identical cDNAs prepared for RT-PCR were used as the templates for qPCR. Specific primer sets were purchased from Takara Bio. qPCR was performed in 25 μL of reaction mixture containing 1× SYBR Premix Ex Taq (Takara Bio), 1× ROX Reference Dye, 0.2 μM of each primer and cDNA. PCR was conducted for 5 s at 95 °C and 31 s at 60 °C for 40 cycles. Gene expression levels were normalized using *gapdh* as an internal control. Results of ABR and qPCR are expressed as the mean ± standard error, and statistical significance was determined by one sample *t*-test.

4.5. Immunohistochemical analysis

Histological sample were made on the before 3-NP treatment and 1 day after vehicle treatment, and 6 h, 1, 2 and 3 days after 3-NP treatment ($n \geq 3$). Rats were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused intracardially with 0.01 M sodium phosphate buffer (pH 7.4) containing 8.6% sucrose, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After decapitation, temporal bones were removed quickly and placed in the same 4% paraformaldehyde fixative. Small openings were made at the round window, oval window and apex of the cochlea. After immersion in the fixative overnight, the temporal bones were decalcified by placement in 5% EDTA and 4% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) at 4 °C for 2 weeks, dehydrated, and embedded in paraffin. Transverse cochlear sections at 5-μm thickness were cut and mounted on glass slides. After rehydration, sections were treated with 0.3% hydrogen peroxide in methanol to quench peroxidase activity. For epitope retrieval, slides were boiled in citrate buffer (pH 6.0) in a microwave. After blocking nonspecific binding with 1% normal goat serum (Vector Laboratories, Burlingame, CA), the slides were incubated with monoclonal anti-CHOP (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:100 dilution at 4 °C overnight. The slides were washed and then incubated with biotinylated anti-mouse IgG (Vector Laboratories) at a 1:200 dilution, and the signal was colorized using VECTASTAIN Elite ABC kit (Vector Laboratories) and DAB Substrate kit for Peroxidase (Vector Laboratories). Some of the slides were counterstained with hematoxylin. Some of the unstained sections were processed for TUNEL histochemical staining using ApopTag Peroxidase *In Situ* Apoptosis Detection kit (Chemicon International, Temecula, CA) according to the manufacturer's protocol. The TUNEL reaction mixture was added to each sample in a humidified chamber, followed by incubation for 1 h at 37 °C for colorization with DAB. For fluorescent immunostaining, the sections were incubated with anti-CHOP at a dilution of 1:50. The sections were subsequently treated with proteinase K (DakoCytomation, Carpinteria, CA) and incubated with Alexa Fluor 568 anti-mouse IgG (Molecular Probes, Eugene, OR) at a 1:1000 dilution. Thereafter, the ApopTag Fluorescein Direct *In Situ* Apoptosis Detection kit (Chemicon) was used according to the manufacturer's protocol. The sections were then covered with PermaFluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA) with DAPI (1 μg/mL; Dojindo, Kumamoto, Japan).

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ORIGINAL ARTICLE

Vestibular function of patients with profound deafness related to *GJB2* mutation

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Abstract

Conclusion: GJB2 mutations are responsible not only for deafness but also for the occurrence of vestibular dysfunction. However, vestibular dysfunction tends to be unilateral and less severe in comparison with that of bilateral deafness. *Objectives:* The correlation between the cochlear and vestibular end-organs suggests that some children with congenital deafness may have vestibular impairments. On the other hand, *GJB2* gene mutations are the most common cause of nonsyndromic deafness. The vestibular function of patients with congenital deafness (CD), which is related to *GJB2* gene mutation, remains to be elucidated. The purpose of this study was to analyze the relationship between *GJB2* gene mutation and vestibular dysfunction in adults with CD. *Methods:* A total of 31 subjects, including 10 healthy volunteers and 21 patients with CD, were enrolled in the study. A hearing test and genetic analysis were performed. The vestibular evoked myogenic potentials (VEMPs) were measured and a caloric test was performed to assess the vestibular function. The percentage of vestibular dysfunction was then statistically analyzed. *Results:* The hearing level of all CD patients demonstrated a severe to profound impairment. In seven CD patients, their hearing impairment was related to *GJB2* mutation. Five of the seven patients with CD related to *GJB2* mutation demonstrated abnormalities in one or both of the two tests. The percentage of vestibular dysfunction of the patients with CD related to *GJB2* mutation was statistically higher than in patients with CD unrelated to *GJB2* mutation and in healthy controls.

Keywords: *Vestibular evoked myogenic potentials, caloric test*

Introduction

Since a correlation between the peripheral auditory and vestibular systems has been identified both anatomically and phylogenetically, a subgroup of children with congenital deafness (CD) may be associated with vestibular and balance impairments [1–3]. Interestingly, the vestibular disturbance in these children gradually disappears as they grow up, probably because of a compensatory mechanism of the central nervous system. However, there have been only a few reports that conducted a detailed analysis of the vestibular function in adults with CD.

CD has been reported in approximately one child per 1000 births [1]. In more than half of these cases, the disease is caused by gene mutation. In particular, mutation in the *GJB2* gene, which encodes Cx26 in the gap junction, is known to be a most common cause (up to 50% of such cases) [2,3]. Gap junction channels enable the neighboring cells to exchange small signaling molecules. Immunohistochemical studies have revealed that Cx26 exists not only in the cochlea but also in the vestibular organs [4]. K^+ cycling involving gap junction protein Cx26 in the vestibular labyrinth, which is similar to that in the cochlea, is thought to play a fundamental role in the endolymph homeostasis and sensory transduction [5]. These findings suggest that mutations in the *GJB2* gene may thus cause vestibular dysfunction.

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In this study, the relationship between *GJB2* gene mutation and vestibular dysfunction in adults with CD was investigated to confirm whether or not there are any abnormalities associated with the vestibular function.

Material and methods

Subjects

The subjects in this prospective study included 21 patients with CD and 10 healthy volunteers. The patients were excluded from the study if they were being treated with ototoxic drugs or if they had a cytomegalovirus infection, bacterial meningitis, external and middle ear pathological findings, or other risk factors for inner ear damage. No participants had syndromic deafness due to pigmentary retinopathy, nephropathy, goiter, or any other diseases. Patients with vestibular dysfunction due to head trauma, brain tumor, Meniere's disease, or other conditions were also excluded from the study. All subjects underwent an otoscopic examination and were found to have a normal tympanic membrane. Audiometric testing was performed in a double-walled, sound-treated booth. All patients gave their informed consent in writing and the study was approved by the Ethics Committee of Juntendo University School of Medicine.

Genetic analysis

DNA was extracted from peripheral blood leukocytes of the subjects. The coding region of *GJB2* was amplified by PCR using the primers *GJB2*-2F 5′-GTGTGCATTCGTCTTTTCCAG-3′ and *GJB2*-2R 5′-GCGACTGAGCCTTGACA-3′. The PCR products were sequenced using the PCR primers and sequence primers *GJB2*-A 5′-CCACGC-CAGCGCTCCTAGTG-3′ and *GJB2*-B 5′-GAA-GATGCTGCTGCTTGTGTAGG-3′. These were visualized using an ABI Prism 310 Analyzer (PE Applied Biosystems, Tokyo, Japan).

Vestibular evoked myogenic potentials

The vestibular evoked myogenic potentials (VEMPs) were measured as described in a previous report [6]. Both sound stimuli of clicks (0.1 ms, 95 dBnHL) and short tone burst (500 Hz; rise/fall time, 1 ms, 95 dBnHL) were presented to each side of the ear through the headphones using a Neuropack evoked-potential recorder (Nihon Kohden Co. Ltd, Tokyo, Japan). The surface electromyographic activity was recorded with the patient in the supine position from symmetrical sites over the upper half of each sternocleidomastoid (SCM) muscle with a reference electrode on the lateral end of the upper sternum. During recording, the subjects were instructed to lift their head up or to turn the contralateral side to induce hypertonicity of the SCM. Thereafter, the electromyographic signals from the stimulated side of the SCM muscle were amplified.

Caloric test

The caloric test in the current study was performed as described elsewhere [7]. Briefly, 2 ml of ice-water (at 4°C) was irrigated in the external auditory meatus to induce a thermal gradient across the horizontal semicircular canal of one ear. The duration of horizontal and vertical nystagmus was recorded. The results were compared between the right and left ears.

