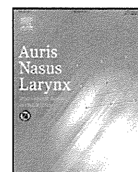
Auris Nasus Larynx xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Auris Nasus Larynx

journal homepage: www.elsevier.com/locate/anl



Cochlin-tomoprotein (CTP) detection test identified perilymph leakage preoperatively in revision stapes surgery

Yuko Kataoka [a,*], Tetsuo Ikezono [b], Kunihiro Fukushima [a], Koji Yuen [c], Yukihide Maeda [a], Akiko Sugaya [a], Kazunori Nishizaki [a]

[a] *Department of Otorhinolaryngology, Head & Neck Surgery, Okayama University Postgraduate School of Medicine, Dentistry and Pharmaceutical Science, Japan*
[b] *Department of Otolaryngology, Saitama Medical University, Japan*
[c] *Yuen Clinic, Japan*

ARTICLE INFO

Article history:
Received 14 March 2012
Accepted 14 September 2012
Available online xxx

Keywords:
Perilymphatic fistula
Stapes surgery
Cochlin
Cochlin-tomoprotein

ABSTRACT

Perilymphatic fistula (PLF) is defined as an abnormal leakage between perilymph from the labyrinth to the middle ear. Symptoms include hearing loss, tinnitus, and vertigo. The standard mode of PLF detection is intraoperative visualization of perilymph leakage and fistula, which ostensibly confirms the existence of PLF. Other possible methods of diagnosis include confirmation of pneumolabyrinth via diagnostic imaging. Recently, a cochlin-tomoprotein (CTP) detection test has been developed that allows definitive diagnosis of PLF-related hearing loss.

We report the case of a 45-year-old man who presented with right-sided tinnitus, hearing loss, and dizziness 30 years after stapes surgery. Middle ear lavage was performed after myringotomy. A preoperative diagnosis of PLF was reached using the CTP detection test. Intraoperative observations included a necrotic long process of the incus, displaced wire piston, and fibrous tissue in the oval window. Perilymph leakage was not evident. The oval window was closed with fascia, and vertigo disappeared within 2 weeks postoperatively. When PLF is suspected after stapes surgery, the CTP detection test can be a useful, highly sensitive, and less invasive method for preoperative diagnosis.

© 2012 Published by Elsevier Ireland Ltd.

1. Introduction

Perilymphatic fistula (PLF) is defined as abnormal leakage between perilymph from the labyrinth to the middle ear. PLF diagnosis has been made with pneumolabyrinth in the inner ear on computed tomography (CT) and T2-weighted magnetic resonance imaging (MRI) [1]. Leakage has been confirmed during open and endoscopic surgery [2,3]. However, PLF diagnosis is clinically difficult because CT, MRI, and perioperative methods are not always able to detect the leakage.

In 2001, cochlin-tomoprotein (CTP), a novel perilymph-specific protein, was identified [4]. CTP is a protein product of *COCH*, which was originally identified from the cochlea-specific cDNA library. Later, its mutation was found to be associated with DFNA9, an autosomal dominant hereditary deafness condition. Three cochlin isoforms were identified; CTP was one of these short 16-kDa isoforms. CTP is found in the functional domain of LCCL in cochlin and is secreted to the perilymph. CTP is highly specific for perilymph. Therefore, a diagnosis of PLF can be made by detection of CTP using Western blotting in lavage of the middle ear [5].

We report a case of right-sided tinnitus, hearing loss, and dizziness manifesting 30 years after stapes surgery. PLF was diagnosed preoperatively using the CTP test in middle ear washings. PLF was not suspected based on clinical manifestations, eardrum examination, and CT. Preoperative diagnosis was possible only because of the CTP test. CTP detection test is a new, highly sensitive, less invasive, and useful method to aid in the diagnosis of PLF.

2. Case report

The patient was a 45-year-old man. In 1980, right stapes surgery had been performed on him and a Teflon wire piston was placed (details of the surgery were uncertain). The patient presented at our hospital with right-sided tinnitus of idiopathic origin. In December 2009, he experienced mild dizziness, but no rotatory vertigo or awareness of hearing loss was evident. In an audiometric test, deterioration of hearing by bone conduction was detected as compared with hearing level recorded during a consultation conducted 20 years previously. Therefore acute mixed hearing loss was suspected.

* Corresponding author at: Department of Otorhinolaryngology, Head & Neck Surgery, Okayama University Postgraduate School of Medicine, Dentistry and Pharmaceutical Science, 2-5-1 Shikata-cho, Kita-ku, Okayama City, Okayama 700-8558, Japan. Tel.: +81 86 235 7307; fax: +81 86 235 7308.
E-mail address: yu-kat@cc.okayama-u.ac.jp (Y. Kataoka).

0385-8146/$ – see front matter © 2012 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.anl.2012.08.001

Please cite this article in press as: Kataoka Y, et al. Cochlin-tomoprotein (CTP) detection test identified perilymph leakage preoperatively in revision stapes surgery. Auris Nasus Larynx (2012), http://dx.doi.org/10.1016/j.anl.2012.08.001

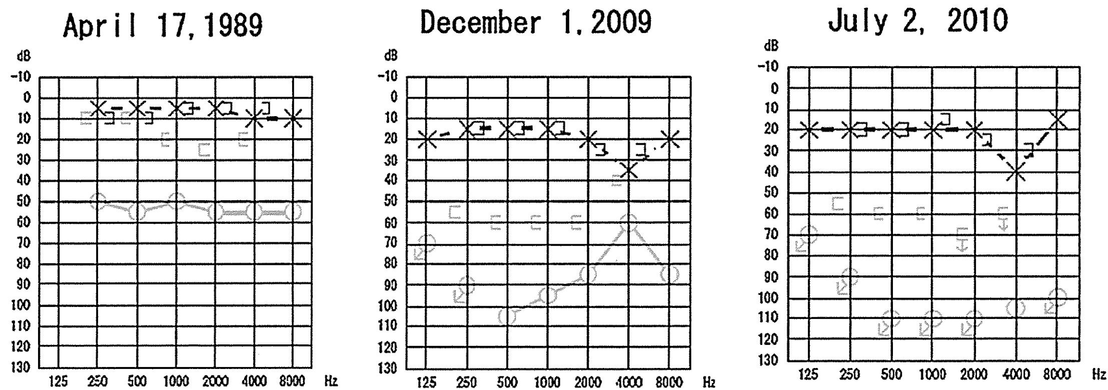


Fig. 1. Audiogram. Hearing levels in December 2009 where lower than those in 1989. In July 2010, vertigo developed, and hearing deteriorated further.

Administration of oral prednisolone (30 mg per day), ATP, and vitamin B12 was initiated. At the end of June 2010, rotatory vertigo and tinnitus appeared and hearing in the right ear deteriorated further (Fig. 1). Pure horizontal nystagmus directed to the left was observed under Frenzel glasses. On physical examination, no fluid was found in the tympanic cavity through the right tympanic membrane.

Hydrocortisone sodium succinate was administered via intravenous drip (500 mg per day) for 10 days tapering starting on July 2, 2010. Rotatory vertigo was gradually relieved, but dizziness continued. On the basis of clinical history, PLF was suspected. We obtained informed consent from him and collected middle ear washings after myringotomy under topical anesthesia and examined by the CTP detection test. The procedure of this test has been reported previously [4]. A CTP-positive signal was observed from the middle ear washings (Fig. 2), confirming the diagnosis of right PLF. After and during the test, no exacerbation of dizziness, tinnitus, or hearing loss was observed.

On November 1, 2010, surgery was performed under general anesthesia. Intraoperative observations included a necrotic long process of the incus, displaced wire piston, and fibrous tissue in the oval window. The body and short process of the incus were in the normal position. The incus and wire were transected and the wire of the piston was visible outside the oval window, but the piston was found lying deep within the vestibule. The footplate of the stapes was not found. Leakage of lymph fluid into the tympanic cavity and around the oval and round windows was not observed. Fibrous adhesions, mucosal hyperplasia, and the wire piston were removed.

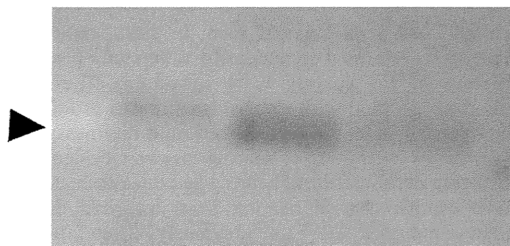


Fig. 2. Detection of cochlin-tomoprotein (CTP) in the middle ear washings. Signals represent CTP in recombinant positive control CTP (left), perilymph (middle), and middle ear washings from the patient (right) by Western blotting.

The oval and round windows were covered with the temporal fascia using fibrin glue to seal the fistula, but no prosthesis was used for the purpose of hearing improvement.

Postoperatively, mild dizziness was observed, but rotatory vertigo and nystagmus disappeared. The dizziness gradually improved and the patient was discharged 12 days after surgery.

3. Discussion

PLF causes inner ear disorders due to perilymph leakage into the tympanic cavity. PLF can be associated with a congenital anomaly, postoperative ear complications, head trauma, or barotrauma, but is most often idiopathic. PLF presents with symptoms of hearing loss, tinnitus, vestibular vertigo or dizziness, popping sounds, streaming tinnitus, and fistula signs. However, it is often indistinguishable from other inner ear diseases.

In some cases of PLF, pneumolabyrinth (air in the inner ear) and liquid leakage into the tympanic cavity can be detected by high-resolution temporal bone CT or T2-weighted MRI [1]. Although the gold standard for PLF diagnosis is intraoperative microscopic or endoscopic visualization, PLF is difficult to identify even during surgery [2,3]. Bakhos et al. [6] and Vincent et al. [7] reported that perilymphatic leakages were identified in 8% and 5.5%, respectively, of cases of revision stapes surgery. Furthermore, in their studies, PLF was suspected preoperatively in 36 cases based on clinical symptoms, but fistula was observed only in 23 cases and in 13 of them, fistula was not diagnosed due to perioperative findings [7].

Proteomic analysis of inner ear proteins identified the unique properties of CTP [4]. CTP is a protein present in perilymph, but not in other body fluids such as cerebrospinal fluid (CSF), serum, saliva, or middle ear mucosa. Therefore, CTP may be considered a specific biochemical marker for perilymph [5].

The sensitivity of the CTP test is 92.3% from middle ear lavage fluid sampled after cochlear fenestration in cochlear implant surgery [8]. While its specificity is 98.2% from middle ear lavage of non-PLF cases without middle ear infections [9]. Analysis of middle ear lavage fluid sampled from patients with middle ear infections may provide false-positive results (e.g., specificity of 93.5%) because of the high protein concentration in the thick pus [9]. In this study, CTP was detected in approximately 1 μl of perilymph present in the middle ear cavity. This method may enable diagnosis of PLF from minimal amounts of leaked perilymph, which is difficult to detect by CT and MRI or perioperatively. This method is also less invasive, as lavage can be performed by myringotomy or puncture of the tympanic membrane.

Several authors have suggested identification of an endogenous perilymph marker such as beta-2 transferrin, beta trace protein, or

Please cite this article in press as: Kataoka Y, et al. Cochlin-tomoprotein (CTP) detection test identified perilymph leakage preoperatively in revision stapes surgery. Auris Nasus Larynx (2012), http://dx.doi.org/10.1016/j.anl.2012.08.001

intrathecal fluorescein [10–12]. Although these markers are also detectable in inner ear fluid, PLF and CSF leakage can be difficult to distinguish because they are not organ specific.

In our case, the wire piston had transferred deep into the vestibule behind the long limb of the incus necrosis. Perilymph leakage occurred, leading to rotatory vertigo and deterioration of hearing. PLF was not initially suspected because 30 years had passed since stapes surgery, and typical symptoms of PLF were not present. In addition, effusion in the tympanic cavity was not detected on examination of the tympanic membrane. Thus, diagnosis of PLF was impossible by visual inspection alone or imaging techniques such as CT and MRI. The CTP detection test was the only method for detecting perilymph leakage in this case.

Our experience suggests that the CTP detection test can be a useful, highly sensitive, specific, and less invasive method to diagnose local manifestations of PLF.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgements

This work was partly supported by a grant from the Ministry of Health, Labor, and Welfare, Japan and the Ministry of Education, Culture, Sports, Science, and Technology, Japan. The authors would like to thank Enago (http://www.enago.jp) for the English language review.

