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Original Article

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Key Words
Meniere's disease
Plasma arginine vasopressin
Plasma osmolality

Abbreviations
95% CI: 95% confidence interval
AVP: arginine vasopressin
ELP: endolymphatic pressure
PLP: perilymphatic pressure

The association of the plasma vasopressin level during attacks with a prognosis of Meniere's disease

Abstract
An elevation of the plasma arginine vasopressin (AVP) level has been frequently observed in Meniere's disease patients. However, little is known regarding the mechanism behind this elevation. The plasma AVP levels in acute phase were determined in 21 Meniere's disease patients and 16 patients with other types of vertigo. The plasma AVP levels of Meniere's disease patients in the acute phase were significantly higher than in those of other vertigo patients ($p<0.01$). In Meniere's disease patients with abnormally high levels of AVP (more than 3.5 pg/ml) in the acute phase, 36% of patients were resistant to conservative treatments for frequent vertigo attacks for the follow-up period of at least 2years. A significant correlation was observed between the plasma AVP in the acute phase and the highest hearing threshold level at a frequency of 1kHz for the follow-up period of at least 1 year ($r=0.45$, $p<0.05$). These results suggest that the elevation in plasma AVP level in the acute phase is associated with the prognosis of Meniere's disease.

Sumario
La elevación del nivel plasmático de vasopresina arginina (AVP) ha sido frecuentemente observado en los pacientes con enfermedad de Meniere. Sin embargo, se sabe poco de los mecanismos detrás de esta elevación. Se determinaron los niveles plasmáticos de AVP durante la fase aguda en 21 paciente con enfermedad de Meniere y en 16 pacientes con otro tipo de vértigo. Los niveles plasmáticos de AVP en los pacientes en la fase aguda de la enfermedad de Meniere fueron significativamente más elevados que aquellos con otro tipo de vértigo ($p<0.01$). De los pacientes con enfermedad de Meniere y niveles anormalmente elevados de AVP (más de 5.5 pg/ml) durante la fase aguda, 36% de ellos fueron resistentes al tratamiento conservador para los ataques frecuentes de vértigo en un periodo de seguimiento de hasta 2 años. Se observó una correlación significativa entre el nivel plasmático de AVP en la fase aguda y el umbral más elevado en la frecuencia de 1kHz durante el periodo de seguimiento de un año ($r=0.45$, $p<0.05$). Estos resultados sugieren que la elevación plasmática de AVP en la fase aguda está asociada con el pronóstico de la enfermedad de Meniere.

The episodic symptoms of Meniere's disease are related to the fluctuating volume/pressure changes within a closed fluid system. Normally, endolymph moves from the cochlea to the endolymphatic sac where it is absorbed. The volume/pressure in the labyrinth is maintained by a variety of subtle regulatory mechanisms under normal conditions (Juhn et al, 1981). Any disturbance in this process can result in the accumulation of fluid within the membranous labyrinth and cochlea. The resulting endolymphatic hydrops distorts the cochlear duct and produces various hearing symptoms. Vertigo also results from endolymphatic pressure (ELP) changes in the labyrinth and/or from changes in the chemical composition of the endolymph fluid that affects neural transmission (Andrew & Strelioff, 1995).

Although there is an interindividual variation in the values of the ELP and perilymphatic pressure (PLP) in the inner ear of normal animals, the ratio of ELP to PLP is approximately 1, and this ratio normally remains constant for individuals in a prone position. The ratio of ELP to PLP in animals with endolymphatic hydrops is significantly higher than that in normal animals in a prone position (Andrew et al, 1991). A non-adapted imbalance between the ELP and PLP subsequently may cause an alteration in the micromechanism of the inner ear or a change in the microvascular blood flow, however it remains unclear how such altered intralabyrinthine pressure may affect the inner-ear function.

It has been reported that the plasma arginine vasopressin (AVP) level decreases after compression of the ELP, followed by decompression induced by an increase in the plasma AVP in animal study. These changes occur without any changes in the plasma osmolality and plasma sodium level (Bartoli et al, 1989, Podda et al, 1999). In addition, AVP may decrease fluid reabsorption in the endolymphatic sac, thereby inducing endolymphatic hydrops in guinea pigs (Kumagami et al, 1998). These results indicate that plasma AVP release may be partially controlled by inner-ear osmoregulatory mechanisms.

AVP receptors are present in the inner ear, and AVP in the inner ear reduces endolymphatic water reabsorption in the endolymphatic sac. In addition, AVP in the endolymphatic sac of the inner ear has the opposite effect to its normal action by reducing aquaporin translocation (Kumagami et al, 1998). The chronic administration of excess levels of AVP in guinea pigs is associated with the development of endolymphatic hydrops (Takeda et al, 2000). In a clinical study, elevated plasma AVP concentrations were observed in patients with Meniere's disease, and the level is significantly higher in the acute phase in comparison to the remission phase (Takeda et al, 1995, Aoki et al, 2005). These findings lead us to the possibility that repeated attacks with the elevation of AVP in acute phase of Meniere's disease as observed in our previous study may thus result in the development of endolymphatic hydrops. However, Meniere's disease patients showed no elevation in the plasma AVP levels during the remission period, and no elevation in the serum AVP, even in the acute phase of Meniere's disease, was observed in another study (Lim et al, 2003). Therefore, the clinical significance of AVP in patients with Meniere's disease remains unclear.

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Table 1. Summary of reporting guideline.

Numerical value	*Class*
0	A (complete control)
1–40	B (Substantial control)
41–80	C (limited control)
81–120	D (insignificant control)
>120	E (poorer control)
secondary treatment initiated	F

Numerical value=(X/Y)×100, rounded to the nearest whole number, where X is the average number of definite spells per month for the 6–24 months after therapy, and Y is the average number of definite spells per month for the six months before therapy.

Table 2. Staging of Meniere's disease. Staging is based on the four-tor average of the pure tone thresholds at 0.5, 1, 2, and 4 kHz of the wor audiogram during the six months before treatment.

Stage	*Four-tone average (dE*
1	≤25
2	26–40
3	41–70
4	>70

Methods

Subjects and study design

In order to study the plasma AVP level during the acute period in Meniere's disease patients and other cases of vertigo, the clinical data of patients who required admission for severe vertigo attacks were retrospectively analyzed using the AAO-HNS guideline (Table 1). We showed this study to all participants and obtained their informed consent, according to the guidelines of the Ethics Committee. This study population consisted of 21 unilateral Meniere's disease patients, as defined by the 1995 guidelines from American Academy of Otolaryngology—Head and Neck Surgery; AAO-HNS (AAO, 1995), and 16 patients with other causes of vertigo due to peripheral origins (Vestibular neuritis, n=5; sudden deafness with vertigo, n=3; idiopathic labyrinth dysfunction, n=8).

Their plasma AVP levels, plasma osmolality, and plasma sodium levels were obtained during the morning between 8:00–10:00 AM in acute phase of vertigo. The blood samples were also collected during the morning between 8:00–10:00 AM from 16 of the 21 Meniere's disease patients and the 16 patients with other causes of vertigo : mentioned above in the remission phase, which was defined by tl absence of vertigo attacks within one month before and after collectir the blood samples. Meniere's disease patients were classified by stagir based on the four-tone average of the pure tone thresholds at 0.5, 1, and 4 kHz, according to the AAO-HNS guidelines (results with audi metric outcome data may be modified by the use of 4 kHz instead 3 kHz) as shown in Table 2. Four patients were classified as stage 4 patients as stage 2, 11 patients as stage 3, and 2 patient as stage The 16 Meniere's disease patients were followed up for at least 2 ye after collection of their blood for measurement of AVP during the vertigo attacks and of the frequency of vertigo attacks. Among the Meniere's disease patients described in our previous study (Aoki et a 2007), 13 patients could be followed up for at least two years, howeve the other five patients could not be followed up for two years f various reasons. Therefore, we added three new patients (50M, 54M and 72M in Table 3) to this study population.

