Cochlear implantation In patients with mitochondrial DNA A3243G mutation

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Summary

Maternally inherited diabetes and deafness (MIDD) is known to be associated with an A to G point mutation at nucleotide pair 3243 in mitochondrial DNA. We performed cochlear implantation (CI) in 4 patients with MIDD. One patient was also diagnosed with cardiomyopathy. The A3243G point mutation was confirmed in all patients, with the heteroplasmy ranging from 6% to 30%. CI was performed in all patients, without any intraoperative or postoperative complications. The scores for open-set monosyllable, word, and sentence recognition were found to be better than the average scores in adult users of cochlear implants with deafness caused by other reasons. A review of the literature also showed that patients with mitochondrial deafness are good candidates for CI.

Introduction

Sensorineural hearing loss (SNHL) occurs in approximately 50% of patients with mitochondrial disorders. Mitochondrial SNHL may be nonsyndromic or syndromic (associated with multiple disabilities ranging from diabetes to stroke-like episodes) and commonly involves the cochlea, predominantly the stria vascularis, hair cells, and spiral ganglion cells [1-4]; this implies that patients with mitochondrial deafness may be good candidates for CI. Sinnathuray et al. [5] have reviewed literatures to show that patients with mitochondrial deafness can be successfully rehabilitated by CI. Herein, we report CI in 4 adult patients with MIDD associated with mitochondrial A3243G mutation.

Material and Methods

Four unrelated patients with deafness associated with an A-to-G transition at np 3243 in the mtDNA who underwent CI were enrolled in the present study. The clinical features and postoperative audiometric findings of these patients were evaluated.

Table 1

No	Age at CI (y)	Gender	Heteroplasmy (%)	Onset HL (y)
1	53	F	30	30
2	46	F	29	28
3	44	F	9	35
4	65	F	6	40

Table 2

	Monosyllable (%)		Word (%)		Sentence (%)	
No	A	A+V	A	A+V	A	A+V
1	36	48	66	74	44	82
2	40	54	68	76	65	89
3	60	82	88	76	88	94
4	70	88	86	95	86	95

Results

The clinical features of the patients are shown in Table I. All of them were women and had diabetes mellitus. One patient (No. 4) also had cardiomyopathy. Their family pedigrees showed maternal inheritance of diabetes and/or hearing impairment over two or three generations. None of their family members showed clinical symptoms characteristic of mitochondrial encephalomyopathy. The age of onset of hearing loss ranged from 28 to 40 years. The percentage of mutant mtDNAs in the peripheral leukocytes ranged from 6% to 30%; hearing loss occurred at a younger age in patients with higher heteroplasmy.

CI was performed in all patients, without any intraoperative or postoperative complications. Since the activation of the implant, they used it successfully and continuously. After the operation, all the patients showed good speech recognition in the open-set monosyllable, word, and sentence tests (Table 2). The average scores of these tests were 52, 77, and 71, respectively, in the hearing-only condition, and 68, 80, and 90, respectively, in the visual-plus-hearing condition. These scores were better than those of adult subjects with deafness caused by other reasons such as meningitis.

Discussion

The present case presentation showed that patients with MIDD associated with A3243G point mutation in the mtDNA showed good speech recognition after CI. Sin-

nathuray et al. [5] reviewed studies of CI in patients with mtDNA mutations in a variety of countries and found that despite the presence of various mitochondrial mutations, this heterogeneous group of cochlear implantees has done well, with 58% of the individuals able to converse on the telephone and most of the remainder showing good open-set speech recognition and no reported complications. These findings suggest that pathologic changes in mtDNA mutations primarily involve a cochlear site; this is consistent with the histopathological findings reported in patients with deafness caused by mitochondrial disorders such as Kearns–Sayre syndrome and MIDD [2-4].

In conclusion, the present study, along with the literature review, indicates that CI is an effective therapy in patients with deafness associated with the A3243G mutation in the mtDNA. The excellent auditory performance after CI suggests that hearing loss associated with this mutation is primarily caused by insult to the cochlear tissue containing rich mitochondria (i.e., hair cells and stria vascularis).

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CASE REPORT

Comparison of Electroaudiometry with cochlear implant in children with inner ear anomaly

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KEYWORDS
Electroaudiometry;
Cochlear implant;
Inner ear anomaly

Summary The promontory stimulation test (PST) using a needle electrode has been used to evaluate the sense of the auditory nerve as a preoperative examination for cochlear implant in adults. Because this is a painful test, it is not suitable for children. It has been reported that children with inner ear anomaly showed poorer outcomes of hearing after cochlear implant. Electroaudiometry developed by Med-El Corporation, which is noninvasive, is a more suitable procedure for young children. Patients were three children less than five years old with inner ear anomaly. Two patients showed common cavity, and one showed narrow IAC with hypoplastic cochlear anomaly. By using Electroaudiometry, we analyzed electro-neural hearing of these children before cochlear implant, and compared their hearing after cochlear implant. Three children seemed to have residural electro-neural hearing because the dynamic range between stimulus level (SL) and uncomfortable level (UCL) was detected by using Electroaudiometry. After cochlear implant, their pure-tone audiograms showed moderate hearing thresholds, and their hearing detection and speech perception improved. These results suggest that Electroaudiometry is available for evaluating electro-neural hearing in young children with inner ear anomaly. It can provide useful information for a successful cochlear implant and evaluation of postoperative performances.

1. Introduction

The promontory stimulation test (PST) using a needle electrode has been used to evaluate the sense of the auditory nerve as a preoperative examination for cochlear implant in adults. It was first described by House and Brackmann in 1974 [1]. This test requires penetration of the tympanic membrane, therefore, it is not suitable for small children. However, before cochlear implant, we need to confirm the level of electro-neural hearing, particularly in small children with inner ear anomaly. It has been reported that they showed poorer outcomes of hearing after cochlear implant [2]. We need to discuss the indication of cochlear implant carefully.

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In 1995, Wagner reported a new technique called Electroaudiometry developed by Med-El Corporation [3]. It is a noninvasive technique that the tip of the electrode is positioned in the external auditory canal, not touching the tympanic membrane.

In this study, we analyzed the electro-neural hearing before cochlear implant in children less than five years old with inner ear anomaly using this technique, and compared their hearing after cochlear implant.

2. Subjects and methods

In this study, we analyzed three children less than five years old with inner ear anomaly at the Department of Otolaryngology-Head and Neck Surgery, University of Tokyo, from January 2005 to December 2006.

The procedure of Electroaudiometry is as follows. The external auditory canal is moistened with a gelatine sponge soaked in saline solution. A ball-shaped electrode is placed in the external auditory canal near the tympanic membrane. The earth electrode is placed on the mastoid. A battery-driven stimulator is fastened to the child's clothing. We hold the remote control unit, choosing a position in the room away from the child. The control unit sends control pulses to the stimulator through an infrared connection (Fig. 1). It generates 512 ms biphasic square wave current, which may be varied from 63 to 2000 Hz. The stimulator confirms current bursts by an acoustic signal. We observe the child's responses carefully. When the child shows a pleasant facial expression in response to the stimulator, we determine that the zone of stimulus level (SL) has been reached. However, when the child does not seem to show this pleasant facial expression in response to the stimulator, we determine that the zone of uncomfortable level (UCL) has been reached.

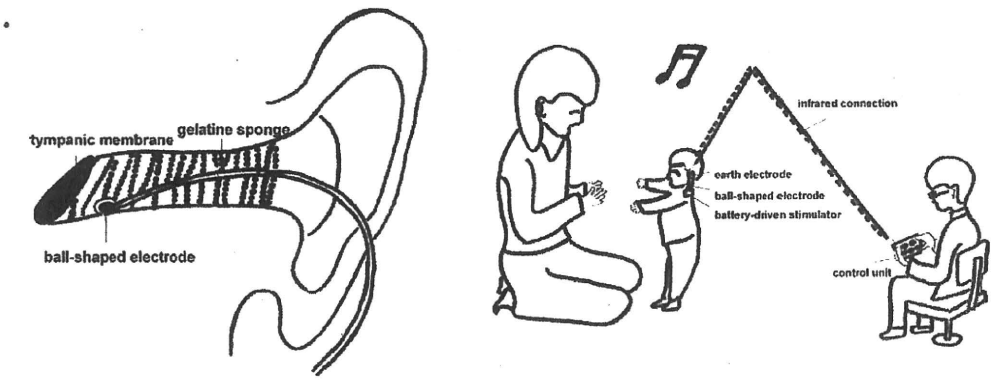

Fig. 1 Electroaudiometry device.