References

[1] Nishizaki K, Yamamoto T, Akagi H, Ogawa T, Masuda Y. Imaging case of the month. Pneumolabyrinth. Am J Otol 1998;19:860–1.
[2] Ogawa K, Kanzaki J, Ogawa S, Tsuchihashi N, Inoue Y, Yamamoto M. Endoscopic diagnosis of idiopathic perilymphatic fistula. Acta Otolaryngol Suppl 1994;514:63–5.
[3] Poe DS, Rebeiz EE, Pankratov MM. Evaluation of perilymphatic fistulas by middle ear endoscopy. Am J Otol 1992;13:529–33.
[4] Ikezono T, Shindo S, Li L, Omori A, Ichinose S, Watanabe A, et al. Identification of a novel Cochlin isoform in the perilymph: insights to Cochlin function and the pathogenesis of DFNA9. Biochem Biophys Res Commun 2004;314:440–6.
[5] Ikezono T, Shindo S, Sekiguchi S, Hanprasertpong C, Li L, Pawankar R, et al. Cochlin-tomoprotein: a novel perilymph-specific protein and a potential marker for the diagnosis of perilymphatic fistula. Audiol Neurootol 2009;14:338–44.
[6] Bakhos D, Lescanne E, Charretier C, Robier A. A review of 89 revision stapes surgeries for otosclerosis. Eur Ann Otorhinolaryngol Head Neck Dis 2010;127:177–82.
[7] Vincent R, Rovers M, Zingade N, Oates J, Sperling N, Devèze A, et al. Revision stapedotomy: operative findings and hearing results. A prospective study of 652 cases from the Otology–Neurotology Database. Otol Neurotol 2010;31:875–82.
[8] Ikezono T, Sugizaki K, Shindo S, Sekiguchi S, Pawankar R, Baba S, et al. CTP (cochlin-tomoprotein) detection in the profuse fluid leakage (gusher) from cochleostomy. Acta Otolaryngol 2010;130:881–7.
[9] Ikezono T, Shindo S, Sekiguchi S, Morizane T, Pawankar R, Watanabe A, et al. The performance of cochlin-tomoprotein detection test for the diagnosis of perilymphatic fistula. Audiol Neurootol 2009;15:168–74.
[10] Buchman CA, Luxford WM, Hirsch BE, Fucci MJ, Kelly RH. Beta-2 transferrin assay in the identification of perilymph. Am J Otol 1999;20:174–8.
[11] Bachmann-Harildstad G, Stenklev NC, Myrvoll E, Jablonski G, Klingenberg O. β-Trace protein as a diagnostic marker for perilymphatic fluid fistula: a prospective controlled pilot study to test a sample collection technique. Otol Neurotol 2011;32:7–10.
[12] Gehrking E, Wisst F, Remmert S, Sommer K. Intraoperative assessment of perilymphatic fistulas with intrathecal administration of fluorescein. Laryngoscope 2002;112:1614–8.

Please cite this article in press as: Kataoka Y, et al. Cochlin-tomoprotein (CTP) detection test identified perilymph leakage preoperatively in revision stapes surgery. Auris Nasus Larynx (2012), http://dx.doi.org/10.1016/j.anl.2012.08.001

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited.

Received: 2011.10.19
Accepted: 2011.11.09
Published: 2011.11.16

Benign paroxysmal positional vertigo related to allergic otitis

Akiko Taura[1], Eriko Ogino[1], Hideaki Ohgita[2], Takayuki Nakagawa[1], Kazuo Funabiki[3], Juichi Ito[1]

[1] Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[2] Kyoto Teishin Hospital, Kyoto, Japan
[3] Osaka Bioscience Institute, Suita, Osaka, Japan

Source of support: This study was supported by a Health and Labor Science Research Grant for Research on Specific Disease (Vestibular Disorders) from the Ministry of Health, Labour and Welfare, Japan (2011)

PERSONAL USE

Summary

Background: To describe a rare case of benign paroxysmal positional vertigo (BPPV) related to allergic otitis.

Case Report: A 38-year-old woman presented with consistent positional vertigo as aggravation of her otitis media and externa. She exhibited positional vertigo like usual BPPV, but persistent geotropic nystagmus was observed. The duration of nystagmus was more than 2 minutes. Her vertigo symptoms were repetitive and each vertigo attack started when her otitis media and externa worsened. She was difficult to treat with usual therapy such as repositioning maneuver or antivertigo drugs. She had some allergy to pollen and her ear symptoms worsened during the seasonal pollen dispersal. Therefore, there might be some relation between her allergy and positional vertigo. This case might be classified as secondary BPPV and we tried to control her allergic otitis media and externa. After taking pranlukast for the treatment of her allergy, her vertigo symptoms were improved.

Conclusions: Her BPPV might be induced by her allergic otitis media and externa. We should consider unusual etiology when we encounter persistent BPPV resistant to normal therapy.

key words: **pranlukast • benign paroxysmal positional vertigo (BPPV) • otitis media**

Full-text PDF: http://www.amjcaserep.com/fulltxt.php?ICID=882102

Word count: 839
Tables: –
Figures: 4
References: 16

Author's address: Akiko Taura, Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Electronic PDF security powered by ISL-science.com

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distrib

BACKGROUND

Benign paroxysmal positional vertigo (BPPV) is well known to be due to detached otoconia, small calcium carbonate particles in the inner ear [1]. However, it is still unclear what causes the otoconia to detach. Some physicians call this disease primary or idiopathic BPPV (i-BPPV) [2]. In contrast, many patients have inner ear disease at the same side of BPPV; they are considered to have secondary BPPV (s-BPPV) [3,4]. We present a case of s-BPPV related to allergic otitis media/externa.

CASE REPORT

A 38-year-old woman presented with recurrent vertigo. She had a history of otitis media/externa in childhood. In her teens, she sometimes had vertigo. From age 25 years, her symptoms worsened and occasionally she required hospitalization to receive drip infusion therapy. She was treated by her local doctor; however, her symptoms grew worse after age 33 years. Therefore, she was transferred to Kyoto University Hospital.

On first examination, her eardrums of both sides were slightly retracted and there was a slight hearing impairment on the right side (Figure 1). CT image revealed poor development of the bilateral mastoid cavity, suggesting recurrent otitis media in childhood; however, it was well pneumatized on the visit to our hospital (Figure 2). Significant directional preponderance (DP) in vestibule-ocular reflex (VOR) gain was not observed. She exhibited positional vertigo; persistent geotropic nystagmus (Figure 3) was observed by infrared video-oculography (VOG). The duration of nystagmus was >2 minutes. Her vertigo symptoms were repetitive; each vertigo attack started when her otitis media/externa worsened. We scaled her symptoms retrospectively as follows: no symptoms, 0; mild vertigo or otitis media/externa, 1; severe vertigo or otitis media/externa, 2 (Figure 4). We tried repositioning maneuver (Lempert), but her symptoms did not improve. Blood examination revealed high antibody level for Japanese cedar and orchard grass. It was noted that her symptoms worsened during these two kinds of pollen dispersal season. She also had mild nasal allergy. Therefore, we suspected that she had allergic otitis media/externa that was causative of her vertigo. We prescribed pranlukast to control her otitis media/externa. All her symptoms including otitis media/externa and positional vertigo got better. After her continuous taking of pranlukast, she was free from severe vertigo attacks and could manage her symptoms under her control.

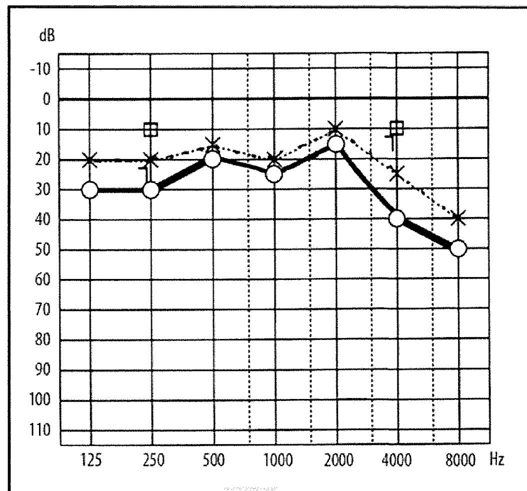


Figure 1. Hearing level on first examination. There was a slight hearing impairment at right side. Thick bar with round mark – right side; dotted line with × mark – left side.

DISCUSSION

Although the main cause of i-BPPV is believed detached otoconia and the duration of nystagmus is within 1–2 minutes, the underlying cause of the condition is still unclear. On the other hand, s-BPPV is thought related to middle ear disease or inner ear disease including neurolabyrinthitis, Ménière disease, and so on. Previous studies have reported that 3 – 66% of total BPPV cases are s-BPPV [2–5]. Earlier studies have also pointed out that a possible relation between chronic middle ear disease and BPPV [6]. During the second half of the twentieth century, the rate of s-BPPV related to middle ear disease decreased from 36% [6] to 0% [3], probably due to improvement of treatment for middle ear disease. However, there remain plenty of patients with recurrent otitis media. Recently, cytokines production has been reported in otitis media [7], and cytotoxic macromolecules pass through round window membrane because of increased membranous permeability in otitis media [8–9]. These molecules might damage the utricular macula and detach otoconia.

Many papers have reported that patients with otitis media effusion (OME) occasionally experience balance disorders. Golz et al. [10] reported that 71% of children with OME

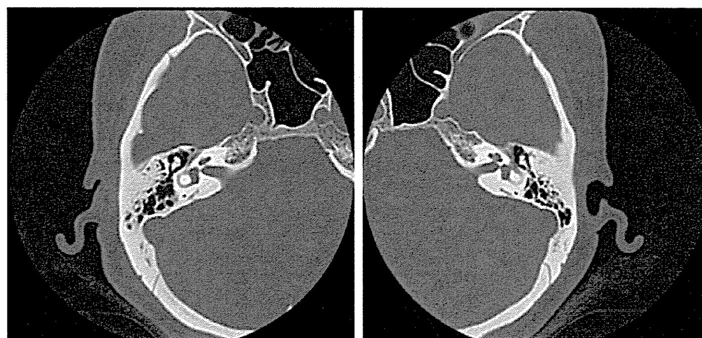


Figure 2. CT image on first examination. Pneumatization of mastoid cavity was good whereas development of both mastoid cavities was poor.

Electronic PDF security powered by ISL-science.com

This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distrib

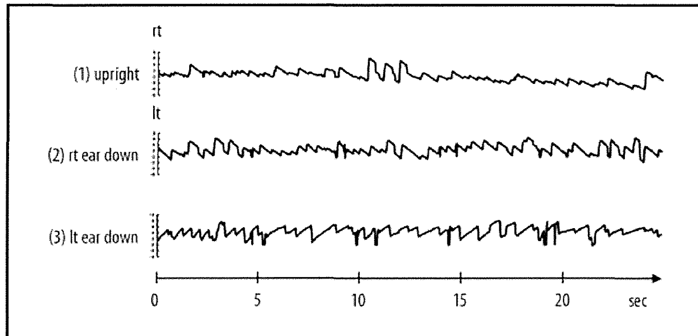


Figure 3. Video-oculography (VOG). The patient had positional and geotropic nystagmus. On the upright and the right ear-down position, she had nystagmus directed to the right side (1) (2). In contrast, she had nystagmus directed to the left side on the left ear-down position (3). The duration of nystagmus at each position was >2 minutes, longer than usual in BPPV.

