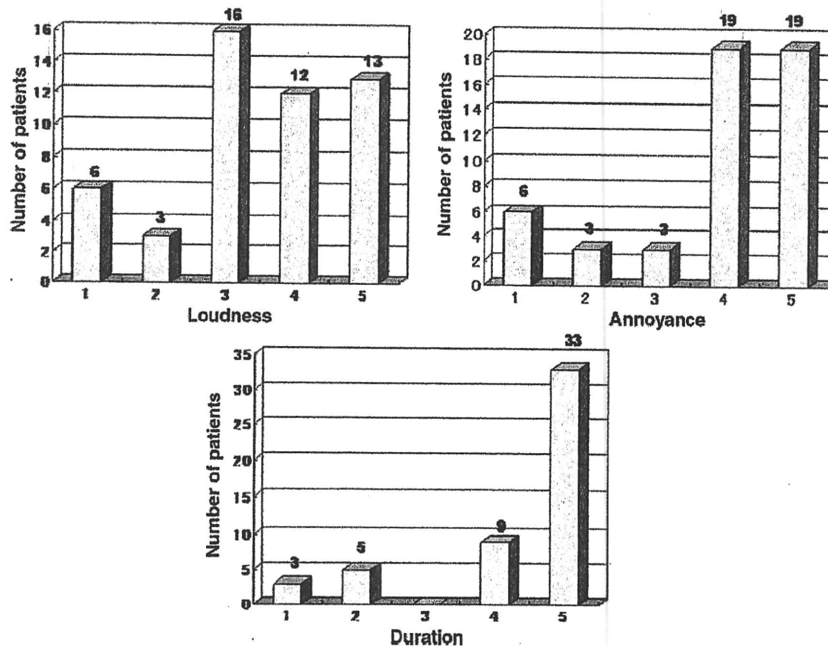

Figure 1. Results of pretreatment tinnitus score. The population in each item of the pretreatment tinnitus score, 'loudness', 'duration' and 'annoyance' was summarized.

according to the Spearman correlation test. When we divided pretreatment tinnitus scores of 'duration' into 'tinnitus-rare' and 'tinnitus-often' groups as above, the pretreatment hearing level in the 'tinnitus-rare' group ($n=8$: 86.0±23.5 dB, range 49.0–115.0 dB) was not different from that in the 'tinnitus-often' group ($n=42$: 74.5±24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: U = 120.0, $p=0.208$) (Figure 3A). When restricted to the 'tinnitus-often' group ($n=42$), the pretreatment hearing level for score 4, 'shorter duration' of tinnitus ($n=9$: 55.0±11.1 dB, range 35.0–69.0 dB) was significantly better than that for score 5, 'longer duration' of tinnitus ($n=33$: 79.8±24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: U = 63.0, $p=0.009<0.01$) (Figure 3B).

(iii) Tinnitus score pretreatment and age of patients

When we divided pretreatment tinnitus scores of 'duration' into 'rare' and 'often' groups as in (i), the age of patients in the 'tinnitus-rare' group ($n=8$: 46.4±22.3 years, range 11–70 years) was not different from that in the 'tinnitus-often' group ($n=42$: 49.1±16.4 years, range 15–80 years) (Mann-Whitney: U = 164.5, $p=0.937$). When restricted to the 'tinnitus-often' group ($n=42$), the age of patients with score 4, 'shorter duration' of tinnitus ($n=9$: 37.6±8.8 years, range 25–52 years) was significantly younger than that with score 5, 'longer duration' of tinnitus ($n=33$: 52.2±16.7 years, range 15–80 years) (Mann-Whitney: U = 59.5, $p=0.007<0.01$).

Discussion

According to previous studies, tinnitus was reported to accompany SSNHL in 74–87% of patients [2,6,13]. In the present study, 88% of SSNHL patients with vertigo complained of tinnitus. In Japan, tinnitus is usually evaluated by the tinnitus scoring questionnaire according to the TRGJ (1993) (Table I) [11]. This questionnaire is easy to handle but does not really provide an objective evaluation. However, among the three items of subjective tinnitus feelings, only 'duration' was significantly correlated with HIR (cf. (i) in Results). This finding suggests that 'duration' could be the most reliable item for tinnitus evaluation of patients with SSNHL. As tinnitus is a quite subjective symptom, the most reliable way of evaluation in the present study could change the status of tinnitus from a non-evaluable complaint of patients to an important prognostic factor for SSNHL.

As summarized in the Introduction, the prognostic value of tinnitus in SSNHL has been controversial until now [2,5,7–10]. In the present study, 'shorter duration' of tinnitus was a positive prognostic factor for hearing after SSNHL, as reported by Cadoni et al. [5], Wilson et al. [7] and Moskowitz et al. [8]. Our data also suggested that 'tinnitus-rare' was a negative prognostic sign for results of treatments of SSNHL, as described by Danino et al. [9] and Ben-David et al. [10]. Taking all these facts together, we assume that tinnitus may lose its prognostic value absolutely, as in the paper by Byl Jr [2], when 'shorter duration' and 'tinnitus-rare' are completely mixed up.

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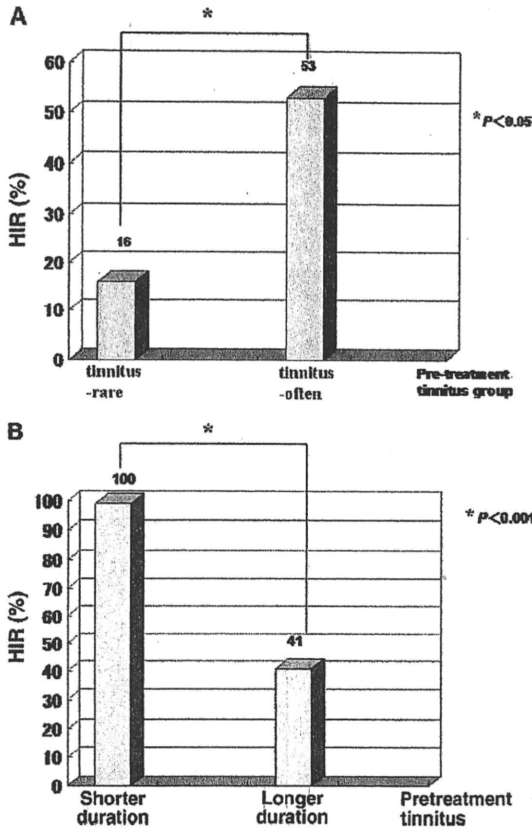

Figure 2. (A) Tinnitus score pretreatment and hearing improvement rate (HIR). The HIR for the 'tinnitus-rare' group (score 1–2) ($n=8$: 16.3±34.0%, range −5.0 to 100.0%) was significantly lower than that for the 'tinnitus-often' group (score 4–5) ($n=42$: 53.2±35.4%, range −19.0 to 119.0%) (Mann-Whitney: U=72.0, $p=0.011<0.05$). (B) When restricted to the 'tinnitus-often' group ($n=42$), the HIR for 'shorter duration' of tinnitus (score 4) ($n=9$: 99.5±9.5%, range 88.0–119.0%) was significantly higher than that for 'longer duration' of tinnitus (score 5) ($n=33$: 40.6±29.3%, range −19.0–100.0%) (Mann-Whitney: U=5.0, $p=1.17E-05<0.001$).

