The function of cochlin has not yet been fully elucidated, but there is a line of evidence that cochlin is a very important protein for inner ear function. Cochlin is an extracellular matrix protein, and a major constituent of the inner ear, comprising 70% of the non-collagenous inner ear protein [Ikezono et al., 2001]. A spatiotemporal expression study of cochlin suggests that it may be deeply related to the maturation of inner ear function [Shindo et al., 2008]. Cochlin has unique isoforms. We reported 3 cochlin isoforms (p63s, p44s and p40s) expressed in the inner ear tissue composed of 16 different protein spots, with charge and size heterogeneity. A 16-kDa short isoform of cochlin, CTP, was identified not in the membranous labyrinth but *only* in the perilymph [Ikezono et al., 2001, 2004]. Full-length cochlin, p63s, has 2 functional domains, an LCCL module of unknown function and vWF-A like domain which might work as a binding domain to collagen. In fact, electron microscopic study revealed the co-localization of cochlin and type II collagen [Mizuta et al., 2008]. Interestingly, the whole molecule of CTP is composed of only an LCCL domain and most of the mutations of cochlin reported in DFNA9 are located in this domain. CTP could be an important molecule by which to understand the function of cochlin and the pathophysiology of DFNA9.

The mechanisms for the formation of these isoforms are unknown, but it is speculated that CTP is cleaved from full-length cochlin p63s by inner ear cells and then secreted into the endo- or perilymphatic space in the inner ear. Alternatively, CTP may be directly coded from a unique *COCH* gene splice variant or from a *COCH* homologue [Kommareddi et al., 2007]. The *COCH* gene was initially isolated by subtractive hybridization and Northern blot. A microarray expression profile analysis showed that *COCH* is preferentially expressed in the inner ear [Robertson et al., 1994; Abe et al., 2003]. Our previous study using rat organs showed that *COCH* gene is expressed preferentially in the inner ear and that expression of full-length cochlin p63s is specific to the inner ear [Li et al., 2005]. Therefore, it is rational to speculate that CTP expression could also be inner ear specific, and indeed CTP is a perilymph specific protein as shown in the present study. The detection limit of the rhCTP was between 0.27 and 0.14 ng/well, and the average detection limit of perilymph was 0.022 μl/well on Western blot analysis. This detection limit could be good for the clinical use of CTP as a diagnostic marker of PLF. We are presently conducting CTP detection tests on PLF suspected cases and evaluating the diagnostic accuracy of the test. The ultimate purpose of this test is to be able to detect the presence of leaked perilymph in the middle ear cavity preoperatively in the outpatient clinic or intra-operatively during exploratory tympanotomy. The leaked perilymph in the middle ear can be microliters in quantity. We collect this minute volume of perilymph by lavaging the middle ear cavity 4 times with 0.3 to approximately 0.4 ml of saline and recovering the fluid. Native CTP in the lavage could be detected by Western blot.

A number of authors have suggested the identification of an endogenous perilymph marker substance. Thalmann et al. [1994] gave a detailed analysis and comparison of plasma, perilymph and CSF protein, using high-resolution 2-dimensional polyacrylamide gel electrophoresis, combined with amino acid sequencing. The majority of proteins were found to be present in perilymph at levels in basic agreement with the total protein gradient between perilymph and plasma (1:35). However, high-density lipoprotein-associated apolipoprotein apo D detected in perilymph was at a 2.1-fold higher concentration than in plasma. This characteristic might make it a marker for PLF.

Previously tested candidate markers such as beta-2 transferrin, Beta-trace protein (prostaglandin D synthase), or intrathecal fluorescein, are markers of CSF leakage. An electrophoretic assay of middle ear fluid for the presence of beta-2 transferrin, a protein unique to CSF, aqueous humor and human perilymph was introduced for the diagnosis of PLF [Bassiouny et al., 1992; Buchman et al., 1999]. In children suspected to have PLF, beta-2 transferrin was detected in 6 of 9 operated ears, all 10 control patients were negative [Weber et al., 1994]. Although this technique holds promise, the dilutional effect of sample handling in preparation may lower beta-2 trannsferrin concentration below the detection limits of the assay. Because of the relative amount of serum and perilymph in a mixed sample, electrophoretic separation of transferrin variants may not be diagnostic [Levenson et al., 1996; Rauch, 2000].

Beta-trace protein (prostaglandin D synthase) has been used as a CSF leakage marker [Bachmann et al., 2002]. Since beta-trace protein is detectable in inner ear fluids in an even higher concentration than in CSF, it is a potential marker for perilymph leakage. However, there are some drawbacks that are pointed out by the author: the normal concentration of beta-trace protein in perilymph and proper cut-off of perilymph detection is not known yet, and it is impossible to distinguish CSF leakage from PLF leakage [Olaf et al., 2005a, 2005b; Risch et al., 2005].

There have been reports of fluorescein use as an exogenous marker for PLF diagnosis. Although it is appealing as a marker, visual detection of faint fluorescence in a small-volume middle ear fluid sample may be subjective, which is the same drawback in conventional visual detection of perilymph leakage. The wide, rapid distribution throughout all physiological fluid compartments makes it an unreliable marker substance in the differential diagnosis of PLF [Poe et al., 1993; Rauch, 2000; Gehrking et al., 2002].

CTP is the first substance that is present in the perilymph, but not in other body fluids such as the CSF, serum and saliva. Therefore, it can be a sensitive biochemical marker for perilymph leakage. Once a well-standardized CTP detection test is established, it could be a definitive objective test for the diagnosis of PLF.

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The Performance of Cochlin-Tomoprotein Detection Test in the Diagnosis of Perilymphatic Fistula

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Key Words
Diagnostic accuracy · Perilymphatic fistula · Hearing loss · Vertigo · Perilymph · *COCH* gene · Cochlin isoform · Cochlin tomoprotein · Human · Specificity · Sensitivity

Abstract
Background: Perilymphatic fistula (PLF), defined as an abnormal communication between the inner and middle ear, presents with a symptomatology of hearing loss and vestibular disorder that is indistinguishable from a number of other inner ear diseases. Methods of diagnosis remain controversial. We have previously shown that Cochlin-tomoprotein (CTP) is selectively detected in the perilymph. To establish a definite diagnostic test for PLF using CTP as a biochemical marker, we examined the diagnostic performance of the CTP detection test. ***Methods:*** CTP detection test was performed by Western blot using recombinant human CTP (rhCTP) as a spiked standard. We evaluated the specificity of the CTP detection test by testing non-PLF cases. To describe the limitations of the test, we tested samples from patients with middle ear infection. We also studied the stability of CTP protein by storing the samples at room temperature (25°C) or 4°C for 55 days. The effects of repeated freezing and thawing were also evaluated. Serially diluted perilymph was tested to find out the detection limit of CTP. ***Findings:*** We have established a standardized CTP detection test using high (0.27 ng) and low (0.13 ng) spiked standards of rhCTP in Western blotting. Middle ear lavages (MEL) from 54 of 55 non-PLF cases were negative in the CTP detection test, i.e. the specificity of the test is 98.2%. MEL from 43 out of 46 cases with chronic suppurative otitis media or middle ear cholesteatoma were negative for CTP. CTP is a stable protein and detection was not affected by the storage, or freezing and thawing. The detection limit of perilymph was 0.161 μl/lane in an average of 5 samples. ***Interpretation:*** CTP is a stable perilymph-specific protein, and this CTP detection could be the first clinically established diagnostic tool to detect PLF with a high specificity. PLF is surgically correctable by sealing the fistula. Appropriate recognition and treatment of PLF can improve hearing and balance in afflicted patients.