In the 16 Meniere's disease patients, we observed the results of tl treatment for Meniere's disease according to the AAO-HNS guid lines, based on a numerical value calculated by the frequency definitive vertigo attacks after treatment for two years in comparisc to that for a period of 6 months before conservative treatment we assessed (Table 1). In addition, we investigated the correlatic

Table 3. Duration of Meniere's disease, four-tone average of audiogram, and plasma AVP levels at admission in our hospital. The reportin result of treatment in 16 Menière's disease patients who could be followed up for at least two years.

age	*sex*	*Duration of disease (year)*	*Fourtone average (dB)*	*pAVP (pg/ml)*	*Class after treatme*
28	F	3	20	2.4	A
31	F	2	11	4.1	B
33	M	3	45	7.2	D, F (M,G)
35	F	1	16	3.7	A
45	F	1	34	3.1	A
46	F	2	14	2.9	A
46	M	4	61	7.9	C
50	M	1	61	3.8	D, F (G)
52	F	2	29	4.8	B
53	F	1	61	5.7	B
54	M	3	45	2.7	B
54	M	2	55	8.4	D, F (M)
59	F	3	45	4.6	B
65	F	3	55	2.3	A
72	M	2	75	9.7	D, F (M)
84	F	5	59	3.8	A

F: female, M: male, duration of disease; duration of Meniere's disease before collecting their blood in the acute phase, Four-tone average: average of 0.5, 1, and 4kHz in pure tone audiogram, F (M); Meniett device was used as secondary treatment initiated due to disability from vertigo, F (M, G); intratympar injection of gentamicin was performed because the vertiginous symptoms did not improve after treatment with the Meniett device.

between the plasma AVP in acute phase and the highest hearing threshold levels in pure tone audiograms for the follow-up period of at least one year in the 21 Meniere's disease patients. They included five Meniere's disease patients reported in previous study, who could not be followed up for two years in this study.

Measurement of plasma vasopressin

The blood for a plasma AVP assay was transferred into an EDTA tube and then i centrifuged at 4°C and the separated plasma stored at −80°C. The plasma AVP was determined by an RIA (Arginine vasopressin RIA kit; Mitsubishi Yuka, Japan). The normal range of the plasma AVP level was 0.3–3.5 pg/ml (average 1.2 pg/ml) based on the data acquired from blood samples collected at 8:00–10:00 AM from 105 healthy subjects (male: 61, female 44; with informed consent) with no history of vestibular or cochlear disease. In addition, the plasma sodium concentration and the plasma osmolality were also measured.

Statistical analysis

The statistical significance of the differences between the groups of patients was determined by the use of a two-tailed unpaired t-test, and correlations among the plasma sodium and the plasma osmolality were analyzed using Spearman's correlation test. The correlations of the plasma AVP levels in acute phase and age with hearing threshold levels at each frequency in pure tone audiogram were also analyzed using Spearman's correlation test. Values of $p<0.05$ were considered to be significant. The illustrated values are presented as the mean and 95% confidence interval (95% CI).

Results

Vasopressin and osmolality analysis

There was no significant difference in the mean age between the Meniere's disease group (n= 16, mean: 46 years old, 95%CI; 49–52) and the other vertigo groups (n=16, mean: 52 years old, 95%CI; 46–57, p=0.12). The blood samples from the Meniere's disease patients were obtained at 1.2 days (95% CI; 0.7–1.6 days) and that of other vertigo patients at 1.9 days (95% CI; 1.1–2.7 days) after the onset of a vertigo attack. No significant difference in the sampling days after the onset of vertigo attacks was observed between either group (p=0.15). According to the clinical data, 56% of Meniere's disease patients and 75% of other vertigo patients experienced nausea or emesis in collecting their blood.

The plasma AVP levels of Meniere's disease patients in the acute phase (n=16, 4.8 pg/ml, 95% CI; 3.7–5.9 pg/ml, Figure 1) was significantly higher than that of the other vertigo patients (n=16, 1.9 pg/ml, 95% CI; 1.5–2.4 pg/ml; $p<0.01$, Figure 1). The average plasma AVP level of the 16 Meniere's disease patients in the remission phase (2.0 pg/ml, 95% CI; 1.5–2.5 pg/ml) was significantly lower than that of the Meniere's disease patients in the acute phase ($p<0.01$, Figure 1). No significant difference was observed in the plasma AVP levels between the Meniere's disease patients in the remission phase and other vertigo patients in the acute phase (p=0.79). The average plasma osmolality of the Meniere's disease group in the acute phase was 293 mOsm/kg H_2O, (n=16, 95% CI; 289-296 mOsm/kg H_2O), which was not significantly different from that of the other vertigo patient group in the acute phase (n=16, 291 mOsm/kg H_2O, 95% CI; 288–294 mOsm/kg H_2O; p=0.61, Figure 1). The average concentration of the plasma sodium of the 16 Meniere's disease patients in the acute phase was 140 mEq/l (95% CI; 139–142 mEq/l), while that of the 16 patients with other vertigo in the acute phase was 141 mEq/l (95% CI; 140–141 mEq/l, Figure 1). There was no significant difference in the concentration of plasma sodium between the two groups (p=0.67, Figure 1). In addition, a significant positive correlation was observed between the plasma sodium level and the plasma osmolality in both groups in the acute phase ($p<0.01$, Figure 1).

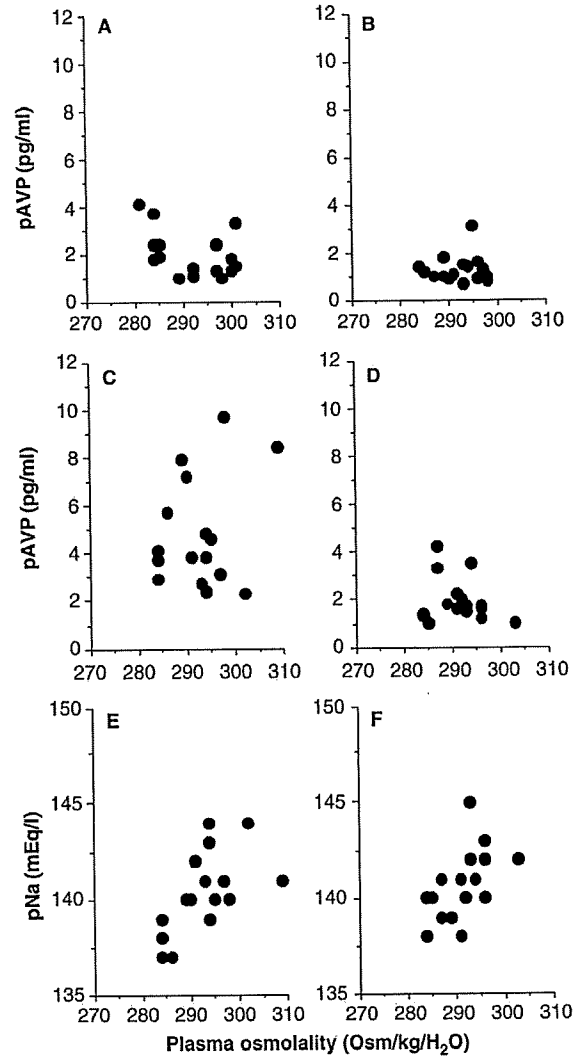

Figure 1. Plasma AVP vs. Plasma osmolality for individual Meniere' disease patients (n=16) and other vertigo patients (n=16) during th remission phase and the acute phase. The plots indicate the actua individual values of plasma AVP of Meniere's disease patients (a) other vertigo patients (b) in the remission phase, Meniere's diseas patients (c), and other vertigo patients (d) in the acute phase. Th correlation between the plasma sodium and plasma osmolality fo Meniere's disease patients (e) and other vertigo patients (f) in the acut phase. A dotted line shows the normal level of plasma AVP (3.5 pg/ml)

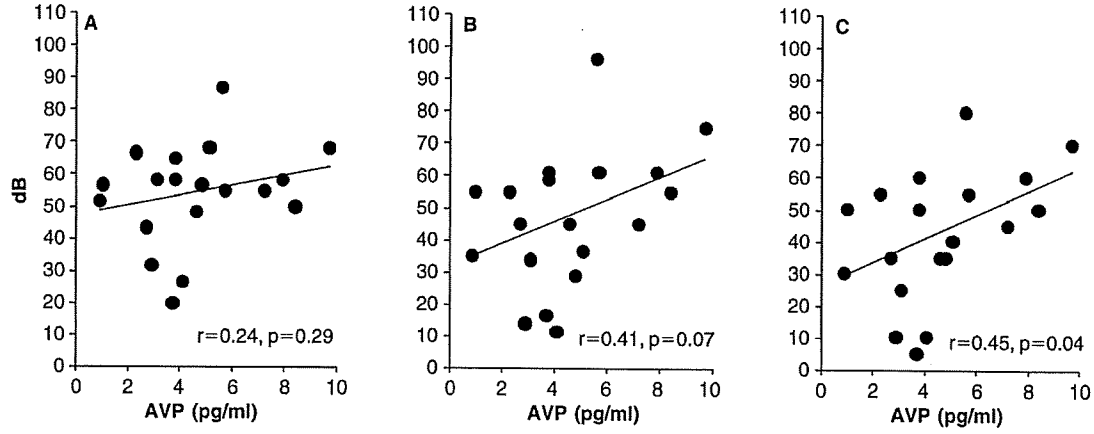

Figure 2. The correlation between the plasma AVP level in acute phase and the hearing thresholds in 21 Meniere's disease patients. A: averag of the highest hearing threshold level at low frequencies (0.125, 0.25 and 0.5 kHz), B: average of the highest hearing threshold level at fou tone frequencies (0.5, 1, 2, and 4kHz), C: the highest hearing threshold level at 1kHz for the follow-up period of at least one year.