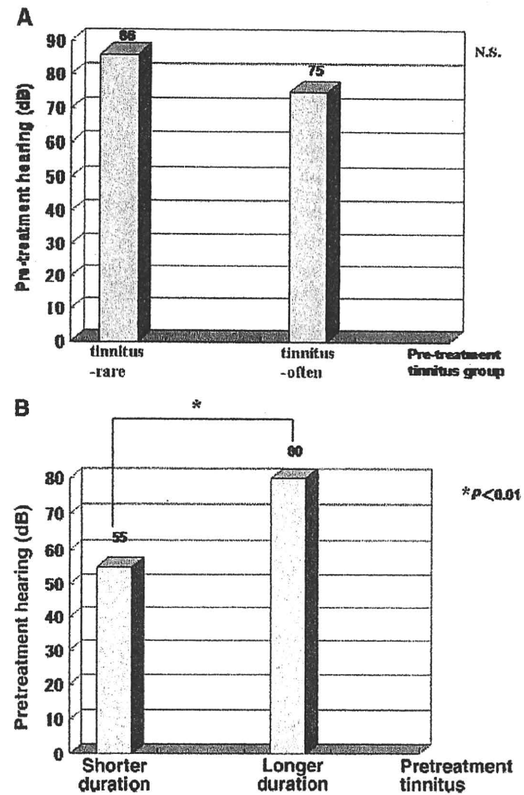

Figure 3. Tinnitus score and hearing level pretreatment. (A) The pretreatment hearing level for the 'tinnitus-rare' group (score 1–2) ($n=8$: 86.0±23.5 dB, range 49.0–115.0 dB) was not significantly different from that of the 'tinnitus-often' group (score 4–5) ($n=42$: 74.5±24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: U=120.0, $p=0.208$). (B) When restricted to the 'tinnitus-often' group ($n=42$), the pretreatment hearing level for 'shorter duration' of tinnitus (score 4) ($n=9$: 55.0±11.1 dB, range 35.0–69.0 dB) was significantly better than that for 'longer duration' of tinnitus (score 5) ($n=33$: 79.8±24.4 dB, range 40.0–115.0 dB) (Mann-Whitney: U=63.0, $p=0.009<0.01$).

When tinnitus was present at the onset of SSNHL, the 'shorter duration' group had a better HIR than the 'longer duration' group (cf. (i) in Results). This may come from the fact that tinnitus duration was significantly shorter in better hearing (cf. (ii) in Results) and/or younger (cf. (iii) in Results) patients at the onset. These findings suggest that the duration of tinnitus and the amount of damage in the inner ear could have a positive relationship in patients with SSNHL accompanied by tinnitus, resulting in a negative relationship with HIR.

On the other hand, the 'tinnitus-rare' group had a poorer HIR than the 'tinnitus-often' group in all the patients with SSNHL (cf: (i) in Results). This may indicate that tinnitus itself may not be a sign for poor hearing prognosis but might be an essential sound for cell survival. Actually, the tinnitus research group of Kitahara and Balaban [14,15] demonstrated that high doses of salicylate could up-regulate a neurotrophic factor, brain-derived neurotrophic factor (BDNF), in the inner ear for cell survival and lead subsequent transcription of a nociceptive cation ion channel receptor, transient receptor potential cation channel superfamily V type 1 (TRPV1) in the inner ear for tinnitus generation. These findings suggest the hypothesis that tinnitus might be a switch-on signal for inner ear cell survival. According to this hypothesis, it could be speculated that causes and/or sites of lesion in SSNHL without tinnitus are absolutely different from those in SSNHL with tinnitus, which is one of the reasons why the prognostic value of tinnitus in SSNHL has been controversial until now [2,5,7–10].

Conclusion

In conclusion, tinnitus at the onset of SSNHL is important as a prognostic factor for hearing. 'Tinnitus-often' is a positive prognostic sign for

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hearing recovery, but 'longer duration' predicts poor results in hearing improvement. Further detailed investigation of tinnitus at the time of SSNHL may elucidate mechanisms of tinnitus generation and may lead to development of effective treatments for tinnitus.

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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Marrow–Middle Ear Connections: A Potential Cause of Otogenic Meningitis

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Hypothesis: We hypothesize that the connections between the hematopoietic bone marrow and middle ear is a potential cause of childhood otogenic meningitis.
Background: Although it is known that there is a causal relationship between otitis media and bacterial meningitis, the relationship has never been satisfactorily established. Human fetal and infant temporal bones prepared for light microscopic evaluation revealed direct connections between the hematopoietic bone marrow and middle ear. We noted this difference in anatomy between the infant middle ear and the adult middle ear.
Methods: We studied 10 temporal bones from 5 infants in each group: meningitis group with otitis media who died of meningitis, control Group 1 without otitis media, and control Group 2 with otitis media who died of diseases other than meningitis. A quantitative analysis of the frequency of connections between the hematopoietic bone marrow and middle ear was performed. The correlation between unabsorbed mesenchyme and otitis media also was investigated.
Results: The frequency of connections was significantly higher in order of the meningitis group, control Group 2, and control Group 1. The degree of unabsorbed mesenchyme tended to be more severe in order of the meningitis group, control Group 2, and control Group 1.
Conclusion: The prevalence of connections between the hematopoietic bone marrow and middle ear in patients with meningitis and otitis media is high. A higher prevalence of connections in infants with otitis media could increase the risk for otogenic meningitis in them. **Key Words:** Childhood otogenic meningitis—Connections between hematopoietic bone marrow and the middle ear—Unabsorbed mesenchyme.