Introduction

Perilymphatic fistula (PLF) is defined as an abnormal communication between perilymph in the labyrinth and the middle ear. Representative symptoms of PLF are

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sudden onset and/or progressive hearing loss with episodic attacks of vertigo; however, reports in the literature have suggested PLF to be putatively involved in a broad spectrum of hearing loss symptoms and balance disorders. PLF can be congenital or acquired, and in the latter it is associated with a traumatic or barotraumatic event resulting in labyrinthine fracture, iatrogenic artifacts (ear surgery), or a disruption of the membranes of the round and/or oval window(s) [Goodhill, 1971; House et al., 1991; Fitzgerald, 2001; Minor, 2003; Weber et al., 2003].

Unlike other causes of sensorineural hearing loss, PLF is surgically correctable by sealing the fistula. Appropriate recognition and treatment of PLF can improve hearing and balance, and hence the quality of life of the afflicted patients. However, despite extensive efforts to establish definitive methods for PLF detection, such as audiometry, electrocochleogram, electronystagmogram and radiological examination, there is as yet no widely accepted specific test for diagnosing PLF [Podoshin et al., 1994; Wall and Rauch, 1995; Nomura, 1994; Black et al., 1992]. The conventional definitive diagnosis of PLF depends on the direct visualization of the perilymphatic leak and fistula, but this is both difficult and highly subjective. The difficulty of making a definitive diagnosis of PLF has caused a long-standing debate regarding its prevalence, natural history, management, and even its very existence [Hughes et al., 1990; Schuknecht, 1992; Friedland and Wackym, 1999].

Previously, by proteomic analysis of inner ear proteins, we found very unique properties of cochlin (encoded by the *COCH* gene and mutated in DFNA9 – a hereditary form of hearing loss), which is expressed abundantly in the inner ear [Robertson et al., 1998; Ikezono et al., 2005; Robertson et al., 2006; Shindo et al., 2008]. We detected 3 cochlin isoforms, p63s, p44s and p40s, in the inner ear tissue and a short 16-kDa isoform named Cochlin-tomoprotein (CTP) in the perilymph [Ikezono et al., 2001, 2004]. Since cochlin was found to be highly specific to the inner ear [Robertson et al., 1994; Abe et al., 2003; Li et al., 2005], we tested the expression specificity of CTP in perilymph; CTP was indeed selectively expressed only in the perilymph, and not in CSF, saliva or serum [Ikezono et al., 2009]. In addition, we reported the molecular mechanisms that regulate the perilymph-specific expression of CTP [Sekine et al., 2009].

In order to establish CTP as a diagnostic marker of PLF, we standardized the CTP detection test using spiked standards of recombinant human CTP (rhCTP) in Western blotting. We evaluated the specificity of the CTP detection test by testing samples from non-PLF cases. To describe the limitations of the test, we evaluated the influence of middle ear infection on the test results. We also studied the stability of CTP protein when samples were stored at room temperature (25°C) or 4°C for as long as 55 days. The effects of repeated freezing and thawing were also evaluated. Serially diluted perilymph was tested to find out the detection limit of CTP. The present study showed that CTP could be the first clinically established biochemical marker to allow a definitive diagnosis of PLF-related hearing loss.

Methods

Standardization of the CTP Detection Test by Western Blot

For Western blot analysis, the rabbit polyclonal anti-CTP antibody (formerly anti-LCCL-C Ab) was prepared as previously described [Ikezono et al., 2004]. In brief, a 14-mer peptide (LSRWSASFTVTKGK) corresponding to residues 114–127 in the LCCL domain was used to generate the antibody. Rabbits were immunized by repeated subcutaneous injections of the KLH-coupled peptides. The serum was purified by a protein A column, followed by peptide-affinity chromatography. The specificity of the antibodies for the corresponding antigenic peptides was confirmed by dot blot analysis and a peptide absorption test (data not shown). The rhCTP was used as a spiked standard in the Western blot. The exact N- and C-terminal sequence of CTP is not yet known. However, a putative CTP sequence predicted from our previous study [Ikezono et al., 2004], located at positions 101–403 of the cDNA and corresponding to amino acid residues 32–132, was amplified by PCR from a human-expressed sequence tag clone, Image ID 27789 (Kurabo); rhCTP was produced using pCR/T7/TOPO/TA expression kits (Invitrogen).

Samples were loaded onto 15% polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were blocked overnight at 4°C in 5% skimmed milk and 0.2% polyoxyethylene sorbitan (Tween-20) dissolved in PBS (pH 7.5). Membranes were then incubated in PBS containing 1% skimmed milk and 0.1% Tween-20 for 2 h at room temperature with the primary antibody (anti-CTP antibody) diluted at 1:1000. After washing with 0.1% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (Dako) diluted at 1:1000 in the same buffer used for the primary antibody reaction. They were washed again, and the reaction was developed with a chemiluminescence reaction kit (ECL advance, Amersham) and then analyzed by an image analyzer LAS-3000 (Fuji Film). Tests were performed and analyzed by well-trained personnel who did not have any information on the clinical background of the patients, to avoid any biased judgments. Test results were expressed as positive or negative by the presence or absence of the anti-CTP antibody reacting protein with the molecular weight that exactly matched the molecular weight of native CTP (16 kDa) on Western blotting.

Method of Sampling

In our previous study, we showed that CTP is selectively expressed in the perilymph, and not in samples of the body fluids, serum, CSF or saliva. The ultimate purpose of this test is to be able to detect the presence of leaked PLF in the middle ear cavity preoperatively in the outpatient clinic. We aimed at establishing an easy-to-perform sampling method. Samples were collected by lavaging the middle ear cavity 3–4 times with the same bolus of 0.3 ml saline and recovering the fluid, and these were defined as middle ear lavage (MEL). MEL was collected from non-PLF cases and those with suppurative otitis media or middle ear cholesteatoma. Samples were centrifuged at 1250 *g* for 1 min, and the supernatants were frozen and stored at –80°C until use; 16 μl MEL was mixed with 8 μl of 3 times concentrated sample buffer (0.188 M Tris buffer, 2.39 mM SDS, 30% glycerol, and 15% of 2-mercaptoethanol) for Western blot analysis.

To test the stability and detection limit of CTP, perilymph was collected from 5 cases of cochleostomy for cochlear implant surgery. We collected the leakage from the cochleostomy using a 27-gauge (0.22 mm internal diameter) blunt-end fine needle. All patients gave their full informed consent, and the study was approved by the Ethics Committee of Nippon Medical School.

Non-PLF Cases

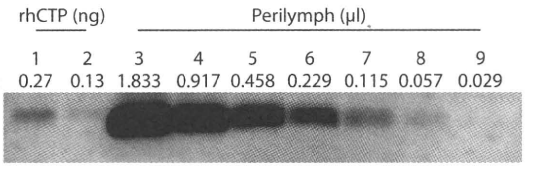
In order to evaluate the specificity of the CTP detection test, we examined MEL from non-PLF cases. In this study, we defined 'non-PLF' as those cases with otosclerosis (which had undergone stapedectomy), profound deafness (cochlear implant surgery) or conductive hearing loss (exploratory tympanotomy). We took MEL prior to the stapedectomy or cochleostomy, or prior to surgical treatment for conductive hearing loss. These cases did not have any symptoms or test results suggestive of PLF (including high-resolution temporal bone target CT scans and intraoperative findings, such as microscopic visualization of perilymph leakage and/or fistula). Patients who had revision stapedectomy, revision cochlear implantation, ossified cochlea or infection of the middle ear were excluded.

Effect of Middle Ear Infection on CTP Detection Test

It is well known that protein-rich samples, such as pus, can cause nonspecific signals on a Western blot. Therefore, we further clarified the influence of the infection in the middle ear on the test results. The MEL from surgically treated chronic suppurative otitis media cases (n = 12) and middle ear cholesteatoma cases (n = 34) were evaluated. None of these cases had any symptoms or test results suggestive of PLF.