Statistical analysis

The data are expressed as the mean ± SD. Statistical analyses were conducted using a non-repeated measures analysis of variance (ANOVA). Significant effects were further analyzed by post hoc multiple comparison tests using the Student-Newman-Keuls test. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Hearing test

The pure-tone averages of 0.5, 1.0, and 2.0 kHz are shown in Table I. The hearing impairments of CD patients ranged from severe (71–95 dB) to profound (> 95 dB). The hearing levels of all controls were at the normal level (< 30 dB; data not shown).

Genetic analysis

GJB2 mutations were found in nine CD patients (Table I). All three mutations have been described previously in association with deafness. Among these mutations, 235delC mutation was found in eight patients. One nonsense mutation (Y136X) and one frameshift mutation (176-191del) were also identified. In six patients with a homozygous *GJB2* mutation and one patient with a compound heterozygous

Table I. Results of hearing level, genetic analysis, and vestibular function of subjects with congenital deafness (CD)

Case no.	Hearing level (dB) Left	Right	Sex	Age (years)	Mutation in *GJB2*	VEMPs	Caloric test
Patients with *GJB2*-related CD							
1	86	98	M	26	Homo 235delC	Right decreased	Left CP
2	106	108	M	25	Homo 235delC	Right decreased	Normal
3	108	106	M	28	Homo 235delC	Right decreased	Normal
4	108	106	M	37	Homo 235delC	Normal	Right CP
5	100	106	M	32	Homo 235delC	Normal	Right poor/left CP
6	80	91	M	25	Homo 235delC	Normal	Normal
7	115	108	M	25	Y136X/235delC	Normal	Normal
Patients without *GJB2*-related CD							
8	98	98	F	24		Left decreased	Bilateral CP
9	98	115	M	26		Normal	Bilateral CP
10	97	97	M	20		Normal	Normal
11	111	108	M	31		Normal	Normal
12	100	104	F	34		Normal	Normal
13	98	95	M	21		Normal	Normal
14	91	91	M	24		Normal	Normal
15	99	101	F	26		Normal	Normal
16	99	95	F	23		Normal	Normal
17	80	68	M	27		Normal	Normal
18	96	95	M	27		Normal	Normal
19	85	73	M	23		Normal	Normal
Patients with heterozygous *GJB2* mutation							
20	73	100	M	25	Hetero 235delC	Normal	Normal
21	97	98	M	25	Hetero 176-191del16	Normal	Normal

CP, canal paresis; Poor, nystagmus was obviously weak.

mutation (case nos 1–7); their profound deafness was thought to be caused by a *GJB2* mutation. No *GJB2* mutation was identified in any of the controls.

Vestibular function

No patients or controls had any subjective symptoms of vertigo. Table I shows the results of the vestibular function in all CD patients. Abnormal responses of VEMPs and the caloric test in CD with a *GJB2*-related mutation were observed in three patients each (case nos 1–5). Three patients with a homozygous *GJB2* mutation showed asymmetrical responses in VEMPs (case nos 1–3). Three patients with a homozygous *GJB2* mutation showed asymmetrical responses in the caloric test (case nos 1, 4, and 5). One of them showed both VEMPs and the caloric test asymmetrical responses (case no. 1). One patient with a homozygous *GJB2* mutation and one patient with compound heterozygous *GJB2* mutation showed normal responses in both VEMPs and the caloric test (case nos 6 and 7). It is notable that five of the six patients with a homozygous 235delC mutation showed no abnormalities in either test. Two heterozygous patients (case nos 20 and 21) showed normal responses in both tests.

Two CD patients with no *GJB2* mutation exhibited abnormal findings for the vestibular tests (case nos. 8 and 9). One patient showed a unilateral reduction in VEMPs and bilateral canal paresis (case no. 8). Bilateral canal paresis was also observed in another patient (case no. 9).

All the controls with normal hearing showed normal responses in both the VEMPs and the caloric test (data not shown).

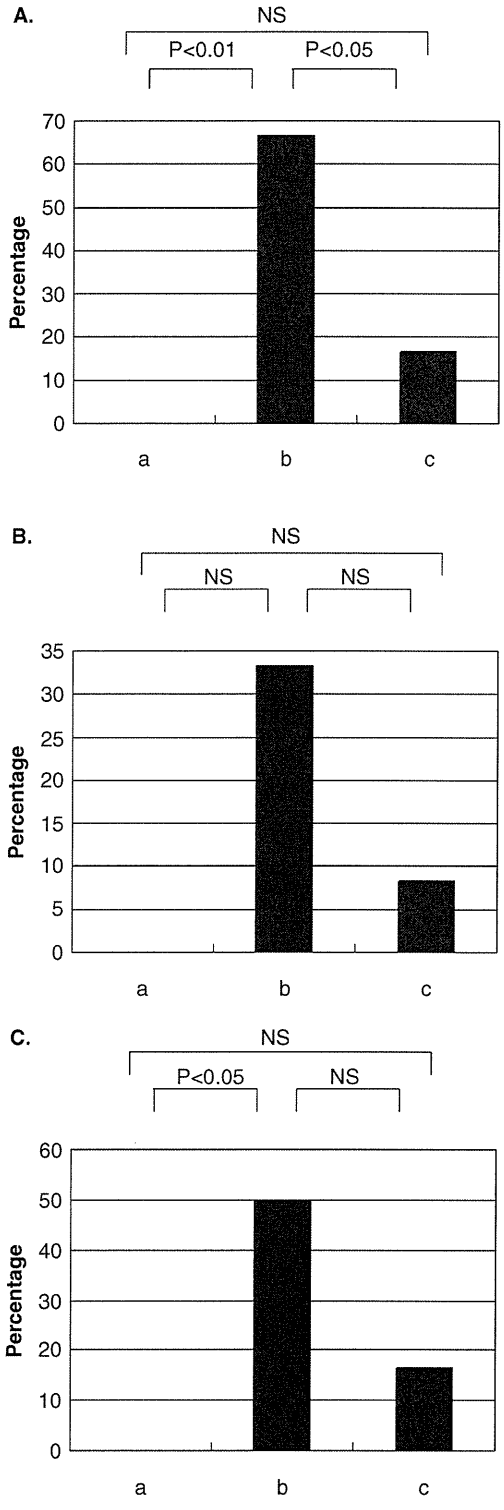

Figure 1. Comparison of the incidence of abnormality in the vestibular tests among the three groups. (A) Percentage showing abnormality in VEMPS and/or caloric test. (B) Percentage showing abnormality in VEMPs. (C) Percentage showing abnormality in the caloric test. a, Controls; b, *GJB2*-related CD subjects; c, CD subjects without *GJB2* mutations.

Statistical analysis of vestibular function in the three groups

Figure 1 shows a comparison of the controls, patients with CD related to a *GJB2* mutation, and those with CD without a *GJB2* mutation. The CD patients with *GJB2* heterozygous mutation were excluded from this statistical analysis, since their symptoms of hearing impairment are not necessarily caused by the *GJB2* mutation alone. Vestibular dysfunction showing an abnormality in VEMP and/or the caloric test significantly increased in patients with *GJB2*-related CD in comparison with those with CD without *GJB2* mutation ($p < 0.05$) and the controls ($p < 0.01$), whereas no difference was observed between CD without a *GJB2* mutation and the controls (Fig. 1A). No differences in the incidence of abnormality in VEMPs were observed among the three groups (Fig. 1B). The incidence of abnormalities in the caloric test in patients with *GJB2*-related CD differed significantly from that in the controls, but the other two comparisons were not significant (Fig. 1C).

Discussion

In this study, vestibular tests were performed in CD patients with or without a *GJB2* mutation by measuring the VEMPs and using the caloric test. Only one report has previously investigated the vestibular function of patients with *GJB2*-related CD [8]. The authors noted that five of the seven patients showed no VEMP responses bilaterally and that only one case had a unilateral pathological response in the caloric test, which led to the conclusion that CD with a *GJB2* mutation is associated with severe saccular dysfunction. However, in the present study, there were no patients showing the absence of both VEMP and a caloric response. Todt et al. [8] showed the existence of *GJB2* mutations that do not cause CD (polymorphisms), thus suggesting a considerable bias. Furthermore, patients with low-grade hearing loss were included in their study. In contrast, all of the *GJB2* mutations detected in the present study are known to cause CD in the Asian population [9]. In addition, the present study included only patients with severe to profound hearing loss, which would therefore clarify the correlation between CD and *GJB2* mutations. Among the seven patients with *GJB2*-related CD, five (71.4%) showed abnormal responses in either or both tests. The incidence was apparently and significantly higher than that in patients with CD without a *GJB2* mutation (2/13: 15.4%). Moreover, the incidence in the controls significantly differed from that in patients with CD related to a *GJB2*

mutation but not in those with CD without *GJB2* mutation. Therefore, these findings support the hypothesis that *GJB2* mutations play a critical role in the disturbance of the vestibular function.