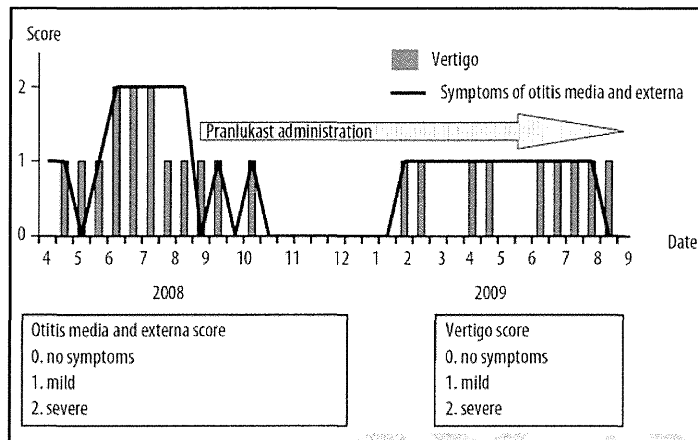


Figure 4. Symptomatic process. The patient's vertigo symptoms occurred coincidentally with aggravation of her otitis media/externa, which corresponded to the seasonal pollen dispersal. Vertical axis – scored severity of her symptoms: 0, no symptom; 1, mild vertigo or mild otitis media/externa requiring medical therapy; 2, severe vertigo or otitis media/externa requiring hospitalization for vertigo, need to insert ventilation tube, and so on.

had abnormal nystagmus and 67% recovered after ventilation tube placement [10]. They also reported that positional nystagmus was found in 22.7% children with OME and inner ear disturbances may be caused by altered pressure effect of the effusion [11].

OME is a multifactorial disease that may result from allergy [12–14]. Yellon et al. [7] reported the presence of cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-alpha in middle ear effusions [7]. Tada et al. [15] revealed that leukotriene could induce middle ear effusion experimentally and pranlukast suppressed cytokine production induced by leukotriene in middle ear. Our patient had some allergy to pollen and her ear symptoms worsened during the seasonal pollen dispersal. Therefore, there might be some relation between her allergy and positional vertigo. This case might be classified as s-BPPV. She was difficult to treat with usual therapy such as repositioning maneuver or antivertigo drugs. These findings are also compatible with s-BPPV, in which nystagmus took longer to resolve than i-BPPV [4,16]. Her allergy to pollen might have caused otitis media/externa and such reaction might have led to her positional vertigo. Therefore, pranlukast was efficacious to relieve otitis media/externa and vertigo. However, several questions still remain such as why her vertigo was positional yet her nystagmus had horizontal canal pattern and the duration was relatively longer, compared to i-BPPV. Further examinations and research are desired.

Conclusions

We present a case of BPPV that was related to allergic otitis and difficult to treat with conventional interventions such as particle repositioning maneuver. However, pranlukast was efficacious against BPPV. We might have to consider unusual etiology when we encounter s-BPPV resistant to normal therapy.

References:

1. Schuknecht HF, Ruby RR: Cupulolithiasis. Adv Otorhinolaryngol, 1973; 20: 434–43
2. Karlberg M, Hall K, Quickert N et al: What inner ear diseases cause benign paroxysmal positional vertigo? Acta Otolaryngol, 2000; 120: 380–85
3. Hughes CA, Proctor L: Benign paroxysmal positional vertigo. Laryngoscope, 1997; 107: 607–13
4. Lee NH, Ban JH, Lee KC et al: Benign paroxysmal positional vertigo secondary to inner ear disease. Otolaryngol Head Neck Surg, 2010; 143: 413–17
5. Park HM, Jung SW, Rhee CK: Vestibular diagnosis as prognostic indicator in sudden hearing loss with vertigo. Acta Otolaryngol Suppl, 2001; 545: 80–83
6. Dix MR, Hallpike CS. The pathology symptomatology and diagnosis of certain common disorders of the vestibular system. Proc R Soc Med, 1952; 45: 341–54
7. Yellon RF, Leonard G, Marucha PT et al: Characterization of cytokines present in middle ear effusions. Laryngoscope, 1991; 101: 165–69
8. Engel F, Blatz R, Kellner J et al: Breakdown of the round window membrane permeability barrier evoked by streptolysin O: possible etiologic role in development of sensorineural hearing loss in acute otitis media. Infect Immun, 1995; 63: 1305–10

Electronic PDF security powered by ISL-science.com

py is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distribution prohibited. This copy is for personal use only - distrib

9. Engel F, Blatz R, Schliebs R et al: Bacterial cytolysin perturbs round window membrane permeability barrier *in vivo*: possible cause of sensorineural hearing loss in acute otitis media. Infect Immun, 1998; 66: 343–46
10. Golz A, Netzer A, Angel-Yeger B et al: Effects of middle ear effusion on the vestibular system in children. Otolaryngol Head Neck Surg, 1998; 119: 695–99
11. Golz A, Westerman ST, Gilbert LM et al: Effect of middle ear effusion on the vestibular labyrinth. J Laryngol Otol, 1991; 105: 987–89
12. Corey JP, Adham RE, Abbass AH, Seligman I: The role of IgE-mediated hypersensitivity in otitis media with effusion. Am J Otolaryngol, 1994; 15: 138–44
13. Maw AR, Bawden R: Factors affecting resolution of otitis media with effusion in children. Clin Otolaryngol Allied Sci, 1994; 19: 125–30
14. Doer F, Yariktas M, Demirci M: The role of allergy in recurrent otitis media with effusion. J Investig Allergol Clin Immunol, 2004; 14: 154–58
15. Tada N, Furukawa M, Ogura M et al: Experimental otitis media with effusion induced by leukotriene D4. Auris Nasus Larynx, 2002; 29: 127–32
16. Mandala M, Santoro GP, Awrey J et al: Vestibular neuritis: recurrence and incidence of secondary benign paroxysmal positional vertigo. Acta Otolaryngol, 2010; 130: 565–67

PERSONAL USE ONLY

Electronic PDF security powered by ISL-science.com

The Laryngoscope
© 2011 The American Laryngological,
Rhinological and Otological Society, Inc.

Adipose Tissue-Derived Stromal Cells Protect Hair Cells From Aminoglycoside

Atsuhiro Yoshida, MD; Shin-Ichiro Kitajiri, MD, PhD; Takayuki Nakagawa, MD, PhD;
Kento Hashido, PhD; Takatoshi Inaoka, MD; Juichi Ito, MD, PhD

Background: Previous studies have demonstrated the therapeutic paracrine activity of adipose tissue-derived stromal cells (ADSCs). This study aimed to examine the ADSC potential for protecting auditory hair cells from aminoglycoside toxicity via paracrine of multiple growth factors and cytokines.

Study Design: Experimental study.

Methods: We assessed hair cell protection from neomycin toxicity by ADSC-derived factors using an explant culture system, in which cochlear explants and ADSCs were separated by a culture mesh insert to avoid direct contact. We measured the levels of growth factors and cytokines in ADSC culture media using an enzyme-linked immunosorbent assay (ELISA).

Results: Neomycin induced severe degeneration of auditory hair cells in cochlear explants, but co-culture with ADSCs significantly increased the number of surviving hair cells in explants. ELISA analysis revealed that ADSCs secreted insulin-like growth factor-1, nerve growth factor, vascular epithelial growth factor, transforming growth factor β1, monocyte chemotactic protein-1, and most prominently hepatocyte growth factor.

Conclusions: These findings demonstrate that ADSCs have the capacity to protect auditory hair cells, and can be a useful strategy to develop therapy for deafness in the clinic. The multiple paracrine growth factors and cytokines secreted by ADSCs might be involved in this effect.

Key Words: Adipose tissue-derived stromal cell, protection, cochlea, cell therapy.

***Laryngoscope*, 121:1281–1286, 2011**

INTRODUCTION

Sensorineural hearing loss (SNHL) is one of the most common disabilities, occurring in 60% of individuals aged over 65 years[1] and in 1/1,000-2,000 newborns.[2] However, therapeutic options for SNHL are limited to hearing aids and cochlear implants for chronic SNHL. For acute SNHL, the most acceptable therapeutic option is systemic glucocorticoid application, but approximately 30% of patients exhibit no response following treatment.[3] In addition, there are currently no secondary choices of therapeutic options that have demonstrated clinical efficacy. A major obstacle for the development of novel therapeutic options for SNHL is the inability to regenerate sensory hair cells in the mammalian cochleae.[4] Therefore, practical strategies have focused on the protection of cochlear hair cells.

Previous experimental studies have demonstrated the efficacy of several growth or neurotrophic factors in the protection of cochlear hair cells against various ototoxic insults. A combination of glial cell line-derived neurotrophic factor and transforming growth factor β1 (TGF-β1) reduces the loss of hair cells due to aminoglycoside ototoxicity.[5] Local application of insulin-like growth factor-1 (IGF-1) into cochleae provides functional and histological protection of cochlear hair cells against noise trauma[6,7] and ischemic injury.[8] More recently, the protective effects of hepatocyte growth factor (HGF) on cochlear hair cells against aminoglycoside toxicity[9] and noise trauma[10] have also been reported.

Transplantation of stem or stem cell-like cells is an alternative strategy for the local, sustained delivery of growth or trophic factors and cytokines into tissues, because they have the capacity to secrete several growth factors and cytokines. For this purpose, stromal cells derived from adipose tissue have been frequently used. Adipose tissue-derived stromal cells (ADSCs) are known to secrete multiple growth factors and cytokines,[11] and ADSC transplantation has contributed to angiogenesis[12,13] or functional restoration of the liver[11] via paracrine of solvable factors. We therefore highlighted the potential of ADSCs for paracrine of multiple growth factors and cytokines, and investigated the effects of ADSC on the protection of cochlear hair cells in an explant culture system where cochlea explants and ADSCs were co-cultured

From the Department of Otolaryngology–Head and Neck Surgery (A.Y., S.-I.K., T.N., K.H., T.I., J.I.), Graduate School of Medicine, Kyoto University, Kyoto; and the Therapeutic Technology R&D Department (K.H.), Medical Technology R&D Division, Olympus Corporation, Tokyo, Japan.

Editor's Note: This Manuscript was accepted for publication December 7, 2010.

This study was supported by a grant-in-aid for Regenerative Medicine Realization, a grant-in-aid for Scientific Research, and a grant from the 21st Century COE program from the Ministry of Education, Science, Sports, Culture and Technology of Japan; a grant-in-aid for Research on Sensory and Communicative Disorders from the Japanese Ministry of Health, Labor and Welfare; and a grant from the Suzuken Memorial Foundation. The authors have no other funding, financial relationships, or conflicts of interest to disclose.

Send correspondence to Takayuki Nakagawa, Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyoku, Kyoto 606-8507, Japan. E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

DOI: 10.1002/lary.21551

without their direct contact. Here, we report on the significant attenuation of aminoglycoside-induced damage in cochlear hair cells co-cultured with ADSCs.

MATERIALS AND METHODS

Animals

Imprinting control region (ICR) mice (Japan SLC, Hamamatsu, Japan) used in this study were cared for in the Institute of Laboratory Animals of the Kyoto University Graduate School of Medicine, Japan. The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols, which were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Isolation and Expansion of ADSCs

Mouse ADSCs were isolated and expanded according to methods described previously.[14] Briefly, femoral adipose tissue samples were obtained from 20 male ICR mice at 8 weeks of age under general anesthesia with midazolam (10 mg/kg; Astellas, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Tokyo, Japan). Samples obtained from five animals were transferred into one conical tube. A total of four conical tubes from 20 mice were used to isolate ADSCs.

Adipose tissues were digested in a lacto-Ringer solution containing 0.035 mg/mL Liberase Blendzyme 3 (Roche Applied Science, Mannheim, Germany) under agitation for 40 minutes at 37°C, then rested for 5 minutes to separate the stromal cell fraction from adipocytes. The stromal cell fraction was filtered through a 100 μm cell strainer (BD Biosciences, San Jose, CA) and centrifuged at 500 $\times$ g for 5 minutes. The cell pellet was resuspended in a lacto-Ringer solution and filtered through a 40 μm cell strainer (BD Biosciences). Isolated cells were seeded at 6×10^4 cells/cm^2 in 10-cm plastic plates and allowed to adhere in basic medium (DMEM/F12 with 10% fetal bovine serum, 50 μg/mL gentamycin, 250 ng/mL amphotericin B) for 24 hours. The medium was changed once daily until the cells were 80% confluent. Nonadherent cells were removed during the medium-change procedure. After the fourth passage, the cells were seeded at 1.6×10^4 cells/mL in 6-well plastic plates and cultured until they were 80% confluent. Culture media were then replaced with DMEM/F12 without supplements. ADSCs were cultured for a further 7 days without medium changes.