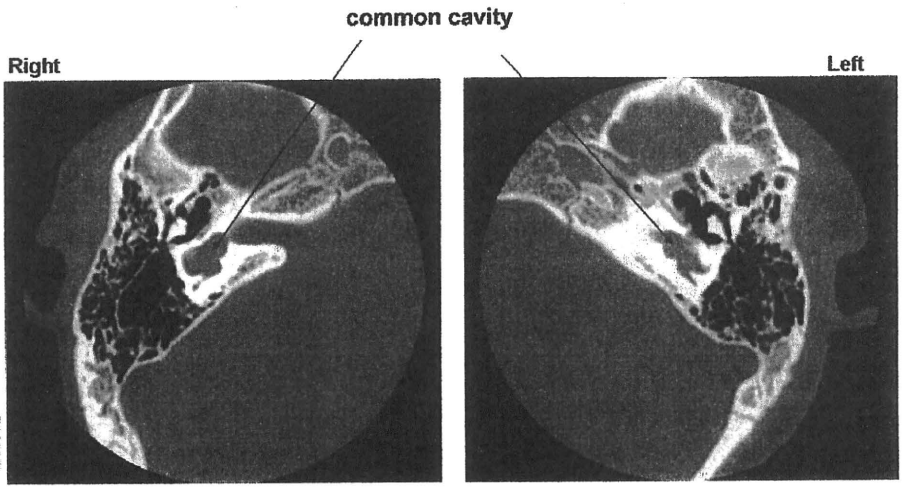

Fig. 2 CT scan showing common cavity in case 1.

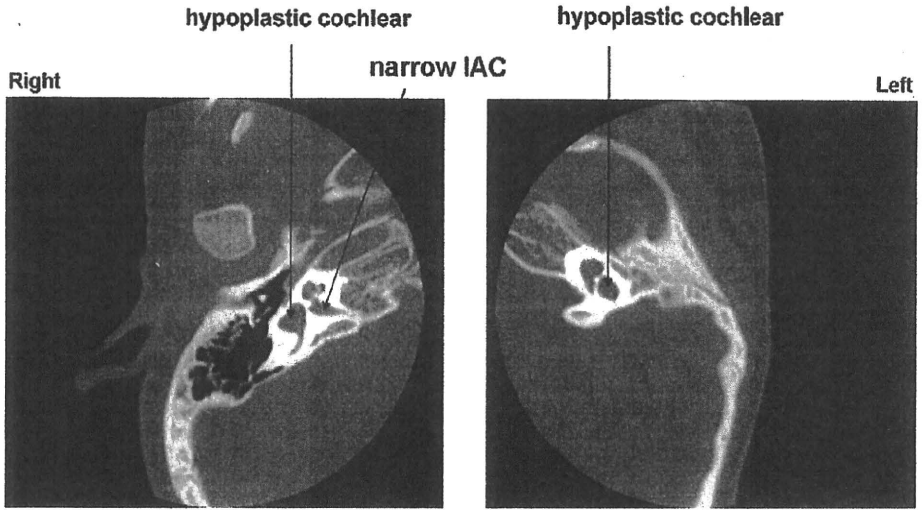

Fig. 3 CT scan showing narrow IAC with hypoplastic cochlear in case 2.

3. Results

3.1. Case reports

3.1.1. Case 1: three years and 9 months old male

He was diagnosed to have a profound hearing loss at the age of two years, and had been using bilateral hearing aids. Because his parents were worried about his limited speech ability, he was brought to our hospital to determine whether cochlear implant was indicated. A CT scan shows that the cochlea and vestible form a common cavity (Fig. 2).

3.1.2. Case 2: four years and 6 months old male

He was diagnosed as having Goldenhar syndrome at his birth and profound hearing loss at the age of 1 month. He had been using bilateral hearing aids, but did no't improve his hearing. He visited our hospital to determine the indication of cochlear implant. A CT scan shows that the cochlea and vestible form a hypoplastic structures, and IAC is short and narrow (Fig. 3).

3.1.3. Case 3: two years and 11 months old female

She was diagnosed to have a profound hearing loss at the age of one year, and had been using bilateral hearing aids, but did no't improve her hearing. She was brought to our hospital to determine whether cochlear implant was indicated. A CT scan shows that the cochlea and vestible formed a common cavity (Fig. 4).

We analyzed their electro-neural hearing to determine whether cochlear implant was indicated as preoperative assessment.

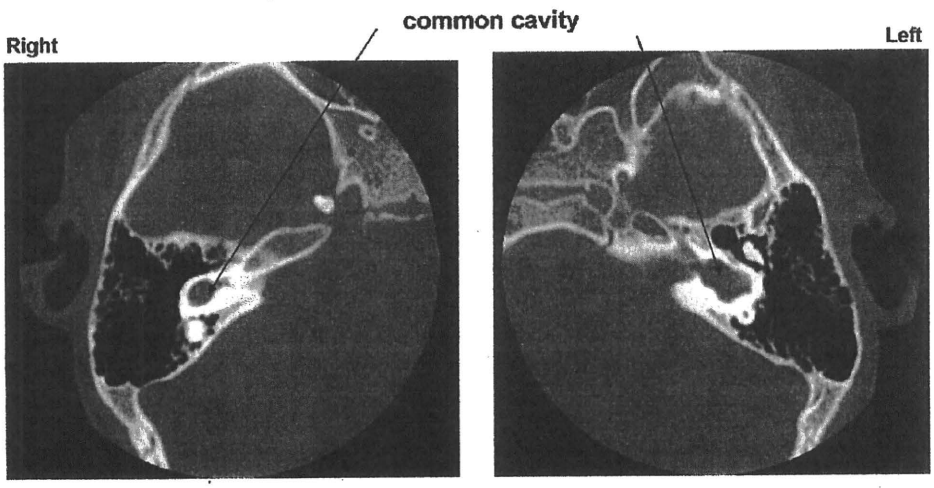

Fig. 4 CT scan showing common cavity in case 3.

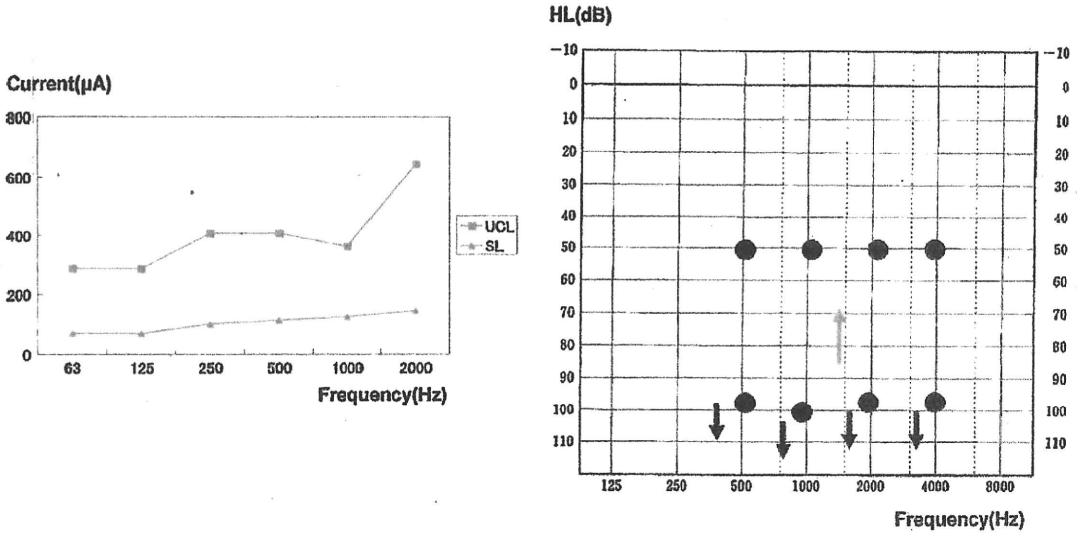

Fig. 5 The results of Electroaudiometry and audiogram in case 1.