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Children constitute a majority of the cases of bacterial meningitis (1). Using light microscopy, Linthicum et al. (2) found that human fetal and infant temporal bones revealed direct connections between the hematopoietic bone marrow and middle ear. They also found that these connections gradually disappeared by 16 to 18 months after birth (2). With respect to potential causes of bacterial meningitis, we conceived the difference in anatomy between the middle ears of infants and adults. We detected the presence of connections between the hematopoietic bone marrow and the middle ear in infants. Therefore, we proposed that the connections between the hematopoietic bone marrow and middle ear were the potential route of bacterial meningitis.

There are 3 possible pathways for the migration of pathogens from the middle ear to the subarachnoidal space in otitis media: 1) hematogenous spread (3); 2) dehiscence (4), which are either anatomic abnormalities (e.g., the posterior fossa along the subarcuate artery) or acquired dehiscences (e.g., iatrogenic injury or temporal bone fractures); and 3) labyrinthitis (5) through the oval or round window membranes. We hypothesized that high frequency of connections between the hematopoietic bone marrow and middle ear is the potential cause of the hematogenously spread otogenic meningitis in infants.

We performed a quantitative analysis of the frequency of these connections to evaluate their correlation with otogenic meningitis. In addition, we studied the

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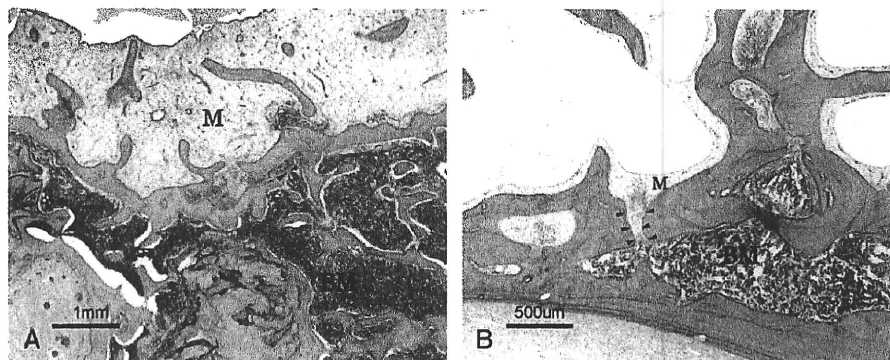

FIG. 1. Marrow–mesenchyme connections (*arrowhead*) in the antrum of a 7-month-old male infant (*A*) and in the mastoid of a 23-month-old male infant (*B*). They died of meningitis due to infection by *H. influenzae*. The unabsorbed mesenchyme (M) varied in thickness. BM indicates hematopoietic bone marrow (hematoxylin and eosin stain; original magnification, ×20 (*A*); original magnification, ×40 (*B*).

correlation between unabsorbed mesenchyme and otitis media.

MATERIALS AND METHODS

Temporal bones from the collection of the University of Minnesota (Minneapolis, MN, USA) were reviewed. This study included 10 temporal bones from 5 infants with clinical and histologic evidence of otitis media who died of meningitis (4 male subjects and 1 female subject; age range, 6–23 mo; mean age ± standard deviation, 12.8 ± 7.4 mo). Cultures of cerebrospinal fluid and blood had grown nontypeable *Hemophilus influenzae* in 2 patients, *H. influenzae* type B in 1 patient, and *Streptococcus pneumoniae* in 2 patients. Meningitis ran a rapid course, and the survival time after admission varied between 1 and 5 days. All temporal bones showed histopathologic evidence suggestive of chronic inflammatory changes (e.g., granulation tissues) and purulent labyrinthitis. During autopsy, none of the patients had any foci of infection that was suggestive as a cause of meningitis. For the control Group 1, 10 temporal bones from 5 infants without clinical and pathologic evidence of otitis media and who had died of diseases other than meningitis (2 male and 3 female subjects; age range, 8–22 mo; mean age ± standard deviation, 15.4 ± 6.4 mo) were selected. The cause of death in the control Group 1 was congenital heart disease. For the control Group 2, 10 temporal bones from 5 infants with clinical and pathologic evidence of otitis media and who had died of diseases other than meningitis (3 male and 2 female subjects; age range, 8–24 mo; mean age ± standard deviation, 15.4 ± 7.3 mo) were selected. The cause of death in the control Group 2 was congenital heart disease. Two temporal bones showed histopathologic evidence suggestive of chronic inflammatory changes and purulent labyrinthitis.

We excluded patients with anatomic abnormalities (e.g., the posterior fossa along the subarcuate artery), acquired dehiscences (e.g., iatrogenic injury or temporal bone fractures), and a history of ear surgery or head trauma.

The temporal bones had been removed at autopsy and fixed in formalin solution. Serial sections were made at a thickness of 20 μm in the horizontal plane. Every tenth section was stained with hematoxylin and eosin and mounted on a glass slide for observation under a light microscope.

We defined bony dehiscences between the hematopoietic bone marrow and the middle ear as the connection (Fig. 1). Every tenth section of each temporal bone from the top to bottom of the cochlea was analyzed for measuring the number of connections between the hematopoietic bone marrow and the middle ear. The middle ear was divided into 3 areas (epitympanum, mesotympanum, and mastoid antrum) to investigate

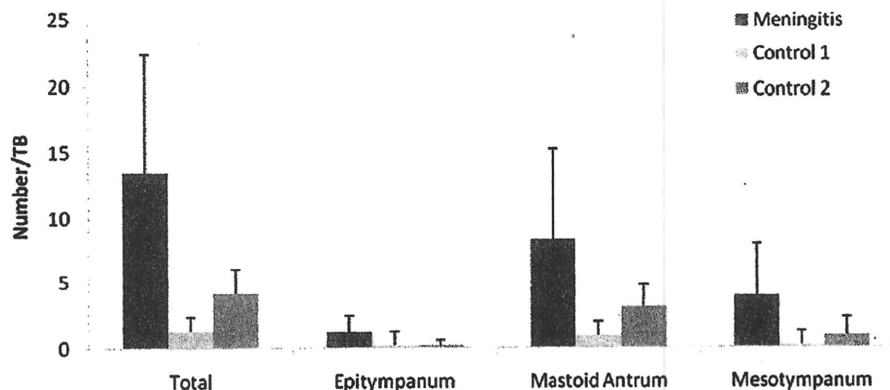

FIG. 2. The mean number of connections between the hematopoietic bone marrow and the middle ear grouped according to anatomic location. TB indicates temporal bone.

the frequency of connections between it and the hematopoietic bone marrow. A four-level grading system was established to score the degree of unabsorbed mesenchyme in sections in which the incudomalleal articulation is seen: none, no unabsorbed mesenchyme is seen; mild, unabsorbed mesenchyme is seen, but its margins do not reach both the malleus and incus; moderate, when unabsorbed mesenchyme such that its margins meet part, but not all, of the malleus and incus is seen; and severe, unabsorbed mesenchyme such that its margins meet the entire area surrounding malleus and incus.