Cochlear Explants

Postnatal day 3, ICR mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected, and the cochleae freed from the surrounding tissue and placed in 0.01-M phosphate buffered saline (pH 7.4). After removing the cochlear lateral wall, the cochlear epithelia were dissected from the cochlear modiolus. The tissue samples were then placed on culture mesh inserts (Falcon, Billerica, MA) and cultured initially in serum-free minimum essential medium (MEM) (Invitrogen, Carlsbad, CA), supplemented with 3 g/L glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/L penicillin G (Wako Pure Chemicals), for 24 hours at 37°C in humidified (95%) air at 5% atmospheric CO_2. In total, 26 cochlear explants were used. As the hair cells in the apex are resistant to aminoglycosides, the basal (60%–80% from the apex) and upper-basal portions (40%–60% from the apex) of the cochlea were used in this study. We used five cochlea explants without any toxic drugs to confirm that this explant culture system did not damage the hair cells.

Neomycin Application and Co-Culture of Cochlear Explants With ADSCs

Neomycin (Wako Pure Chemicals) was added to the culture wells of 6-well plastic plates containing ADSCs at a final concentration of 1 mM. To avoid attachment between ADSCs and cochlear explants, the latter (n = 9) were transferred with culture-mesh inserts to culture wells containing ADSCs and 1 mM neomycin. In this co-culture study, we used ADSCs from the same origin. Other cochlear explants (n = 12) were transferred with culture-mesh inserts to culture wells containing fresh DMEM/F12 supplemented with 1 mM neomycin, and served as controls. Cultures were maintained for 24 hours, then fixed for 15 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Hair Cell Protection Assay

Cochlear explants were provided for the immunostaining of myosin VIIa and F-actin staining with phalloidin. Specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1:500; Proteus Bioscience Inc., Ramona, CA). Alexa-Fluor 568 goat anti-rabbit IgG (1:500; Invitrogen) was used as the secondary antibody. Specimens were then incubated in fluorescein isothiocyanate-conjugated phalloidin (1:80; Invitrogen) and viewed with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany).

To quantify cochlear hair cell damage, inner hair cells (IHCs) and outer hair cells (OHCs) with organized stereocilia were counted over 200-μm-longitudinal distances from two separate regions in the basal and upper-basal portions, respectively. The average value was used as the data for each culture. All values are expressed as the mean $\pm$ 1 standard error (SE). Differences in the number of hair cells with organized stereocilia between specimens co-cultured with ADSCs and control specimens cultured without ADSCs were analyzed by unpaired t tests. P values <.05 were considered to be statistically significant.

Measurement of Growth Factors and Cytokines in Culture Media

We expanded ADSCs from the four conical tubes into four separate culture wells. Following a 7-day culture of ADSCs, 500 μL of culture media were collected from each culture well, and used for an enzyme-linked immunosorbent assay (ELISA) of growth factors and cytokines using the assay kits shown in Table I. ELISA analyses were performed according to the manufacturers' instructions. Triplicate measurements were performed for each sample, and an average taken. All values are expressed as the mean $\pm$ 1 SE.

RESULTS

Hair Cell Protection by ADSCs

We estimated the protective effects of ADSC on cochlear hair cells against neomycin toxicity using an explant culture system. Previously, we established an explant culture model to analyze aminoglycoside toxicity on cochlear hair cells, in which we confirmed that any damage was not seen in the hair cells without toxic drug treatments, and a 24-hour culture with 1 mM neomycin destroyed approximately 70% of IHCs and OHCs in the explants.[9] These were deemed suitable conditions to evaluate the protective effects on cochlear hair cells. Morphology of the hair cell surface in cochlear explants is shown in Figure 1. In control specimens that were cultured with 1 mM neomycin alone, both IHCs and OHCs were severely degenerated in the basal (Fig. 1d–1f) and

Protein	Kit Name	Supplier
BDNF	Human BDNF Quantikine kit	R&D systems, Minneapolis, MN
BMP2	Human BMP2 Quantikine kit	R&D systems, Minneapolis, MN
EGF	EGF Mouse, ELISA Kit, Quantikine	R&D systems, Minneapolis, MN
bFGF	Human FGFb Quantikine kit	R&D systems, Minneapolis, MN
HGF	HGF, DuoSet(R)ELISA Development kit	R&D systems, Minneapolis, MN
IGF-1	Mouse IGF-1 Quantikine ELISA Kit	R&D systems, Minneapolis, MN
NGF	NGF Emax (R) ImmunoAssay System	Promega, Madison, WI
PDGF	Mouse/Rat PDGF-BB Quantikine ELISA Kit	R&D systems, Minneapolis, MN
TGF-b1	Canine, Mouse, Rat, Porcins TGF-b1 Quantikine kit	BenderMedSystems, Vienna, Austria
VEGF	VEGF, Mouse, ELISA Kit, RayBio	BenderMedSystems, Vienna, Austria
G-CSF	Mouse G-CSF Single Analyte ELISArray Kit	SABiosciences, Frederick, MD
GM-CSF	Mouse GM-CSF Platinum ELISA	BenderMedSystems, Vienna, Austria
M-CSF	M-CSF ELISA	Peprotech, Rocky Hill, NJ
MCP-1	Mouse MCP-1 kit	BenderMedSystems, Vienna, Austria
MIP-1a.	MIP-1α(Ccl3)ELISA Kit, Single Analyte ELISArray	Peprotech, Rocky Hill, NJ
MIP-1b	MIP-1β(Ccl4) ELISA Kit, Single Analyte ELISArray	Peprotech, Rocky Hill, NJ
SDF-1	Mouse SDF-1, Quantikine kit	R&D systems, Minneapolis, MN

TABLE I.
Measured Growth Factors, Cytokines, and Enzyme-Linked Immunosorbent Assay Kits.

BDNF: Brain-derived nuerotrophich factor, BMP2: Bone morphogenetic protein-2, EGF: Epidermal growth factor, bFGF: basic fibroblast growth factor, HGF: Hepatocyto growth factor, IGF-1: Insulin-like growth factor-1, NGF: Nerve growth factor, PDGF: Platelet-derived growth factor, TGF-b1: Transforming growth factor-beta1, VEGF: Vascular endothelial growth factor, G-CSF: Granulocyte-colony stimulating factor. GM-CSF: Granulocyte-macrophage colony-stimulating factor, M-CSF: Macrophage colony-stimulating factor, MCP-1: Monocyte chemotactic protein-1MIP-1a: Macrophage inflammatory protein-1 alpha, MIP-1b: Macrophage inflammatory protein-1beta, SDF-1a Stromal cell-derived factor-1alpha.

upper-basal portions (Fig. 1j–1l), as observed in our previous investigation.[9] In the basal portion of cochlear explants, 4.7 ± 0.5 IHCs and 12.4 ± 1.5 OHCs were present in a 200-μm length region, and the numbers of surviving IHCs and OHCs were 4.8 ± 0.9 and 12.1 ±1.9 in a 200-μm length region of the upper-basal portion of cochlear explants, respectively.

In contrast to control specimens, IHCs and OHCs were well maintained in both the basal (Fig. 1a–1c) and upper-basal portions (Fig. 1g–1i) of cochlear explants following co-culture with ADSCs. The numbers of surviving IHCs and OHCs in the basal portion were 20.3 ± 1.1 and 61.1 ± 4.6 in a 200-μm length region, and those in the upper-basal portion were 19.0 ± 0.7 and

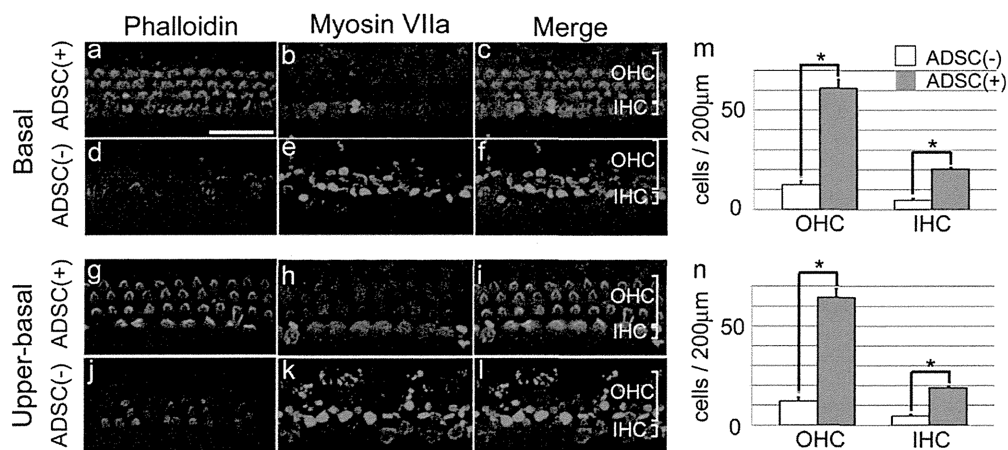


Fig. 1. Hair cell protection by paracrine effects of adipose tissue-derived stromal cells (ADSCs) against aminoglycoside toxicity. Basal (a–f) and upper-basal portions (g–l) of cochlear epithelia cultured with neomycin are shown. Phalloidin staining and immunostaining for myosin VIIa demonstrate that inner hair cells (IHCs) and outer hair cells (OHCs) are well maintained in cochleae cultured with ADSCs (a–c, g–i), whereas severe degeneration is found in both IHCs and OHCs in cochleae cultured without ADSCs (d–f, j–l). Quantitative analyses reveal significant differences in surviving IHC and OHC numbers between the two groups in the basal (m) and upper-basal (n) portions, respectively (*). Scale bar in (a) represents 50 μm (a–l). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

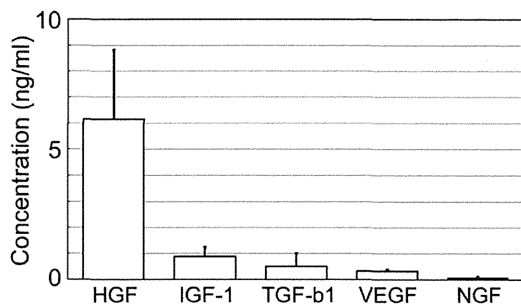


Fig. 2. Paracrine factors in ADSC-conditioned media. Hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF) were detected by enzyme-linked immunosorbent assay (ELISA). Bars represent 1 standard error.

64.4 ± 4.2. Differences in numbers of IHCs and OHCs between co-cultured and control specimens were statistically significant ($P < 0.01$ for each condition) (Fig. 1m–1n), demonstrating that co-culture with ADSCs significantly attenuates neomycin-induced damage of cochlear hair cells. This suggests that ADSCs may secrete protective factors for cochlear hair cells into culture media.

Growth Factors and Cytokines Secreted by ADSCs

To examine the ability of ADSCs to secrete growth or trophic factors and cytokines, we measured the protein levels of several factors in culture media collected after a 7-day culture of ADSCs using ELISA. Analyses revealed the presence of five growth factors: HGF, IGF-1, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and TGF-β1 at detectable levels for each assay kit (Fig. 2). The protein levels of brain-derived neurotrophic factor, bone morphogenic protein-2, epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor were undetectable by ELISA, whereas the protein level of HGF was 6154.62 ± 2673.18 pg/mL. The levels of IGF-1, TGF-β1, VEGF, and NGF were 889.69 ± 355.21, 502.00 ± 502.00, 327.53 ± 63.90, and 76.53 ± 33.89 pg/mL, respectively. For cytokines, ELISA analyses revealed the presence of one cytokine: monocyte chemotactic protein-1 (MCP-1), whereas the protein levels of other cytokines, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, macrophage inflammatory protein-1α, β (MIP-1α, β), and stromal cell-derived factor-1α (SDF-1α), were not detectable. The level of MCP-1 was 6310.67 ± 2101.91 pg/mL. These findings demonstrate that HGF, IGF-1, VEGF, NGF, TGF-β1 and MCP-1 are secreted from ADSCs.