Testing the Stability of CTP

In everyday clinical settings, collected samples may not be frozen immediately. We therefore evaluated if the results of the CTP detection test were affected by storage conditions that could lead to protein degradation. We tested diluted perilymph (1:20 with saline) kept at room temperature (25°C) or in a refrigerator at 4°C for 1, 2, 6, 8, 9, 12, 13, 15, 16, 19, 20, 23, 27, 34, 41, 48 or 55 days; 4 μl diluted saline was mixed with sample buffer (24 μl total volume) and 22 μl sample, i.e. 0.18 μl of perilymph/lane, was loaded on to the gel. In addition, MEL could be tested multiple times by Western blotting or by an alternative method to confirm the test results. We performed the CTP detection test of diluted perilymph after repeatedly freezing (–70°C) and thawing (25°C) for 10 times.



Lane	Sample	Amount of sample/lane
1:	rhCTP	0.27 ng
2:	rhCTP	0.13 ng
3:	perilymph	1.833 μl
4:	perilymph	0.917 μl
5:	perilymph	0.458 μl
6:	perilymph	0.229 μl
7:	perilymph	0.115 μl
8:	perilymph	0.057 μl
9:	perilymph	0.029 μl

Fig. 1. The detection limit of serially diluted perilymph samples using a standardized CTP detection test to define spiked standards. We loaded rhCTP as high and low spiked standard (lanes 1, 2) and serially diluted perilymph samples (lanes 3–9). When the intensity of the band in samples tested was below the high standard signal, the result was considered to be negative. The intensity of the band in lane 8 is below the high spiked standard (lane 1); thus, lane 8 was considered to be negative. The detection limit of CTP in the diluted perilymph (0.115 μl/lane; lane 7) is shown.

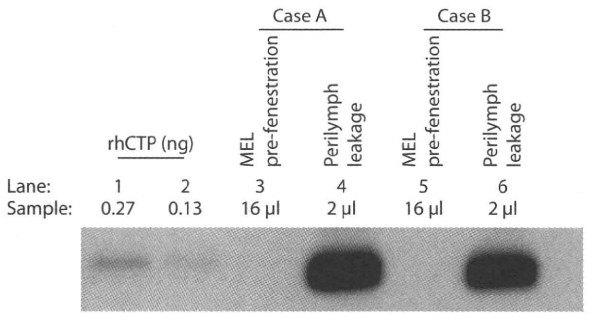
Detection Limit

Five serially diluted perilymph samples were tested independently to establish the detection limit of CTP. We mixed 4 μl perilymph with 28 μl saline and 16 μl of 3 times concentrated sample buffer. This mixture was serially diluted with sample buffer. Diluted samples were heated to 100°C for 10 min. Then 22 μl of these samples were loaded onto the gel and the volume of loaded perilymph samples was calculated as follows: 1.833, 0.917, 0.458, 0.229, 0.115, 0.057, 0.029 (μl/lane).

Results

Standardized CTP Detection System

As previously reported, the detection limit of the serially diluted rhCTP was between 0.27 and 0.13 ng/well. These 2 amounts of rhCTP were set as the high and low spiked standards, respectively, and were the amounts electrophoresed each time when we tested the samples



Lane	Sample	Amount of sample/lane
1:	rhCTP	0.27 ng
2:	rhCTP	0.13 ng
3:	Case A: MEL pre-fenestration	16 µl of MEL
4:	Case A: Perilymph leakage	2 µl of perilymph
5:	Case B: MEL pre-fenestration	16 µl of MEL
6:	Case B: Perilymph leakage	2 µl of perilymph

Fig. 2. The result of CTP detection from non-PLF cases and the perilymph (samples from 2 cochlear implant surgery cases). MEL taken prior to the fenestration and the perilymph leakage from the cochleostomy were subjected to the CTP detection test. MEL taken before fenestration did not have any signal, whereas CTP was detected at 16 kDa in perilymph samples.

Table 1. CTP detection in non-PLF samples

	Total	CTP positive	CTP negative
Prior to stapedectomy	35	1	34
Prior to cochleostomy	12	0	12
Exploratory tympanotomy	8	0	8
Total	55	1	54

Table 2. Effect of middle ear infection on CTP detection test

	Total	CTP positive	CTP negative
Chronic suppurative otitis media	12	1	11
Middle ear cholesteatoma	34	2	32
Total	46	3	43

(fig. 1). When a high standard was detected, we accepted the result; otherwise, samples were re-evaluated. When the intensity of the band in samples tested was below the high-standard signal, the result was considered to be negative. Low spiked standard was used to estimate of the protein transfer efficiency. The molecular weight of rhCTP exactly matched that of native CTP (16 kDa) on Western blot. Inter-assay and intra-assay reproducibility was tested and confirmed (data not shown).

CTP Detection from non-PLF Cases

MEL from 34 of 35 cases prior to stapedectomy, 12 of 12 cases prior to cochleostomy, and 8 of 8 cases during exploratory tympanotomy were negative for CTP. In total, 54 MEL from 55 non-PLF cases were negative for CTP (table 1); therefore, the specificity of the CTP detection test for the diagnosis of PLF is 98.2%.

Figure 2 shows the results of CTP detection from non-PLF cases and the perilymph. Samples of MEL taken prior to fenestration and the perilymph leakage from the cochleostomy of 2 cochlear implant surgery cases were subjected to the CTP detection test. MEL taken before fenestration did not have any signal, whereas CTP was detected at 16 kDa in perilymph samples.

Effect of Middle Ear Infection on the CTP Detection Test

MEL from 11 out of 12 cases with chronic suppurative otitis media and 32 of 34 cases of middle ear cholesteatoma were negative for CTP (table 2). Thus, the specificity of the CTP detection test is 93.5%.

Stability Test of CTP

We tested samples stored at 25°C or 4°C for 1, 2, 6, 8, 9, 12, 13, 15, 16, 19, 20, 23, 27, 34, 41, 48, 55 days. In the Western blot, CTP was detected in all 34 samples tested. The intensity of CTP signals did not change remarkably. After repeated freezing and thawing (10 times), the intensity of CTP signals did not change (data not shown). These results suggest that CTP is a stable protein, and the results of CTP detection test by Western blotting would not be altered by storage conditions within this rage.

Detection Limit of CTP

Five serially diluted perilymph samples were tested to show the detection limit. Detection limits were 0.229 µl/lane (2 samples) and 0.115 µl/lane (3 samples), which gives an average of 0.161 µl/lane (fig. 1). This detection limit could be useful in the clinical application of CTP as a diagnostic marker of PLF.

Discussion

We previously analyzed the expression of CTP in various human bodily fluids, including the serum, CSF, saliva and perilymph [Ikezono et al., 2009]. All bodily fluid samples, except the perilymph, were negative for CTP. These results strongly suggest that CTP is expressed specifically and exclusively in the perilymph, from amongst these 4 kinds of bodily fluids that may be present in a healthy or diseased middle ear, and that CTP can be considered to be a specific biochemical marker for PLF. Recently, we reported the molecular mechanisms that regulate the perilymph-specific expression of CTP [Sekine et al., 2009]. We performed RNA ligation-mediated amplification of cDNA ends (RLM-RACE) using RNA isolated from the inner ear and spleen of rats, which are known to express abundant cochlin mRNA. We detected a novel short mRNA (a spliced variant), which includes the LCCL domain. This short mRNA was detected in the inner ear, and not in spleen.