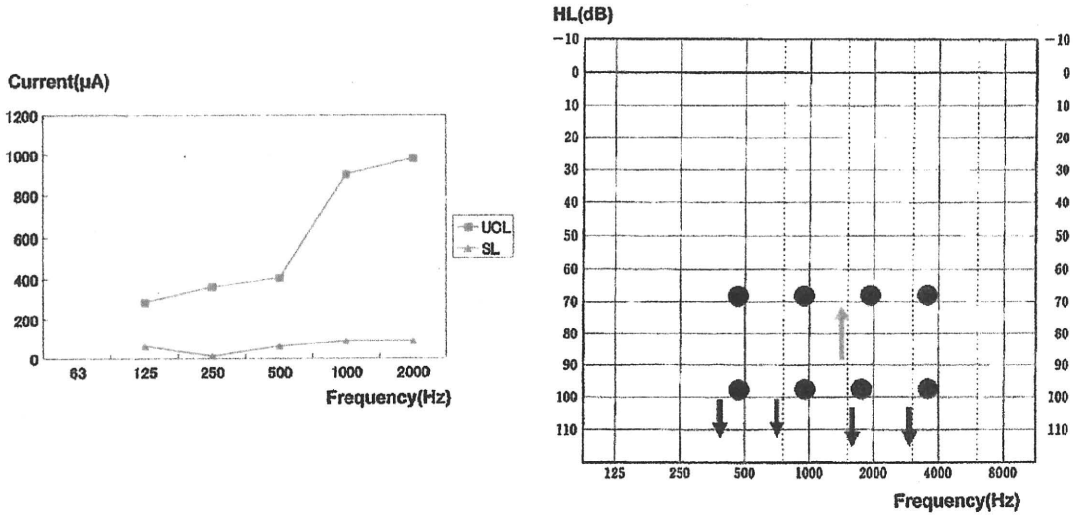

Fig. 6 The results of Electroaudiometry and audiogram in case 2.

We chose the right ear in case 1 and case 3 considering the cerebral dominant hemisphere. We chose left ear in case 2 because the shape of the left side cochlea remained normal compared with that of the right side as revealed by temporal bone CT. The graphic illustrations of stimulus level (SL) and uncomfortable level (UCL) of the three patients are shown in Figs. 5–7. We were able to determine the narrow dynamic range (DR) between SL and UCL in the three patients. We predicted that their electro-neural hearing were inadequate, but confirmed the potential preservation of inadequate spiral ganglion cells.

They were implanted with Nucleus CI 24 M (Cochlear Ltd, Lane Cove NSW, Australia). There were no complications following their cochlear implant, and they showed good postoperative sound perception and vocalization during rehabilitation. One year after the cochlear implant, we were able to recognize their good results that pure-tone audiograms revealed significant improvement of hearing, as shown in Figs. 5–7.

4. Discussion

Promontory stimulation test (PST) using a needle electrode is generally used as a preoperative examination for cochlear implant in adults. A needle electrode is penetrated through the tympanic membrane and placed against the promontory.

Because this is a painful test, it is not suitable for children. Wagner reported a new test called Electroaudiometry developed by Med-El Corporation,

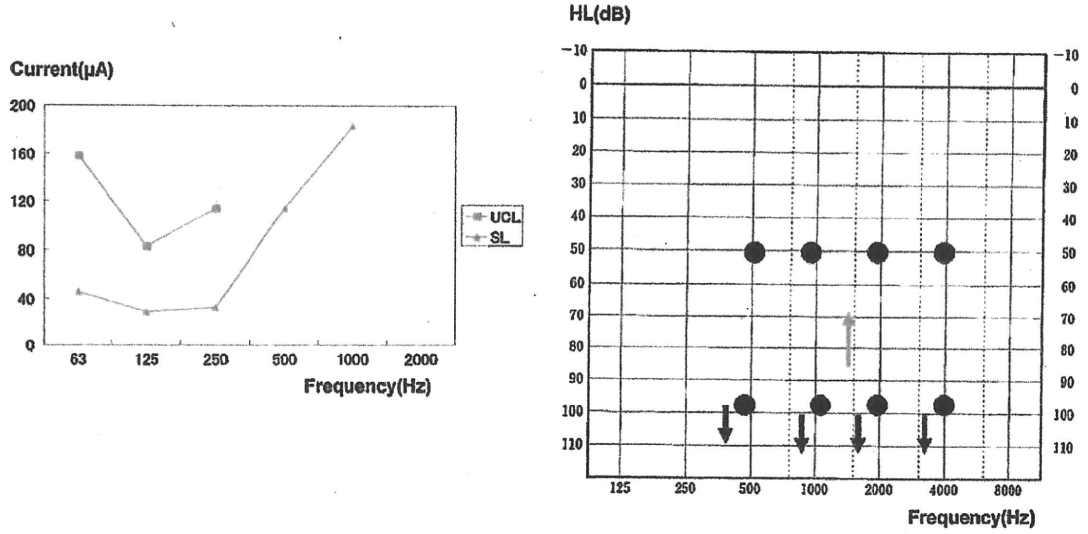

Fig. 7 The results of Electroaudiometry and audiogram in case 3.

and Lesinski compared the electrical stimulation results of ear-canal electrode and promontory needle electrode [4]. Electroaudiometry is less invasive than PST, because the ball-shaped electrode is only placed in the external auditory canal near the tympanic membrane. However, it tends to omit this preoperative examination for cochlear implant in adults and in children without anomaly of the inner ear, because these patients may have normal spiral ganglion cells, and the trial does not have an association with the success of cochlea implant. But we demonstrate to investigate in detail whether it is suitable and efficient for children with inner ear anomaly in whom cochlear implant must be determined immediately. Our study is to assess the electro-neural hearing in children less than five years with inner ear anomaly.

According to Sennaroglu, inner ear anomaly represents approximately 20% of the etiology of profound hearing loss [5]. It has been reported that children with inner ear anomaly showed poorer outcomes of hearing after cochlear implant.

This is mainly due to an abnormal anatomy, that is the cochlear nerve may be absent in inner ear anomaly. Other problems are an abnormal course of the facial nerve, and a defect at the end of the IAC [6]. As a result of these problems, patients with inner ear anomaly may develop meningitis in the postoperative period. In most cases we must be ready to make modifications in the surgical approach because these difficulties may lead to unpredictable complications. Therefore, cochlear implant in children with inner ear anomaly is difficult for surgeons.

To success cochlear implant, we demonstrate the use of Electroaudiometry before cochlear implant as a preoperative assessment and prediction of postoperative performance in children with inner ear anomaly.

In our study, we were able to assess a good outcome in children less than five years old with inner ear anomaly. On the basis of this assessment, we performed cochlear implant. They showed good postoperative sound perception and vocalization during rehabilitation, as indicated by their preoperative assessment. We also found that electro-neural hearing was associated with the number of cochlear nerves, because of good hearing obtained after cochlear implant. This indicates that cochlear nerves remain intact, but the amount of neural tissues is significantly decreased in the inner ear in congenital anomalies. We hypothesize the potential preservation of inadequate spiral ganglion cells despite of hair cell degeneration in congenital anomalies. Further study is necessary to investigate the abnormal anatomy of the inner ear in children with inner ear anomaly, and we propose the use of Electroaudiometry as a preoperative assessment for young children.

5. Conclusion

In conclusion, we propose the use of this noninvasive method, called Electroaudiometry, to assess the integrity of the auditory nerve before cochlear implant in children with inner ear anomaly.