DISCUSSION

Cell transplantation can contribute to the functional restoration of various organs through two different mechanisms. One is the differentiation of transplants into functional cells and the formation of functional connections with host cells. The second is paracrine of therapeutic factors from transplanted cells that promote self-repair systems in host organs. Several cell types have had their potential tested for use as transplants in cell-based therapy. Of these, mesenchymal stromal cells have often been used because of their ready and easy availability from bone marrow or adipose tissue, particularly in the case of autologous cells.[15] Recent studies have also demonstrated the high potential of human ADSCs for the secretion of various growth factors.[11] Furthermore, ADSCs have some advantages over bone marrow-derived stromal cells (BMSCs). First, collecting ADSCs is easier and less invasive than collecting BMSCs. Complications of bone marrow aspiration include osteomyelitis and accidental puncture of adjacent vital organs. Second, we can collect larger amount of cells at one time from adipose tissue than from bone marrow. It is possible that multiple passages of ADSCs are not needed before there are enough cells to be transplanted. Third, autologous fat transplantation have been performed for improving facial and body contour depressions and scars since 1898,[16] and the safety of adipose tissue transplantation is well established. For such reasons, we investigated the paracrine effects of ADSCs in the protection of mammalian cochlear hair cells.

In this study, we tested the potential of ADSCs in the rescue of cochlear hair cells from aminoglycoside toxicity ex vivo. To exclude the possibility of ADSCs' penetration into cochlea explants, the cochlear explants were separated from ADSCs by culture mesh inserts to avoid direct contact. We demonstrated that co-culture with ADSCs significantly promotes the survival of cochlear hair cells in explants against neomycin toxicity. We then measured the protein levels of several growth and trophic factors in the culture media of ADSCs using ELISA, revealing the presence of IGF-1, VEGF, TGF-β1, NGF, and robust amounts of HGF, which has protective effects on cochlear hair cells against neomycin toxicity[9] and noise-induced damage.[10] IGF-1 also demonstrates important protective effects on cochlear hair cells against noise trauma[6,7] and ischemic injury.[8] VEGF is associated with attenuation of noise-induced damage on cochlear hair cells,[17] whereas TGF-β1 exhibited hair cell protection against aminoglycosides in combination with glial cell line-derived neurotrophic factor.[5] In short, most ADSC-secreted growth factors presented in the current study have the potential to protect cochlear hair cells, which strongly supports our hypothesis about the paracrine effects of ADSCs.

In comparison with previous studies showing the direct effects of growth factors on hair cell protection, the levels of growth factors demonstrated in the present study were considerably low. HGF levels (6 ng/mL) were the highest of all growth factors measured in this study, but were still less than HGF levels reported to demonstrate protective effects on cochlear hair cells in explant cultures against neomycin toxicity.[9] Although we used the same explant culture system in both studies, our previous work showed significant protective effects of as much as 20 ng/mL HGF on cochlear hair cells against

1 mM neomycin, with no protection provided by 4 ng/mL HGF.[9] Based on these findings, we speculate that the protection observed in the present study was achieved by the synergistic effects of multiple growth factors secreted from ADSCs, although we cannot exclude the possibility that the recombinant HGF we used in the previous report had weaker activity than HGF ADSCs secreted in this study because of the difference in the post-transcriptional regulation. Indeed, TGF-β1 enhanced the protective effects of glial cell line-derived neurotrophic factor on cochleae against aminoglycosides,[5] which supports our synergistic hypothesis.

We also measured the levels of several cytokines in ADSC culture media, because cytokines derived from ADSCs could exert protective effects on hair cells. In the present study, ELISA analysis demonstrated the secretion of MCP-1 from ADSCs to the culture media. MCP-1 is a small cytokine belonging to adipocytokines that is also known as chemokine (C-C motif) ligand 2. MCP-1 displays chemotactic activity to monocytes and macrophages. Recently, MCP-1 was also known to play an important role in the regulation of metabolism[18] and in the pathogenesis of neurodegenerative diseases.[19] In the inner ear, the involvement of MCP-1 has been demonstrated in the process of inflammatory responses following exposure to otitis media pathogens[20] and acoustic overstimulation.[21] In addition, previous studies have suggested that migration of macrophages into cochlear tissue following traumatic insults contributes to the protection of cochlear tissues[21,22] However, direct effects of MCP-1 on hair cells have not been elucidated. Therefore, it is unclear whether MCP-1 secreted from ADSCs plays positive or negative roles in hair cell protection against neomycin toxicity. However, the present finding indicates that ADSCs could modulate inflammatory responses in the cochlea under pathological conditions via paracrine of MCP-1.

The cell transplantation approach can be advantageous at some points when we compare it with local application of growth factors. First, transplanted cells can keep secreting growth factors for a long period. Second, the secretion volume can be regulated by cross-talk with surrounding cells including damaged cells. Third, the cells may secrete unknown growth factors that have protective effect on hair cells. Fourth, the growth factors transplanted cells secrete can have higher activity than recombinant growth factors because they were regulated by physiological post-transcriptional modifications. Although these are advantages of the cell transplantation approach, it requires surgical procedure for opening the cochlear fluid space. One possible application of ADSC transplantation is hybrid cochlear implantation,[23] where low frequency auditory stimuli are transmitted to the auditory primary neurons by the remaining hair cells, and high frequency auditory stimuli are transmitted to the auditory primary neurons by direct electrical stimulation via cochlear implant devices. In the case of hybrid cochlear implantation, the preservation of residual hearing at low frequency regions is critical. It is therefore important to protect cochlear hair cells from invasive surgery. During cochlear implant surgery, the cochlear bony wall is opened to insert a cochlear implant electrode; at this stage, therapeutic cells could be transplanted into the cochlea. Because the capacity of ADSCs to protect cochlea hair cells was demonstrated in this study, ADSCs are expected to be applied to clinical use in case of hybrid cochlear implantation in future.

CONCLUSION

The present findings demonstrate that ADSCs have the capacity to protect cochlear hair cells via paracrine effects, suggesting that ADSC transplantation into cochleae may be a useful strategy for the protection of cochlear hair cells in vivo. As a next step, we will examine the effects of ADSC transplantation for cochlear protection using animal models in vivo.

Acknowledgments

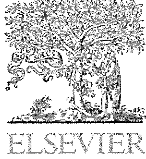
The authors thank Tomoko Kita for help and instruction in ELISA analyses, and Yayoi S. Kikkawa, Norio Yamamoto, and Yushi Hayashi for help and instruction in cochlear explant cultures.

BIBLIOGRAPHY

1. Lopez-Torres Hidalgo J, Boix Gras C, Tellez Lapeira J, Lopez Verdejo M, del Campo del Campo J, Escobar Rabadan F. Functional status of elderly people with hearing loss. *Arch Gerontol Geriatr* 2009;49:88–92.
2. Fortnum H, Summerfield A, Marshall D, Davis A, Bamford J. Prevalence of permanent childhood hearing impairment in the United Kingdom and implications for universal neonatal hearing screening: questionnaire based ascertainment study. *BMJ* 2001;323:536–540.
3. Ogawa K, Takei S, Inoue Y, Kanzaki J. Effect of prostaglandin E1 on idiopathic sudden sensorineural hearing loss: a double-blinded clinical study. *Otol Neurotol* 2002;23:665–668.
4. Chardin S, Romand R. Regeneration and mammalian auditory hair cells. *Science* 1995;267:707–711.
5. Kawamoto K, Yagi M, Stover T, Kanzaki S, Raphael Y. Hearing and hair cells are protected by adenoviral gene therapy with TGF-beta1 and GDNF. *Mol Ther* 2003;7:484–492.
6. Iwai K, Nakagawa T, Endo T, et al. Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. *Laryngoscope* 2006;116:529–533.
7. Lee K, Nakagawa T, Okano T, et al. Novel therapy for hearing loss: delivery of insulin-like growth factor 1 to the cochlea using gelatin hydrogel. *Otol Neurotol* 2007;28:976–981.
8. Fujiwara T, Hato N, Nakagawa T, et al. Insulin-like growth factor 1 treatment via hydrogels rescues cochlear hair cells from ischemic injury. *Neuroreport* 2008;19:1585–1588.
9. Kikkawa Y, Nakagawa T, Tsubouchi H, et al. Hepatocyte growth factor protects auditory hair cells from aminoglycosides. *Laryngoscope* 2009; 119:2027–2031.
10. Inaoka T, Nakagawa T, Kikkawa Y, et al. Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs. *Acta Otolaryngol* 2009;129:453–457.
11. Banas A, Teratani T, Yamamoto Y, et al. IFATS collection: in vivo therapeutic potential of human adipose tissue mesenchymal stem cells after transplantation into mice with liver injury. *Stem Cells* 2008;26: 2705–2712.
12. Kondo K, Shintani S, Shibata R, et al. Implantation of adipose-derived regenerative cells enhances ischemia-induced angiogenesis. *Arterioscler Thromb Vasc Biol* 2009;29:61–66.
13. Nakagami H, Maeda K, Morishita R, et al. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. *Arterioscler Thromb Vasc Biol* 2005;25:2542–2547.
14. Fujimura J, Ogawa R, Mizuno H, Fukunaga Y, Suzuki H. Neural differentiation of adipose-derived stem cells isolated from GFP transgenic mice. *Biochem Biophys Res Commun* 2005;333:116–121.
15. Zuk P, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;7:211–228.
16. Pulagam S, Poulton T, Mamounas E. Long-term clinical and radiologic results with autologous fat transplantation for breast augmentation: case reports and review of the literature. *Breast J* 2006;12:63–65.
17. Picciotti P, Fetoni A, Paludetti G, et al. Vascular endothelial growth factor (VEGF) expression in noise-induced hearing loss. *Hear Res* 2006;214: 76–83.
18. Rull A, Camps J, Alonso-Villaverde C, Joven J. Insulin resistance, inflammation, and obesity: role of monocyte chemoattractant protein-1 (or

CCL2) in the regulation of metabolism. *Mediators Inflamm* 2010;2010. pii: 326580.
19. Gerard C, Rollins BJ. Chemokines and disease. *Nat Immunol* 2001;2: 108–115.
20. Moon SK, Woo JI, Lee HY, et al. Toll-like receptor 2-dependent NF-kappaB activation is involved in nontypeable Haemophilus influenzae-induced monocyte chemotactic protein 1 up-regulation in the spiral ligament fibrocytes of the inner ear. *Infect Immun* 2007;75:3361–3372.
21. Sautter NB, Shick EH, Ransohoff RM, Charo IF, Hirose K. CC chemokine receptor 2 is protective against noise-induced hair cell death: studies in CX3CR1(+/GFP) mice. *J Assoc Res Otolaryngol* 2006;7:361–372.
22. Okano T, Nakagawa T, Kita T, et al. Bone marrow-derived cells expressing Iba1 are constitutively present as resident tissue macrophages in the mouse cochlea. *J Neurosci Res* 2008;86:1758–1767.
23. Gantz B, Turner C. Combining acoustic and electrical hearing. *Laryngoscope* 2003;113:1726–1730.

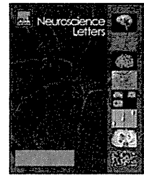
Neuroscience Letters 534 (2013) 18–23



Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet



Otospheres derived from neonatal mouse cochleae retain the progenitor cell phenotype after ex vivo expansions

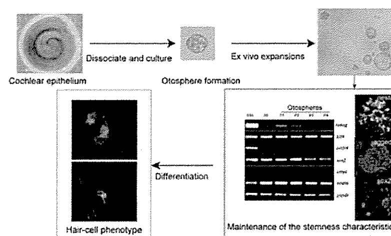
Xiang-Xin Lou, Takayuki Nakagawa*, Hiroe Ohnishi, Koji Nishimura, Juichi Ito

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

HIGHLIGHTS

- ► Otospheres are generated from the cochlear epithelia of neonatal mice.
- ► Otospheres after ex vivo expansions retained the stemness characteristics.
- ► Otospheres after ex vivo expansions differentiated into hair-cell phenotypes.
- ► The activity for self-renewal in otospheres was weakened by ex vivo expansions.
- ► Otospheres after ex vivo expansions are useful for studying hair cell regeneration.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 25 May 2012
Received in revised form 16 October 2012
Accepted 3 December 2012

Keywords:
Stem cell
Hair cell
Regeneration
Sphere
Self-renewal
Differentiation

ABSTRACT

Because of their limited regenerative potential, cochlear hair cell loss is one of the major causes of permanent hearing loss in mammals. However, recent studies have shown that postnatal cochlear epithelia retain the progenitor cells that form otospheres. Otospheres are capable of self-renewing and differentiating into inner ear cell lineages, thereby suggesting a promising source for hair cell regeneration. We investigated retention of the progenitor cell phenotype in otospheres after ex vivo expansion, which is crucial for transplantation approaches. Reverse transcriptase-polymerase chain reaction and immunocytochemical analyses showed that otospheres derived from neonatal mice retained expression of stem and cochlear cell markers. After in vitro differentiation, otosphere-consisting cells differentiated into hair cell phenotypes after ex vivo expansion. However, the capacity of otospheres for self-renewal weakened with subsequent generations of ex vivo expansion. Our results indicate that ex vivo expanded-otospheres are useful experimental tools for studying hair cell regeneration in transplantation approaches and that the mechanisms for retention of the progenitor cell phenotype in otospheres should be investigated.
© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Sensorineural hearing loss is a common and often irreversible disability. One of the major reasons for poor recovery from hearing loss is the apparent lack of regenerative capacity in cells of the mammalian cochleae, particularly hair cells. However, hearing recovery via hair cell regeneration occurs in avian species [2,15]; this has encouraged researchers to seek a method for regeneration in mammalian cochlear hair cells. Several novel therapeutic strategies, including gene and/or stem cell therapy, have been investigated for achieving hair cell regeneration in mammalian cochleae [5,14,21].