GJB2 mutations cause profound deafness and the associated mechanism has been discussed in several studies [10,11]. A recent study showed that *GJB2* is indispensable in the normal development of the organ of Corti and normal hearing on the basis of the study in *Gjb2* dominant-negative mutant mice [12]. Despite the widespread expression of Cx26 in both the cochlear and vestibular organs [4], the vestibular function impairment of the patients with a *GJB2* mutation is not as severe as the hearing dysfunction observed in the present study. Two hypotheses have been proposed to explain this inconsistency between hearing and balance function. One hypothesis is based on the fact that two temporal bone studies performed in patients with *GJB2*-related hearing impairment in the previous study revealed that one patient had mild vestibular hydrops and saccular degeneration, while another patient had a dysplastic neuroepithelium of the saccule [13,14]. This suggests that a *GJB2* mutation can cause morphological dysplasia in not an entire organ, but in part of the vestibular organs. This is contrast to the cochlea of these patients, which showed nearly total dysplasia of the organ of Corti. These histopathological studies support the results of the vestibular dysfunction of patients with *GJB2*-related CD in the present study. The other hypothesis is based on the presence of several connexins such as Cx26, Cx30 (encoded by *GJB6*), Cx31 (encoded by *GJB3*), and Cx32 (encoded by *GJB1*) in the inner ear. A previous study showed all of these connexins to be distributed in the vestibular organs [15]. Cx30 gene knockout mice had hair cell loss in the saccule, which was restored by the over-expression of the Cx26 gene [16]. Therefore, the specific loss of Cx30 causes vestibular dysfunction, which can be compensated by other types of connexins. The present clinical study in which a complete defect of Cx26 resulted in a definitive but partial dysfunction of vestibular end organs can be explained by the compensation of other connexins normally expressed in the vestibule. Further studies are required to clarify the relationship between connexins and the vestibular function.

Although there was a statistically significant difference in the objective examination of the vestibular function among patients with *GJB2*-related CD, those with CD without a *GJB2* mutation, and healthy controls, none of these subjects had any vestibular symptoms regardless of the presence or absence of a *GJB2* mutation. The peripheral vestibular dysfunction predicted in individuals with the *GJB2* mutation may be compensated by the central vestibular system in young patients with deafness, as shown in the present study. However, aging is known to affect both the peripheral and central vestibular system [17]. In patients with a *GJB2* mutation, the vestibular symptoms may progress with aging. Another problematic point regarding patients with CD related to *GJB2* mutations is cochlear implantation, which has been reported to cause vestibular dysfunction, such as a reduction of the caloric responses [18] and a decrease in the VEMP responses [19]. It is thought that the mechanical damage caused by the insertion of the electrode may induce vestibular dysfunction [20]. In the present study, four patients with *GJB2*-related deafness showed unilateral vestibular dysfunction, while only one of them had bilateral dysfunction. Therefore, it should be emphasized that the assessment of the vestibular function in patients with *GJB2*-related CD is important to determine which side of the ear should be selected to insert the cochlear implant.

Conclusions

A *GJB2* mutation is responsible not only for deafness but also for vestibular dysfunction. However, such vestibular dysfunction is likely to be unilateral and less severe in patients with a *GJB2* mutation than in those with bilateral deafness.

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実験動物を用いた内耳細胞治療研究へのアプローチ

神谷　和作・池田　勝久

Experimental Approaches to Inner Ear Cell Therapy Using Laboratory Animals

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Recently, a number of clinical studies on cell therapy have been reported and used in clinical practice for several intractable diseases. Inner ear cell therapy for sensorineural hearing loss also has been studied using some laboratory animals, although to date reports on successful hearing recovery have been few.

Previously, we developed a novel rat model of acute sensorineural hearing loss due to fibrocyte dysfunction induced by a mitochondrial toxin and performed cell therapy with bone marrow mesenchymal stem cells (MSCs). In this study, we injected MSCs into the lateral semicircular canal; a number of these stem cells were then detected in the injured area in the lateral wall. Rats with transplanted MSCs in the lateral wall demonstrated a significantly higher hearing recovery ratio than the untreated controls. These results suggested that mesenchymal stem cell transplantation into the inner ear may be a promising therapy for patients with sensorineural hearing loss due to degeneration of cochlear fibrocytes.

In this article, we review studies on inner ear cell therapy using some laboratory animals including rodents such as mice and rats, and primates such as cynomologus monkeys (Macaca fascicularis).

Key words : inner ear, cell therapy, sensoryneural hearing loss, stem cell

はじめに

感音性難聴の原因は多岐にわたるが，近年の遺伝子改変動物開発技術の向上や多種のモデル動物の開発により多くの病態メカニズムが解明に近づいている．すべての先天性疾患の中でも頻度の高い遺伝性難聴においては，難聴家系や突然変異難聴マウスの遺伝子解析によって多くの遺伝性難聴原因遺伝子が同定されている．初期に発見された遺伝性難聴の原因の多くは内耳有毛細胞の変性または機能的・形態的異常であったため，多くの研究者が有毛細胞を中心に難聴の病態メカニズム解明に取り組んできた．哺乳類の有毛細胞は再生能力を持たないため遺伝子導入などによる有毛細胞再生の誘導も盛んに研究されてきた[1)2)]．その一方で内耳への細胞移植による有毛細胞の修復の試みも行われているが，特殊なリンパ液で満たされた内耳の構造的特徴から，聴力を保持しつつ標的部位に移植細胞を到達させ分化させることは容易ではない．そのため有毛細胞の修復には多種のモデル動物を用いた多くの検討実験が必要と考えられる．近年有毛細胞以外にも蝸牛線維細胞などの機能異常が単独で難聴病態の引き金となることも明らかとなっており，多様な治療戦略が求められている．幹細胞の損傷部への移動能力や組織環境（ニッシェ，niche）による分化誘導を十分に検討すれば細胞治療は内耳組織変性に対する治療にも応用可能と考えられる．著者らの報告では実験的に蝸牛線維細胞のみに傷害を与えたラットへ半規管外リンパ液を経由した細胞液還流法を用いることにより，損傷部の修復と聴力回復率を高めることに成功した[3)]．現在はヒト疾患に近い遺伝性難聴モデル動物への各種の幹細胞移植に取り組んでいる．また，サル類を用いた細胞移植アプローチの検討も今後応用性を高めるためには非常に重要であるため現在，カニクイザルによる検討を行っている．各種のモデル動物の特徴を考慮した細胞移植実験検討を

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積み重ねることにより，将来的には有毛細胞も標的とした多様な難聴に対する聴力回復も不可能ではないと考えられる．本稿では各種実験動物を用いた内耳への細胞治療研究に関する知見について報告する．

内耳細胞治療実験に用いられる実験動物

外傷，騒音，感染，薬物障害，血流障害，加齢に起因する聴覚障害動物モデルは多く開発されており，細胞治療研究のための有用な実験モデルとして活用することができる．著者らはミトコンドリア阻害薬を用いて蝸牛線維細胞のみに損傷を与えるモデルラットを開発し，この細胞移植実験に成功している．しかしこのような実験的に内耳損傷を誘導した動物モデルがヒトと同等な内耳組織障害および機能的障害を忠実に再現しているかという点に関しては実証することは困難である．これに対し原因タンパク質がすでに特定されている遺伝子改変動物または突然変異動物はヒト遺伝性難聴の病態の多くが一致していると考えられる．細胞移植によりそのタンパク質が担う機能を回復させることができれば，幹細胞が正常に分化し失われていたタンパク質機能を取り戻した結果として聴力が回復したことを実証しやすい．有毛細胞の変性が顕著にみられるモデル動物としては，アッシャー症候群原因遺伝子（Pcdh15[4]，Cdh23[5]，Sans[6]，Harmonin[7]，MyosinVIIa[8]など）の突然変異動物あるいは遺伝子改変動物が，明白な表現型を持つため有毛細胞の研究に広く用いられている．これらの進行性の組織変性は重度であり有毛細胞の変性から連鎖的にラセン神経節細胞の消失へとつながる場合も多い．そのため細胞治療による細胞の生着・分化の検討は可能であるが聴力改善の検討は現段階で容易ではないと思われる．蝸牛線維細胞を標的とした場合，有毛細胞変性を伴わず蝸牛線維細胞のみに変性を持つ Brn4 欠損マウス[9]，Otospiralin 欠損マウス[10]が有効であると考えられる．これらの聴力改善の可能性は有毛細胞を標的とした細胞治療より格段に高いと思われる．ヒト遺伝性難聴でもっとも高頻度に出現するコネキシン26の遺伝子欠損マウスおよび優性阻害トランスジェニックマウス[11]は同遺伝子が蝸牛線維細胞および支持細胞に主に発現するため，著者らの行った骨髄間葉系幹細胞移植も有効であると考えられる．