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Plasma Vasopressin and V2 Receptor in the Endolymphatic Sac in Patients With Delayed Endolymphatic Hydrops

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Objective: There are some kinds of sicknesses provoked by inadequate adaptation to physical and/or psychogenic stress in daily life. Delayed endolymphatic hydrops (DEH) is an inner ear disease like Ménière's disease (MD) characterized by episodic vertigo in the setting of preexisting unilateral deafness that especially occurs in civilized people with a stressful lifestyle. Its otopathologic finding was demonstrated to be inner ear endolymphatic hydrops through a temporal bone study in 1976, as in the case with MD in 1938. To elucidate the relationship between stress and the inner ear, we examined the plasma antidiuretic stress hormone vasopressin (pAVP) and its type 2 receptor (V2R) expression in the endolymphatic sac in patients with DEH.
Study Design: A prospective molecular biological study.
Methods: Between 1998 and 2007, we enrolled 20 patients with ipsilateral DEH to examine their pAVP during remission from vertigo attacks. Plasma vasopressin was also examined in 87 patients with unilateral MD and 30 control patients with chronic otitis media. Using the real-time polymerase chain reaction method with tissue samples obtained during surgery, we examined V2R mRNA expression in the endolymphatic sac in 6 patients with ipsilateral DEH, 9 patients with unilateral MD, and 6 control patients with acoustic neuroma.
Results: Plasma vasopressin (1.5 times versus controls; unpaired t test, $p = 0.140$) and V2R mRNA expression in the endolymphatic sac (35.8 times versus controls; unpaired t test, $p = 0.002$) were higher in patients with DEH compared with those with acoustic neuroma. There were no significant differences in pAVP or V2R expression in the endolymphatic sac between DEH and MD. Patients with DEH showed a significantly negative correlation between pAVP and V2R (Pearson test, $r = -0.92$, $p = 0.009$) as in those with MD (Pearson test, $r = -0.68$, $p = 0.043$).
Conclusion: Civilized people are frequently exposed to stress in their daily life, and pAVP can easily become elevated at any time. Therefore, a negative feedback system between pAVP and V2R in the endolymphatic sac may function for inner ear fluid homeostasis against stress-induced increases in pAVP. For the pathogenesis of endolymphatic hydrops resulting in vertigo attacks in patients with DEH as well as MD, pAVP may represent a matter of consequence, but V2R overexpression in the endolymphatic sac could be much more essential as a basis for these diseases. **Key Words:** Delayed endolymphatic hydrops—Endolymphatic sac—Ménière's disease—Stress—V2 receptor.

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There are some kinds of sicknesses provoked by inadequate adaptation to physical and/or psychogenic stress in daily life. Vertigo attacks of Ménière's disease (MD) due to the inner ear abnormality represent a common example. Delayed endolymphatic hydrops (DEH) due to inner ear abnormality similar to MD is characterized by episodic vertigo in the setting of preexisting unilateral deafness and occurs in people with a stressful lifestyle (1). However, it is very difficult to prove a significant relationship between stress and inner ear abnormality because the definition of stress is too obscure for a scientific analysis of these aspects.

Since the otopathologic finding in DEH was demonstrated to be inner ear endolymphatic hydrops by a temporal bone study in 1976 (2), as in cases with MD in 1938 (3,4), it has gradually become understood that inner ear end organs, including the endolymphatic sac, regulate the fluid homeostatic system via water metabolism–related molecules such as vasopressin and aquaporin (5). Subsequently, it was proposed that the pathogenesis in DEH as well as MD could be inner ear endolymphatic hydrops due to a disorder of water metabolism–related molecules.

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In the present study, to elucidate the relationship between stress and the inner ear abnormality, we tested the hypothesis that the plasma antidiuretic stress hormone vasopressin (pAVP) and its type 2 receptor (V2R) in the endolymphatic sac will be increased in patients with DEH compared with controls.

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 no. 0424).

DIAGNOSIS AND ENROLLMENT

Patients were eligible for enrollment if they had received a clinical diagnosis of DEH or MD according to the 1995 American Academy of Otolaryngology—Head and Neck Surgery criteria (6). These criteria can be briefly described as follows: 1) Repeated attacks of vertigo: a definitive spell is spontaneous vertigo lasting at least 20 minutes. A mixed type of spontaneous nystagmus is observed during attacks. 2) Fluctuating cochlear symptoms: the hearing test usually reveals profound sensorineural hearing loss or deafness in the affected ear (ipsilateral type of DEH) or marked fluctuation of the threshold in the low and middle tone range contralateral to the affected ear (contralateral type of DEH). 3) Exclusion of other causes: to exclude other disorders, a thorough history, neurological, neurotological, and magnetic resonance imaging examinations were performed. Intractable DEH was designated in cases where various forms of medical and psychological management failed for at least 6 months. Medical management included diuretics, β-histine, diphenidol, dimenhydrinate, and diazepam, which were thought to be effective for persistent symptoms in DEH (7).

Patients designated as having intractable DEH had endolymphatic sac drainage, if there was no reason for declination of surgery. The technical details of this surgery were described before (8–10).

LABORATORY EXAMINATION FOR PLASMA VASOPRESSIN

Patients and Controls

Between 1998 and 2007, we enrolled 20 patients at Osaka University Hospital with ipsilateral type of DEH to examine their pAVP level. We also enrolled 87 patients with unilateral MD and 30 patients with chronic otitis media (OM) without any direct inner ear damage. Before collecting blood samples, we were given permission from all the patients with DEH, MD, and OM. Blood samples in all 3 groups were collected in the early morning of the day of surgery. Endolymphatic sac drainage was performed as an inner ear surgery for DEH and MD, and tympanoplasty was performed as a middle ear surgery for OM. There were no significant differences in patients' background (sex and age) among DEH (M/F = 10:10, 36.0 ± 2.5 yr), MD (M/F = 38:49, 47.2 ± 1.4 yr), and OM (M/F = 19:11, 45.4 ± 2.5 yr) except for age (patients with DEH were the youngest of all).

Patients with MD did not have any vertigo attacks and did not take any medicine for endolymphatic hydrops after hospitalization. Patients in all 3 groups took the same kind of nonrestricted meals before surgery and had no water from the morning on the day of surgery. Patients' conditions of medication, meals, and water intake at the collection of blood samples were almost the same in all 3 groups and were thought to have no influence on the pAVP level.

Procedures

The blood for a pAVP assay was transferred into an ethylenediaminetetraacetic acid tube and centrifuged at 4°C, and the separated plasma was stored at −80°C. The pAVP was determined by radioimmunoassay (arginine vasopressin radioimmunoassay kit; Mitsubishi, Tokyo, Japan). The normal pAVP level ranged from 0.3 to 4.2 pg/ml (mean, 2.25 pg/ml) based on the data acquired by blood samples collected at 8:00 to 10:00 AM from 105 healthy subjects with their informed consent (61 men, 44 women) who had no history of vestibular or cochlear disease (11).

MOLECULAR EXAMINATION FOR VASOPRESSIN RECEPTOR

Patients and Controls

Before surgery, we obtained permission for collection of the endolymphatic sac tissue during surgery from 6 of 20 patients with ipsilateral DEH and from 9 of 87 patients with unilateral MD mentioned. We also prepared 6 patients with acoustic neuroma (AN) without any direct endolymphatic sac damage as controls. Tissue samples from a part of the endolymphatic sac in groups, DEH, MD, and AN, were collected during surgery (endolymphatic sac drainage for DEH and MD groups and acoustic neuroma removal surgery for the AN group). There were no significant differences in patients' background (sex and age) among DEH (M/F = 3:3, 34.8 ± 4.2 yr), MD (M/F = 4:5, 47.9 ± 4.9 yr), and AN (M/F = 3:3, 53.0 ± 6.5 yr) except for age (patients with DEH were the youngest of all).

Tissue Preparation

For real-time polymerase chain reaction (PCR; DEH = 1–6, unilateral MDs = 1–9, ANs = 1–6) and Western blotting (DEH = 4–6, MDs = 7–9, ANs = 4–6), tissues were obtained from the endolymphatic sac during endolymphatic sac drainage for DEH and MD groups or from AN removal surgery for the AN group, placed 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, Alameda, CA, USA).