Abbreviations: ICR, Institute of Cancer Research; DMEM, Dullbecco's modified Eagle medium; DAPI, 4', 6-diamidino-2-phenyliodole; iPS cells, induced pluripotent stem cells; ES cells, embryonic stem cells; RT-PCR, reverse transcriptase-polymerase chain reaction; SE, sensory epithelia.

* Corresponding author at: Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyoku, Kyoto 606-8507, Japan. Tel.: +81 75 751 3346; fax: +81 75 751 7225.
E-mail addresses: louxiangxin@yahoo.com.cn (X.-X. Lou), tnakagawa@ent.kuhp.kyoto-u.ac.jp (T. Nakagawa), h_oonishi@ent.kuhp.kyoto-u.ac.jp (H. Ohnishi), k_nishimura@ent.kuhp.kyoto-u.ac.jp (K. Nishimura), ito@ent.kuhp.kyoto-u.ac.jp (J. Ito).

0304-3940/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neulet.2012.12.001

Stem cell-based therapy has gained considerable attention as a potential treatment for several irreversible diseases, including sensorineural hearing loss. In particular, the establishment of induced pluripotent stem (iPS) cells, which are generated from one's own cells with a pluripotency similar to that of embryonic stem (ES) cells [20], highlights the importance of developing stem cell-based therapy. Recently, studies showed the possibility of generating inner ear cell lineages, including hair cells, from mouse ES and iPS cells [7,14], thereby suggesting the potential of PS cells as a transplant source for hair cell regeneration.

In addition, recent studies have indicated the presence of endogenous stem or progenitor cells in rodent cochlear epithelia [19,23]. Numerous reports have shown that rodent postnatal cochlear cells have the capacity for sphere formation, similar to that of neural stem cells [3,8,9,12,13,16,17,25,26]. Spheres generated from cochlear cells, namely otospheres, have the capacity to self-renew and differentiate into cochlear cells, including hair cells. More recently, dissociated cells from fetal human cochleae have been demonstrated to have the capacity for generating otospheres [1]. Furthermore, the generation of otospheres is possible from post-mortem cochlear specimens [18], suggesting the possible therapeutic potential of harvesting otospheres from post-mortem human cochleae.

To consider the use of otospheres in transplantation approaches, at least 3 passages of ex vivo expansion are required to obtain sufficient numbers of otospheres. Therefore, it is important to determine whether otospheres retain their progenitor phenotype after ex vivo expansion. To this end, we investigated the progenitor cell phenotype of otospheres after ex vivo expansion and tested their capacity for differentiation into hair cell phenotypes.

2. Materials and methods

2.1. Animals

Postnatal day 1 Institution of Cancer Research (ICR) mouse pups (Japan SLC, Hamamatsu, Japan) from different litters were used. The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols, which were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Generation of otospheres

Dissociated cell cultures were obtained from postnatal day 1 ICR mice under aseptic conditions. Sensory epithelial sheets were isolated from the cochleae in Hank's buffered salt solution (HBSS, pH 7.4; Invitrogen, Carlsbad, CA) at 4 °C. Tissues were subjected to 0.125% trypsin in 0.1 M phosphate-buffered saline (PBS, pH 7.4; Invitrogen) for 15 min at 37 °C, and then blocked using a trypsin inhibitor and a DNase I solution (Sigma, St. Louis, MO). The pellets were suspended in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1; Invitrogen) supplemented with N2 and B27 supplements (Invitrogen), epidermal growth factor (20 ng/ml), basic fibroblast growth factor (10 ng/ml), insulin-like growth factor-1 (50 ng/ml; all growth factors obtained from R&D Systems, Minneapolis, MN), ampicillin (50 ng/ml; Sigma) and heparin sulfate (50 ng/ml; Sigma). The suspension was passed through a 70-μm cell strainer (BD Biosciences, San Jose, CA) into 6-well plastic Petri dishes (Greiner Bio-One, Monroe, NC). After 3 days of culturing, the cell suspension was re-plated in new Petri dishes. Otospheres were sequentially identified after 4–5 days of culturing.

2.3. Ex vivo expansion of otospheres

Ex vivo expansion of otospheres was repeated at intervals of 5–7 days. For ex vivo expansion, otospheres were dissociated using 0.125% trypsin. A 40-μm cell strainer (BD Biosciences) was used to collect dissociated cells. The cell suspensions (1×10^4 cells) were re-plated in 6-well plastic Petri dishes. Primary otospheres were defined as P1 and those obtained after 1, 2, or 3 additional passages were defined as P2, P3, or P4 generation otospheres, respectively.

2.4. Proliferation assays

The numbers of total otospheres and solid spheres were counted at each generation. The solid spheres were identified according to their morphology [3]. The difference in the total sphere number and the proportion of solid spheres compared to the total number of spheres among the passages was examined by analysis of variance (ANOVA) and Tukey–Kramer tests.

2.5. Induction of otosphere differentiation

To study the capacity for differentiation, P4 generation otospheres were dissociated using 0.125% trypsin (Invitrogen) and transferred to 0.1% gelatin (Sigma)-coated 4-well tissue culture plates (Greiner Bio-One). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2 in differentiation media consisting of (DMEM)/F12 (1:1) (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 50 ng/ml ampicillin (Sigma). The fate of the differentiated cells was examined by immunocytochemistry after 7 days of culturing.

2.6. Reverse transcription-polymerase chain reaction

Total RNA was isolated from mouse ES cells (G4-2, generously donated by Dr. Hitoshi Niwa, Riken CDB, Kobe, Japan), cochlear epithelia from postnatal day 1 mice, and each generation of otospheres using RNeasy Mini Kits (QIAGEN, Valencia, CA). Next, 500 ng of total RNA was reverse-transcribed using Superscript III (Invitrogen). We examined expression of *nanog*, *oct3/4*, *klf4*, *c-myc*, *sox2*, and *nestin* as markers for stem cells, and expression of *pax2*, *bmp7*, *brn3.1*, *myosin VIIa*, *p27kip1*, and *espin* as cochlear lineage markers. The primers used in this study are shown in Table 1. The reverse transcription-polymerase chain reaction (RT-PCR) experiments were independently repeated 3 times with the same total RNA material.

2.7. Immunostaining

Specimens were fixed with 4% paraformaldehyde for 15 min at room temperature. Cochlear epithelia from postnatal day 1 mice were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), and subsequently sectioned in 10-μm slices on a cryostat. Specimens were incubated with primary antibodies overnight at 4 °C, followed by incubation with secondary antibodies for 60 min at room temperature. At the end of the staining procedures, counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) was performed. Fluorescein isothiocyanate-conjugated phalloidin (Invitrogen) was used to visualize F-actin. Specimens were viewed using a confocal microscope (Leica Microsystems, Wetzlar, Germany). The primary antibodies used were as follows: mouse monoclonal antibodies for Oct3/4 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), nestin (1:500, BD Biosciences), and espin (1:200, BD Biosciences); rabbit polyclonal antibodies for myosin VIIa (1:500, Proteus Biosciences, Ramona, CA) and p27kip1

Table 1
Primers for RT-PCR analyses.

Genes	Primers	
	Forward	Reverse
nanog	AGG GTC TGC TAC TGA GAT GCT CTG	CAA CCA CTG GTT TTT CTG CCA CCG
oct3/4	TCT TTC CAC CAG GCC CCC GGC TC	TGC GGG CGC ACA TGG GGA GAT CC
klf4	CCA ACT TGA ACA TGC CCG GAC TT	TCT GCT TAA AGG CAT ACT TGG GA
cmyc	TGA CCT AAC TCG AGG AGG AGC TGG AAT C	TTA TGC ACC AGA GTT TCG AAG CTG TTC G
sox2	TAG AGC TAG ACT CCG GGC GAT GA	TTG CCT TAA ACA AGA CCA CGA AA
nestin	GAT CGC TCA GAT CCT GGA AG	AGA GAA GGA TGT TGG GCT GA
pax2	CCC ACA TTA GAG GAG GTG GA	GAC GCT CAA AGA CTC GAT CC
bmp7	TCT TCC ACC CTC GAT ACC AC	GCT GTC CAG CAA GAA GAG GT
brn3.1	GTC TCA GCG ATG TGG AGT CA	TCA TGT TGT TGT GCG ACA GA
myosin VIIa	CAC TGG ACA TGA TTG CCA AC	ATT CCA AAC TGG GTC TCG TG
p27kip1	ATT GGG TCT CAG GCA AAC TC	TTC TGT TCT GTT GGC CCT TT
espin	ACC TAC GTA CGG TGC AAA CC	AGT GAC TGG AGG AGC AGG AG
gapdh	GGG TGT GAA CCA CGA GAA AT	ACA GTC TTC TGG GTG GCA GT

(1:200, NeoMarkers, Fremont, CA); and goat polyclonal antibodies for sox2 (1:200, Santa Cruz Biotechnology) and jagged1 (1:200, Santa Cruz Biotechnology).

3. Results

3.1. Otosphere generation

Dissociated cells from the cochlear epithelial sheets of postnatal day 1 mice formed the primary otosphere after 5–7 days of culturing; this was defined as the P1 generation sphere. For ex vivo expansion, otospheres were dissociated, re-plated, and reformed into otospheres after 5–7 days. Otospheres obtained from 1, 2, or 3 additional passages were designated the P2, P3, or P4 generation otospheres, respectively. Otospheres were divided into 3 types: solid, transitional, or hollow spheres, according to morphology [3] (Fig. 1A). Solid spheres are condensed, small in size, and have the potential for self-renewal, whereas hollow spheres are the largest and lack self-renewal capacity [3]. At each generation, all 3 types of otospheres were observed.

3.2. Proliferative activity

To assess the self-renewal potential, the total yield of otospheres and solid spheres was determined at each generation in 6 independent experiments. There were significant differences in the total yield of otospheres among generations (Fig. 1B). The number of total otospheres significantly increased from the P1 (93.5 ± 14.2; mean ± standard deviation) to P2 generation (109.8 ± 4.8). However, after the P2 generation, the number of otospheres significantly decreased as the number of passages increased (Fig. 1B). The numbers of otospheres in the P3 and P4 generations were 83.5 ± 6.6 and 58.1 ± 9.5, respectively. The proportion of solid spheres was calculated in each generation (Fig. 1C). As expected, P1 generation otospheres exhibited the highest proportion of solid spheres. The proportion of solid spheres decreased in reverse relation to increasing numbers of passages. Differences in the proportion of solid spheres between P1 and P2, P3, or P4 and between P2 and P4 were statistically significant. These findings indicate that the capacity of otospheres for self-renewal weakens during subsequent ex vivo expansions.