Association of the plasma AVP with a prognosis of Meniere's disease

There was a significant correlation between the plasma AVP in acute phase and the highest hearing threshold levels at a frequency of 1kHz for the follow-up period of at least one year in 21 Meniere's disease patients (r=0.45, p=0.04, Figure 2C, Table 4). The plasma AVP levels in acute phase also showed weak correlations with the four-tone average of the highest hearing threshold levels at 0.5, 1, 2, and 4 kHz for follow-up period (r=0.41, p=0.07, Figure 2B, Table 4) and the highest hearing threshold levels at 2kHz for the follow-up period (r=0.43, p=0.06, Table 4). However, there was no significant correlation between the plasma AVP levels in acute phase and the average of the highest hearing thresholds levels at low frequencies of 0.125, 0.25, and 0.5 kHz for the follow-up period (r=0.24, p=0.29, Figure 2A). The plasma AVP level was not correlated with age, however, the hearing threshold level increased with age except for threshold in 8 kHz (Table 4).

Among 16 Meniere's disease patients who could be followed up for at least two years, four patients had been distressed by frequent vertigo attacks resistant to conservative treatments, such as a low-salt diet and diuretic drugs (Table 3). The frequent vertigo attacks in three of those patients (33M, 54M, and 72M in Table 3) were managed by the Meniett Low-Pressure Pulse Generator, which restored the balance in the hydrodynamic system of the inner ear b applying low-pressure pulses to the middle ear (Gates et al, 2004) In addition, two patients (33M and 50M in Table 3) underwent a intratympanic injection of gentamicin for the chemical ablation o the vestibular system (Minor et al, 2004) because the vertiginou symptoms did not improve after treatment with the Meniett devic alone. The plasma AVP levels of the four Meniere's disease patient in acute phase were more than the normal upper level (3.5 pg/ml) whereas all of the Meniere's patients with normal AVP levels i acute phase (less than 3.5 pg/ml) could be managed with conserva tive treatments (Table 3).

Discussion

The elevation of AVP during Meniere's attacks never seemed t accompany a change of plasma sodium and osmolality, whereas proportional relationship between the plasma sodium and plasm osmolality was preserved in patients with Meniere's disease in th acute phase as well as in patients with other types of periphera vertigo in this study. This result may indicate that such patients hav functions to maintain the fluid balance normally. Although severa factors that induce elevated AVP release have been identifie including nausea and emesis (Rowe et al, 1979), there was n

Table 4. The correlations among the plasma AVP in acute phase, age, and the highest hearing threshold levels in pure-tone audiograms for th follow-up period of at least one year.

				Frequency in pure tone audiogram (kHz)						
		AVP	*age*	*0.125*	*0.25*	*0.5*	*1*	*2*	*4*	*8*
AVP	*r*	1	0.11321	0.18846	0.17989	0.32511	0.45042	0.43117	0.28182	0.0814
	p-value	–	0.63463	0.42621	0.4479	0.16191	*0.04627*	0.05769	0.22868	0.73297
age	r	0.11321	1	0.45172	0.52147	0.56368	0.5644	0.63021	0.65376	0.39514
	p-value	0.63463	–	*0.04556*	*0.01837*	*0.00964*	*0.00953*	*0.0029*	*0.00177*	0.08465

r: correlation coefficient.

significant difference in occurrence of nausea and emesis during vertigo attack between Meniere's disease and other vertigo patients in this study. The release of AVP has been reported to be associated with overactivation of the major stress regulation system by psychosocial stress (Scott et al, 1988). We previously reported that the life-change unit (LCU) score to identify the life events that they recognized as stressful in the Meniere's disease group was not significantly higher than that in the other vertigo group. Our results did not support the hypothesis that the elevation in the plasma vasopressin level in Meniere's attacks is caused by the stress status (Aoki et al, 2007). It therefore remains unclear how the elevation of the plasma AVP in Meniere's disease patients may be associated with the vertigo attacks.

The AVP secretion is mainly controlled by cardiovascular volume receptors, whereas the extra-vascular volume receptors in the inner ear may play a role in controlling the water balance not only in the ear but also partially in the whole body (Bartoli et al, 1989). The system in the inner ear may be associated with the release of AVP, which may represent a finer bias of homeostatic control, especially when plasma osmolality is stable. In an animal study, electrical vestibular stimulation and caloric stimulation also increased plasma AVP levels, suggesting that imbalance of intervestibular activity induced by vestibular activation or inhibition may increase plasma AVP levels in animals (Horii et al, 2004). However we could not find the elevation of plasma AVP levels in other peripheral vertigo patients in acute phase. Kitahara et al showed an overexpression and hyperactivity of vasopressin-2 receptors (V2R) in the endolymphatic sac in intractable Meniere's disease patients, thus speculating that susceptibility of the V2R-overexpressed endolymphatic sac to elevation of plasma AVP may be essential for the pathogenesis of Meniere's disease attack (Kitahara et al, 2008). In order to evaluate the precise mechanism for the elevation of the plasma AVP in Meniere's disease patients, further clinical basic studies may be needed. However, our study and other reports may support the possibility that testing of plasma AVP levels may provide helpful information for distinguishing between Meniere's disease and various other inner-ear diseases.

The abnormal elevation of AVP in some patients with Meniere's disease in acute phase may cause inner-ear disorders. This possibility may be supported by the findings of an animal study that demonstrated that AVP produces endolymphatic hydrops, resulting in hearing loss due to a decrease in the endolymphatic potential in an endolymphatic hydrops ear (Lohuis et al, 1999). Takeda et al demonstrated that high levels of plasma AVP (> 5.0 pg/ml) were often observed in patients between 6 and 19 years of age with the early onset of profound hearing loss. Moreover, persistent elevations of plasma AVP were frequently observed in patients with clinical signs of the development of the delayed endolymphatic hydrops (Takeda et al, 2008). In this study, 36% of Meniere's disease patients with abnormally high levels of the AVP (> 3.5 pg/ml) in the acute phase were resistant to symptomatic control of vertigo by conservative treatment. In contrast, all Meniere's disease patients with normal levels of the AVP in acute phase could be managed by conservative treatments. Although, because of the small number of cases, we need further study before making any definitive conclusions, we speculate that the elevation of the AVP may be associated with the Meniere's attack, resulting in irreversible formation of the endolymphatic hydrops.

On the other hand, there was a significant correlation between plasma AVP in acute phase and the highest hearing threshold lev at a frequency of 1 kHz for the follow-up period of at least one y It is highly possible that an elevation of the AVP levels during Menie attacks may influence a prognosis of hearing threshold level. Howe there are still numerous controversial statements regarding pathoph iology and management of Meniere's disease, and additionally th is no means of predicting how the hearing loss may progress. Evaluat the association between plasma AVP and prognosis of Meniere's dise may therefore provide helpful information for understand Meniere's disease patients.

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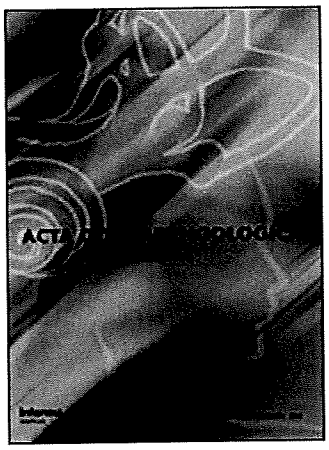

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Subclinical deviation of the subjective visual vertical in patients affected by a primary headache

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ORIGINAL ARTICLE

Subclinical deviation of the subjective visual vertical in patients affected by a primary headache

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Abstract

Conclusion. Our results suggest that patients with migraine or tension-type headache have subclinical deviations of the subjective visual vertical, which may be associated with their subjective imbalance *Objectives.* Patients affected by migraine or tension-type headache often complain of unsteadiness. However, they rarely show a clinical significance in the objective examinations of their equilibrium. We investigated the equilibrium functions in patients affected by migraine or tension-type headache *Subjects and methods.* We investigated the neurotological findings of 17 patients with migraine, 20 patients with tension-type headaches, and 16 patients without headache. All patients in this study experienced vertigo or dizziness before they underwent the examination; however, they never had vertigo attacks for more than 1 month before the examination. All patients in this study were tested during headache-free intervals *Results.* There was no significant difference in the hearing levels of pure tone audiometry, the canal palsy percentage of bithermal caloric test, and the body sway in posturography among the three groups ($p > 0.05$, Mann-Whitney U test). The average values in absolute deviations of subjective visual vertical (SVV) in patients with tension-type headache ($1.3 \pm 1.1°$) and patients with migraine ($1.5 \pm 1.2°$) were significantly larger in comparison with those of patients without headache (0.6 ± 0.4) ($p < 0.05$, Mann-Whitney U test). Intra-individual varaiances of the SVV in patients with primary headache were significantly larger than those in patients without headache ($p < 0.05$, Mann-Whitney U test).