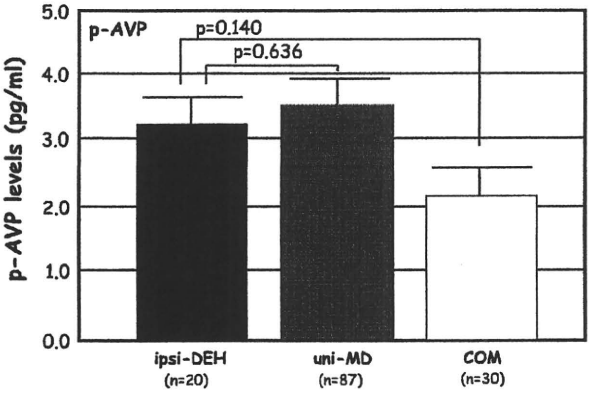

FIG. 1. Plasma vasopressin levels in patients with DEH compared with control patients. Plasma vasopressin was 1.5 times higher in patients with ipsilateral DEH (ipsi-DEH; n = 20; 3.10 ± 0.58 pg/ml) than in control chronic otitis media (COM) patients (n = 30; 2.11 ± 0.38 pg/ml) during the early morning of the day of surgery, although this difference was not statistically significant (unpaired *t* test, $p = 0.140$). There were no significant differences between the pAVP levels in patients with ipsi-DEH and unilateral MD (uni-MD; n = 87; 3.47 ± 0.35 pg/ml; unpaired *t* test, $p = 0.636$).

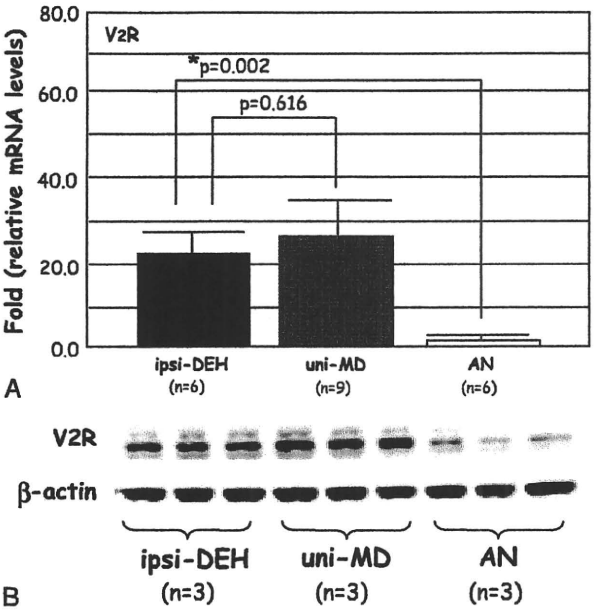

FIG. 2. V2 receptor mRNA and protein expression levels in the endolymphatic sac in patients with DEH compared with control patients. *A*, V2 receptor mRNA expression in the endolymphatic sac was 35.8 times significantly higher in patients with ipsilateral DEH (ipsi-DEH; n = 6; 22.21 ± 5.28-fold) than in control AN (AN) patients (n = 6; 0.62 ± 0.10-fold) as evaluated by real-time PCR (unpaired *t* test, $*p = 0.002$). There were no significant differences between the V2R mRNA expression levels in patients with ipsi-DEH and unilateral MD (uni-MD; n = 9; 27.86 ± 8.19-fold; unpaired *t* test, $p = 0.616$). *B*, V2R protein expression in the endolymphatic sac was also higher in patients with DEH and MD than in control AN patients as evaluated by Western blotting.

Briefly, samples were homogenized 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, Nutley, NJ, 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, Inc., Foster City, CA, USA), 30 μl of 25 mmol/L $MgCl_2$, 4 μl of 25 mmol/L of each deoxynucleotide triphosphate, 5 μl of 100 μmol/L 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 minutes, 48°C for 30 minutes, and 95°C for 5 minutes in a 9600 Thermocycler (Applied Biosystems).

Reverse Transcription–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 transcription–PCR. Samples without reverse transcription were also forwarded for PCR as negative controls to make sure of no genomic DNA contamination.

TABLE 1. *Raw data for 6 patients with DEH (A) and 9 patients with MD (B)*

	pAVP, pg/ml	V2R mRNA, fold	V freq, per mo	H level, dB	Duration, mo
(A)					
Ipsi-DEH 1	1.0	40.74	2.0	115.0	36
Ipsi-DEH 2	1.4	25.11	1.0	100.0	60
Ipsi-DEH 3	1.7	28.54	1.3	101.3	18
Ipsi-DEH 4	4.7	18.78	4.3	112.5	45
Ipsi-DEH 5	6.9	18.33	4.0	115.0	30
Ipsi-DEH 6	11.6	1.76	1.0	115.0	60
(B)					
Uni-MD 1	0.5	64.78	1.0	30.0	34
Uni-MD 2	0.8	69.28	1.0	45.0	60
Uni-MD 3	1.3	13.72	1.3	66.3	84
Uni-MD 4	2.0	20.18	3.3	60.8	98
Uni-MD 5	2.7	1.90	1.7	70.0	48
Uni-MD 6	2.7	32.38	7.3	57.5	18
Uni-MD 7	3.5	29.52	8.0	66.5	30
Uni-MD 8	4.2	17.32	4.0	60.0	48
Uni-MD 9	6.0	1.70	2.0	58.5	60

The raw data for 6 patients with ipsilateral DEH (ipsi-DEH) and 9 patients with unilateral MD (uni-MD) include the pAVP level, V2R mRNA expression level in the endolymphatic sac, vertigo frequency (V freq), hearing level (H level), and duration of disease (Duration) before surgery. In both ipsi-DEH (*Pearson test, $r = -0.92$, $p = 0.009$) and uni-MD patients (**Pearson test, $r = -0.68$, $p = 0.043$), there were significantly negative correlations between pAVP and V2R mRNA expression in the endolymphatic sac.