3.3. Otosphere characteristics

We examined the expression of stem cell markers in otospheres from each generation using RT-PCR and immunostaining. The expression pattern in the otospheres was compared with the expression patterns in mouse ES cells or cochlear sensory epithelia from postnatal day 1 mice. ES cells expressed stem cell markers, including *nanog*, *klf4*, *oct3/4*, *sox2*, and *nestin* (Fig. 2). *c-myc* expression was not observed in any cell, including ES cells (Fig. 2). Otospheres also expressed *nanog*, *sox2*, *klf4*, and *nestin*, and the expression pattern was similar among the generations (Fig. 2). The expression pattern of stem cell markers in otospheres, as observed by RT-PCR analyses, was identical to that in cochlear sensory epithelia of neonatal mice (Fig. 2). In immunohistochemical analyses, sox2 and jagged1 were widely observed in the supporting cells in the neonatal cochlear epithelia (Fig. 3A and B), which is consistent with previous findings [10,11]. Nestin was robustly expressed in the tympanic border cells underneath the basilar membrane

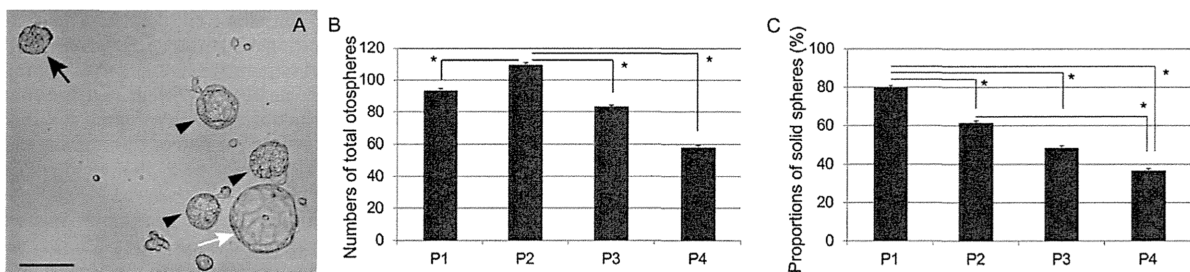


Fig. 1. Otosphere morphology (A), total otosphere numbers (B), and proportion of solid otospheres (C) after ex vivo expansion. (A) Otospheres are divided into 3 types: solid (black arrow), transitional (arrowheads), or hollow spheres (white arrow). Scale bar represents 100 μm. (B) The number of total otosphere at the P2 generation is significantly higher than that at the P1, P3, or P4 generations (*$p < 0.05$). (C) The proportion of solid spheres at the P1 generation is higher than at the P1, P3, or P4 generations (*$p < 0.05$). Bars in panels B and C indicate 1 standard deviation.

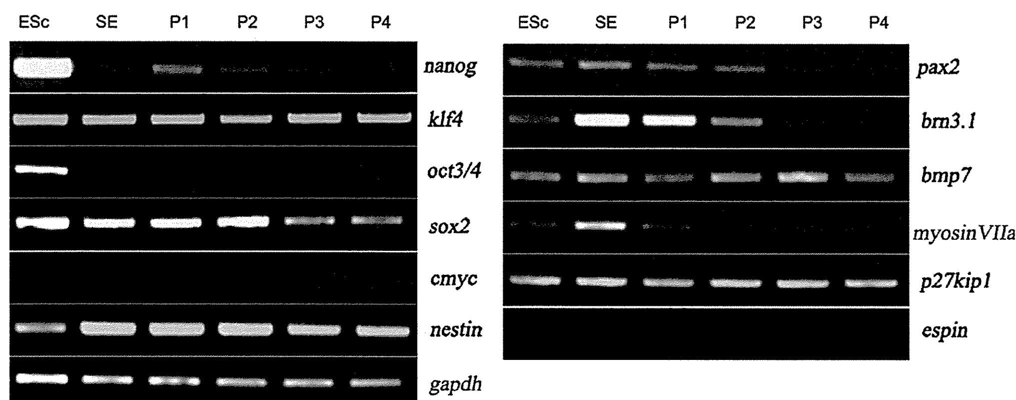


Fig. 2. RT-PCR analyses for several stem cell and developing cochleae markers in ES cells (ESc), neonatal cochleae sensory epithelia (SE), and P1–P4 generation otospheres.

and in the osteospiral lamina (Fig. 3C), which is in line with previous findings [22]. Weak immunoreactivity was observed in some supporting cells, including Deiters and inner border cells (Fig. 3C). No Oct3/4 expression was detected in the cochlear epithelial cells. Similar to postnatal day 1 cochlear epithelia, P4 generation otospheres expressed sox2, jagged1 (Fig. 3F–H), and nestin, but not Oct3/4. Sox2 expression was detected in $30 \pm 3.4\%$ of the P4 generation otospheres, and nestin was expressed in $91 \pm 12.4\%$ of the otospheres. Jagged1 was expressed in all P4 generation otospheres. In summary, our results show that the characteristics of a population of supporting cells in neonatal cochleae, which may be the origin of otospheres, are retained in the otospheres after ex vivo expansions.

We also examined the expression of pax2, brn3.1, and bmp7 as markers for the early developmental stage of cochlear epithelia. In RT-PCR assessments, these markers were found in all specimens (Fig. 2). Expression of p27kip1 and myosin VIIa, which appears in cochlear epithelia in mice approximately on embryonic day 12, was also detected in all specimens (Fig. 2). Conversely, the expression of espin, a mature hair cell marker, was observed only in the cochlear sensory epithelia (Fig. 2). In immunohistochemical analyses, p27kip1 immunoreactivity was observed in the supporting cells in the neonatal cochlear epithelia (Fig. 3D), and myosin VIIa and espin expression was observed in hair cells (Fig. 3E). P4 generation otospheres expressed p27kip1 and myosin VIIa, but not espin (Fig. 3I and J). All P4 generation otospheres contained p27kip1-expressing cells, and 31.7 ± 6. Six percent of the otospheres contained myosin VIIa-positive cells. These findings indicate that the otospheres maintained the characteristics of cochlear lineages after ex vivo expansion and contained no mature hair cells. In summary, after ex vivo expansion, otospheres retained the characteristics of both stem and cochlear cells.

3.4. *Differentiation capacity*

To study the capacity of P4 generation otospheres to differentiate, otospheres were dissociated and transferred into differentiation media [8]. After 7 days in culture in the differentiation media, the fate of the dissociated cells was assessed by immunocytochemical analyses. Of the cultured dissociated otosphere cells, $4.4 \pm 1.2\%$ remained positive for nestin with the morphology of immature neurons (Fig. 4A). Sox2 expression was observed in $18.1 \pm 3\%$ of the cultured cells (Fig. 4B). p27kip1 expression was found in $90.3 \pm 2.8\%$ of the cultured cells (Fig. 4C). The expression of myosin VIIa was observed in $0.5 \pm 0.2\%$ of cells, and double staining for espin and myosin VIIa was observed in a limited numbers of cultured cells (Fig. 4D–I). Co-localization of F-actin labeling with phalloidin and espin indicated the presence of the stereocilia-like structures in the cultured cells (Fig. 4J–L). These findings indicate that P4 generation otospheres have the capacity to differentiate into hair cells.

4. Discussion

Cochlear epithelia of neonatal mice contain cells with the ability to form otospheres when cultured in non-adherent conditions [3,9,12,25,26]. Otospheres have been shown to have the capability for self-renewal and differentiation into multiple lineages [13,25]. Importantly, hair cell-like cells are constantly generated from otospheres without the use of specific culture conditions, which may be advantageous over stem cells or stem cell-like cells from other origins. Somatic stem cells derived from the bone marrow [6], neural tissues [21], or olfactory tissues [4] require specific conditions, including transplantation into damaged cochleae or gene transfer,

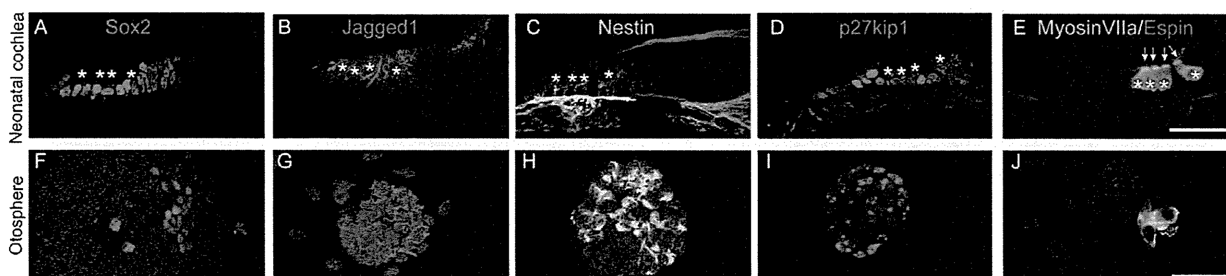


Fig. 3. Immunostaining for sox2, jagged1, nestin, p27kip1, myosin VIIa, and espin in neonatal cochleae sensory epithelia (panels A–E) and P4 generation otospheres (panels F–J). Asterisks indicate the location of hair cells, and the arrows in panel E indicate espin expression at the top of hair cells. Scale bars represent 50 μm.

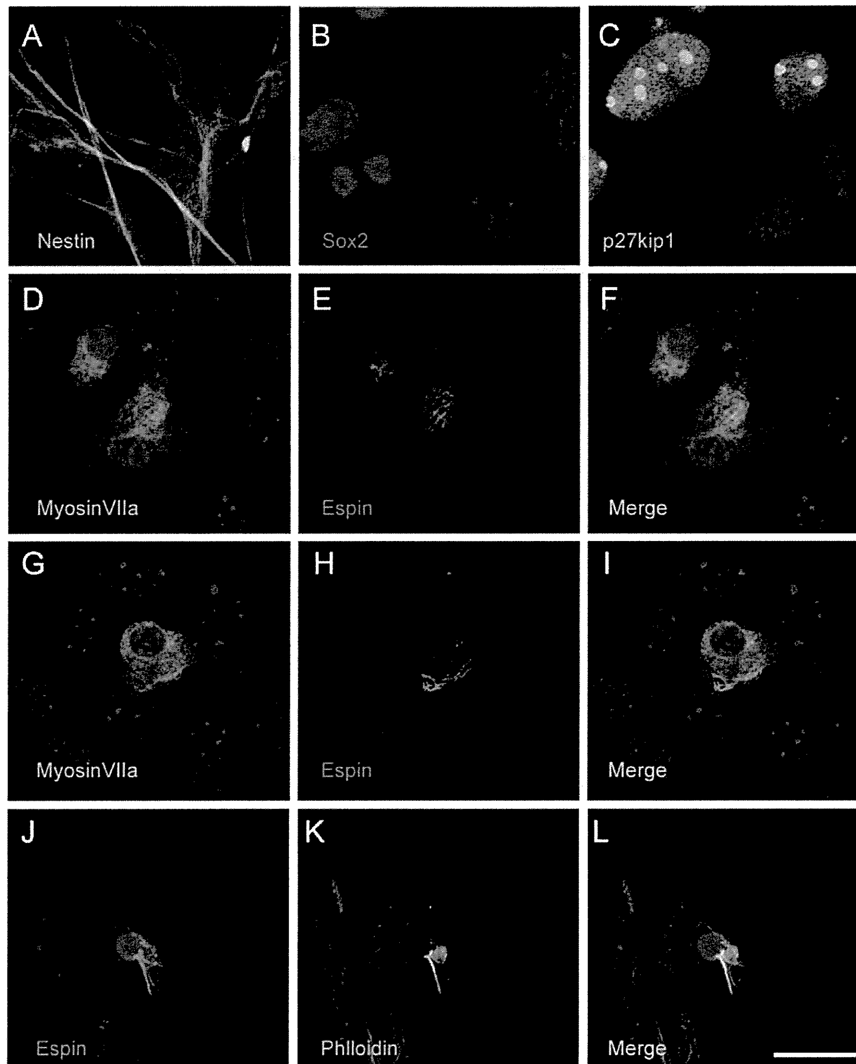


Fig. 4. Otosphere-derived cells after culturing in differentiation media for 7 days. Nestin or sox2 expression is observed in some cells (panels A and B). The majority of cells are positive for p27kip1 (panel C), whereas some cells co-express myosin VIIa and espin (panels D–I). Espin co-localizes with F-actin labeling and phalloidin (panels J–L). Scale bar represents 20 μm.

to generate hair cell-like cells. In ES or iPS cells, the generation of hair cell-like cells required co-culture with chicken utricle stromal cells [14], or transplantation into chick auditory epithelia [7]. Therefore, otospheres may be a better source for transplantation aimed at hair cell regeneration.