Keywords: *Migraine, tension-type headache, dizziness, subjective visual vertical*

Introduction

Patients with primary headache often complain of dizziness. However, its pathological mechanism has been not evaluated. Migraine has been recognized as causing benign recurrent vertigo and has been associated with several vestibular and cochlear syndromes [1–3]. Some reports demonstrated that patients with migraine have subclinical dysfunctions in the vestibular spinal reflex system, which may be partially due to the subclinical damage to otolith macula [4,5].

Patients with tension-type headache also often report on balance disorders. However, their balance impairments have been underestimated in the clinical objective examination. The tenderness of muscle around the cranium and cervical muscle in patients affected by tension-type headache is known to be significantly higher than in normal subjects [6]. In fact, patients with chronic cervical pain and concomitant dizziness have balance disturbance, as compared with healthy subjects. The altered tenderness of cervical muscle can result in abnormal proprioceptive information [7]. It is highly possible that the abnormal information may influence the proprioceptive cues for postural control, resulting in induction of the unsteadiness. However, the pathophysiological mechanism of dizziness in patients affected by tension-type headache remains unclear.

Clinical studies emphasize that the subjective visual vertical (SVV) is a sensitive tool for detecting an imbalance in otolith function [8,9]. Normal subjects can adjust an illuminated rod in an otherwise completely dark room to vertical within a mean error of $< 2°$. The ability depends primarily on proprioceptive and vestibular cues, the latter usually

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equated with the tonic afferent input from otolith organs and central graviceptive pathways [10,11]. The purpose of this study was to investigate the SVV in patients affected by migraine or tension-type headache to evaluate their subjective vertical, which may be related to the subjective imbalance.

Subjects and methods

Study populations

The study population consisted of 104 patients <70 years old in our clinic. They had experienced vertigo or dizziness; however, they never had vertigo attacks for more than 1 month before they underwent the examination for this study. We performed clinical neurotological examination on all patients, comprising audiogram, posturography, caloric test, vestibular-evoked myogenic potential (VEMP), and SVV after obtaining informed consent from patients and the local Ethical Committee's agreement. An inquiry regarding their headache revealed that 88 of the 104 patients in this study had a history of headache and 16 patients had not experienced headache. Based on the International Headache Society Criteria (2004) [12], we diagnosed 20 patients affected by frequent episodic tension-type headache, 17 patients affected by migraine (4 migraine with aura, 13 migraine without aura), and 51 patients affected by non-identical headache.

In the patients with primary headache, 8 cases of 17 dizzy patients with migraine had more than one migraine attack per month in the mean frequency of attacks. All dizzy patients with tension-type headache were diagnosed as experiencing episodic tension-type headache, the mean frequency of which was more than 15 days per month. All patients with migraine or tension-type headache in this study underwent all examinations during headache-free periods.

In patients with tension-type headache, two have had vertigos diagnosed as vestibular neuritis, two as benign paroxysmal positional vertigo (BPPV), and one as Meniere's disease. Four patients with migraine have been diagnosed with Meniere's disease, two with migraine-related vertigo, and one with vestibular neuritis. The diagnoses of patients without prevalence of headache included Meniere's disease (one case), vestibular neuritis two), and BPPV (one). The remaining patients have never been diagnosed definitely. The Meniere's disease was diagnosed by the 1995 guidelines from the Committee of Hearing and Equilibrium (American Academy of Otolaryngology-Head and neck Surgery; AAO-HNS) [13], and migraine-related vertigo was diagnosed by the criteria of Furman et al. [3].

Pure tone audiometry

Audiometry with air- and bone-conduction thresholds was performed in all patients at the first consultation in our clinic. We estimated an average hearing level of 0.125, 0.25, 0.5, and 1 kHz as the low-tone threshold hearing level, and an average hearing level of 0.5, 1, 2, and 4 kHz as the four-tone threshold hearing level. The data of the average hearing levels were calculated with the air-conduction threshold of the worst side in each patient.

Static posturography

Fore/aft and lateral body sway were assessed during upright stance (shoeless, feet closed, arms by the sides) using a stabilometric platform (G5500; Anima Co. Ltd, Japan). Body sway was recorded with eyes open and closed, and each condition was recorded for 1 min. The size of the environmental area inside the path defined as ENV and the sum of the path lengths defined as locus length (LNG) were calculated.

Caloric testing

The canal palsy (CP) percentage was calculated based on the results of a bithermal air caloric test at 24°C and 50°C. The caloric test was evaluated for side difference using the maximal slow phase velocities of nystagmus for cold and warm caloric stimulus (25% difference of CP being considered significant). The bilateral hypofunction (maximal slow phase velocities of nystagmus for cold and warm caloric stimulus should not exceed 10°/s) was defined as 100% in CP.

VEMPs electromyogram activity

Electromyogram (EMG) activity was recorded from the upper half of the bilateral sternocleidomastoid (SCM) muscle using surface electrodes, with a reference electrode on the upper edge of the sternum and a ground electrode on the forehead. During each recording session, the subject was instructed to rotate the head towards the contralateral side of the tested ear to keep the SCM muscle under tension. Rarefaction clicks of 0.1 ms duration were presented at a rate of five per second through a headphone. The click intensities were 135 dBSPL referred to the perceptual threshold for normal subjects (0 dB nHL; 40 dB peak-equivalent SPL). The EMG signal was amplified and bandpass filtered (5–2000 Hz), and usually the responses to 100 stimuli were averaged. The response consists of an initial positivity or inhibition (p13) followed by a

negativity or excitation (n23). We measured the amplitude of the p13n23 response for the VEMP.

Measurements of SVV

Subjects were seated upright and their head was held in place by a chin-rest. A 15 cm long rod of charged fluorescent tape 5 mm wide was placed in front of each one at a distance of 1 m. It was settled in height to eye level and in complete darkness (i.e. no frame, no disc) first, and the starting position of the rod was tilted to either the left or right (approximately 40°). Subjects were required to adjust the rod without time constraints to the gravitational vertical with potentiometer by rotating handle. Performance in SVV adjustments was expressed as the deviation from gravitational vertical (0°) measured in degrees. Deviations to the left (counter-clockwise) were counted as negative and deviations to the right (clockwise) as positive. For the SVV measurement, recordings consisted of eight consecutive trials in each patient and the actual values are plotted in Figure 1. The data of the SVV in results are shown as absolute value of the SVV deviations to both sides.

Statistical analysis

Non-parametric Mann-Whitney U test was performed on the parameters of the above examinations among groups because of unequal variances of the data among the groups and the small number of patients in this study. The values are shown as the mean and 1 standard deviation (SD) in this text. The correlation between the LNG of posturography and the deviation of the SVV was analyzed using Spearman's correlation test. F test was used for analysis of inter-individual or intra-individual variances in the deviation of SVV. A level of significance of $p < 0.05$ was adopted throughout the study.

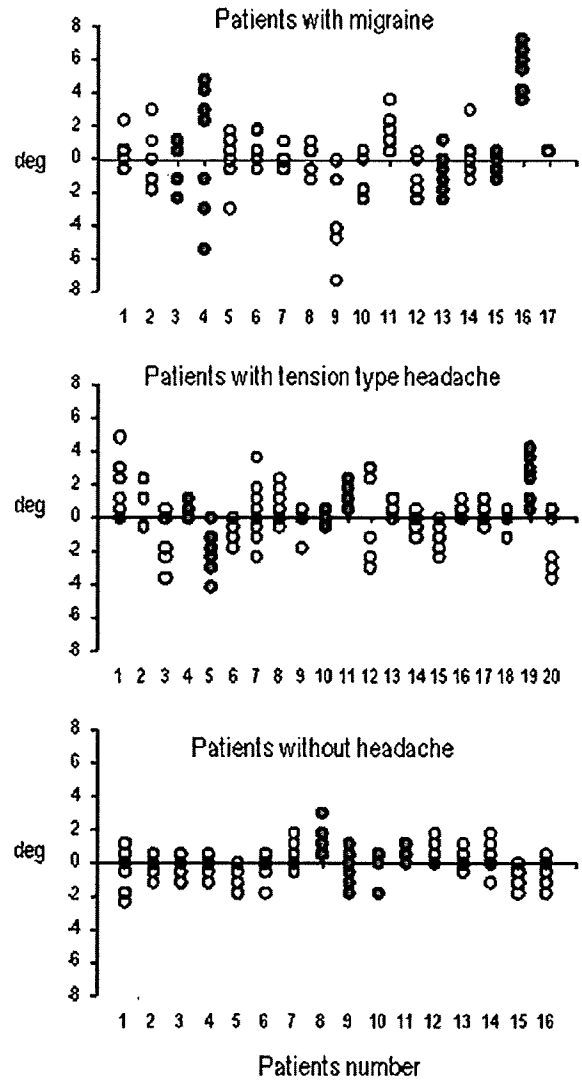

Figure 1. Actual individual values of eight SVV measurements in all patients are shown. The data of patients with diagnoses of peripheral vestibular disorders in each group are shown with closed circles. Deviations to the left (counter-clockwise) were plotted as negative and deviations to the right (clockwise) were plotted as positive.