All histopathologic grade was scored without knowledge of any clinical information. The scoring was rigidly performed by 3 different physicians. Statistical evaluation was performed using the unpaired t test, and $p < 0.05$ was considered significant.

RESULTS

The frequency of connections between the hematopoietic bone marrow and the middle ear was significantly different among these 3 groups ($p = 0.002$ in the meningitis group and control Group 1; $p = 0.009$ in the meningitis group and control Group 2; $p = 0.001$ in the control Group 1 and control Group 2). The frequency of connections in all locations was significantly higher in the meningitis group (total, 13.5 ± 8.9; mastoid antrum, 8.4 ± 6.8; mesotympanum, 3.9 ± 4.1; epitympanum, 1.2 ± 1.2), control Group 2 (total, 4.2 ± 1.8; mastoid antrum, 3.1 ± 1.7; mesotympanum, 0.9 ± 1.3; epitympanum, 0.2 ± 0.4), and control Group 1 (total, 1.3 ± 1.6; mastoid antrum, 0.9 ± 1.4; mesotympanum, 0.2 ± 0.4; epitympanum, 0.2 ± 0.6) (in that order; Fig. 2).

In all groups, the frequency of connections was high at the mastoid antrum, mesotympanum, and epitympanum (in that order). The details of the mesotympanum were as follows: Eustachian tube, 2.5 ± 3.1, 0.3 ± 0.1, and 0.2 ± 0.4; tympanic sinus, 1.4 ± 0.8, none, and 0.2 ± 0.4; and facial recess, 1.3 ± 0.6, 0.3 ± 0.1, and 0.5 ± 0.7 in the meningitis group, control Group 1, and control Group 2, respectively.

The degree of unabsorbed mesenchyme tended to be more severe in the meningitis group, control Group 2, and control Group 1 (in that order; Table 1; Fig. 3).

DISCUSSION

Connections between the hematopoietic bone marrow and unabsorbed mesenchyme of the middle ear are a normal developmental finding in the perinatal period. Unabsorbed mesenchyme is gradually absorbed as the mastoid cell system matures. Occasionally, these connections remain patent in infants.

TABLE 1. *Degree of unabsorbed mesenchyme*

Group	None	Mild	Moderate	Severe
Meningitis	0	2 (20%)	3 (30%)	5 (50%)
Control 1	5 (50%)	3 (30%)	2 (20%)	0
Control 2	0	4 (40%)	6 (60%)	0

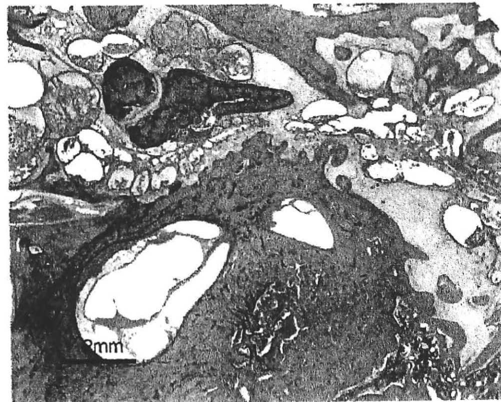

FIG. 3. Extensive unabsorbed mesenchyme (severe degree) in the middle ear and mastoid of an 18-month-old female infant who died of meningitis due to infection by *S. pneumoniae* (hematoxylin and eosin stain; original magnification, ×10).

Mesenchyme refers to the nonepithelial cells that lie between and are derived from the mesothelial and epithelial layers of the embryo (6). The middle ear is the only area in which the primitive mesenchyme can be found after birth. This feature is not seen in other organs that are well developed at birth (7). Mesenchyme may be involved in the middle ear and mastoid pneumatization process, but its role and function in the developing middle ear are controversial.

The relationship between these connections and unabsorbed mesenchyme has not been examined. Takahara et al. (8) found that mesenchyme disappeared almost completely in normal infants by the age of 1 year. Many studies have shown that unabsorbed mesenchyme is associated with otitis media (7,9–11), similar to our findings. Kasemsuwan et al. (7) revealed that unabsorbed mesenchyme was seen in infants with otitis media aged older than 2 years. In addition to otitis media, unabsorbed mesenchyme was related to congenital morphologic ear anomalies and syndromes as well as pulmonary diseases but not to congenital heart diseases (7). Therefore, we selected patients who died of congenital heart diseases as the control group. Connections between the hematopoietic bone marrow and middle ear were seen from 15 weeks of gestation to 19 months of age (2). This study showed that patients with otitis media had a significantly higher frequency of connections than patients without otitis media. Our findings suggested that there was a significant correlation between unabsorbed mesenchyme and the presence of these connections. Mesenchymal cells can give rise to various types of connective tissues (e.g., fibrous tissue, bone, cartilage, and tendons), blood cells, muscle, lymph vessels, lymph glands, and endothelium of the vascular system (12). These connections may remain patent because these unabsorbed mesenchymal cells lose the ability to differentiate into various types of tissues. However, we could not explain why patients with otitis media who died of meningitis had

a significantly higher frequency of connections than patients with otitis media who died of congenital heart diseases.

The area of the temporal bone where the mesenchyme was absorbed most rapidly was the mesotympanum. The slowest absorption of the mesenchyme was seen in the mastoid antrum (7,8). In this study, the mastoid antrum (in which the mesenchyme was absorbed most slowly) had the largest number of connections. There was a significant correlation between these results and our findings.

The frequency of connections in the meningitis group was significantly higher than that in the control group. These connections are possible pathways enabling hematogenous spread from the middle ear to the subarachnoidal space in otitis media. We believe that high incidence of connections increases the risk for otogenic meningitis in infants. Linthicum et al. (2) suggested that unabsorbed mesenchyme helps to protect the middle ear against bacterial invasion in the postnatal period. We suggest that the mesenchyme, whose absorption was disrupted by the otitis media, lost the ability to protect the middle ear against bacterial invasion.