According to our calculations, an adequate number of otospheres for transplantation experiments can be harvested from at least 3 passages of ex vivo expansion from 1 cochlea. However, it is important to determine whether otospheres retain their characteristics after ex vivo expansions. Thus, in the present study, the characteristics of otospheres after 3 passages of ex vivo expansion were compared with those of the cochlear sensory epithelium and of otospheres from earlier generations. The current findings show that after ex vivo expansion, otospheres retain the characteristics of cochlear progenitor cells, of which origins may be cochlear supporting cells [19], as well as the capability to differentiate into hair cell phenotypes, suggesting the feasibility for their use in transplantation approaches. However, the potential of otospheres for self-renewal weakened with subsequent ex vivo expansions. Savary et al. demonstrated promotion of sphere formation activity upon Notch activation [17], which may be associated with the role of Notch signaling in the maintenance of cochlear progenitor cells during development [24]. The molecular mechanisms responsible for the diminished capacity for self-renewal in otospheres after ex vivo expansion should be investigated further to evaluate the potential use of otospheres in hair cell regeneration.

Conflict of interests statement

The authors declare no competing financial interests.

Acknowledgements

The research was supported by a Grant-in-Aid for Regenerative Medicine Realization and a Grant-in-Aid for Scientific Research

from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

References

[1] W. Chen, S.L. Johnson, W. Marcotti, P.W. Andrews, H.D. Moore, M.N. Rivolta, Human fetal auditory stem cells can be expanded in vitro and differentiate into functional auditory neurons and hair cell-like cells, Stem Cells 27 (2009) 1196–1204.
[2] J.T. Corwin, D.A. Cotanche, Regeneration of sensory hair cells after acoustic trauma, Science 240 (1988) 1772–1774.
[3] M. Diensthuber, K. Oshima, S. Heller, Stem/progenitor cells derived from the cochlear sensory epithelium give rise to spheres with distinct morphologies and features, Journal of the Association for Research in Otolaryngology 10 (2009) 173–190.
[4] K.L. Doyle, A. Kazda, Y. Hort, S.M. McKay, S. Oleskevich, Differentiation of adult mouse olfactory precursor cells into hair cells in vitro, Stem Cells 25 (2007) 621–627.
[5] M. Izumikawa, R. Minoda, K. Kawamoto, K.A. Abrashkin, D.L. Swiderski, D.F. Dolan, D.E. Brough, Y. Raphael, Auditory hair cell replacement and hearing improvement by Atoh1 gene therapy in deaf mammals, Nature Medicine 11 (2005) 271–276.
[6] S.J. Jeon, K. Oshima, S. Heller, A.S. Edge, Bone marrow mesenchymal stem cells are progenitors in vitro for inner ear hair cells, Molecular and Cellular Neuroscience 34 (2007) 59–68.
[7] H. Li, G. Roblin, H. Liu, S. Heller, Generation of hair cells by stepwise differentiation of embryonic stem cells, Proceedings of the National Academy of Sciences of the United States of America 100 (2003) 13495–13500.
[8] X. Lou, Y. Zhang, C. Yuan, Multipotent stem cells from the young rat inner ear, Neuroscience Letters 416 (2007) 28–33.
[9] B. Malgrange, S. Belachew, M. Thiry, L. Nguyen, B. Rogister, M.L. Alvarez, J.M. Rigo, T.R. Van De Water, G. Moonen, P.P. Lefebvre, Proliferative generation of mammalian auditory hair cells in culture, Mechanisms of Development 112 (2002) 79–88.
[10] E.C. Oesterle, S. Campbell, Supporting cell characteristics in long-deafened aged mouse ears, Journal of the Association for Research in Otolaryngology 10 (2009) 525–544.
[11] E.C. Oesterle, S. Campbell, R.R. Taylor, A. Forge, C.R. Hume, Sox2 and JAGGED1 expression in normal and drug-damaged adult mouse inner ear, Journal of the Association for Research in Otolaryngology 9 (2008) 65–89.
[12] J. Oiticia, L.C.M. Barboza-Junior, A.C. Batissoco, K. Lezirovitz, R.C. Mingroni-Netto, L.A. Haddad, R.F. Bento, Retention of progenitor cell phenotype in otospheres from guinea pig and mouse cochlea, Journal of Translational Medicine 8 (2010) 119.
[13] K. Oshima, C.M. Grimm, C.E. Corrales, P. Senn, R. Martinez Monedero, G.S. Geleoc, A. Edge, J.R. Holt, S. Heller, Differential distribution of stem cells in the auditory and vestibular organs of the inner ear, Journal of the Association for Research in Otolaryngology 8 (2007) 18–31.
[14] K. Oshima, K. Shin, M. Diensthuber, A.W. Peng, A.J. Ricci, S. Heller, Mechanosensitive hair cell-like cells from embryonic and induced pluripotent stem cells, Cell 141 (2010) 704–716.
[15] B.M. Ryals, E.W. Rubel, Hair cell regeneration after acoustic trauma in adult Coturnix quail, Science 240 (1988) 1774–1776.
[16] E. Savary, J.P. Hugnot, Y. Chassigneux, C. Travo, C. Duperray, T. Van De Water, A. Zine, Distinct population of hair cell progenitors can be isolated from the postnatal mouse cochlea using side population analysis, Stem Cells 25 (2007) 332–339.
[17] E. Savary, J.C. Sabourin, J. Santo, J.P. Hugnot, C. Chabbert, T. Van De Water, A. Uziel, A. Zine, Cochlear stem/progenitor cells from a postnatal cochlea respond to Jagged1 and demonstrate that notch signaling promotes sphere formation and sensory potential, Mechanisms of Development 125 (2008) 674–686.
[18] P. Senn, K. Oshima, D. Teo, C. Grimm, S. Heller, Robust postmortem survival of murine vestibular and cochlear stem cells, Journal of the Association for Research in Otolaryngology 8 (2007) 194–204.
[19] S.T. Sinkkonen, R. Chai, T.A. Jan, B.H. Hartman, R.D. Laske, F. Gahlen, W. Sinkkonen, A.G. Cheng, K. Oshima, S. Heller, Intrinsic regenerative potential of murine cochlear supporting cells, Scientific Reports 1 (2011) 26.
[20] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[21] I. Tateya, T. Nakagawa, F. Iguchi, T.S. Kim, T. Endo, S. Yamada, R. Kageyama, Y. Naito, J. Ito, Fate of neural stem cells grafted into injured inner ears of mice, Neuroreport 14 (2003) 1677–1681.
[22] R. Watanabe, M.H. Morell, J.M. Miller, A.C. Kanicki, K.S. O'Shea, R.A. Altschuler, Y. Raphael, Nestin-expressing cells in the developing, mature and noise-exposed cochlear epithelium, Molecular and Cellular Neuroscience 49 (2012) 104–109.
[23] P.M. White, A. Doetzlhofer, Y.S. Lee, A.K. Groves, N. Segil, Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells, Nature 441 (2006) 984–987.
[24] N. Yamamoto, W. Chang, M.W. Kelley, Rbpj regulates development of prosensory cells in the mammalian inner ear, Developmental Biology 353 (2011) 367–379.
[25] M.V. Yerukhimovich, L. Bai, D.H. Chen, R.H. Miller, K.N. Alagramam, Identification and characterization of mouse cochlear stem cells, Developmental Neuroscience 29 (2007) 251–260.
[26] Y. Zhang, S.Q. Zhai, J. Shou, W. Song, J.H. Sun, W. Guo, G.L. Zheng, Y.Y. Hu, W.Q. Gao, Isolation, growth and differentiation of hair cell progenitors from the newborn rat cochlear greater epithelial ridge, Journal of Neuroscience Methods 164 (2007) 271–279.

Otology & Neurotology
35:e84–e89 © 2014, Otology & Neurotology, Inc.

In Vivo Imaging of Mouse Cochlea by Optical Coherence Tomography

*Yosuke Tona, *Tatsunori Sakamoto, *Takayuki Nakagawa, †Tsunemichi Adachi, *Mirei Taniguchi, *Hiroko Torii, ‡Kiyomi Hamaguchi, *Shin-ichiro Kitajiri, and *Juichi Ito

**Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto; †Adachi ENT Clinic, Kobe; and ‡Department of Otolaryngology, Tenri Hospital, Tenri, Japan*

Hypothesis: Cochlear pathology can be evaluated in living animals using optical coherence tomography (OCT).
Background: The current imaging methods available for the detailed analysis of cochlear pathology in a clinical setting provide only limited information. Thus, a cochlear imaging modality with high definition is needed for improving the diagnosis of cochlear pathology. OCT has been used in other fields for obtaining high-resolution subsurface images, and its use could potentially be extended to the analysis of cochlear pathogenesis.
Methods: *Slc26a4*$^{(-/-)}$ mice, which generate endolymphatic hydrops, and their littermates were used in this study. Auditory function was monitored by the auditory brainstem responses (ABR). After the mice were placed under general anesthesia, OCT images of the cochlea were captured. The cochlea was subsequently dissected out and histologically evaluated. Three or 7 days later, the wild-type mice cochleae were visualized again.
Results: In ABR assessments, *Slc26a4*$^{(-/-)}$ mice showed severe hearing loss, while no significant hearing loss was found in *Slc26a4*$^{(+/-)}$ or *Slc26a4*$^{(+/+)}$ mice. OCT demonstrated normal morphology in the cochlea of both *Slc26a4*$^{(+/-)}$ and *Slc26a4*$^{(+/+)}$ mice, including the location of Reissner's membrane. Meanwhile, in *Slc26a4*$^{(-/-)}$ mice, obvious dislocation of Reissner's membrane was observed, indicating severe endolymphatic hydrops. These findings in the OCT images were consistent with the histologic results for the cochlear morphology, as observed with hematoxylin and eosin staining. Three or 7 days later, wild-type cochleae were successfully visualized using OCT, and no otitis media or labyrinthitis was observed.
Conclusion: OCT can be applied in the detection of endolymphatic hydrops in living mice, indicating the potential of OCT for cochlear imaging analyses for clinical use in the near future.
Key Words: Endolymphatic hydrops—In vivo imaging—Optical coherence tomography—Slc26a4.
Otol Neurotol **35**:e84–e89, 2014.

The development of novel cochlear imaging modalities for improving the detailed analysis of cochlear pathology is an urgent issue because these new imaging modalities will be crucial for the development of new therapeutic options for inner ear diseases. At present, only limited information on intracochlear structures can be obtained clinically. For instance, magnetic resonance images (MRIs) after intratympanic or systemic application of gadolinium-based contrasting agents have been used to detect endolymphatic hydrops in patients who are suspected of having Ménière's disease (1–3). However, the image resolution of MRI is limited in visualizing the details of cochlear pathogenesis. Thin-sheet laser imaging microscopy and related microscopic techniques provide high-resolution images of tissues, including cochlea, without sectioning (4,5). However, this technique requires tissue to be transparent and fluorescent, and thus, its application in rodents and other larger animals, including humans, is difficult.

Optical coherence tomography (OCT) is a noninvasive technique for obtaining subsurface images of opaque materials at a high spatial resolution of 1 to 15 μm (6). The basis of OCT is the use of infrared light to penetrate into the scattering medium (7). This approach is analogous to

Address correspondence and reprint requests to Tatsunori Sakamoto, M.D., Ph.D., Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University 54, Shogoin Kawahara-cho, Sakyo-ku, Kyoto, Japan, 606-8507; E-mail: sakamoto@ent.kuhp.kyoto-u.ac.jp

Conflicts of Interest and Sources of Funding: This work was partly supported by the Innovative Techno-Hub for Integrated Medical Bioimaging of the Project for Developing Innovation Systems, from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan; the Hospital-Company collaboration support project for developing/improving problem-solving-type medical equipment from the Ministry of Economy, Trade and Industry (METI), Japan; and the Promotion Grant for Translational Research at Kyoto University Hospital (FY2010). The authors declare no conflicts of interest.

Copyright © 2014 Otology & Neurotology, Inc. Unauthorized reproduction of this article is prohibited.