The conventional gold standard of PLF detection is the intraoperative microscopic visualization of perilymph leakage and fistula, which ostensibly confirms the existence of PLF. If the patient does not have PLF, leakage will not be detected. However, since the surgical procedure itself can induce seepage that accumulates in the concave-shaped round and oval window niches, this could be misinterpreted as perilymph leakage [Nomura, 1994; Friedland and Wackym, 1999]. The difficulty of making a definitive diagnosis of PLF has caused a long-standing debate regarding PLF [Hughes et al., 1990; Schuknecht, 1992; Friedland and Wackym, 1999].

The appropriate recognition and treatment of PLF can improve hearing and balance in the afflicted patients. Our ultimate goal has been to establish a clinical test to allow a definitive diagnosis of PLF using CTP as a biochemical marker. It should be a clinically useful and specific test for the 'preoperative' diagnosis of PLF, in order to avoid unnecessary exploratory surgery. At the same time, this method has to be applied to a variety of clinical scenarios in PLF, wherein the leakage could take place in the oval or round window, fractured bony labyrinth, or minor fissures [Kohut et al., 1986]. Moreover, the leakage could be intermittent, ongoing or could have ceased with the leaked perilymph pooled in the middle ear. Therefore, we used MEL for collecting the samples from the middle ear in which the sampling was easily performed in an outpatient setting, only by the conventional method of myringotomy under local anesthesia. Saline lavage should include all the perilymph from wherever the perilymph leaked out or became pooled.

Detection of the target protein in a Western blot is affected by the efficiency of protein transfer. Transfer efficiency depends on factors such as the composition of the gel, complete contact of the gel with the membrane, the position of the electrodes, the transfer time, size and composition of proteins, field strength and the presence of detergents. In the present study, we have standardized the CTP detection test through defining high and low spiked standards as 0.27 and 0.13 ng rhCTP, respectively. When a high standard was detected, we accepted the result; otherwise, samples were re-evaluated. When the intensity of the band in samples tested was below the high standard signal, the result was considered to be negative. The average detection limit of CTP in 5 serially diluted perilymph samples was 0.161 μl/lane. This means that the test can detect CTP if there is 3.3 μl perilymph in 0.3 ml MEL (amount of perilymph contained in the diluted sample of the detection limit: $0.161 \times 24/22 = 0.176$ μl; perilymph in the total MEL: $0.176 \times 300/16 = 3.3$ μl). This detection limit could be used in the clinical application of CTP as a diagnostic marker of PLF.

MEL should contain middle ear mucosal secretion and other substances normally expressed in the middle ear cavity. Since these substances may cause false-positive reactions to the antibody, we tested MEL from non-PLF cases. In this study, we defined 'non-PLF' as those cases with otosclerosis (who had undergone stapedectomy), profound deafness (cochlear implant surgery), or conductive hearing loss (exploratory tympanotomy). We took MEL prior to the stapedectomy or cochleostomy, or prior to surgical treatment of conductive hearing loss. None of these cases had any symptoms or test results suggestive of PLF (including high-resolution temporal bone target CT scans and intraoperative findings). We detected anti-CTP antibody reacting protein at 16 kDa in 1 otosclerosis case. The diagnostic performance of CTP detection test for the diagnosis of PLF was found to have a specificity of 98.2%. We are now trying to evaluate the sensitivity of the test by performing the CTP detection test in 'definite PLF cases', such as traumatic stapes injury.

There are limitations to this test. Analysis of MEL collected from patients with middle ear infections can give a false-positive result (as in this study), where the high protein concentration of the thick pus was the most likely cause. Specificity of CTP detection test decreases to 93.5% when testing infected ears. We have reported that CTP was not detectable in 28 serum samples [Ikezono et

al., 2009], and was not detected in multiple hemolyzed samples (data not shown). However, to ensure the accuracy of the test, MEL samples should ideally be kept frozen after removing the cells or tissue debris by the centrifuge to provide the minimum protein concentration.

Protein markers such as CTP may become degraded through the process of storage prior to the detection test or during the handling of the samples. The result of the test may vary if the marker is easily degradable protein. We have tested the stability of CTP by storing the diluted sample (1:20 with saline) at room temperature or at 4°C for 17 time points maximum of 55 days. CTP was detected in all 34 samples tested, without remarkable changes in the intensity of CTP signals. In addition, CTP was stable after repeated freezing (–70°C) and thawing (25°C) for 10 repetitions. CTP has enough stability in the various storage conditions in hospitals, and it is responsive to multiple measurements after thawing.

Conclusion

CTP is a stable perilymph-specific protein, for which we have established a standardized CTP detection test. This is the first clinically established diagnostic tool for the detection of PLF with a high specificity. In PLF, inner ear damage is affected by the speed, duration of the perilymph leakage, the site of the leakage and other biological factors. Hence, these patients' symptoms, physiological test results and outcomes of treatment are widely variable. Using this CTP detection test, a definitive diagnosis of PLF can be made and appropriate therapeutic options for this surgically correctable disease taken into consideration. Further studies will be needed to provide insight into the etiology, pathomechanisms, prevalence and natural history of PLF, and these may lead to the development of therapeutic and preventative strategies for acute, late-onset and debilitating neuro-otological problems.

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Local Drug Delivery to Inner Ear for Treatment of Hearing Loss

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Abstract: Sensorineural hearing loss (SNHL) is one of the most common disabilities in our society. Experimentally, many candidates for use as therapeutic molecules have been discovered. However, a considerable obstacle to clinical application is the lack of an effective method for drug delivery to the cochlea. In order to overcome this obstacle, there needs to be development of a local cochlear drug delivery system. Advances in pharmacological technology have provided various drug delivery systems that use biomaterials, and which can be utilized for local drug delivery to the cochlea. Indeed, recent studies have demonstrated the potential of synthetic and natural biomaterials for local drug delivery to the cochlea, indicating that the clinical application of such local drug delivery systems could be used in the near future for therapeutic treatments. Recent progress in cell therapy research also offers a novel drug delivery method for the cochlea. In addition, transplantation of stem cells into the cochlea has been demonstrated to provide protective effects for the auditory function. Transplantation of genetically engineered cells has also resulted in the sustained delivery of aimed therapeutic molecules within the inner ear. Although problems involving clinical application still need to be resolved, these drug delivery systems for the inner ear may hold the future therapeutic options for treatment of SNHL.

Key Words: Drug delivery system, cochlea, biodegradable polymer, cell transplantation, gene therapy.

THERAPEUTIC TARGETS FOR TREATMENT OF HEARING LOSS

Sensorineural hearing loss (SNHL) is one of the most prevalent disabilities in our society. Sound stimuli are received by auditory hair cells (HCs) in the bony, snail-shaped cochlea, followed by transduction of the sound stimuli by the HCs to neural signals. Spiral ganglion neurons (SGNs), which are auditory primary neurons, are located in the central bony axis of the cochlea and responsible for transmitting auditory signals to the central auditory system. Excessive noise, ototoxic drugs, genetic disorders and aging all contribute to the causes of SNHL. Severe to profound SNHL affects 1 in 1000 newborns, and another 1 in 2000 children before they reach adulthood. About 60% of individuals older than 70 years will manifest SNHL. Previous studies on human temporal bones have demonstrated that the loss of HCs and/or SGNs is a major cause of SNHL [1]. Protecting HCs and SGNs from irreversible degeneration is therefore a primary objective due to the limited regeneration capacity of these cells. Acute SNHL sometimes responds to drug treatment; however, there are no therapeutic options for chronic SNHL except for hearing aids and cochlear implants, which are small devices that are surgically implanted into the cochlea in order to stimulate SGNs. However, the success of cochlear implants depends on the remaining SGNs and with their loss, this severely compromises the efficacy of this technique. HCs and SGNs are therefore the major targets for the treatment of SNHL.