Results

There was no significant difference in the low-tone average and the four-tone average in pure tone audiogram among the three groups in this study ($p > 0.05$, Table I).

There was no significant difference in the LNG and the ENV of posturography and CP percentage of bithermal caloric test among the three groups ($p > 0.05$, Table I). We defined no response of bilateral VEMP or laterality more than double in the amplitude of the p13n23. In patients with tension-type headache, 30% showed abnormal VEMP responses; however, the abnormal rate was not significantly different from that of patients with migraine (29%) and patients without headache (25%).

The SVVs of patients with migraine ($1.5 \pm 1.2°$) and tension-type headache ($1.3 \pm 1.1°$) were significantly larger, compared with the average SVV of patients without headache (0.6 ± 0.4, $p < 0.05$, Table I). We also calculated the average deviations of the SVV in the participants except for patients who were diagnosed as having Meniere's disease, BPPV, and vestibular neuritis. The average of the SVV deviaitions in 12 patients with migraine and 15 patients with tension-type headache except for patients with diagnoses were $1.3 \pm 1.1°$ and $1.2 \pm 0.7°$, which was

Table I. Neurotological findings in patients with migraine, tension-type headache or without headache.

Parameter	Migraine	Tension-type headache	No headache
Age	42±17	46±17	44±10
Sex (F:M)	11:6	14:6	11:5
Hearing levels (dB)			
Four-tone average	20.4±16.1	18.2±18.2	18.2±9.3
Low-tone average	29.9±17.0	25.7±22.3	27.8±7.2
CP%	10.1±9.2	9.5±13.8	11.1±8.5
LNG (cm)			
Eyes open	92.4±34.2	99.1±48.4	87.3±23.1
Eyes closed	129.4±61.7	148.2±85.6	109.9±43.7
ENV (cm^2)			
Eyes open	6.5±6.7	5.8±4.4	4.8±7.1
Eyes closed	9.9±12.6	9.4±8.5	6.1±8.9
VEMP abnormal rate	29%	30%	25%
SVV (°)	1.5±1.2*	1.3±1.1*	0.6±0.4

Data show mean and standard deviation.
*Significant differences from patients without headache at $p<0.01$.

significantly larger than that in 11 patients without headache except for patients with diagnoses (0.7± 0.2°) ($p<0.05$).

Thirteen of 17 patients with migraine and 13 of 20 patients with tension-type headache showed a deviation of >2° at least once of 8 times in SVV measurements in each patient, while only 3 of 16 patients without headache showed >2° at least once of 8 times in SVV measurements in each patient (Figure 1). To assess the intra-individual variability in the deviations of SVV, we used the F test to determine whether the three groups in this study had different variances in the distribution of the SDs of eight trials in each patient. There was a significant difference in the intra-individual variability between patients with migraine and patients without headache ($F=9.6$, $p<0.01$), and between patients with tension-type headache and patients without headache ($F=5.0$, $p<0.01$). There was no significant difference in the intra-individual variability between patients with migraine and patients with tension-type headache ($F=1.9$, $p>0.05$). The inter-individual variability of the average deviations of SVV in patients with migraine and patients with tension-type headache were significantly larger than those in patients without headache ($F=21.5$, $F=16.2$, $p<0.01$).

The deviation of the SVV was significantly correlated with the LNG of posturography in eyes closed condition in patients with tension-type headache ($r=0.69$, $p<0.01$); although not in patients with migraine ($r=0.30$, $p>0.05$) or without headache ($r=0.25$, $p>0.05$), as shown in Figure 2. There was a significant correlation between the LNG and the deviations of the SVV in patients with tension-type headache except for patients with diagnoses ($r=0.81$, $p<0.01$).

Discussion

In general, patients affected by tension-type headaches often complain of a sensation of floating and drift rather than rotary vertigo. However, their balance impairments have been underestimated in the clinical objective examination because their balance disorders are often ambiguous and virtually non-specific. Kayan and Hood reported that vestibular symptoms including both dizziness and vertigo occur in 54% of patients with migraine compared with 30% of patients with tension-type headache [1]. A recent study demonstrated that the prevalence of vertigo or dizziness was 51.7% in patients with migraine and 31.5% in healthy subjects [14]. It is speculated that patients with migraine have a higher prevalence of balance disorders, while the prevalence of balance disorders in patients with tension-type headache is similar to that in the general population. It is not easy to evaluate the causal relationship between tension-type headache and the balance disorders because such postural instability can be induced by variable causes in the general population.

Despite that there was no significant difference among the three groups in the severity of vestibular disorders that were examined by the bithermal caloric test, the VEMP test, and posturography. The average deviations of the SVV in patients affected by tension-type headache or migraine were significantly larger than those in patients without headache. However, the average deviations of the SVV in both groups of patients with primary headache were within the normal range obtained by healthy subjects (mean 0.57°, normal range; 0–2.06° ($n=208$)). More than 65% of patients with primary headache showed more than 2° at least once in eight trials in each patient, while 18% of patients

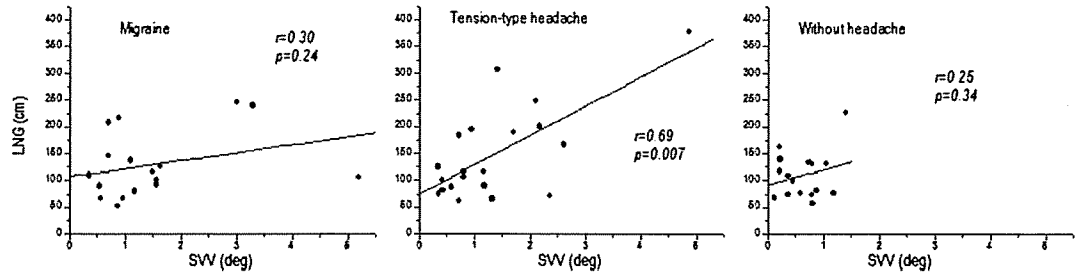

Figure 2. The correlation between the deviation of SVV and the LNG in eyes closed condition. *r*, correlation coefficient, *p*, *p* value.

without headache showed more than 2° at least once in eight trials in each patient. In addition, the inter-individual variances of deviations of the SVV in patients with migraine or tension-type headache were also greater than those in patients without headache. Our results therefore suggest that the affection of migraine or tension-type headache frequency is exerted over the subjective verticality independent of vestibular dysfunctions. However, further studies are needed to evaluate the causal relationship between headache and subjective imbalance.

The maintenance of the postural balance is accomplished by a minimum of three sensory inputs from the visual, proprioceptive, and vestibular receptors. The proprioceptive system is usually important for postural control when the vestibular function is normal. However, patients affected by tension-type headache may depend more on visual cues for postural control because they have proprioceptive alterations induced by cervicofacial muscle contraction [15]. If dizzy patients with impaired vestibular function are affected by tension-type headache, the erroneous input from the neck possibly produces a sensory mismatch resulting in the postural disturbance.

For unilateral utricular lesions, Tabak et al. found a permanent deviation of the SVV of about 2.2° after unilateral vestibular differentiation. It is known that the deviation of SVV is gradually attenuated due to the mechanism of central compensation [16]. In this study, 25% of patients with tension-type headache showed more than 2° in the average deviation in the SVV. There was a significant correlation between the deviation of the SVV and the LNG with eyes closed in the posturography in patients with tension-type headache. Schoenen et al. reported that some patients affected by tension-type headache have deficiency in the central control system of muscle contractions [17]. The abnormal cervical signals induced by peripheral and central origins may influence visual perception of roll–tilt, resulting in the deviation of the SVV [18]. The abnormal signals may induce the subjective unsteadiness.