The prevalence of connections between the hematopoietic bone marrow and the middle ear in patients with meningitis and otitis media is high. A higher prevalence of these connections in infants with otitis media could increase the risk for otogenic meningitis in them.

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ORIGINAL ARTICLE

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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.

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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-

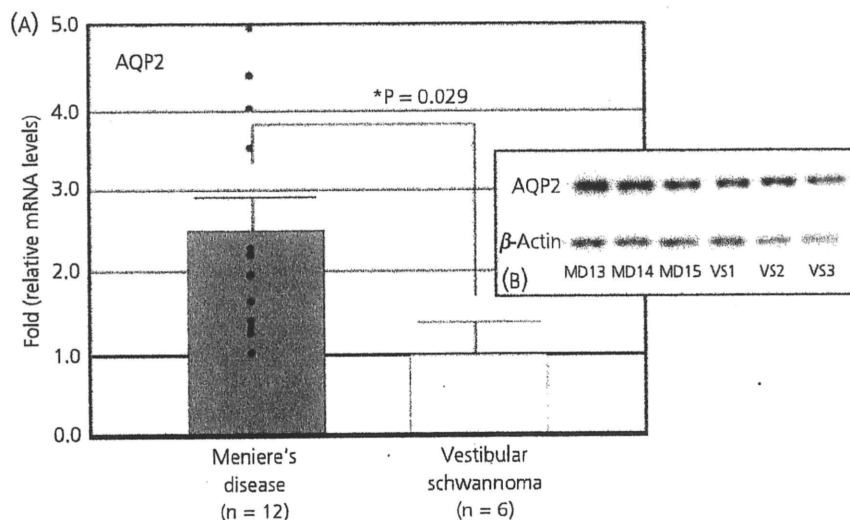

Fig. 1. Aquaporin-2 (AQP2) mRNA and protein expression levels in the endolymphatic sac in Meniere's disease patients compared with control patients. (A) The relative AQP2 mRNA expression in the endolymphatic sac was significantly higher in Meniere's disease (MD) patients (n = 12; 2.52 ± 0.40-fold) than 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.

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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-

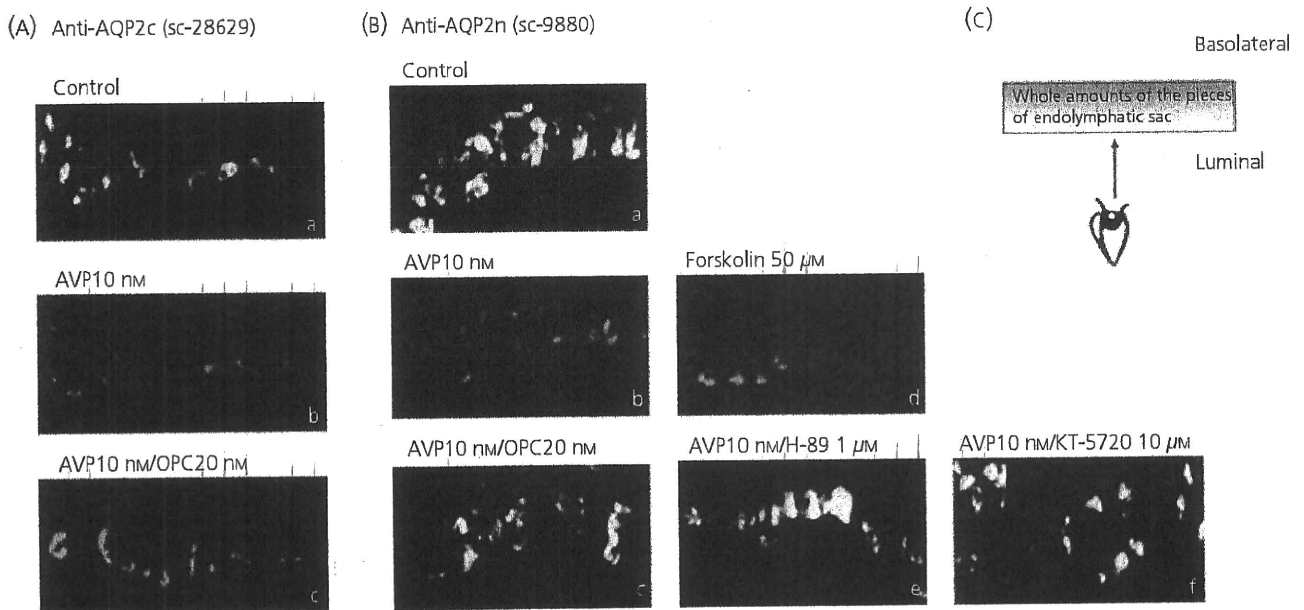

Fig. 2. Intracellular translocation of aquaporin-2 (AQP2)-like immunoreactivity (-LIR) via V2 receptor (V2R)-cAMP-protein kinase A (PKA) system in the human endolymphatic sac. Using both kinds of antibodies, AQP2c for C-terminal (A) and AQP2n for N-terminal (B), AQP2-LIR was detected in the luminal side 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).