WHY IS LOCAL DRUG DELIVERY REQUIRED FOR THE INNER EAR?

Based on the backgrounds described above, studies are being conducted with the hopes of providing an alternative means of biological therapy. Thus far, research has identified a number of candidates for use as therapeutic molecules. Experimentally, protective effects of neurotrophins have been demonstrated [2,3], and inhibitors of apoptosis and glutamate antagonists have also been shown to have the ability to promote HC survival [4-6]. Recently, it has been found that local application of genes by virus vectors induces HC regeneration in the mammalian auditory epithelium [7,8], and additionally, by silencing the mutant gene *via* RNA interference, can restore hearing loss in the genetic mouse model [9]. These therapeutic strategies are attractive and promising for the restoration of SNHL. However, clinical application is still quite limited. The problem of how to deliver such therapeutic molecules to the inner ear has been a considerable obstacle in the development of treatments for SNHL. One of the reasons for the difficulty of drug delivery involves the limited blood flow to the cochlea [10]. In addition, the blood–inner ear barrier, which inhibits the transport of drugs from serum to the inner ear, represents a fundamental obstacle to the use of systemic applications [11]. The inner ear tissues are isolated from the surrounding organs by a bony construction, which allows for the topical introduction of drugs or genes. Based on these considerations, local application has generally been the preferred method for drug administration to the inner ear. The sustained delivery of therapeutic molecules is also critical for the efficient treatment of the cochlea, as bioactive molecules usually require a period of minutes or hours over which they produce their pharmacological actions. Consequently, a number of researchers are currently working to solve these problems and develop methods for the local direct application of these molecules into the cochlea [12].

STRATEGIES FOR LOCAL DRUG DELIVERY

The cochlea is connected to the tympanic cavity by the round window membrane (RWM). When substances are applied intratympanically, the assumption is that they will enter the scala tympani through the RWM and then be dis-

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tributed throughout the cochlear fluids. The idea of using a topical application of medicine to the inner ear is not new, as local anesthetics and aminoglycosides were applied decades ago, with the compounds passing through the tympanic membrane into the tympanic cavity during the treatment of the inner ear disorders [13,14]. Intratympanic injections have been used for local application of aminoglycosides or steroids during therapy for Ménière's disease and sudden hearing loss. There are a number of clinical reports showing the efficacy of intratympanic injections of these drugs [see review in reference 12]. However, it is very difficult to predict the amounts of drugs that will actually reach the cochlear fluid space. Some reports have indicated that this method can lead to varying results during therapeutic treatment of Ménière's disease [15-17]. While intratympanic injection is a simple and easy method to perform, unfortunately, a controlled and sustained release of drugs cannot be achieved using this method. The pharmacokinetics of drug entry into cochlear fluids is crucial to determine the efficacy of the method for drug delivery into cochlear fluids [12]. Salt and Plontke have indicated importance of sustained delivery of drugs on the RWM by means of perilymph sampling from various regions of the cochlea [18] and computer simulation [19].

Implantable mini-pumps have also frequently been used for local drug delivery to the cochlea in animal experiments [20]. Several clinical reports have described the efficacy of local glucocorticoid application when using a semi-implantable mini-pump [21,22]. However, the use of an implantable mini-pump has not been widely adopted, given the need for surgical procedures similar to tympanoplasty that must be done in order to place the mini-pump. The use of a local viral gene transfer as a sustained treatment of the inner ear can provide sufficient protection from noise, drug toxicity and re-perfusion injury [23-28]. Today, adenoviral vectors or adeno-associated viral vectors are the most widely used for cochlear gene transfer, because of the high efficiency for the transfection, the availability of high titers, and the ease of production. However, their use can potentially initiate an immune response that results in the destruction of the recipient's cochlear cells.

The use of biomaterials for local drug delivery has recently gained attention as an alternative to the implantable mini-pumps or gene transfer using virus vectors. In general, biodegradable polymers containing therapeutic molecules are placed on the RWM, with the therapeutic molecules released into the cochlear fluids from the polymers in a controlled manner *via* the RWM [12,28].

DRUG DELIVERY *VIA* BIOMATERIALS

In the past decade, pharmaceutical technologists have paid increasing attention to controlled or sustained release technology using biomaterials for the delivery of drugs in order to avoid side effects and achieve sufficient drug levels in tissues. In an effort to develop a controlled-release system, a variety of methods using synthetic and natural materials have been undertaken. Recent publications have reported the use of a controlled-release system for local drug delivery to the inner ear. Two synthetic materials, siloxane-based polymers [29] and polylactic/glycolic acid (PLGA) polymers [30], and several natural materials, which include hyaluronic acid [31] and gelatin [32-34], have been used for this purpose.

Siloxane-based polymers have been used for years in medical applications that involve contact with the human body. In the clinic, silicone-transdermal patches have been widely used. In this system, drug release is controlled by its diffusion through the silicone network [35]. The actual release rate is determined by the composition of the polymer. This system is particularly suitable for application of lipophilic and low-molecular weight molecules. Arnold *et al.* [29] have utilized this system for local application of beclomethasone into the cochlear fluids. When using this system, the silicone microimplant remains on the RWM, although it does not induce functional and histological damage in the cochlea. Therefore, repeated treatments require that there is extirpation of the material used during the procedure.

Encapsulating bioactive molecules in PLGA or polylactic acid (PLA) particles has been also used as a method of controlled-release application. Water-insoluble, low-molecular weight agents have been encapsulated in PLGA or PLA microparticles and nanoparticles [36,37]. PLGA and PLA are familiar substances to surgeons, as they are the materials that make up absorbable sutures. Tamura *et al.* [30] examined the potential of PLGA nanoparticles for drug delivery to the cochlea using guinea pigs. To evaluate the use of PLGA nanoparticles (140 to 180 nm in diameter) in the cochlea, rhodamine, which is a red fluorescent dye, was encapsulated and then following local application onto the RWM, its overall distribution was evaluated. PLGA nanoparticles containing rhodamine were observed in the cochlea, indicating that PLGA nanoparticles can penetrate through the RWM. Rhodamine is released from PLGA nanoparticles after penetration of the particles through the RWM. Compared to a silicone microimplant, PLGA nanoparticles have the advantage of being able to be repeatedly applied, as the PLGA is dissolved by hydrolysis. However, there is a limitation with regard to the variation of the drugs that can be applied, since the process of encapsulation in the PLGA particles requires that compounds must be dissolved in acetone. Therefore, this method is not suitable for the delivery of proteins or peptides.

GELATIN HYDROGEL

Gelatin is a commonly used natural polymer that is derived from collagen. In the clinic, gelatin polymers have been widely used as hemostats. Recently, gelatin-based controlled-release systems have been developed [38]. During the fabrication process, the isoelectric point of gelatin can be modified to yield either a negatively charged acidic gelatin or a positively charged basic gelatin. This allows for electrostatic interactions to take place between charged therapeutic molecules and gelatin of the opposite charge, leading to the formation of polyion complexes. The significance of such a system is that it provides the ability for application of water-soluble, comparatively high-molecular weight proteins and peptides. Additionally, this method is also capable of being used for the delivery of plasmid DNA [39]. In this system, therapeutic molecules are released by enzymatic degradation of gelatin polymers, for which the rates can be determined based on the crosslinking density of the gelatin polymers.