Thirteen of 17 patients with migraine showed more than 2° at least once in eight SVV measurements in each patient. In addition, the intra-individual variance of the SVV in patients with migraine was significantly larger than that in patients without headache. While the average deviation was not correlated with the LNG in posturography unlikely in patients with tension-type headache. Because the static posturography in this study was not necessarily sensitive enough to detect the deficiency of the vestibular functions, the association between the balance impairments and deviations of the SVV in patients with migraine remains unclear. However, some reports have demonstrated that unilateral otolith dysfunctions [19] and a subclinical cerebellar or brainstem dysfunction [20] may exist in some patients with migraine. The dysfunction may therefore be associated with the deviation of the SVV in patients with migraine in this study.

In conclusion, these results suggest that patients affected by migraine or tension type headaches may therefore have a subclinical abnormality of the subjective visual vertical, thus resulting in the occurrence of a subjective imbalance.

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

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The Laryngoscope

Transplantation of Bone Marrow-Derived Neurospheres Into Guinea Pig Cochlea

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Objectives/Hypothesis. To investigate the potential of neurally induced bone marrow stromal cells (BMSCs) as transplants for replacement of spiral ganglion neurons.

Methods. BMSCs were harvested from the femurs and tibias of adult guinea pigs. BMSCs were cultured with neural induction media and formed spheres. The capacity of BMSC-derived spheres for neural differentiation was examined by immunocytochemistry in vitro. BMSC-derived spheres were injected into the modiolus of the intact cochleae or those locally damaged by ouabain, followed by histological and functional analyses.

Results. In vitro analysis revealed a high capacity of BMSC-derived spheres for neural differentiation. After transplantation into the cochlear modiolus, the survival and neural differentiation of BMSC-derived spheres was observed in both the intact and damaged cochleae. In intact cochleae, transplants settled in various portions of the cochlea, including the cochlear modiolus, whereas in damaged cochleae, transplants were predominantly observed in the internal auditory meatus. Transplantation of BMSC-derived spheres resulted in no functional recovery of the cochlea or protection of host spiral ganglion neurons.

Conclusions. The present findings indicate that BMSC-derived spheres can be a source for replacement of spiral ganglion neurons, although further manipulations are required for functional recovery.

Key Words: Allograft, cell therapy, cochlea, neurosphere, spiral ganglion neuron, regeneration.

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INTRODUCTION

Treatment options for sensorineural hearing loss are currently limited to cochlear implants and hearing aids. Hence, there is a requirement for alternative means of biological therapy, including cell-based therapy. Indeed, recent studies have indicated that cell-based therapy could be utilized as a therapeutic option for inner ear disorders.[1–3] Spiral ganglion neurons (SGNs), primary auditory neurons, are located in the modiolus of the cochlea and transmit sound stimulation to the central auditory system. The loss of SGNs, therefore, compromises auditory function. In addition, SGN loss also reduces the effectiveness of cochlear implants, which can improve impaired hearing by stimulating SGNs. SGNs are, therefore, a primary target for cell transplantation in the auditory system.

Bone marrow stromal cells (BMSCs) are a heterogeneous population of stem/progenitor cells with pluripotent capacity to differentiate toward a neuronal phenotype,[4,5] and consequently the possible use of BMSCs for the treatment of neurological diseases has acquired enormous importance. BMSCs have great potential as therapeutic agents, because they are easy to isolate and expand. Previously, the potential of BMSC transplantation for the treatment of inner ear disorders has been investigated.[6–9] These previous studies have demonstrated that undifferentiated BMSCs are able to settle in the cochlea and have a high capacity for migration. However, limited numbers of transplants differentiated into neurons after transplantation into the intact or damaged cochlea,[6–8] which indicates that neural induction of BMSCs before transplantation is required for SGN replacement by BMSC transplantation.

The aim of this study was to elucidate the neural expression profile of neurally induced BMSCs of guinea pigs and their ability to retain neural differentiation potential when transplanted into the intact or damaged cochleae of guinea pigs. In addition, we examined the capacity of neurally induced BMSCs for functional and histological replacement of SGNs.

MATERIALS AND METHODS

Experimental Animals

A total of 18 Hartley-strain guinea pigs were purchased from Japan SLC Inc. (Hamamatsu, Japan). The Animal

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Research Committee of the Graduate School of Medicine, Kyoto University, Kyoto, Japan, approved all of the experimental protocols. Animal care was carried out under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

BMSCs

Bone marrow was isolated from the femurs and tibias of 6- to 8-week-old guinea pigs (n = 4, Japan SLC Inc.). Under general anesthesia with midazolam (8 mg/kg, intramuscular injection) and xylazine (8 mg/kg, intramuscular injection), the epiphyses of the femurs and tibias were removed, and the marrow was flushed out into a 100-mm culture dish. The isolated bone marrow, composed of hematopoietic and stromal cells, was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Thermo Trace Ltd., Noble Park, Victoria, Australia) and 1% antibiotic-antimycotic (Invitrogen) at 37°C with 5% CO_2. The medium was changed twice weekly until the cells were 80% confluent. Nonadherent cells were removed during the medium-change procedure. The BMSCs were passaged three to five times before use. BMSCs at this stage were defined as undifferentiated.

Neural Induction of BMSCs

For neural induction, cultured BMSCs were enzymatically detached from culture dishes. The BMSCs were plated into 100-mm culture dishes at a density of 2×10^6 cells/well, and cultured in serum-free DMEM/F-12 medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/mL of basic fibroblast growth factor (bFGF, Invitrogen), and 20 ng/mL of epidermal growth factor (EGF, Invitrogen). We added the same amounts of bFGF and EGF every 3 days. After 7 days of culture, BMSC-derived spheres were collected and fixed with 4% paraformaldehyde (PFA) for 15 minutes. The characteristics of BMSC-derived spheres were examined by immunohistochemistry for nestin and Musashi-1. Anti-nestin mouse monoclonal antibody (1:500; BD Biosciences, San Jose, CA) or anti-Musashi rabbit polyclonal antibody (1:500; Chemicon, Billerica, MA) was used as the primary antibody. Alexa Fluor 488-conjugated anti-mouse donkey IgG (1:1000; Invitrogen) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (1:1000; Invitrogen) were used as the secondary antibodies. We counted the numbers of spheres and the number of marker-positive spheres in five randomly selected fields (3.4 mm^2 in area), and then calculated the ratio of nestin or Musashi-1 expressing spheres to the total number of spheres. Four independent cultures were performed.

In Vitro Neural Differentiation

To investigate the ability of BMSC-derived spheres to neurally differentiate, BMSC-derived spheres were plated onto 8-well chamber slides at a density of 100 spheres/well in serum-free DMEM/F-12 medium supplemented with B27, retinoic acid (1 mM, Sigma, St. Louis, MO), and dibutyryl cyclic adenosine monophosphate (AMP) (1 mM, Sigma). After 7 days of culture, the cells were fixed with 4% PFA for 15 minutes and immunostained for beta-III tubulin. Anti–beta-III tubulin rabbit polyclonal antibody (1:500; Sigma) was used as the primary antibody, and Alexa Fluor 488-conjugated anti-rabbit donkey IgG (1:1000; Invitrogen) was used as the secondary antibody. We counted the total numbers of the cells and the number of beta-III tubulin-positive cells in five randomly selected fields (3.4 mm^2 in area), and then calculated the ratio of beta-III tubulin expressing cells to the total number of cells. Four independent cultures were performed.

Transplantation Procedure

After labeling with DiI (Invitrogen, 5 μg/mL), the cell suspension of BMSC-derived spheres (10^5 cells in 10 μL DMEM) was injected into the cochlear modiolus of guinea pigs weighing 300 to 330 g as described previously.[10] Briefly, under general anesthesia with midazolam and xylazine, a small hole was made on the left otic bulla to expose the round window niche and the basal turn of the cochlea. After cochleostomy in the basal turn of the cochlea, a glass pipette, which was connected to a microsyringe (Hamilton, Reno, NV), was inserted into the cochlear modiolus of the basal portion of the cochlea. The glass pipette was removed 1 minute after completion of the infusion. Finally, the cochleostomy site was closed with a fat graft and then covered with fibrin glue.

BMSC-Derived Sphere Transplantation

BMSC-derived spheres were transplanted into intact or damaged cochleae of guinea pigs weighing 300 to 330 g. Four weeks after transplantation, four intact guinea pigs were transcardially perfused with phosphate buffered saline (PBS) at pH 7.4, followed by 4% PFA, and sacrificed under general anesthesia with an overdose of pentobarbital. The temporal bones were immediately dissected out and immersed in the same fixative for 4 hours at 4°C.