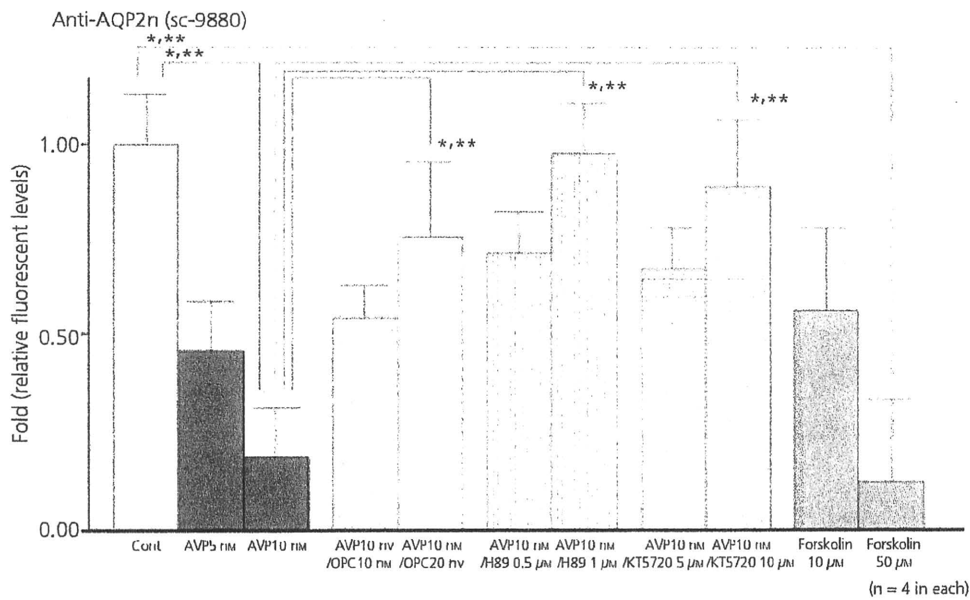

Fig. 3. Statistical analysis of aquaporin-2 (AQP2)-like immunoreactivity (-LIR) intensity in the endolymphatic sac. Changes in all the relative fluorescence intensity of AQP2n-LIR in Fig. 2B were confirmed statistically. The blockade of vasopressin (AVP) effects by OPC31260, H-89 or KT-5720 was demonstrated 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).

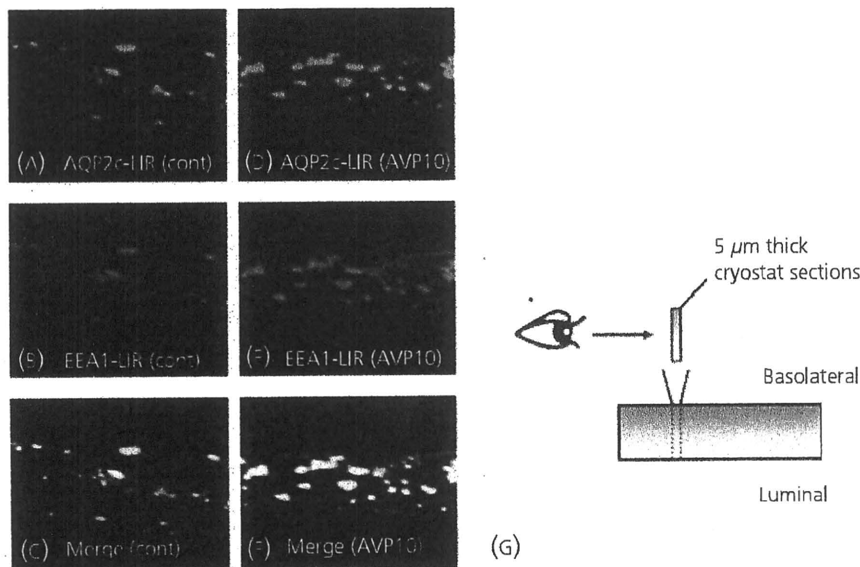

Fig. 4. Co-localisation of aquaporin-2 (AQP2)- and early endosome antigen (EEA)1-like immunoreactivity (-LIR) in the human endolymphatic sac. Using both kinds of antibodies, AQP2c for aquaporin-2 C-terminal domain (A, D: green) and EEA1 for early endosome antigen-1 (B, E: red), co-localisation was shown 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

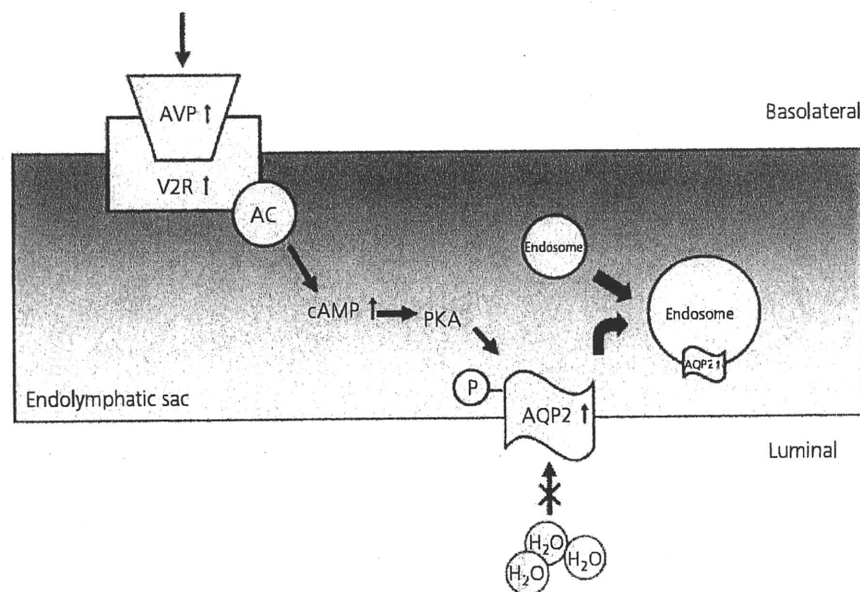

Fig. 5. Schematic representation of a hypothetical mechanism for endolymphatic hydrops generation in Meniere's disease via stress hormone vasopressin (AVP) and water channel aquaporin-2 (AQP2). Plasma AVP elevation and subsequent V2 receptor (V2R)-cAMP-protein kinase A (PKA) activation in the endolymphatic 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.

a small increase in plasma vasopressin (Fig. 5). Additionally, AQP2 was not resolved but stored within the cytoplasmic endosome in the endolymphatic sac after AVP stimulation. This finding indicates that AQP2 can be translocated again to the luminal side by medical treatments, which may lead new ideas to cure patients with Meniere's disease.

Finally, we would like to speculate about the possible causes for 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 (21,22). Among these insults, immune-mediated responses in the inner ear endo-organs, such as the endolymphatic sac, stria vascularis and spiral ligament, are considered to be the main bases for the fluid homeostatic disorder in Meniere's disease (23,24). Certain virus infections, such as varicella-zoster, Epstein–Barr and adenovirus infections, of the endolymphatic sac in early childhood represent other bases for the dysfunction of endolymph absorption (25,26). Taken together with the present data, it is suggested that autoimmune responses and/or virus infections could cause damage to the V2R regulatory genes in the endolymphatic sac, resulting in V2R-cAMP-PKA-AQP2 activation and endosomal trapping of AQP2. In the V2R-cAMP-PKA-AQP2 activated inner ear, endolymphatic hydrops could gradually be generated and Reissner's membranes could become ruptured (27) after even a small elevation in plasma vasopressin as a result of stress, thereby resulting in attacks of Meniere's disease. The laterality of Meniere's disease could not be decided by the level of plasma vasopressin but by the laterality of inner ear molecular pathology mentioned above. The data of V2R up-regulation in Meniere's disease in the previous study (4) and AQP2 up-regulation in Meniere's disease in the present study were obtained from human *in vivo*. However, all the evidence for AQP2 translocation in the endolymphatic sac in the present study was demonstrated in humans *in vitro*. Therefore, there are limitations in the present study and further genetic studies in mice and clinical observations in patients with Meniere's disease are required to confirm the significant relationship among the inner ear molecular pathology, endolymphatic hydrops and Meniere's attacks.