蝸牛線維細胞を標的とした骨髄間葉系幹細胞移植

蝸牛ラセン靭帯およびラセン板縁を構成する蝸牛線維細胞はナトリウムポンプとギャップジャンクションによる蝸牛内イオンの能動輸送および受動輸送という単純な機能を担っている．しかしながら蝸牛線維細胞の傷害は複数の先天性および後天性難聴の主要因となることが示され，その重要性が近年示唆されている．とくにヒト非症候性難聴 DFN3 の原因因子 Brn4 の遺伝子欠損マウス[9]や otospiralin 欠損マウス[10]では蝸牛線維細胞の変性を主要因とした聴力低下が実証され，有毛細胞を含むコルチ器と同様に正常聴力を維持するうえで重要性の高い細胞群であることが明確に示された．また複数の加齢性難聴モデル動物においても蝸牛線維細胞の変性が他の細胞に先立ち開始することが報告されている[12)～14)]．また蝸牛線維細胞は単一細胞としての機能が単純であるにもかかわらず内耳機能における重要性が高いという点から，高度に分化した有毛細胞に比べて細胞治療が成功する可能性が格段に高いと考えられる．これらのことから蝸牛線維細胞は多種の感音難聴に対する新規治療法確立への重要な標的となりうると考えられる．著者らは薬剤

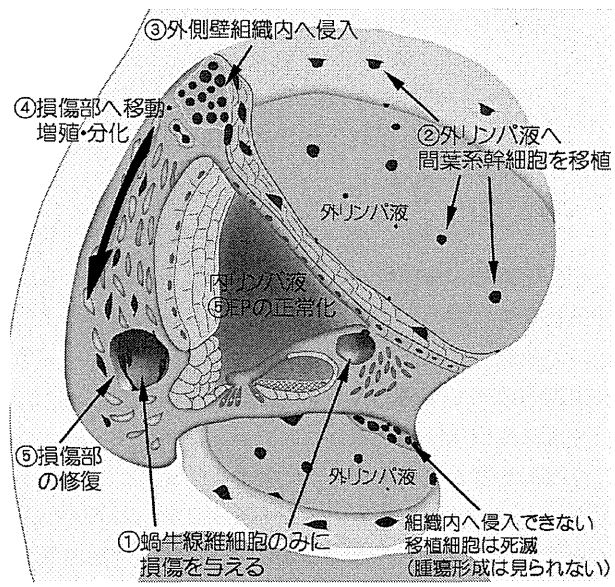

図 1　蝸牛線維細胞をターゲットとした骨髄間葉系幹細胞移植での損傷部の修復および推測された移植細胞の移動経路
薬剤投与によりらせん靭帯およびらせん板縁に選択的に損傷を与え①，その後外リンパ液へ骨髄から採取した間葉系幹細胞を半規管からの還流により投与②した結果，投与した間葉系幹細胞が外側壁組織内へ進入し④，移動・増殖・分化④により損傷部の修復⑤を促進し高周波数音域の聴力回復率が有意に上昇した．（Reprinted from Am J Pathol Am J Pathol 2007, **171**：214 ～ 226 with permission from the American Society For Investigative Pathology）

局所投与により蝸牛線維細胞の二点にのみ限局的なアポトーシスを起こすモデルラットを開発し[15)16)]，半規管からの骨髄間葉系幹細胞の外リンパ液還流投与を行った．その結果，移植 11 日後の高音域（40 KHz）の聴力回復が有意に促進し，外側壁の蝸牛線維細胞損傷部に多数の移植細胞が観察された．組織内には腫瘍化を示す移植細胞は観察されなかった．移植細胞は蝸牛外側壁の頂回転側，外リンパ液に面している部分で多くみられ，この部位を中心に蝸牛組織に侵入し損傷部まで移動したと考えられる．損傷部ではコネキシンの発現とともに隣接細胞と接合する移植細胞が観察され，イオン輸送経路の回復による内リンパ液 K^+濃度の正常化が聴力回復に寄与したと推測される（図 1）[3)]．

内耳への細胞投与法

著者らの初期の移植検討実験では，ラット蝸牛管付近より細胞液投与を試みた際はどの部位でも手術による永続的な聴力低下がみられ，蝸牛組織には繊維化が認められた．著者らは Iguchi らの方法[17)]を参考にラットの後半規管および外側半規管にそれぞれ小孔を開け，片側から微小チューブを挿入し細胞液（1×10^5cells/20 μl）での 10 分間の還流を行った．この方法では手術による聴力低下はほとんどみられず，大量の細胞を蝸牛内に導入することができるため内耳細胞治療に適した投与法であると思われる．また新生児難聴スクリーニング直後の早期治療を想定した内耳への投与方法として，Iizuka ら[18)]は生後 0 日齢の幼若マウスへ微小ガラス管を用いて遺伝子治療用ウイルス液を非侵襲的に外リンパ液内へ注入することに成功している．同方法は非侵襲性を必要とする幼若個体への細胞注入にも応用可能であると考えられる．この方法では外リンパ液の漏出がほとんどないため，少量であれば非侵襲的に細胞液を注入することができる．細胞移植用としてはガラス管先端の直径をパッチクランプ用のプラーで微調整することで利用可能と考えられる．

サル類を用いた細胞治療研究の重要性

げっ歯類を用いることにより，新規な細胞治療法の開発や多くの分子生物学的，生理学的データの取得が期待できる．一方でげっ歯類ではその生理・代謝機能が必ずしもヒトを忠実に反映していない部分もあり，ヒトへの外挿面で必ずしも一致した効果を得られない可能性も考えられる．たとえばヒトと同様サル類でも出生直後に外部の音刺激を入力することができると考えられるが，マウスやラットでは生後約10日齢までは内耳が未成熟であるため音の入力が開始しない．新生児難聴スクリーニング直後の細胞治療を目的とした検討の場合などはとくに成熟レベルによる細胞治療の有効性や安全性が大きく異なることが予想されるため，実験用サルによる安全性・

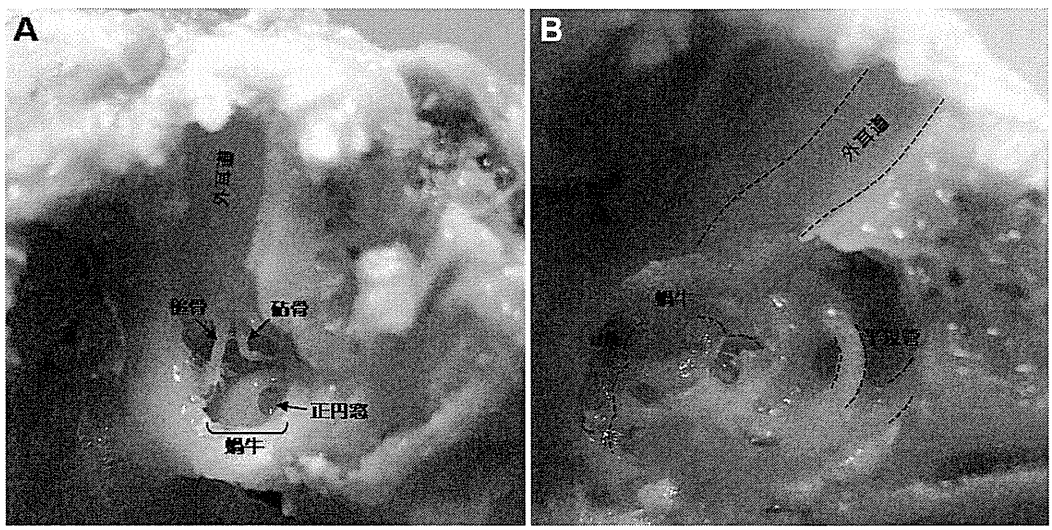

図 2　成熟カニクイザルの内耳
A．上顎側より削開し外耳道，耳小骨，蝸牛を露出した．
B．さらに内耳周囲を削開し蝸牛内部および半規管を露出した．ヒトとほぼ同様の内耳構造およびその周囲構造がみられる．