Ten guinea pigs received local application of ouabain (5 μL at a concentration of 5 mM in saline; Sigma), which causes SGN degeneration,[9] through the round window membrane under general anesthesia with midazolam and xylazine. One week after application, the electrically evoked auditory brainstem response (eABR), which has been used for functional evaluation of SGNs, was measured before cell transplantation as previously described.[10] Eight animals that showed no eABRs were used in the following experiments. Immediately after the eABR measurements, four animals received transplantation of BMSC-derived spheres similar to intact guinea pigs, and the other four animals received an injection of the culture media and were used as controls. Four weeks later, the cochlear specimens were collected after eABR recording.

Specimens (10-μm thick) were prepared using a cryostat after decalcification with 0.1 M ethylenediaminetetraacetic acid in PBS for 3 weeks at 4°C. Then, immunostaining for beta-III tubulin was performed, followed by nuclear staining with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; 2 μg/mL PBS, Invitrogen). Specimens were viewed with a Leica TCS-SPE confocal laser-scanning microscope (Leica Microsystems Inc., Wetzlar, Germany). Five midmodiolar sections (each separated by 30 μm) were provided for quantitative analyses from each tissue sample. We defined the cells that were positive for DiI with a distinct nucleus identified by DAPI as surviving transplants. The numbers of transplants were counted in the internal auditory meatus and in five cochlear compartments (the modiolus, the scala vestibuli, the scala media, the scala tympani, and the lateral wall). We also counted the numbers of beta-III tubulin-positive transplants, and calculated the ratio of beta-III tubulin expressing transplants to total surviving transplants. In addition, the densities of SGNs in the Rosenthal canals were quantified in ouabain-treated specimens as described previously.[11] All data are presented as the mean ± 1 standard deviation.

Statistical Analyses

We statistically compared the total numbers of surviving transplants and the ratios for beta-III tubulin expression

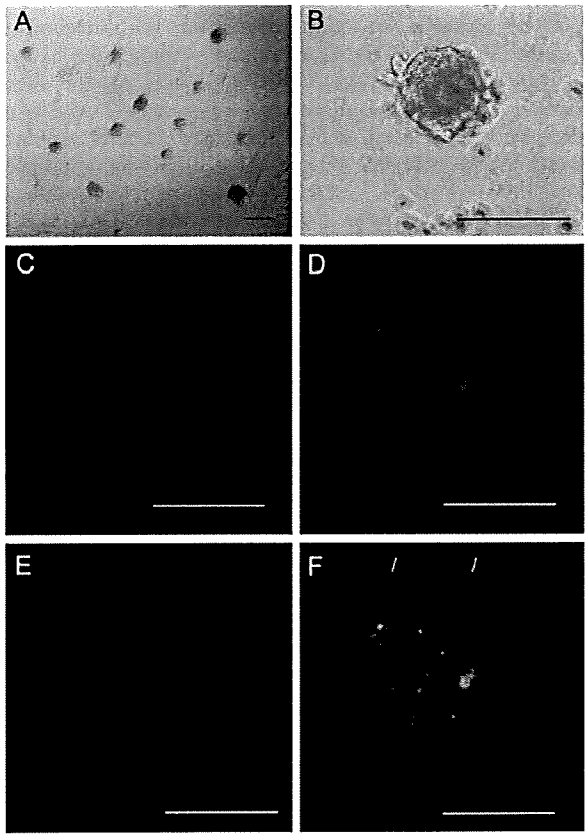

Fig. 1. BMSC-derived spheres. (A, B) Phase contrast images. (C) 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) staining. (D) Immunostaining for nestin. (E) Immunostaining for Musashi-1. (E) Merged image. Scale bars = 500 μm.

between transplants in damaged cochleae and those in intact cochleae, using unpaired *t* tests. The difference in the locations of surviving transplants between damaged and intact cochleae was examined by two-way analysis of variance. In damaged models, the difference in the density of remaining SGNs between control and transplanted cochleae were compared using unpaired *t* tests. *P* values of $< .05$ were considered to be statistically significant.

RESULTS

Neural Induction of BMSCs

After 2 to 3 days in vitro, BMSCs began to form spheres. On day 7, $1.28 \pm 0.71 \times 10^4$ spheres were identified in each culture dish (Fig. 1A, 1B). Immunohistochemistry revealed the expression of nestin and Musashi-1 in the BMSC-derived spheres (Fig. 1C–1E). The expression of nestin was found in 91.9 ± 4.7% of total BMSC-derived spheres, and that of Musashi-1 was found in 93.6 ± 2.9%, suggesting that neurospheres were generated from guinea pig BMSCs.

In Vitro Neural Differentiation

We transferred the BMSC-derived spheres into differentiation medium containing retinoic acid and dibutyryl cyclic AMP to examine their capacity for neural differentiation. Sphere-forming cells attached to culture dishes and the cells migrated from the sphere (Fig. 2A). Then, some of the cells extended processes (Fig. 2B). On day 7, 89.2 ± 2.8% of the cells expressed beta-III tubulin (Fig. 2C, 2D), indicating that BMSC-derived spheres have the capacity to differentiate into neurons.

Transplantation Into Intact Cochleae

DiI-positive transplants were found in all intact cochleae following transplantation of BMSC-derived spheres. In each midmodiolar section, 74.1 ± 44.4 transplants were found. Transplants were located in multiple regions of the cochlea, predominantly in the scala tympani and the modiolus (Fig. 3A, 3C). Transplants were rarely found in the internal auditory meatus. The expression of beta-III tubulin was observed in 18.6% ± 6.4% of transplants (Fig. 3B).

Transplantation Into Damaged Cochleae

DiI-positive transplants were also identified in all transplanted cochleae that had been damaged by ouabain. The number of surviving transplants in each midmodiolar section was 72.1 ± 53.1. There was no significant difference in the number of surviving transplants between intact and damaged cochleae, whereas the locations of surviving transplants in the damaged cochleae significantly differed from those in the intact cochleae ($P = .007$). In the damaged cochleae, the settlement of transplants was observed in the modiolus, similar to observations in the intact cochleae; however, the most prominent region for settlement of transplants

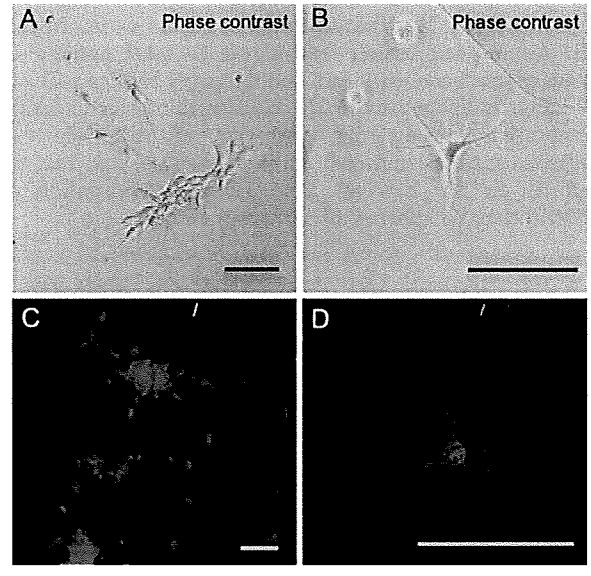

Fig. 2. Neural induction of BMSC-derived spheres in vitro. (A) Phase contrast image on day 3 in vitro. (B) Phase contrast image on day 7 in vitro. (C, D) Immunostaining for beta-III tubulin and nuclear staining with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI). Scale bars = 20 μm.

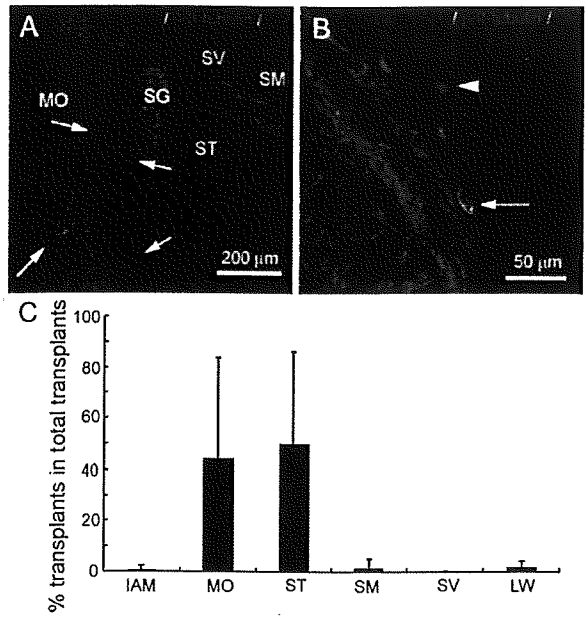

Fig. 3. Transplantation into the intact cochleae. (A) Transplants labeled by DiI (red) are located in the modiolus (MO) of the cochlear basal portion (arrows). (B) Transplant labeled by DiI (arrow) is positive for beta-III tubulin and another (arrowhead) is negative. (C) The locations of transplants in the cochlea and in the internal auditory meatus (IAM). SV = scala vestibule; SG = spiral ganglion; SM = scala media; ST = scala tympani; LW = lateral wall. Bars represent a standard deviation.

was the internal auditory meatus (Fig. 4A, 4C). The expression of beta-III tubulin was observed in 24.1 ± 5.3% of transplants in the damaged cochleae (Fig. 4B). There was no significant difference in the ratio of beta-III tubulin expressing transplants to total number of transplants between intact and damaged cochleae. These findings indicated that SGN degeneration prior to transplantation caused the migration of BMSC-derived spheres into the internal auditory meatus, and had no effects on the survival and neural differentiation of BMSC-derived spheres after transplantation.