In conclusion, in the pathogenesis of inner ear hydrops resulting in vertigo attacks accompanied by hearing loss and tinnitus in Meniere's disease, plasma vasopressin elevation as a result of stress and subsequent V2R-cAMP-PKA-AQP2 activation and endosomal trapping of AQP2 in the endolymphatic sac might be essential as the basis of this disease.

Meniere's disease, associated with vertigo, fluctuating hearing loss and tinnitus as a result of inner ear pathology, has been proposed to occur especially in people living a stressed lifestyle (28). Indeed, this disease is provoked by a poor adaptation to physical and/or psychogenic stress in daily life (29). We consider that the results obtaned in the present study will encourage continued investigations that help to ascertain ideal treatments for the inner ear in Meniere's disease, ranging from psychotherapy for leading a stressless life to gene therapy for stress hormone receptor-related molecules via an endolymphatic sac approach.

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BEHAVIORAL ASSESSMENT AND IDENTIFICATION OF A MOLECULAR MARKER IN A SALICYLATE-INDUCED TINNITUS IN RATS

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Abstract—Tinnitus is a non-observable phantom sensation. As such, it is a difficult condition to investigate and, to date, no effective treatment has been developed. To approach this phantom sensation, we aimed to develop a rat behavioral model of tinnitus using salicylate, an active component of aspirin known to induce tinnitus. We also aimed to establish a molecular marker of tinnitus by assessing the expression of transient receptor potential cation channel superfamily V-1 (TRPV1) in the rat auditory pathway during salicylate-induced tinnitus. Animals were trained to perform "an active avoidance task": animals were conditioned by electrical footshock to move to the other side of the conditioning box when hearing a sound. Animals received a single injection of saline or salicylate (400 mg/kg i.p.) and false positive responses were measured 2 h after injection as the number of movements during a silent period. The number of responses in salicylate-treated animals was highest when the conditioned stimulus was 60 dB sound pressure level (SPL) and 16 kHz. This indicates that animals could feel tinnitus 2 h after salicylate injection, equivalent to that induced by 60 dB SPL and 16 kHz. By means of real-time PCR and western blot analysis, TRPV1 expression was significantly upregulated in spiral ganglion cells 2 h after salicylate injection and this upregulation together with the increase in the number of false positive responses was significantly suppressed by capsazepine (10 mg/kg i.p.), a specific antagonist of TRPV1. This suggests that salicylate could induce tinnitus through activation of TRPV1 in the rat auditory pathway. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: animal model, TRPV1, spiral ganglion, dorsal cochlea nucleus.

Many people have experienced a sensation of ringing in their ears when no external sound is present. Typically, this sensation of tinnitus is associated with a reversible cause and subsides over a period of time ranging from a few seconds to a few days. However, in 5–15% of the general population, the tinnitus is unremitting (Heller, 2003). Chronic tinnitus is more prevalent among seniors (12% after the age of 60) than in young adults (5% in the age group 20–30), but can occur at any age. In 1–3% of the general population, tinnitus is perceived as loud enough to affect the quality of life (Eggermont and Roberts, 2004). However, no effective therapeutic strategy for such intractable tinnitus has been established.

There are at least two obvious reasons why it is still so hard for clinicians to treat intractable tinnitus. One reason is that, since Jastreboff and Sasaki (1994) proposed an animal behavioral model of tinnitus based on an active avoidance task of drinking water with electrical footshock, better feasibility of the model has been discussed. The first purpose of the present study was, therefore, to develop a rat behavioral model to enable the objective evaluation of tinnitus. Salicylate, an active component of aspirin, is well known to induce an acute and transient type of tinnitus (Cazals, 2000; Eggermont and Roberts, 2004). According to the recent good work of climbing a pole with electrical footshock by Guitton et al. (2003), our model using salicylate was developed with modifications of a much easier active avoidance task. These previous and present tinnitus models will be addressed again in the first paragraph in Discussion.

The other reason is that the molecular mechanism of tinnitus generation in the auditory pathway has not been clarified yet. The second purpose of the present study was, therefore, to identify molecular markers to understand the molecular mechanism of salicylate-induced tinnitus in the rat auditory pathway. Salicylate inhibits cyclo-oxygenase activity (Christie et al., 1998) and cyclo-oxygenase inhibition leads to the *in vitro* activation of a nociceptive receptor transient receptor potential cation channel superfamily V-1 (TRPV1) (Fosslien, 1998; Hwang et al., 2000; Caterina et al., 1997; Benham et al., 2003). TRPV1 is located in the mouse inner ear ganglia and is upregulated *in vivo* after noxious challenges of kanamycin (Kitahara et al., 2005a). Furthermore, cochlear background activity is increased by inner ear perfusion of capsaicin, a TRPV1 agonist and is suppressed by capsazepine, a TRPV1 specific antagonist (Zhou et al., 2006). We examined changes in mRNA and protein levels of TRPV1 in the salicylate-treated rat auditory pathway. Although salicylate-induced tinnitus is acute and transient, we believe that by elucidating the molecular mechanism of salicylate-induced tinnitus we can provide insight into chronic intractable tinnitus.

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Abbreviations: ABR, auditory brainstem response; BRCx, brain cortex; B2m, beta-2 microglobulin; CT, cycle threshold; DCN, dorsal cochlear nucleus; DMSO, dimethyl sulfoxide; RT, room temperature; SG, spiral ganglion; SPL, sound pressure level; TRPV1, transient receptor potential cation channel superfamily V-1.