有効性の評価が将来的に非常に重要になると考えられる．マウスやラットで開発された細胞治療法を臨床応用へ近づけるためには，サル類（カニクイザル等）を用い，それらの安全性評価データをヒトへ外挿する必要がある．サル類を用いた細胞治療実験では，マウスやラットによる基礎データをもとに有効かつ安全な移植方法や移植細胞の種類，成熟ステージを評価することが重要であると考えられる．現在，カニクイザル頭部を用いた細胞治療アプローチに関する検討実験を行っている（図 2）．カニクイザル側頭骨は手術の際に削開を進める角度などがヒトと多少異なるが，内耳構造やその周囲構造に共通点が多く細胞移植手術による半規管や正円窓へのアプローチもヒトのモデルとして非常に有効であると考えられる．

各種実験動物の特徴

マウスに関していえば，近年膨大な種類の遺伝子改変マウスが各国でされ，データベース化や共有研究資材として分配されているものもある．病態変化や細胞移植後の変化を分子生物学的手法により解析する場合は情報や各種実験ツールの多さからマウスが他の実験動物と比較し圧倒的に有用性・汎用性が高いと考えられる．各種幹細胞の入手もマウスでは比較的容易である．しかし内耳への細胞の局所投与を必要とする場合はアプローチの方法やターゲットとする疾患に対し最適な病態モデルを得るため，マウス以外のげっ歯類（モルモット，スナネズミ，ラット）も十分有効に活用できる．半規管経由の細胞移植に関していえば，げっ歯類では外側半規管および後半規管が側頭骨表面に露出しているためアプローチが容易である．

サル類に関していえば，研究コスト面での負担が大きいが臨床応用へ向けての安全性・有効性評価の面で必須の実験動物といえる．カニクイザル（*Macaca fascicularis*）は我が国でも保有する施設が多数存在し，多くの動物実験に利用されている．前述したように内耳周辺の組織構造，生理機能がヒトとほぼ同等と考えられるため，げっ歯類で有効性が実証された細胞治療法をカニクイザルにおいて有効性および安全性を再評価することが臨床応用に向けて重要であると考えられる．

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難聴に対する内耳細胞治療法の開発

Inner ear cell therapy for sensorineural hearing loss

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◎内耳は特殊なリンパ液で満たされた独特な構造をもち，血液-内耳関門とよばれる血管系を有するため，内耳有毛細胞やその周辺細胞への薬物的アプローチが難しい．しかし，移動能・多分化能を兼ね備えた幹細胞による内耳細胞治療の方法が確立すれば，難聴の根本的治療への有効なツールになると考えられる．近年の内耳再生医療に関する基礎研究分野は，*in vitro* での有毛細胞への分化誘導において年々進歩している．最近では培養シャーレ内で鳥類細胞から聴毛を有する有毛細胞への分化誘導も可能となっており[1]，細胞工学的分野では一定の成果が得られている．しかし，それらの細胞を移植により内耳組織へ生着させ，同時に機能的補足や組織修復によって聴力回復を誘導する細胞治療の試みは成功例が少なく，引用度の高い論文での報告も少ない．聴力回復を目的とした内耳細胞治療法を開発するためには移植細胞の生着と機能発現を同時に考慮し，内耳の解剖学的特徴および各細胞の生理学的特徴を十分に理解することが重要であると考えられる．著者らの報告では，実験的に蝸牛線維細胞のみに傷害を与えたラットへ半規管外リンパ液を経由した細胞液灌流法を用いることにより，損傷部の修復と聴力回復率を高めることに成功した[2]．現在は，ヒト疾患に近い遺伝性難聴モデル動物への各種の幹細胞移植に取り組んでいる．蝸牛線維細胞のような，修復が困難ではないが聴力維持に不可欠な細胞を標的に検討を積み重ねることにより，将来的には有毛細胞も標的とした多様な難聴に対する聴力回復も不可能ではないと考えられる．本稿では，とくに各種幹細胞や遺伝子改変動物を用いた内耳への細胞治療に関する知見について報告する．

Key word：内耳，蝸牛，有毛細胞，蝸牛線維細胞，骨髄間葉系幹細胞

背景

難聴の原因は多岐にわたるが，近年の遺伝子改変動物開発技術の向上や多種のモデル動物の開発により，多くの病態メカニズムが解明に近づいている．すべての先天性疾患のなかでも頻度の高い遺伝性難聴においては，難聴家系や突然変異難聴マウスの遺伝子解析によって多くの遺伝性難聴原因遺伝子が同定されている．初期に発見された遺伝性難聴の原因の多くは内耳有毛細胞の変性または機能的・形態的異常であったため，多くの研究者が有毛細胞を中心に難聴の病態メカニズム解明に取り組んできた．哺乳類の有毛細胞は再生能力をもたないため遺伝子導入などによる有毛細胞再生の誘導も盛んに研究されてきた[3,4]．その一方で，内耳への細胞移植による有毛細胞の修復の試みも行われているが，特殊なリンパ液で満たされた内耳の構造的特徴から，聴力を保持しつつ標的部位に移植細胞を到達させ分化させることは容易ではない．そのため有毛細胞の修復にはモデル動物を用いた多くの検討実験が必要と考えられる．近年，有毛細胞以外にも蝸牛線維細胞などの機能異常が単独で難聴病態の引き金となることも明らかとなっており，多様な治療戦略が求められている．幹細胞の損傷部への移動能力や組織環境(ニッシェ，niche)による分化誘導を十分に検討すれば，細胞治療は未だ根本的治療法の存在しない内耳組織変性に対する治療にきわめて有効であると考えられる．

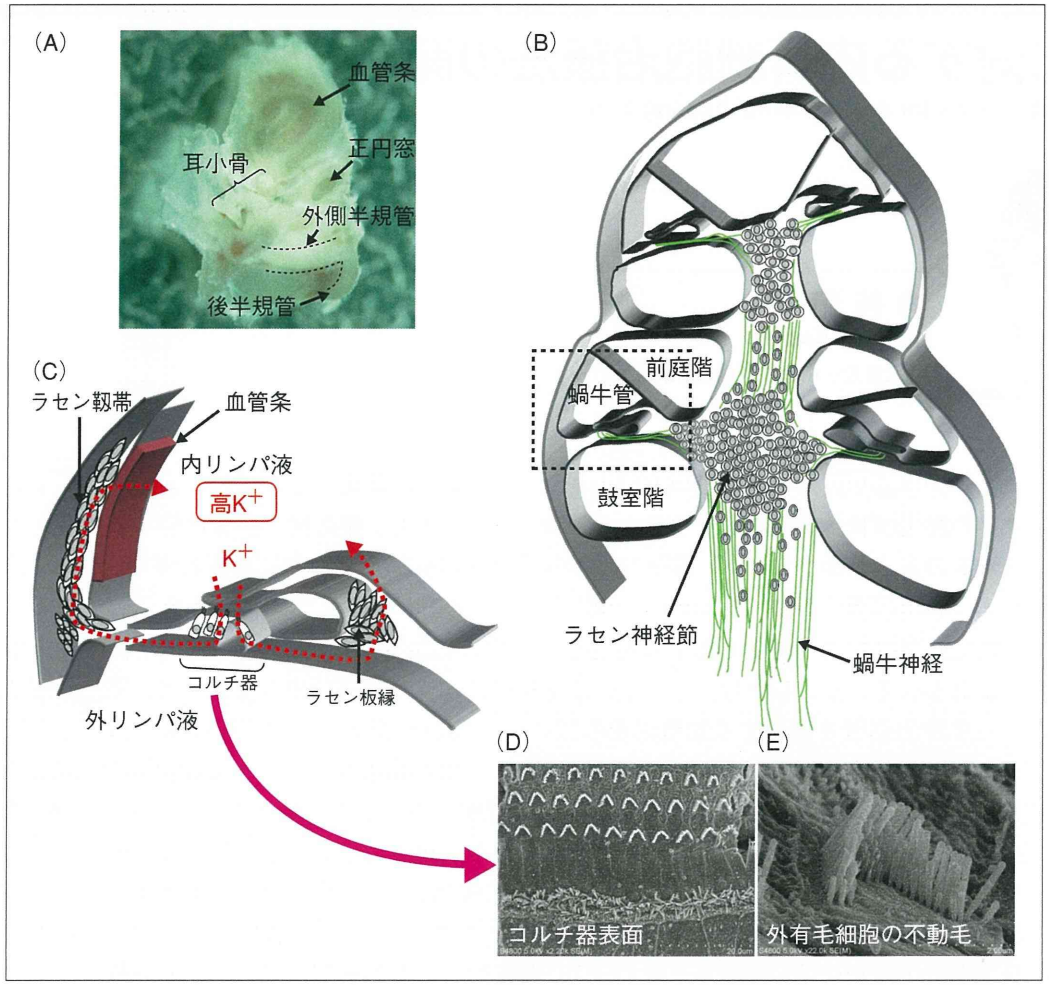

図 1　内耳の構造および K^+リサイクリングシステム

A：成熟マウス内耳．上部の血管条とともに螺旋構造を示す部位が蝸牛．B：蝸牛の断面．C：K^+リサイクリングシステム．ラセン靱帯，ラセン板縁の蝸牛線維細胞および血管条細胞による内リンパ液高 K^+濃度を維持するためのイオン輸送システム．これにより蝸牛内リンパ電位(EP)が恒常的に維持される．D：有毛細胞を含むマウスコルチ器の表面構造(走査電子顕微鏡)．上部 3 列の外有毛細胞，下部一列の内有毛細胞が整然と並ぶ．E：成熟マウス外有毛細胞の不動毛(走査電子顕微鏡)．V 字型に配置した 3 列の長さの異なる不動毛で構成され，基底膜を介した外リンパ液の振動に起因するこれらの傾きにより K^+が細胞内へ流入し，聴覚シグナルが生まれる．