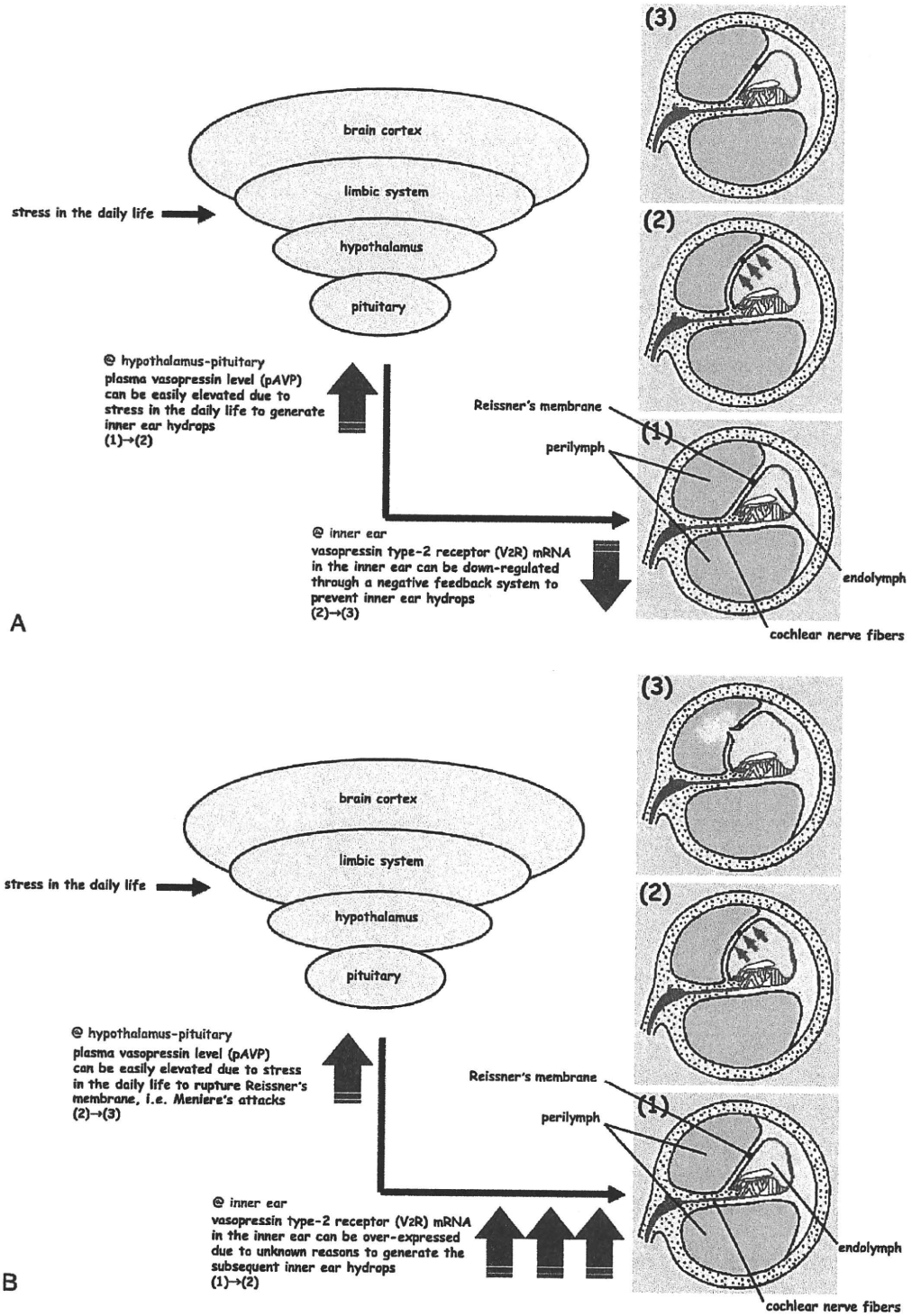

FIG. 3. Schematic representation of a hypothetical mechanism for endolymphatic hydrops generation and subsequent attacks of DEH and MD via pAVP and V2R. *A*, Healthy subjects: civilized people are frequently exposed to stress in their daily life, and pAVP can easily become elevated at any time (1)→(2). Therefore, a negative feedback system between pAVP and V2R in the inner ear may function for inner ear fluid homeostasis against stress-induced increases in pAVP (2)→(3). *B*, Patients with MD: autoimmune responses and/or virus infections in early childhood can lead V2R overexpression and hypersensitivity of cyclic AMP–linked signaling in the inner ear (1). After years, endolymphatic hydrops can gradually be generated (2). In the V2R-overexpressing and cyclic AMP–hypersensitized inner ear, the Reissner membrane can rupture after even a small elevation of pAVP due to stress, thereby resulting in attacks of MD (3). Upward arrows indicate upregulation; downward arrows, downregulation; number of arrows, strength of regulation.

Polymerase chain reaction products were electrophoresed on 3% Seakem GTG agarose gel (FMC Bioproducts, Rockland, ME, 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

Polymerase chain reactions were performed in the presence of the oligonucleotide primers for V2R (NM: 000054; forward: actgtgaggatgacgctagtgattg; reverse: ggacacgctgctgctgaaag) (Takara, Kyoto, Japan) and β_2-microglobulin (*B2M*) (NM: 004048; forward: cgggcatt cctgaagctga; reverse: ggatggatgaaacccagacacatag) (Takara) 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 mmol/L $MgCl_2$, 4 μl of each deoxynucleotide triphosphate (blended with 2.5 mmol/L of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate and 5 mmol/L of 2′-deoxyuridine 5′-triphosphate), 2.5 μl of each gene-specific primer (5 μmol/L), 0.5 μl of AmpErase UNG (0.5 U), 0.25 μl of AmpliTaq Gold (1.25 U), and 5 μl (250 ng) of complementary DNA in a final volume of 50 μl. The conditions for the real-time PCR were as follows: 50°C for 2 minutes, 95°C for 12 minutes, and 35 cycles at 95°C for 15 seconds and 60°C for 1 minute 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 predetermined 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 normalized 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 $ddCT = (CT_{target} - CT_{B2M})_A - (CT_{target} - CT_{B2M})_B$ (12). For example, changes in the gene expression of V2R in endolymphatic sac in DEH compared with AN were quantified as the fold range: 2^{-ddCT} ($ddCT = [CT_{V2R} - CT_{B2M}]_{DEH} - [CT_{V2R} - CT_{B2M}]_{AN}$).

Western Blotting

Samples from the endolymphatic sac were homogenized on ice with a polytron homogenizer (PCU-11; Kinematica, Bohemia, NY, USA) in 20 mmol/L HEPES (pH 7.2), 25 mmol/L NaCl, 2 mmol/L EGTA, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 25 mmol/L β-glycerophosphate, 0.2 mmol/L dithiothreitol, 1 mmol/L phenylmethanesulfonyl fluoride, 60 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1% Triton X-100). After incubating at 4°C for 30 minutes, homogenates were sonicated (Sonifier 250; Branson Ultrasonics, Danbury, CT, USA) on ice for 1 minute and centrifuged at 10,000 × *g* at 4°C for 30 minutes. 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 the sample buffer containing final concentrations of 50 mmol/L Tris (pH 6.7), 2% sodium dodecyl sulfate, and 2% mercaptoethanol. Twenty micrograms of protein extracts was boiled for 10 minutes, cooled to room temperature, and loaded on 10% sodium dodecyl sulfate–polyacrylamide gels. Equal amounts of protein in each sample were further checked by immunoblotting with β-actin monoclonal antibody (diluted to 1:500; Oncogene Research Products, Cambridge, MA, USA).

Proteins were transferred to Hybond–polyvinylidene difluoride membranes (Amersham, 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 (BSA) and normal goat serum (NGS) in 0.3% Triton X-100 in PBS for 3 hours; antisera against V2R (diluted to 1:500; sc-18100-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 24 hours; 0.1 mol/L PBS for 30 minutes; horseradish peroxidase–conjugated secondary antibody (Dako, Carpinteria, CA, USA) in 1% BSA and NGS in 0.3% Triton X-100 in PBS for 3 hours; 0.1 mol/L PBS for 30 minutes. Protein bands were visualized using an enhanced chemiluminescence detection kit and Hyperfilm MP (Amersham) and analyzed using Scion Image software (Scion Corp., Frederick, MD, USA).

STATISTICAL ANALYSIS

Statistical differences of patients' backgrounds (sex and age) among DEH, MD, and controls were examined by Kruskal-Wallis test among all 3 and then Mann-Whitney *U* test between each 2 (independent variables). Statistical differences of the data among DEH, MD, and controls in Figures 1 and 2 were determined by Kruskal-Wallis test among all 3 and then unpaired *t* test between each 2 (independent variables). Correlations between 2 parameters in Table 1 were analyzed by Pearson test. A multivariate analysis (a multiple regression analysis) was conducted to determine the relative predictive value of pAVP, V2R mRNA levels, and duration of DEH or MD for vertigo frequency. $p < 0.05$ were considered to indicate statistical significance. All the statistical analyses in the present study were performed using SPSS version 14.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

The raw data for 6 patients with ipsilateral DEH and 9 patients with unilateral MD, including their pAVP level,

V2R mRNA expression level in the endolymphatic sac, vertigo frequency (number of attacks per month), hearing level (four-tone average of 0.25, 0.5, 1, and 2 kHz) and duration of disease before surgery, are shown in Table 1.

The pAVP level was 1.5 times higher in patients with DEH (n = 20; 3.10 ± 0.58 pg/ml) than in control OM patients (n = 30; 2.11 ± 0.38 pg/ml), although this difference was not statistically significant (unpaired *t* test, $p = 0.140$; Fig. 1). There were no significant differences between the pAVP levels in patients with DEH and MD (n = 87; 3.47 ± 0.35 pg/ml; unpaired *t* test, $p = 0.636$).

The V2R mRNA expression level in the endolymphatic sac was 35.8 times higher in DEH patients (n = 6; 22.21 ± 0.58-fold) than in control AN patients (n = 6; 0.62 ± 0.10-fold), and this difference was statistically significant (unpaired *t* test, $p = 0.002$; 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 patients with DEH and MD (n = 9; 27.86 ± 8.19-fold; unpaired *t* test, $p = 0.616$).

In both groups of patients with DEH (Pearson test, $r = -0.92$, $p = 0.009$) and MD (Pearson test, $r = -0.68$, $p = 0.043$), there were significantly negative correlations between pAVP and V2R mRNA expression in the endolymphatic sac (Table 1). A multivariate analysis (a multiple regression analysis) showed no significant predictive value of pAVP (DEH, $p = 0.87$; MD, $p = 0.78$), V2R mRNA levels (DEH, $p = 0.97$; MD, $p = 0.81$), or duration of DEH or MD (DEH, $p = 0.69$; MD, $p = 0.26$) for vertigo frequency. However, both pAVP (>2.5 pg/ml) and V2R mRNA expression in the endolymphatic sac (>10.00-fold) were relatively higher in Patients 4 and 5 with DEH and in Patients 6 to 8 with MD who experienced relatively more frequent vertigo attacks (≥4.0 attacks/mo).