The potential use of the gelatin hydrogel system was initially investigated for cochlear delivery of brain-derived neurotrophic factor (BDNF) [32]. BDNF plays a crucial role in the development of the inner ears [38] and in the maintenance of the auditory function [41]. In addition, previous studies have demonstrated the effects of local BDNF application when using an osmotic mini pump [3] or adenovirus [26]. We measured BDNF concentrations in the cochlear fluid after placing a gelatin hydrogel that contained this agent onto the RWM [32]. The results revealed a sustained delivery of BDNF into the cochlear fluid *via* the hydrogel over a seven-day period. The functional and histological protection of the SGNs by BDNF that was applied through the gelatin hydrogel was then examined using a guinea pig model of SGN degeneration. The measurement of electrically evoked auditory-brainstem responses, which reflect SGN function, demonstrated that BDNF delivered *via* gelatin hydrogels was able to significantly reduce the threshold elevation [32]. Histological analysis demonstrated an increased survival of SGNs due to BDNF application through gelatin hydrogels. These findings indicate that gelatin hydrogel can be utilized for drug delivery to the cochlea.

Subsequently, we examined the efficacy of cochlear delivery of insulin-like growth factor-1 (IGF1) for the protection of auditory HCs against acoustic trauma [33]. IGF1 is a mitogenic peptide that plays essential roles in the regulation of growth and development in the inner ear [42]. In addition, previous studies on the inner ear have suggested the possibility of inner ear protection by IGF1 [43,44]. Moreover, recombinant human IGF-1 (rhIGF1) has already been approved for clinical use. Therefore, we selected rhIGF1 as a suitable trophic factor for local cochlear application using a gelatin hydrogel. Local rhIGF1 application through the gelatin hydrogel prior to noise exposure has been shown to efficiently protect the hearing from noise trauma. Additionally, histological analysis also revealed that local rhIGF-1 treatment ameliorated the loss of HCs [33].

Our ultimate goal is the clinical use of a local rhIGF1 application using gelatin hydrogel as a therapeutic option for the treatment of SNHL. Therefore, we examined whether post-traumatic application of rhIGF1 to the cochlea *via* gelatin hydrogels could attenuate noise-induced hearing loss. The results demonstrated that functional and histological efficacy of local rhIGF1 treatment on the attenuation of noise-induced hearing loss occurred in a dose-dependent manner [34]. We also measured IGF1 concentrations in the cochlear fluid, cerebrospinal fluid (CSF) and serum after placing a gelatin hydrogel containing rhIGF1 onto the RWM of guinea pigs. The results demonstrated that there was sustained delivery of rhIGF1 into the cochlear fluid, in addition to no alterations of the IGF1 levels in CSF and serum [34]. There were also no adverse effects due to local rhIGF1 treatment found in any of the experimental animals. These findings document both the effectiveness and the safety of local rhIGF1 treatment using gelatin hydrogels for noise-induced hearing loss.

CELL TRANSPLANTATION

Chronic SNHL is usually incurable because of the loss of HCs and SGNs, and which at the present time is irreversible. Therefore, an alternative means of biological therapy, including cell therapy is required. Indeed, recent studies have indicated that cell therapy could be utilized to regenerate HCs [45] and SGNs [46]. In contrast, cell transplantation is an alternative that can be used as a method for drug delivery where the transplanted cells for this purpose have the ability to survive and generate therapeutic agents. Several stem cells have been reported to have the ability to secrete trophic factors [47-49]. Cell transplantation has been used as a means of delivering peptides or proteins into the central nervous system, demonstrating its viable use as a delivery vehicle for therapeutic molecules [50,51].

Iguchi *et al.* have reported on the ability of neural stem cell-derived cells being used for the production of BDNF and glial cell line-derived neurotrophic factor (GDNF) after engraftment into the cochlea [47]. In addition, transplantation of neural stem cells into the cochlea has the potential of being able to attenuate HC damages due to transient ischemia of the cochlea [48]. Bone marrow derived cells also have the potential for secreting trophic factors. Implantation of bone marrow stromal cells has been reported to contribute to functional recovery of the brain [52] and spinal cord [53] by means of producing trophic factors. Furthermore, previous studies have revealed the potential of bone marrow derived cells surviving in the cochlea [54,55]. Yoshida *et al.* have demonstrated a significant increase in the protein level of GDNF in cochlear specimens and the prevention of HC death due to transient cochlear ischemia by engraftment of hematopoietic stem cells [49]. These findings indicate that cell transplantation into the cochlea may be a novel strategy for treatment of SNHL by providing a means for local application of trophic factors within the cochlea.

Transplantation of cells that have been genetically manipulated ex vivo has been used as a means of delivering peptides or proteins into the central nervous system [56-58]. In comparison with the stem cell transplantation that has been described above, this strategy has an advantage in that aimed gene-encoded products are applicable. In addition, use of non-viral vectors for ex vivo gene transfer potentially could resolve the problem of viral vector toxicity in cochlear gene therapy. Therefore, we conducted an examination of the efficacy of cell-gene delivery in the application of therapeutic molecules into the cochlea [59]. NIH3T3 cells were chosen as a delivery vehicle for the gene. NIH3T3 cells are a well-established fibroblast cell line, thus, it is easy to optimize conditions for gene transfer and to select gene-expressing cells for use *in vitro*. In addition, such fibroblasts are available from various human sources, which may be advantageous for extending future clinical investigations. NIH3T3 cells were transfected with the BDNF gene using lipofection, with the cells expressing the BDNF gene being selected for use. We examined the potential for transplanting transfected NIH3T3 cells into the inner ear of the mouse. Immunohistochemistry and Western blotting demonstrated the survival of the grafted cells within the cochlea, and a BDNF-specific enzyme-linked immunosorbent assay revealed a significant increase in BDNF production in the inner ear following cell transplantations [59]. These findings indicate that cell-gene delivery with non-viral vectors may be applicable for the local, sustained delivery of therapeutic

molecules into the cochlea. Cell-gene delivery of therapeutic molecules into the inner ear is suitable for protection of inner ear cells against gradually progressive degeneration. Presbycusis, which is an age-related hearing loss, may also need to be included as one of the targets for cell-gene therapy. BDNF application *via* cell-gene delivery could be an effective strategy for survival promotion of SGNs in cases involving cochlear implants, which require the opening of the cochlea for the purpose of inserting an electrode.

CONCLUSIONS

The lack of effective methods for drug delivery to the cochlea has been a considerable obstacle with regard to developing novel therapeutic strategies for SNHL. However, recent findings in studies examining drug delivery systems using biomaterials and cell therapy demonstrate the efficacy of these strategies for cochlear drug delivery, which in the future may contribute to the establishment of novel therapeutic strategies for SNHL.

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Insulin-like growth factor 1 protects vestibular hair cells from aminoglycosides

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This study investigated the therapeutic potential of insulin-like growth factor-1 (IGF-1) for vestibular hair cells using explant cultures of mouse utricles. After incubation with the ototoxic drug gentamicin, explants from neonatal mouse utricles were cultured in medium containing IGF-1 at various concentrations. Histological evaluation revealed significant increases in the number of surviving hair cells cultured with IGF-1 at concentrations reflecting a clinical setting. Immunostaining for trio-binding protein and espin showed the maintenance of functional structures in hair bundles at the apex of surviving hair cells. An FM1-43 assay indicated the presence of mechanoelectrical transduction channels in surviving hair cells. These findings indicate that IGF-1 may protect the functionality of vestibular hair cells against drug-induced injury. *NeuroReport* **22:38–43 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The peripheral vestibular apparatus consists of two otolith organs and three semicircular canals. Vestibular sensory epithelia report the magnitude and direction of angular and linear motion of the head to the central nervous system. Normal sensory processing through the vestibular system is largely unconscious, and we become aware of it only when it malfunctions. Dysfunction of the peripheral vestibular apparatus has an incapacitating influence on every aspect of our lives, and drug-induced damage can cause serious disability as it often leads to bilateral dysfunction.