内耳の形態的特徴と K^+リサイクリングシステム

哺乳類の内耳は蝸牛と前庭器で構成され，骨で覆われた内部には鼓室階と前庭階を満たす外リンパ液と，蝸牛管を満たす内リンパ液が存在する(図 1)．内リンパ液ではつねに高 K^+濃度(150 mM)が維持されており，これにより生じる電位を蝸牛内リンパ電位(endocochlear potential：EP)とよぶ．内リンパ液に面している有毛細胞は，このEP という電位が存在するために，音の振動から聴毛に存在する機械電気変換(mechanoelectrical transduction：MET)チャネルの開口に伴って脱分極を起こすことができる．EP を維持するために重要な役割を担っているのが蝸牛線維細胞と血管条であり，これらはコネキシンで構成されるギャップジャンクション，Na^+，K^+-ATPase，Na^+，K^+，$2Cl^-$共輸送体などによってイオン輸送を行い，内リンパ液の高 K^+状態を維持している(図 1)．このイオン輸送システムは K^+リサイクリングシステムとよばれ，これが正常に機能しなければ EP は低下し，たとえ有毛細胞機能が正常であっても脱分極は起こらず，その後の聴覚系神経の活動電位は発生しない．

内耳細胞治療での標的細胞

多くの研究者が内耳再生の分野でもっとも力を入れているのは有毛細胞の再生であるが，これは有毛細胞が再生能をもたない細胞であるからである．同細胞は，臨床のみならず科学的にも分化や機能的メカニズムに対する興味が集中している．しかし，有毛細胞は高度に分化した細胞であり，特殊化した巨大な繊毛の形成や極性の保たれた蛋白質局在を必要とするため，内耳で細胞を生着させ，正常に分化および機能発現させるための最初のターゲットとしては困難が予想される．蝸牛内には有毛細胞以外にも正常聴力の維持に必須な細胞(ラセン神経節細胞，コルチ器支持細胞，血管条細胞，蝸牛線維細胞)が存在し，これらをターゲットとした細胞治療の検討も重要であると考えられる．著者らの研究では，比較的機能がシンプルではあるにもかかわらず蝸牛の機能としては重要な働きを担う蝸牛線維細胞に着目し，同細胞を内耳細胞治療の第1のターゲットとして研究に着手した．

蝸牛線維細胞を標的とした骨髄間葉系幹細胞移植

蝸牛ラセン靱帯およびラセン板縁を構成する蝸牛線維細胞は，ナトリウムポンプとギャップジャンクションによる蝸牛内イオンの能動輸送および受動輸送という単純な機能を担っている．しかし，蝸牛線維細胞の傷害は複数の先天性および後天性難聴の主要因となることが示され，その重要性が近年示唆されている．とくにヒト非症候性難聴DFN3の原因因子Brn4の遺伝子欠損マウス[5]やotospiralin欠損マウス[6]では蝸牛線維細胞の変性を主要因とした聴力低下が実証され，有毛細胞を含むコルチ器と同様に正常聴力を維持するうえで重要性の高い細胞群であることが明確に示された．また，複数の加齢性難聴モデル動物においても，蝸牛線維細胞の変性が他の細胞に先立ち開始することが報告されている[7-9]．また，蝸牛線維細胞は単一細胞としての機能が単純であるにもかかわらず内耳機能における重要性が高いという点から，高度に分化した有毛細胞に比べて細胞治療が成功する可能性が格段に高いと考えられる．これらのことから蝸牛線維細胞は，多種の感音性難聴に対する新規治療法確立への重要な標的となりうると考えられる．著者らは，薬剤局所投与により蝸牛線維細胞の二点にのみ限局的なアポトーシスを起こすモデルラットを開発し[10,11]，半規管からの骨髄間葉系幹細胞の外リンパ液還流投与を行った．その結果，移植11日後の高音域(40kHz)の聴力回復が有意に促進され，外側壁の蝸牛線維細胞損傷部に多数の移植細胞が観察された．組織内には，腫瘍化を示す移植細胞は観察されなかった．移植細胞は蝸牛外側壁の頂回転側，外リンパ液に面している部分で多くみられ，この部位を中心に蝸牛組織に侵入し損傷部まで移動したと考えられる．損傷部ではコネキシンの発現とともに隣接細胞と接合する移植細胞が観察され，イオン輸送経路の回復による内リンパ液K^+濃度の正常化が聴力回復に寄与したと推測される[2](図2)．

内耳細胞治療に用いる幹細胞

ここ数年いくつかの内耳細胞移植研究に用いられてきたのがES細胞(embryonic stem cell，胚性幹細胞)であり，同細胞は有毛細胞への分化も大いに期待できる．しかし他臓器同様，移植後に奇形腫(teratoma)様の形態がみられるなど，不安定性も示唆されている[12]．また，成体細胞から作成することができる人口多能性幹細胞，iPS細胞(induced pluripotent stem cells)もES細胞同様，有毛細胞への分化の可能性をもつことで期待できるが，いまだ移植実験の報告はない．同細胞もES細胞同様，分化の不安定性を考慮する必要がある．著者らが内耳移植実験に用いた骨髄間葉系幹細胞はES細胞，iPS細胞ほどの多分化能はもたないが，分化が安定しており，著者らの移植実験でも奇形腫など腫瘍化に関連した形態はみられなかった．

2007年にCorwinらの研究チームはニワトリ内耳由来の増殖・継代・凍結保存可能な間葉系細胞から動毛，不動毛をもつ有毛細胞を作成した[1]．それまで*in vitro*においては有毛細胞特異的マーカーを発現させた報告はあったが，形態的にも有毛細胞と同様の聴毛を形成させた報告ははじめてである．次段階としては哺乳類細胞，とくにヒト

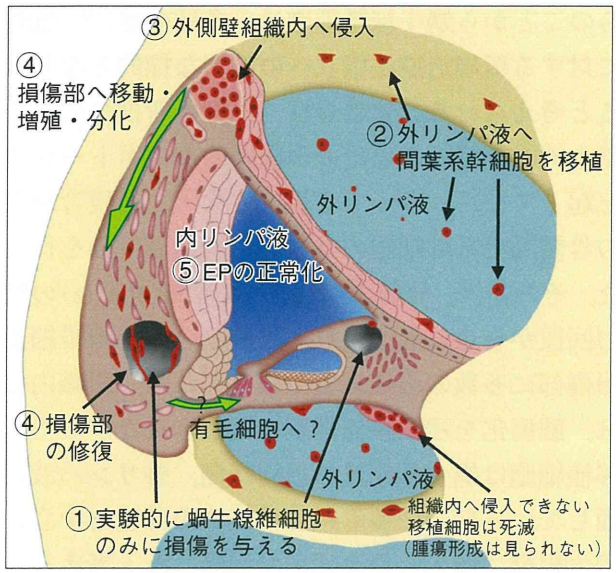

図 2　蝸牛線維細胞をターゲットとした骨髄間葉系幹細胞移植[2)]

著者らの行った細胞治療法において観察された蝸牛線維細胞損傷部の修復および推測された移植細胞の移動経路.