Effects of Transplantation on Cochlear Function

We used eABR recording to monitor SGN function. All the animals receiving eABR evaluation showed no responses before an injection of a cell suspension or a culture medium. Four weeks postoperation, positive eABRs were identified in two of four animals in each group. Thresholds of eABRs in the two animals that showed positive responses in the transplanted group were 300 and 400 μA, and those in the sham-operated group were 250 and 650 μA. These findings indicated that transplantation of BMSC-derived spheres into the cochlear modiolus induced no significantly functional recovery of the cochlea.

We quantified SGN densities after cell transplantation or sham operation to evaluate the effects of BMSC-derived spheres on enhancement of the survival of remaining host SGNs. Local ouabain application caused severe degeneration of SGNs, especially in the basal turn of cochleae. No significant differences in the SGN density of the basal, second, or third turn of cochleae were found between transplanted and sham-operated specimens (Fig. 5), indicating that transplantation of BMSC-derived spheres did not promote the survival of remaining host SGNs, which is consistent with eABR results.

DISCUSSION

The present findings demonstrate that guinea pig BMSCs are able to form spheres that have the capacity to differentiate into neurons in vitro. We aimed to replace SGNs, which are located in the cochlear modiolus, with BMSC-derived neurons. We thus directly injected BMSC-derived spheres into the modiolus of intact or damaged cochleae of guinea pigs. For accurate introduction of the cells into the cochlear modiolus, the size of the cochlea is a critical issue. Previously, we tried to introduce transplants into the cochlear modiolus of mice,[12] in which the success rate for the settlement of the transplants was not satisfactory. In addition, functional evaluation following cell transplantation is virtually impossible. On the other hand, guinea pig[10,13] or chinchilla[6] model systems exhibited better settlement

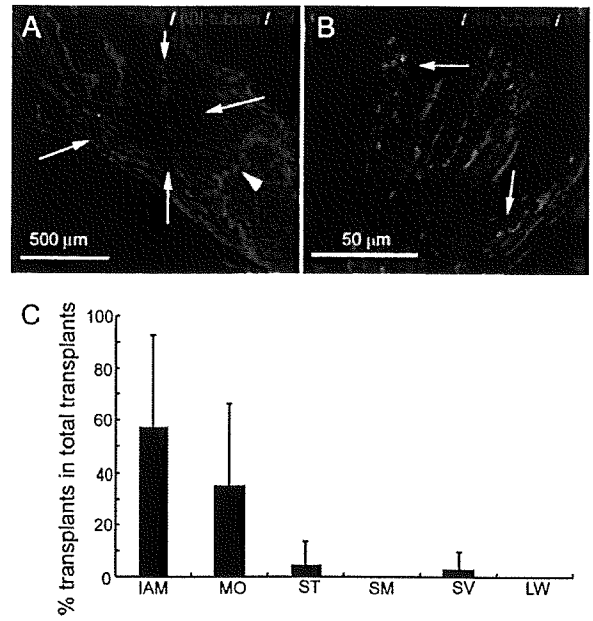

Fig. 4. Transplantation into the damaged cochleae. (A) In the internal auditory meatus (IAM), transplants labeled by DiI (red) are observed (arrows). An arrowhead indicates the location of the glial-schwann junction. (B) A transplant labeled by DiI (arrow) is positive for beta-III tubulin and another (arrowhead) is negative. (C) The locations of transplants in the cochlea and in the internal auditory meatus (IAM). MO = modiolus; ST = scala tympani; SM = scala media; SG = spiral ganglion; SV = scala vestibule; LW = lateral wall. Bars represent a standard deviation.

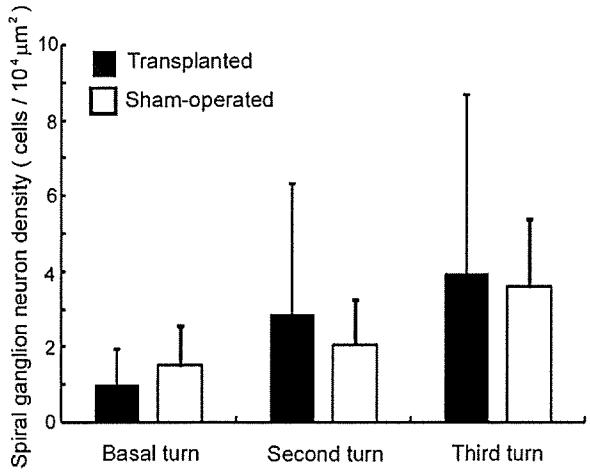

Fig. 5. Densities of remaining spiral ganglion neurons in the basal, second, or third turn of transplanted or sham-operated cochleae. There are no significant differences in the density of spiral ganglion neurons between transplanted and sham-operated cochleae.

of transplants in the cochlear modiolus and enabled functional evaluation using eABRs.[10,13] Moreover, our refined technique for cell introduction into the cochlear modiolus of guinea pigs caused no significant elevation of eABR thresholds.[10] Based on these previous findings, we used guinea pigs as experimental animals in the present study.

After transplantation of BMSC-derived spheres into the intact or damaged cochleae, BMSC-derived neurons were found in various portions of cochleae, including the cochlear modiolus. These findings indicate that BMSCs can be an alternative source of transplants for replacing SGNs. However, measurements of eABRs in the present study revealed no significant improvements of eABR thresholds after transplantation of BMSC-derived spheres. Previously, we demonstrated the recovery of eABR thresholds after transplantation of embryonic stem (ES) cell-derived neural progenitors in a different injury model.[13] There are several possible explanations for this lack of functional recovery following transplantation of BMSC-derived spheres. One is insufficient neurite elongation from BMSC-derived neurons to the central nervous system. Another possibility relates to different subtypes of neurons that are generated from BMSC-derived spheres. Previous studies have demonstrated that glutamatergic neurons are generated from both ES cells[14] and BMSCs,[15] meaning that both cell types have the capacity for differentiation into glutamatergic neurons. To achieve functional SGN restoration by transplantation of BMSC-derived spheres, additional treatments are required to enhance elongation of neurites from BMSC-derived neurons or to induce differentiation of BMSC-derived spheres into glutamatergic neurons.

Interestingly, the localization of transplants was different between the intact and damaged cochleae. In the intact cochleae, a number of transplants were found in the scala tympani. We injected BMSC-derived spheres through the scala tympani.[10] Therefore, transplants that were found in the scala tympani may have originated from the leakage of injected cell suspensions. In the intact cochlea, there are limited spaces in the cochlear modiolus, because host SGNs and auditory nerves are present, which may cause the leakage of injected cell suspensions into the scala tympani. On the other hand, the loss of host SGNs may result in an increase of spaces for transplants in the cochlear modiolus. Hence, in the damaged cochleae limited numbers of transplants were observed in the perilymphatic spaces including the scala tympani. In the damaged cochleae a number of transplants were found not only in the cochlear modiolus but also in the internal auditory meatus. Transplants in the internal auditory meatus may migrate from the cochlear modiolus, which is an injected site. The degeneration of SGNs could make a path from the cochlear modiolus of the basal portion to the internal auditory meatus, or stimulate production of chemotactic factors that promote the migration activity of BMSC-derived spheres. Future studies should be performed to determine the mechanisms of migration of BMSC-derived spheres into the internal auditory meatus.

CONCLUSION

The present findings demonstrate that BMSCs are a preferable source of neurospheres and that BMSC-derived spheres retain the ability for neural differentiation after transplantation into the cochlea. Functional restoration of damaged cochleae was not achieved by transplantation of BMSC-derived spheres, although a number of transplant-derived neurons settled in the cochlea and in the internal auditory meatus. To achieve functional restoration of SGNs by transplantation of BMSC-derived spheres, additional treatments including local application of neurotrophic or growth factors may be required.

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