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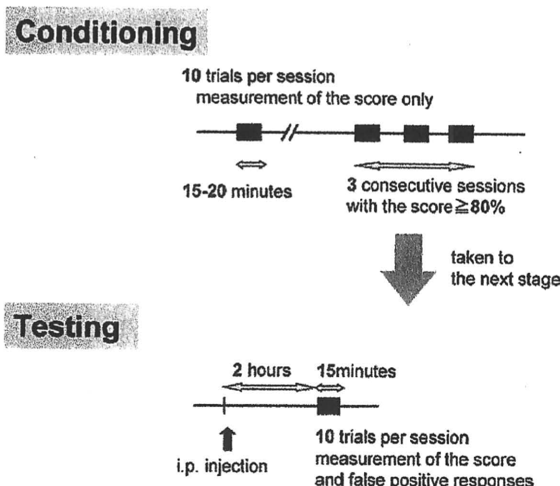

Fig. 1. Schematic representation of the behavioral protocol. In "conditioning," animals were conditioned to move in response to a sound stimulation. The conditioning procedure requires up to seven sessions lasting 15–20 min. When conditioned (criterion, three consecutive sessions with an active avoidance score ≥80%), animals were taken to the next stage, "testing" (day 0). The "testing" behavioral protocol consisted of a daily measurement (on four consecutive days, days 1–4) of the correct responses to sound (active avoidance score) and moves during inter-trial periods (false positive responses) in a 15 min session. Saline or salicylate was injected daily 2 h before the testing session.

EXPERIMENTAL PROCEDURES

Experimental procedures involving animals were performed according to animal ethical guidelines and were approved by Osaka University, School of Medicine (certificate number: 0755). All efforts were made to minimize the suffering to animals and to limit the number of animals used. A total of 99 male Wistar rats (Japan SLC, Hamamatsu, Shizuoka, Japan), weighting between 150 and 200 g, were used. Animals were individually housed in a temperature-controlled room on a constant 12 h light/dark cycle. All behavioral tests were conducted during the animals' activity period (dark phase) at approximately the same time each day. Food and tap water were available throughout the experiments.

Behavioral assessment

Animals were trained to perform "an active avoidance task," according to the protocol of Guitton et al. (2003) (Fig. 1). Both "conditioning" and "testing" were performed in a conditioning box that had an electrified floor, which was divided in two by a low wall, 3 cm high. The conditioning box was in a soundproof room. A 5 s pure tone sound was used as the "conditioned stimulus" and a 3.7 mA electrical footshock was given for a maximum of 30 s as the "unconditioned stimulus." The interval between conditioned and unconditioned stimuli was 1 s. The footshock was stopped when animals correctly escaped from the unconditioned shock to the opposite side of the cage. The inter-trial interval or silent period was at least 1 min. The level of performance over 10 trials, or "active avoidance score," was assessed by the ratio of how many times the rat moved correctly in response to the conditioned sound. In the "conditioning" stage, animals were considered to be conditioned when the active avoidance score reached at least 80% in three consecutive sessions. When conditioned, animals were taken to the next "testing" stage (day 0).

Testing was performed once daily for 4 days, at the same time each day (day 1–4). Animals received a single daily injection of saline or sodium salicylate (400 mg/kg i.p.) (Sigma, St. Louis, MO, USA) for 3 days (day 1–3), according to the previous reports of Jastreboff and Sasaki (1986); Rüttiger et al. (2003) and Im et al. (2007). Injections were performed 2 h before behavioral measurements. On the fourth day, they received no treatment. The behavioral protocol consisted of a daily measurement of the active avoidance score and of false positive responses. The "false positive responses" represent the number of movements to the opposite side of the cage during the inter-trial interval, when there was no sound. Trials were randomized and electric footshocks were presented only if animals didn't move in response to sounds. Whatever the results of the active avoidance score and false positive responses were, each session included 10 trials and lasted 15 min. Both the active avoidance score and false positive responses were measured in the same session.

The most appropriate conditioned stimulus was determined by the following pilot experiments. Animals were divided into four groups (n=6 in each group) according to conditioned stimuli of 4, 10, 16 or 40 kHz (60 dB sound pressure level (SPL)), and false positive responses were measured on the third day after salicylate injection. Animals were also divided into three groups (n=6 in each group) according to conditioned stimuli of 20, 60 or 80 dB SPL (16 kHz) and false positive responses were measured as above.

Auditory brainstem response (ABR) recording

The ABR was measured with a Neuropack-4 (Nihon Koden Co., Shinjuku, Tokyo, Japan). The active platinum electrode was inserted at the vertex, and reference electrodes at both pinnae of the ears. Binaural, open fielded stimuli of click were generated through a TDH-49 headphone attached to the animal's ears. The rat ABR consisted of a series of III to V vertex-positive peaks within the first 6 ms from the onset of the stimulus, and they were called I to V. The III wave was detected at the lowest stimulus intensity, so the threshold was defined as the lowest stimulus intensity to elicit a reliable III wave.

Hematoxylin and eosin staining

Serial inner ear sections from saline controls and salicylate-treated rats were stained with hematoxylin and eosin to determine if salicylate treatment caused any overt damage to the inner ear. Morphological structures in the organ of Corti and the spiral ganglion (SG) were microscopically observed.

Analysis of mRNA levels

Animals were divided into five groups: a saline i.p. injection control group, a 2 h post-salicylate i.p. injection group, a 12 h post-salicylate injection group, a 24 h post-salicylate injection group, and a 72 h post-salicylate injection group (n=6 in each group).

The procedures of tissue preparation for real-time PCR have already been described in our previous papers (Kitahara et al., 2005a,b). Animals were deeply anesthetized with pentobarbital and the SG, dorsal cochlear nucleus (DCN) and brain cortex (BRCx) were immediately dissected under a stereomicroscope in chilled buffered saline and then frozen in dry ice powder. The DCN region is thought to be one of the main structures involved in tinnitus (Eggermont and Roberts, 2004) and it was carefully dissected according to the coordinates of the Paxinos and Watson brain atlas; rostral coordinate, bregma: −10.52 mm, and caudal coordinate, bregma: −11.60 mm (Paxinos and Watson, 1986). The BRCx region does not include the auditory cortex. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions.

PCR was performed using oligonucleotide primers for TRPV1 (Takara Bio Inc., Otsu, Shiga, Japan) and beta-2 microglobulin (B2m) (Takara), as shown in Table 1, and products were quantified by SYBR Green PCR reagents (Applied Biosystems, Foster City, CA, USA). B2m was assayed as a control housekeeping gene. The PCR mixture included 10 μl of 2× SYBR Premix Taq,