DISCUSSION

For more than 10 years, it has been pointed out that the pAVP levels in patients with endolymphatic hydrops, including MD and DEH, during remission (13) as well as attacks (11), were significantly higher than those in patients with vertigo because of nonendolymphatic hydrops such as benign paroxysmal positional vertigo and vestibular neuronitis. It was also revealed that systemic injection of vasopressin induced bilateral endolymphatic hydrops and hearing deterioration in guinea pigs (14). These findings led us to the hypothesis that a high level of pAVP is one of the causes of inner ear endolymphatic hydrops in patients with MD and DEH. In contrast, however, it was reported that the pAVP levels in patients with unilateral MD did not differ significantly from those in healthy volunteers (15). Furthermore, the hypothesis of a high level of pAVP, which should have equal effects on both ears, contradicts the fact that 70% to 80% of patients with MD are unilateral (16). In fact, there were no significant differences between the pAVP levels in patients with unilateral and bilateral MD in our recent study (17). Therefore, the above hypothesis is insufficient for explaining the pathogenesis of endolymphatic hydrops, which has remained unsolved since endolymphatic hydrops was demonstrated in patients with MD in 1938 (3,4) and in patients with DEH in 1976 (2).

Regarding vasopressin receptors, V2R molecules have been detected in rat (18,19) and human (18,20) inner ear end organs. 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 pAVP and V2R in the endolymphatic sac attenuated the membranous turnover via cyclic AMP–dependent signaling in a contrasting manner with the kidney in rats (18), and then these interactions also translocated aquaporin 2 from the luminal side to the basolateral side in a contrasting manner with the kidney in human (our unpublished observations). These previous findings indicate that V2R and cyclic AMP–linked signaling could suppress the endolymphatic fluid absorption in the endolymphatic sac.

In the present study, we first detected 35.8-fold higher V2R mRNA expression in the endolymphatic sac in patients with ipsilateral DEH compared with controls, whereas the pAVP level was only elevated by 1.5 times. There were no significant differences in the pAVP or V2R expression in the endolymphatic sac between ipsilateral DEH and unilateral MD. These findings in the present study suggest that V2R overexpression in the endolymphatic sac in ipsilateral DEH as well as unilateral MD could attenuate the membrane turnover and cause the endolymphatic fluid overflow into the endolymphatic space after even a small increase in pAVP. In other words, patients with unilateral V2R overexpression in the endolymphatic sac could develop unilateral endolymphatic hydrops, resulting in attacks of vertigo in the overexpressing ear after even a small elevation in pAVP due to stress in their daily life.

Second, we detected a significantly negative correlation between pAVP and V2R expression in the endolymphatic sac in DEH as well as MD, consistent with the previous study on intact rats (19). These findings suggest that a negative feedback system between pAVP and its receptor in the endolymphatic sac could function for inner ear fluid homeostasis against stress-induced increases in pAVP (Fig. 3A). A negative feedback system between the hypothalamus-pituitary and inner ear has not been clarified yet. However, the direct neuroanatomic interactions between the hypothalamus-pituitary and inner ear were demonstrated electrophysiologically (21,22) and morphologically (23). Inner ear volume and/or pressure changes modified the pAVP secretion (24–26), which indicates that the negative feedback system is really working. Anyway, this negative correlation may explain why the previous studies of pAVP in MD and DEH produced a large variety of results and have been quite controversial (10, 11,13,15).

Finally, we would like to speculate about the possible causes for attacks associated with inner ear abnormality in

MD and DEH. It has been reported that MD is usually triggered by immune, infectious, traumatic, or other insults to the inner ear in association with a small misplaced malfunctioning endolymphatic sac (27–29). Among these insults, immune-mediated responses in the inner ear end organs, such as the endolymphatic sac, stria vascularis, and spiral ligament, are thought to be the main bases for the fluid homeostatic disorder in MD (30,31). 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 (32,33). Taken together with the present data, it is suggested that autoimmune responses and/or virus infections could modify the V2R regulatory system in the endolymphatic sac, resulting in V2R overexpression there (Fig. 3B-1). Years after such insults to the inner ear, hardworking people tend to become frequently exposed to stress in their daily life (1). In the V2R-overexpressing inner ear, endolymphatic hydrops could gradually be generated (Fig. 3B-2), and the Reissner membrane could rupture (2) after even a small elevation in pAVP due to stress, thereby resulting in attacks of MD and DEH (Fig. 3B-3).

CONCLUSION

Civilized people are frequently exposed to stress in their daily life, and pAVP can easily become elevated at any time. Therefore, a negative feedback system between pAVP and its receptor, V2R, in the endolymphatic sac may function for inner ear fluid homeostasis against stress-induced increases in pAVP. For the pathogenesis of endolymphatic hydrops resulting in vertigo attacks in MD and DEH, pAVP may represent a matter of consequence, but V2R overexpression in the endolymphatic sac could be much more essential as a basis for these diseases.

The results of the present study encourage us to continue our investigations and ascertain ideal treatments for the inner ear in patients with DEH as well as MD, ranging from psychotherapy for leading a stressless life to gene therapy for the stress hormone receptor V2R regulation in the inner ear.

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ORIGINAL ARTICLE

Analysis of gene expression profiles along the tonotopic map of mouse cochlea by cDNA microarrays

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Abstract

Conclusion: This study demonstrated differential gene expression profiles along the axis of the mouse cochlea. It also suggests the mechanism that establishes the tonotopic organization. *Objectives:* The molecular basis of the tonotopic mapping of the mammalian cochlea remains unclear. We therefore examined the genes that were abundantly expressed in either the apex or the base of mouse cochlea. *Materials and methods:* Apical and basal portions of cochlea, which contained the organ of Corti and spiral ganglion neurons, were independently dissected from 10 adult C57BL/6 mice, and their total RNAs were respectively isolated. The gene expression profiles of each of these two pools were examined and compared to each other by the complementary DNA microarray technique. *Results:* Of 20 289 probes tested, 64 genes were found to be expressed in the apical portion more than fivefold more abundantly than in the basal portion, and 77 genes were vice versa. Of interest, the genes of several functional proteins such as β2- and γ2-subunits of $GABA_A$ receptors dominated the basal part, indicating that these molecules may be involved in high-frequency tuning of the hair cells and/or ganglion neurons.

Keywords: *Cochlea, tonotopic mapping, cDNA microarray, gene profile, mouse*

Introduction

The cochlea of the inner ear is the peripheral auditory organ that converts vibrations of sounds to electrical signals and transmits them to the central nervous system. These tasks are achieved mainly by hair cells, which are the primary receptors for hearing and the constituents of the organ of Corti [1–3]. The mechanical stimulation by sounds opens mechano-sensitive channels at the tip of the hair cells' stereocilia, which allows influx of K^+ from endolymph to the cells and excites them. Afterwards, a neurotransmitter glutamate is released from the hair cells and stimulates the dendrites of the spiral ganglion neurons [2]. The neurons further activate auditory nuclei of brain via their axons. Therefore, two components of the cochlea, i.e. the organ of Corti containing hair cells and the ganglion neurons, are essential for hearing.

One of the features of the cochlea is that this organ is tonotopically orientated: the hair cells at the apical portion are sensitive to low frequencies and those at the basal side vice versa. Previous studies suggested that this monotonic tuning map involved various elements in different species. In amphibians, reptiles, and birds, stimulation of hair cells caused the oscillation of their membrane potential. Its frequency, called 'electrical resonant frequency,' is unique to a hair cell at a respective portion along the axis of the cochlea. The oscillation serves as the electrical filter to maximize a hair cell's response to sounds of particular frequencies and is at least partially determined by differential expression of variants of Ca^{2+}-activated K^+ channels along the tonotopic map [4–7]. The length of hair bundles is longer in the hair cells of the apex, the mechanism that tunes the bundles sensitive to lower frequencies. Furthermore, synaptic vesicles are specialized to fuse a membrane of the hair cell most efficiently at its characteristic frequencies [8]. In mammals, the stiffness of the basilar membrane varies along the cochlear axis, which plays a pivotal role in amplifying a specific frequency of sound-induced vibration in

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each portion [9]. The adaptation time constant of mechano-electrical transduction current becomes faster from the apex to the base, which imposes a bandpass filter on transduction and improves the signal-to-noise ratio near threshold [10]. In spite of this evidence, the precise machinery determining tonotopic organization is still poorly understood.