由来細胞から有毛細胞を *in vitro* で作成する技術が期待できる．著者らも同方法によるマウス内耳間葉系細胞の調整を試みており，この方法で増殖させ調整した有毛細胞前駆細胞が移植に有効である可能性がある．同論文では有毛細胞が間葉系由来の細胞から分化誘導可能であることを証明しており，他の *in vitro* 研究においても骨髄間葉系幹細胞が有毛細胞への分化能をもつことが示唆されている[13)]．このことは，著者らの示した骨髄間葉系幹細胞の内耳移植が有毛細胞を標的とした治療にも有用である可能性を示唆している．

内耳細胞治療実験に適した難聴モデル動物

外傷，騒音，感染，薬物障害，血流障害に起因する聴覚障害動物モデルは多く開発されており，これらも細胞治療の対象とすることができる．著者らはミトコンドリア阻害薬を用いて蝸牛線維細胞のみに損傷を与えるモデルラットを開発し，この細胞移植実験に成功している．しかし，このような実験的に内耳損傷を誘導した動物モデルがヒトと同等な内耳組織傷害および機能的障害を忠実に再現しているかという点に関しては，実証することは困難である．これに対し原因蛋白質がすでに特定されている遺伝子改変動物または突然変異動物は，ヒト遺伝性難聴と病態の多くが一致していると考えられる．細胞移植によりその蛋白質が担う機能を回復させることができれば，幹細胞が正常に分化し失われていた蛋白質機能を取り戻した結果として聴力が回復したことを実証しやすい．有毛細胞の変性が顕著にみられるモデル動物としては，アッシャー症候群原因遺伝子(Pcdh15[14)], Cdh23[15)], Sans[16)], Harmonin[17)], Myosin Ⅶa[18)]など)の突然変異動物あるいは遺伝子改変動物が，明白な表現型をもつため有毛細胞の研究に広く用いられている．これらの進行性の組織変性は重度であり，有毛細胞の変性から連鎖的にラセン神経節細胞の消失へとつながる場合が多い．そのため細胞治療による細胞の生着・分化の検討は可能であるが，聴力改善の検討は現段階で容易ではないと思われる．蝸牛線維細胞を標的とした場合，有毛細胞変性を伴わず蝸牛線維細胞のみに変性をもつ Brn4 欠損マウス[5)]，Otospiralin 欠損マウス[6)]が有効であると考えられる．これらの聴力改善の可能性は，有毛細胞を標的とした細胞治療よりも格段に高いと思われる．ヒト遺伝性難聴でもっとも高頻度に出現するコネキシン 26 の遺伝子欠損マウスおよび優性阻害トランスジェニックマウス[19)]は，同遺伝子が蝸牛線維細胞および支持細胞におもに発現するため著者らの行った骨髄間葉系幹細胞移植の応用も有効であると考えられる．

内耳への細胞投与法

著者らの初期の移植実験では，蝸牛管付近より細胞液投与を試みた際はどの部位でも手術による永続的な聴力低下がみられ，蝸牛組織には線維化が認められた．蝸牛リンパ液は半規管リンパ液と直接交通しているため，著者らは Iguchi らの方法[20)]を参考に，ラットの後半規管および外側半規管にそれぞれ小孔を開け(図 3)，片側から微小チューブを挿入し細胞液(1×10^5 cells/20 μl)での 10 分間の還流を行った．この方法では手術による聴力低下はほとんどみられず，大量の細胞を蝸牛内に導入することができるため，内耳細胞治療に適した投与法であると思われる．また，新生児難聴スクリーニング直後の早期治療を想定した内耳への投与方法として，Iizuka らは生後 0 日齢の幼

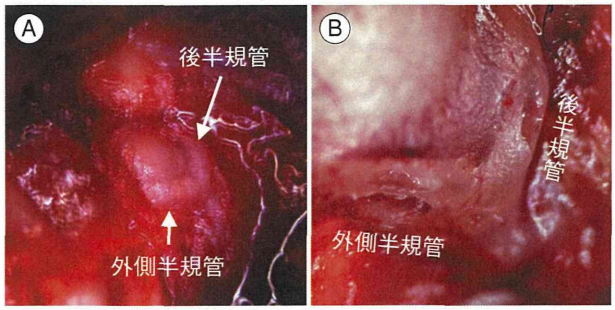

図 3　半規管からの細胞投与法
A：経半規管細胞移植時に露出された成熟マウス半規管．B：後半規管および外側半規管に細胞液を灌流するための小孔を開け，一方に微小チューブを挿入し細胞液を注入，もう一方より外リンパ液を排出する．

若マウスへ，微小ガラス管を用いて遺伝子治療用ウイルス液を非侵襲的に外リンパ液内へ注入することに成功している[21]．同方法は，非侵襲性を必要とする幼若個体への細胞注入にも応用可能であると考えられる．この方法では外リンパ液の漏出がほとんどないため，少量であれば非侵襲的に細胞液を注入することができる．細胞移植用としては，ガラス管先端の直径をパッチクランプ用のプラーで微調整することで利用可能と考えられる．

血液内耳関門と移植細胞に対する拒絶

遺伝性難聴など遺伝子変異を原因とする聴覚障害に対する細胞治療としては，正常な遺伝子をもつ幹細胞による他家移植による細胞置換が考えられるが，そのなかで重要と考えられるのが移植の拒絶である．内耳の毛細血管には，脳と同様に過剰な免疫系から組織を保護する血液-内耳関門というシステムがあり，容易に移植細胞を拒絶することはないと考えられる．この特徴のため，免疫抑制剤なしに他家移植を行える可能性がある．著者らは F344 系ラット由来の骨髄間葉系幹細胞を SD 系ラットへの移植に用いたが，組織内に侵入した移植細胞のなかで拒絶を示す像はきわめて少なかった[2]．このことは，他系統間であっても内耳細胞移植が成立する可能性を示唆している．

おわりに

内耳は他臓器と比較して組織容量が小さく隔絶された組織であるため，実験後の移植細胞の動態が解析しやすいという利点がある．内耳への導入方法，移植に最適な細胞の選抜などが発展していけば，内耳の細胞変性を伴うあらゆるタイプの難聴において細胞治療が実現できると考えられる．

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COCHLEAR OUTER HAIR CELLS IN A DOMINANT-NEGATIVE CONNEXIN26 MUTANT MOUSE PRESERVE NON-LINEAR CAPACITANCE IN SPITE OF IMPAIRED DISTORTION PRODUCT OTOACOUSTIC EMISSION

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Abstract—Mutations in the connexin26 gene (GJB2) are the most common genetic cause of congenital bilateral non-syndromic sensorineural hearing loss. Transgenic mice were established carrying human Cx26 with the R75W mutation that was identified in a deaf family with autosomal dominant negative inheritance [Kudo T et al. (2003) Hum Mol Genet 12:995–1004]. A dominant-negative *Gjb2* R75W transgenic mouse model shows incomplete development of the cochlear supporting cells, resulting in profound deafness from birth [Inoshita A et al. (2008) Neuroscience 156:1039–1047]. The Cx26 defect in the *Gjb*2 R75W transgenic mouse is restricted to the supporting cells; it is unclear why the auditory response is severely disturbed in spite of the presence of outer hair cells (OHCs). The present study was designed to evaluate developmental changes in the *in vivo* and *in vitro* function of the OHC, and the fine structure of the OHC and adjacent supporting cells in the R75W transgenic mouse. No detectable distortion product otoacoustic emissions were observed at any frequencies in R75W transgenic mice throughout development. A characteristic phenotype observed in these mice was the absence of the tunnel of Corti, Nuel's space, and spaces surrounding the OHC; the OHC were compressed and squeezed by the surrounding supporting cells. On the other hand, the OHC developed normally. Structural features of the lateral wall, such as the membrane-bound subsurface cisterna beneath the plasma membrane, were intact. Prestin, the voltage-dependent motor protein, was observed by immunohistochemistry in the OHC basolateral membranes of both transgenic and non-transgenic mice. No significant differences in electromotility of isolated OHCs during development was observed between transgenic and control mice. The present study indicates that normal development of the supporting cells is indispensable for proper cellular function of the OHC. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: hereditary deafness, connexin26, *Gjb2*, outer hair cell, prestin, electromotility.

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Abbreviations: C_m, membrane capacitance; C_v, nonlinear capacitance; Cx26, connexin26; DAPI, 4′,6-diamidino-2-phenylindole; DPOAE, distortion product otoacoustic emission; GJB2, connexin26 gene; OHC, outer hair cell; P, postnatal day; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

The organ of Corti in mammals is a complex three-dimensional structure containing both sensory and supporting cells sitting on the basilar membrane. The supporting cells, including the pillar cells and Deiter's cells, form a rigid scaffold adjacent to and surrounding the outer hair cell (OHC) and confer essential mechanical properties for efficient transmission of stimulus-induced motion of the hair cells between the reticular lamina and the basilar membrane. Although development of pillar cells and the formation of a normal tunnel of Corti are required for normal hearing (Colvin et al., 1996), the physiological function of the supporting cells in postnatal development remains unclear.