Hair cells are the primary mechanotransducers of the peripheral vestibular apparatus. A bundle of enlarged microvilli, stereocilia, is present at the apex of each hair cell, and the tilt of these bundles induces the opening of mechanoelectrical transduction (MET) channels, which is the primary step in the sensing of head movement by the vestibular system. The survival of hair cells is therefore crucial for the maintenance of vestibular functions. In mammalian inner ears, cochleae have limited capacity for spontaneous regeneration. Although mammalian vestibular epithelia can undergo hair cell regeneration [1–5], this is not sufficient for functional recovery [1,4–6].

The protection of hair cells is therefore a practical strategy for the treatment of the mammalian vestibular epithelium. Earlier studies have indicated the potential of growth factors, including insulin-like growth factor-1 (IGF-1), for the protection and regeneration of vestibular hair cells [7–11]. This study focused on IGF-1 and examined these effects using explant culture systems with special attention to the functionality of hair cells.

Methods

Animals

ICR mice (Japan SLC Inc., Hamamatsu, Japan) were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan. Experimental protocols were approved by the Animal Research Committee of Kyoto University Graduate School of Medicine (MedKyo10119), and complied with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Explant culture

Postnatal day 3 mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected out and the utricles were removed from the surrounding tissue in 0.01 M phosphate-buffered saline, pH 7.4, which was supplemented with 0.2% glucose. The otoconial membranes were gently removed with a fine needle. Explants of utricle sensory epithelia were placed intact on type I collagen-coated cover glass (Iwaki, Tokyo, Japan) and maintained in 24-well culture plates (Iwaki) in Dulbecco's modified Eagle's medium (Invitrogen, Eugene, Oregon, USA), supplemented with 6 g/l glucose

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(Wako Pure Chemicals, Osaka, Japan) and 1.5 g/l penicillin G (Wako Pure Chemicals), at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h.

Gentamicin application and insulin-like growth factor-1 protection assay

The aminoglycoside antibiotic gentamicin was used as an ototoxic agent. To reflect clinical situations, we applied IGF-1 after intoxication with gentamicin. Initially, utricle explants were cultured in medium containing 0.5 mM gentamicin (Nacalai Tesque, Inc., Kyoto, Japan) for 24 h. The explants were then transferred to medium containing recombinant human IGF-1 (Astellas, Tokyo, Japan) at concentrations of 0, 0.01, 0.1, or 1.0 μg/ml, with five to six utricles incubated at each concentration for another 48 h. We determined IGF-1 concentrations in the media according to our earlier observation of IGF-1 concentrations in the perilymph after its local application onto the round-window membrane using gelatin hydrogels [12]. The maximum concentration of IGF-1 in this study was equivalent to that in the perilymph after local application.

Four explants immediately after dissection and four explants immediately after incubation with gentamicin were obtained for the hair cell survival assay. In this assay, explants were fixed for 15 min with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Specimens were incubated with Alexa-Fluor 488-conjugated phalloidin (1 : 250; Invitrogen) to label F-actin, and then viewed with a TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). Quantitative analysis of the hair cell number in utricles was performed according to the method described earlier [5,8,13]. Initially, each utricle was observed under low magnification. Two frames ($100 \times 100\ \mu m^2$) were set in the center area (the central region in Fig. 1e). Additional frames were made around the two central frames, keeping within the sensory epithelium as much as possible. We then randomly chose two frames that were not adjacent to the center frames (the peripheral region in Fig. 1e). The hair cell numbers in each frame were counted, and the average of each region was used as the data for the specimen.

Differences in hair cell numbers among IGF-1 concentrations were statistically tested by analysis of variance with Scheffe's method. P values less than 0.05 were considered statistically significant.

Hair bundle of surviving hair cells

To examine the functional structures of the hair bundles in surviving hair cells, we performed immunostaining for trio-binding protein (TRIOBP) and espin, which are crucial for the MET function of hair cells. TRIOBP is an actin-bundling protein that is selectively located in the rootlet of hair bundles [14], and espin is an actin-bundling protein that colocalizes with F-actin in hair bundles [15]. The expression of these proteins in the hair bundles of utricle hair cells was examined in utricles immediately after dissection, immediately after 24-h incubation with gentamicin, and after culture with 1.0 μg/ml IGF-1 ($n = 4$ for each condition).

After fixation with 4% PFA, specimens were examined by immunohistochemistry. The primary antibodies were rabbit polyclonal antibodies against TRIOBP (1 : 1000; Shin-ichiro Kitajiri, Kyoto University, Japan) and espin (1 : 100; James Bartles, Northwestern University, Evanston, Illinois, USA), and the secondary antibody was Alexa-Fluor 594 donkey anti-rabbit immunoglobulin G (1 : 500; Invitrogen). At the end of the staining procedures, the specimens were incubated with Alexa-Fluor 488-conjugated phalloidin and viewed with a TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc.).

To evaluate the MET function of surviving hair cells, we carried out labeling with FM1-43FX dye (Invitrogen), which passes through MET channels at hair bundles. Explants immediately after dissection, immediately after 24-h incubation with gentamicin, and after culture with 1.0 μg/ml IGF-1 were used for this purpose. The explants were transferred to culture media supplemented with FM1-43 at a concentration of 5 μM for 10 s. During the incubation with FM1-43, we applied mechanical stimulation, during which frequency was 1–2 Hz and intensity was approximately 100 V, as described earlier [4]. After fixation with 4% PFA, the specimens were examined with a TCS-SP2 laser-scanning confocal microscope. Four independent assays were performed in each condition.