To identify the functional molecules that underlie the tuning property of the mammalian cochlea, in this study we examined and compared gene expression profiles in the organ of Corti and spiral ganglion neurons between the apical and basal portions of the cochlea by microarray technology. The genes that were found to be differentially expressed along the tonotopic map were of the proteins involved in a variety of signal transductions such as ionic transports, neurotransmission, and protein modification.

Materials and methods

Tissue dissection and RNA extraction

All animals were handled in accordance with the guidelines of the Animal Care Committee of Osaka University Medical School. Ten C57BL/6 mice aged 6 weeks were euthanized by decapitation, and wetted with 70% ethanol to prevent contamination of samples with RNase-rich hairs. Cochleae were rapidly removed from the temporal bone and transferred to a 5 ml bath of RNA-later solution (Ambion, Austin, TX, USA). After removing the otic capsule, spiral ligament, and stria vascularis from the cochlea, the organ of Corti and modiolus were dissected and separated into apical and basal segments. All of these procedures were performed in RNA-later solution on a chilled plate to prevent RNA degradation. The pooled samples were homogenized with a micro-homogenizer (Microtec, Chiba, Japan) for 15 s, and total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of extracted total RNA was confirmed with bioanalyzer biosizing gel in which the 28S, 18S, and 5S ribosomal bands were clearly visible (data not shown) by using an Agilent Technologies 2100 Bioanalyzer-Bio Sizing (Agilent Technologies, Palo Alto, CA, USA).

RNA labeling and microarray hybridization

Experiments were conducted according to the manufacturer's instructions for CodeLink Expression Bioarrays (Amersham Bioscience, Piscataway, CA, USA). Total RNA was used to produce single-stranded cDNA in the presence of T7-24 Oligo(dT) promoter primer and reverse transcriptase (RT). After double-stranded cDNA synthesis with DNA polymerase I and RNase H, the cDNA was prepared as the template in an *in vitro* transcription reaction to produce the target cRNA. The *in vitro* transcription reaction was performed in the presence of biotinylated nucleotides to label the target cRNA. A bacterial mRNA was used as positive control for the cDNA synthesis and in vitro transcription reaction. Each step of the procedure was monitored using these control mRNAs.

The biotin-labeled cRNA was fragmented randomly by incubation in the presence of magnesium for 20 min at 94°C. Fragmented target cRNA (10 μg) was used for hybridization with a CodeLink Uniset Mouse 20K Bioarray chip (Amersham), which was spotted by 20 289 probes including 108 positive control genes and 300 negative control genes.

Microarray analysis

Hybridized microarray chips were washed and processed using a direct detection method of the biotin-labeled transcripts by Streptavidin-Cy5 conjugates. Slides were scanned by CodeLink scanning software (Motorola Life Sciences, Pasadena, CA, USA). Images for each slide were quantified using the CodeLink expression analysis software (Motorola Life Sciences). The signal intensity for each spot was calculated by summation of the pixel intensities in each spot, and subtracting the local background (based on the median pixel intensity of the area surrounding each spot). In each slide, signal intensities were normalized by dividing the intensity of each spot (after background subtraction) by the median signal intensity of all spotted probes.

Results

Total RNAs isolated from either the apical or basal portion of the cochlea were assayed by cDNA microarray technique. We tested 20 289 probes for each experiment. When the signal intensity of the probe in the apex was more than fivefold stronger or weaker than that of the base, we concluded that the difference was significant. We identified that 64 probes provided higher intensity for the pool of the apical part than for that of the basal part. Of these genes, the products of 39 genes (61%) had been named whereas the others were categorized to expressed sequence tag (EST) sequences (25 genes: 39%) (Figure 1a). On the other hand, we found that 77 genes were much more abundant in the base than in the apex: 60 genes (78%) had been named and 17 genes (22%) belonged to EST sequences (Figure 1b). We classified some of the genes, of which products had been physiologically characterized, into functional categories (Tables I and II). Accession

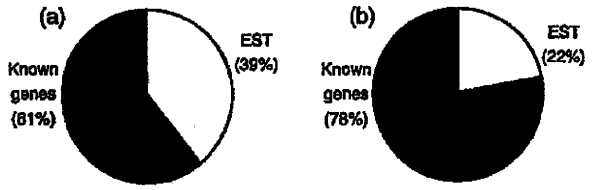

Figure 1. Classification of genes. (a) Sixty-four genes with higher expression in the apex. Of these genes, 39 genes (61%) had been named whereas the others were categorized to expressed sequence tag (EST) sequences (25 genes, 39%). (b) Seventy-seven genes with higher expression in the base. 60 genes (78%) had been named and 17 genes (22%) belonged to EST sequences.

numbers of the aforementioned EST sequences are shown in Table III.

Of the genes dominant in the base, five genes were of ion transporters and the products of seven genes were related to neurotransmission. In contrast, the group of the genes highly expressed in the apex contained only one cDNA of ion transporter and nothing associated with neurotransmission.

Discussion

Using the cDNA microarray technique we determined that 141 genes were differentially expressed between the apical and the basal portions of the cochlea. Although the expression pattern of mRNA does not always correlate with that of the protein, our finding may provide a mechanistic insight into the system underlying the tonotopic organization of the mammalian cochlea.

Among the highly expressed gene in the base, five genes were in the ion transporter group and seven genes were related with neurotransmitter, while among the highly expressed genes in the apex, only one gene was classified as an ion transporter and there were no genes associated with neurotransmitters. It is conceivable that the basal, high-frequency neurons

Table I. Genes showing higher expression in the apex by categories.

Function	Accession no.	Gene	Ratio (apex:base)
Structural cytoskeleton	NM_009406.1	Troponin I, cardiac (TNNI 3)	10.6
	NM_010867.1	Myomesin 1 (MYOM1)	5.3
	AJ002521.1	Myosin heavy chain 2A (MYH2)	5.3
	NM_010052.1	Delta-like 1 homolog (Drosophila) (DLK1)	7.1
Cell adhesion	NM_008402.1	Integrin alpha V (CD51) (ITGAV)	9.3
Membrane transport	NM_008429.1	Potassium inwardly-rectifying channel, subfamily J, member 9 (KCNJ9)*	14.9
	NM_021889.2	Synaptotagmin 9 (SYT9)	5.8
	NM_009205.1	Solute carrier family 3, member 1 (SLC3A1)	12.1
Transcription factor or nucleic acid synthesis and modification	NM_011623.1	Topoisomerase (DNA) II alpha (TOP2A)	5.5
	NM_009392.1	T-cell leukemia, homeobox 2 (TLX2)	6.1
	NM_010569.2	Inversin (INVS)	7.4
	NM_009238.1	Sry-box containing gene 4 (SOX4)	6.0
	NM_010635.1	Kruppel-like factor 1 (Brythroid) (KLF1)	5.4
	NM_053111.1	Eosinophil-associated ribonuclease 6 (EAR6)	5.0
	NM_007894.1	Eosinophil-associated ribonuclease 1 (EAR1)	5.7
Protein modification	NM_008079.1	Galactosylceramidase (GALC)	7.1
	NM_019775.2	Carboxypeptidase B2 (CPB2)	8.8
	NM_145217.1	Diras family, GTP-binding ras-like 1 (DIRAS1)	5.5
Signal transduction	NM_011611.1	Tumor necrosis factor receptor superfamily, member 5 (TNFRSF5)	5.3
Hormone	NM_008117.1	Growth hormone (GH)	7.1
	NM_009889.1	Glycoprotein hormones, alpha subunit (CGA)	20.0
	NM_011164.1	Prolactin (PRL)	12.6
Immune or inflammatory response	NM_018866.1	Chemokine (C-X Motif) ligand 13 (CXCL13)	6.0
	NM_008327.1	Interferon activated gene 202A (IFI202A)	5.6
Others	NM_010841.1	Metallothionein-like 5, testis-specific (TESMIN) (MTL5)	10.1
	NM_013868.2	Heat shock protein family, member 7 (CARDIOVASCULAR) (HSPB7)	6.7

*Genes related to ion exchange.