Gap junction proteins in the cochlear supporting cells are believed to allow rapid removal of K^+ away from the base of hair cells, resulting in recycling back to the endolymph (Kikuchi et al., 1995). In addition to these effects on K^+, gap junction proteins act to mediate Ca^{2+} and anions such as inositol 1,4,5-trisphosphate, ATP, and cAMP as cell-signaling, nutrient, and energy molecules (Beltramello et al., 2005; Zhao et al., 2005; Piazza et al., 2007; Gossman and Zhao, 2008). In the developing postnatal cochlea, Tritsch et al. (2007) further found that within a transient structure known as Kolliker's organ, ATP can bind to P2X receptors on the inner hair cells, thus causing depolarization and Ca^{2+} influx, while also mimicking the effect of sound.

In the organ of Corti, most gap junctions are assembled from connexin (Cx) protein subunits, predominantly connexin 26 (Cx26, *Gjb2* gene) and co-localized Cx30 (Forge et al., 2003; Zhao and Yu, 2006). Mouse models have confirmed that Cx26 encoded by *Gjb2* is essential for cochlear function (Cohen-Salmon et al., 2002; Kudo et al., 2003). A dominant-negative *Gjb2* R75W transgenic mouse model shows incomplete development of the cochlear supporting cells, resulting in profound deafness from birth (Inoshita et al., 2008). Characteristic ultrastructural changes observed in the developing supporting cells of the *Gjb2* R75W transgenic mouse model include (i) the absence of the tunnel of Corti, Nuel's space, or spaces surrounding the OHCs; and (ii) reduced numbers of microtubules in the pillar cells. On the other hand, the development of the OHCs, at least from postnatal day 5 (P5) to P12 was not affected. The Cx26 defect in the *Gjb2* transgenic mouse is restricted to the supporting cells; it is thus difficult to explain why the auditory response is extensively disturbed despite the presence of the OHCs.

The present study was designed to evaluate developmental changes in the *in vivo* and *in vitro* function of the OHC together with the ultrastructure of the OHC and its adjacent

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supporting cells in the R75W transgenic mouse, to provide a better understanding of the functional properties of the supporting cells, and to gain new insights into the molecular and physiological mechanisms of *Gjb2*-based deafness.

EXPERIMENTAL PROCEDURES

Animals and anesthesia

All mice used for this study were obtained from a breeding colony of R75W transgenic mice (Kudo et al., 2003) and maintained at the Institute for Animal Reproduction (Ibaraki, Japan). R75W transgenic mice were maintained on a mixed C57BL/6 background and intercrossed to generate R75W transgenic animals. The animals were genotyped using DNA obtained from tail clips and amplified with the Tissue PCR Kit (Sigma, Saint Louis, MO, USA). The animals were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg, Ohara Pharamaceutical Co., Ltd., Tokyo, Japan) and xylazine (10 mg/kg) in all experiments. All experiment protocols were approved by the Institutional Animal Care and Use Committee at Juntendo University School of Medicine, and were conducted in accordance with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Distortion product otoacoustic emission

All electrophysiology was performed within an acoustically and electrically insulated and grounded test room. Distortion product otoacoustic emission (DPOAE) responses at 2f1–f2 were measured through the meatus using a measuring system (model ER-10B, Etymotic Research Inc., Elk Grove Village, IL, USA) with a probe developed for immature mice according to a previous paper (Narui et al., 2009). DPOAE stimuli were administered at two primary frequencies, f1 and f2, such that f1<f2. DPOAE input/output functions at f2=12, 30, and 45 kHz with f2/f1=1.2 were constructed. At each frequency pair, primary levels L1 (level of f1 tone) and L2 (level of f2 tone) were increased incrementally by 5 dB steps from 30 to 80 dB (f2=12 kHz and 30 kHz), and 30 to 70 dB (f2=45 kHz) with L1=L2. The DPOAE threshold level was defined as the dB level at which the 2f1–f2 distortion product was more than 10 dB above the noise level.

Non-linear capacitance

OHCs were obtained from acutely dissected organs of Corti from both transgenic and non-transgenic mice according to a previous report (Abe et al., 2007). Briefly, cochleae were dissected, and the organs of Corti were separated from the modiolus and stria vascularis. The organs were then digested with trypsin (1 mg/ml) in external solution (100 mM NaCl, 20 mM tetraethylammonium, 20 mM CsCl, 2 mM $CoCl_2$, 1.52 mM $MgCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 5 mM dextrose (pH 7.2), 300 mosmol/L, in order to block ionic conductance) for 10–12 min at room temperature and transferred into 35 mm plastic dishes (Falcon, Lincoln Park, NJ, USA) with 2 ml external solution. OHCs were isolated by gentle trituration. The dish was mounted on an inverted microscope (IX71; Olympus, Tokyo, Japan).

The patch pipette solution contained 140 mM CsCl, 2 mM $MgCl_2$, 10 mM ethyleneglycoltetraacetic acid, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2), 300 mosmol/L (adjusted with dextrose).

The cells were whole-cell voltage-clamped with an Axon (Burlingame, CA, USA) 200 B amplifier using patch pipettes having initial resistances of 3–5 MΩ. Series resistances, which ranged 5–20 MΩ, remained uncompensated for membrane capacitance (C_m) measurements, though corrections for series resistance voltage errors were made offline.

Data acquisition and analysis were performed using the Windows-based patch-clamp program jClamp (SciSoft, New Haven, CT, USA).

The C_m functions were obtained 1 min after establishment of the whole-cell configuration. C_m was assessed using a continuous high-resolution (2.56 ms sampling) two-sine voltage stimulus protocol (10 mV peak at both 390.6 and 781.2 Hz) superimposed onto a voltage ramp (200 ms duration) from −150 to +150 mV (Santos-Sacchi et al., 1998; Santos-Sacchi, 2004). The capacitance data were fit to the first derivative of a two-state Boltzmann function (Santos-Sacchi, 1991).

$$C_m = Q_{max}\frac{ze}{kT}\frac{b}{(1+b)^2} + C_{lin}$$

$$b = \exp\left(\frac{-ze(V_m - V_{pkcm})}{kT}\right)$$

where Q_{max} is the maximum nonlinear charge moved, V_{pkcm} is voltage at peak capacitance or half-maximum charge transfer, V_m is membrane potential, z is valence, C_{lin} is linear membrane capacitance, e is electron charge, k is Boltzmann's constant, and T is absolute temperature. For analyses, we quantified C_v, peak, an estimate of maximum voltage-dependent, nonlinear capacitance, as the absolute peak capacitance minus linear capacitance.

Histology

The mice were perfused with 4.0% paraformaldehyde (PFA) and 2.0% glutalaldehyde (pH 7.4) in 0.1 M phosphate buffer (PB). The inner ears were dissected and immersed in fixative overnight at room temperature. Decalcification was completed by immersion in 0.12 M ethylenediaminetetraacetic acid with gentle stirring at room temperature for a day. The cochleas were flushed again with buffer prior to perfusion with a warm solution of 10% gelatin. They were chilled on ice, thus allowing the gelatin to solidify, and then cut in half under a dissecting microscope. The half cochleas were rinsed (four times for 1 min each) with warm PB (40 °C) to remove residual gelatin. The specimens were post-fixed 1.5 h in 2.0% OsO_4 in 0.1 M PB, then dehydrated through graded ethanols and embedded in Epon. Semithin sections (1 μm) were stained with Toluidine Blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscopy (HITACHI H7100, Japan).

Immunohistochemistry

The cochleae were removed after cardiac perfusion with 4% PFA (pH 7.4), placed in the same fixative at room temperature for 1 h, decalcified with 0.12 M ethylenediaminetetraacetic acid (pH 7.0) at 4 °C overnight. The specimens were dehydrated through graded concentrations of alcohol, embedded in paraffin blocks and sectioned into 5 μm thick slices. The sections were washed in several changes of 0.01 M phosphate-buffered saline (PBS; pH 7.2), blocked with 2% bovine serum albumin in 0.01 M PBS for 30 min, and then were incubated for 1 h at room temperature with goat polyclonal antibodies to Prestin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Kitsunai et al., 2007) diluted in 0.01 M PBS+1% bovine serum albumin. The following day, the tissues were rinsed with 0.01 M PBS, incubated for 1 h at room temperature with a Alexa-Fluor-594 conjugated donkey anti-goat (1:1000; Molecular Probes, Eugene, OR, USA), rinsed with 0.01 M PBS, and then mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Labeling was viewed using a confocal laser scanning microscope (LSM510 META, Carl Zeiss, Esslingen, Germany), and each image was analyzed and saved using the ZeissLSM image Browzer (Carl Zeiss).

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