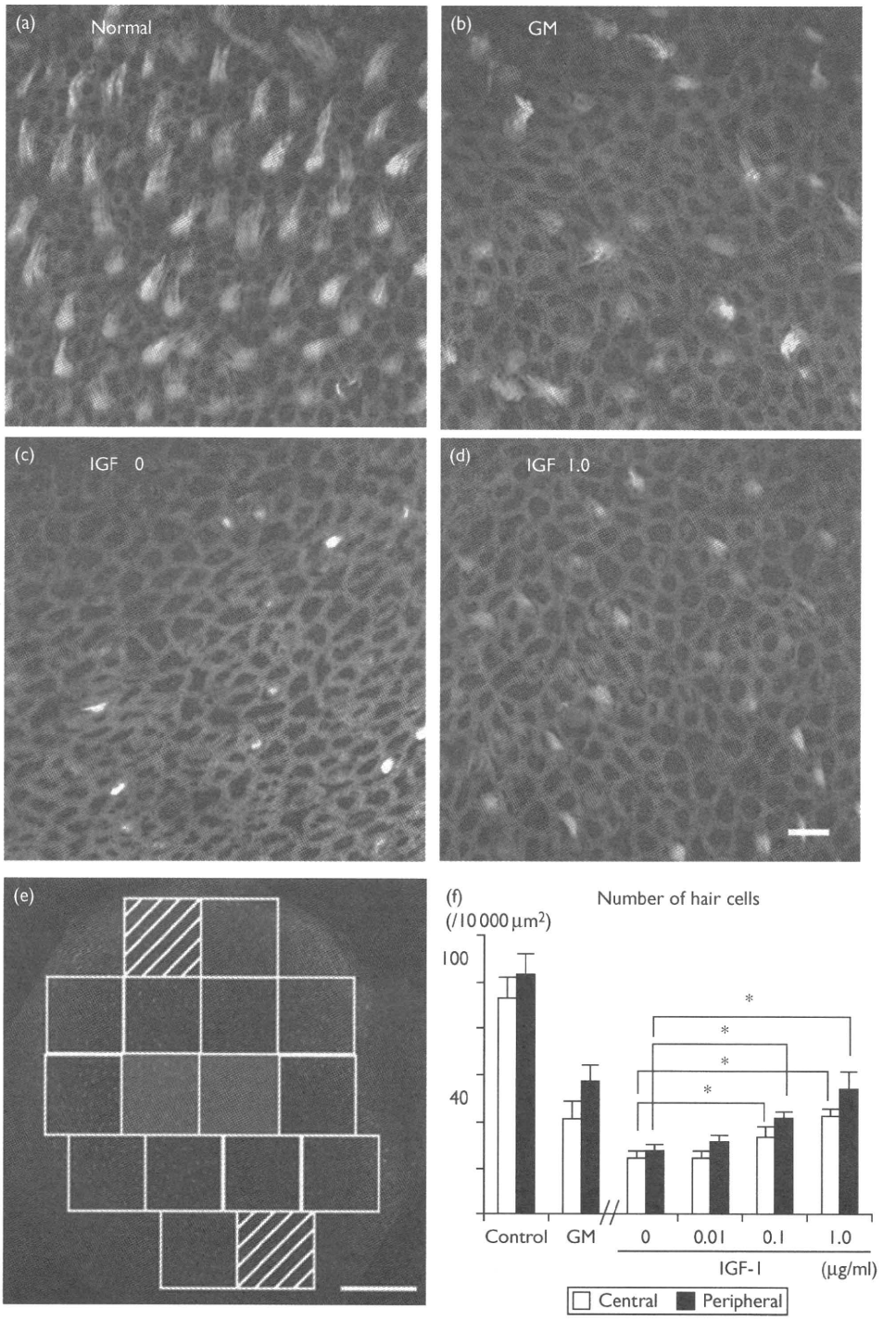
Results

Insulin-like growth factor-1 protection assay

In comparison with normal utricles that were fixed immediately after dissection (Fig. 1a), incubation with 0.5 mM gentamicin for 24 h caused approximately one-half of all hair cells to be lost (Fig. 1b) in both the central and peripheral regions. After an additional 48-h culture with no extra IGF-1 supplementation, extended loss of hair cells occurred in both central (Fig. 1c) and peripheral regions. Supplementation of the culture medium with 0.1 or 1.0 μg/ml IGF-1 resulted in increased survival of hair cells in these regions (Fig. 1d).

Quantitative analyses revealed statistically significant increases in hair cell numbers in explants treated with 0.1 or 1.0 μg/ml IGF-1 in both central and peripheral regions (Fig. 1f). Notably, supplementation with 1.0 μg/ml IGF-1 largely inhibited the hair cell loss that occurred during an additional 48-h culture after gentamicin exposure. These findings indicate that IGF-1 has protective effects on vestibular hair cells against gentamicin toxicity. However, no increase in hair cell numbers was observed in IGF-1-treated specimens in comparison with those in explants immediately after incubation with gentamicin (Fig. 1f), suggesting that little or no hair cell regeneration was induced by IGF-1.

Fig. 1

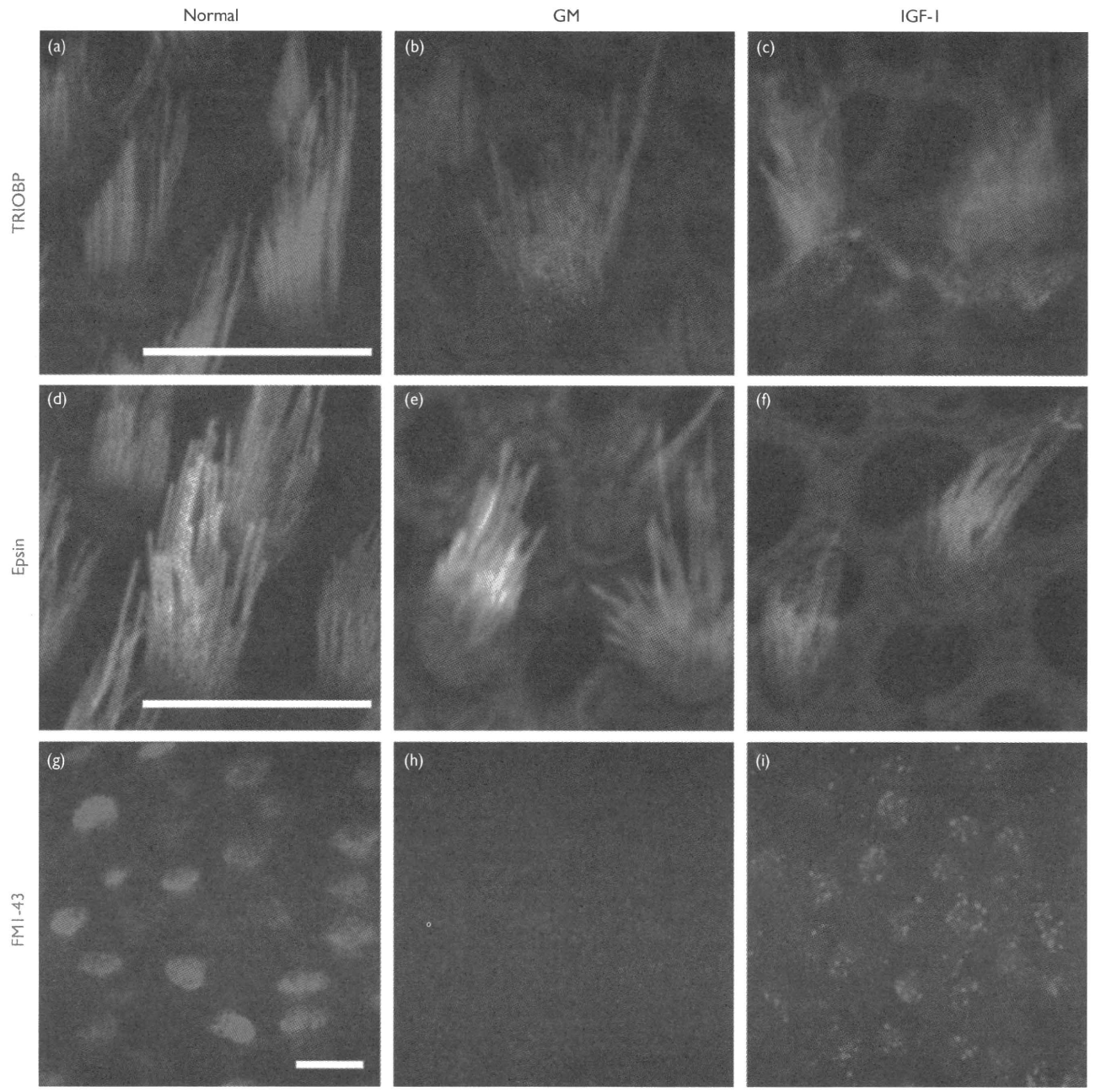


Surface images of the central regions in utricle explants immediately after dissection (a), incubation with 0.5 mM gentamicin (GM) for 24 h (b), following additional 48-h culture with no extra insulin-like growth factor 1 (IGF-1) (c) or with 1.0 μg/ml IGF-1 (d). Scale bar in (d) represents 10 μm for (a–d). In (e), grey squares represent the central regions in a sensory epithelium, and shaded portions show the peripheral regions. Scale bar represents 100 μm. Quantitative analyses (f) revealed severe loss of hair cells after 24-h incubation with GM in comparison with controls, which were immediately after dissection. Significant increases in hair cell numbers (*$P < 0.05$) were found in explants treated with 0.1 or 1.0 μg/ml IGF-1 in both central and peripheral regions.

Hair bundle of surviving hair cells

In normal utricles, TRIOBP was distributed in the rootlet of hair bundles (Fig. 2a), and colocalization of espin and F-actin was also identified (Fig. 2d). In utricles, immediately after gentamicin exposure, the expression of TRIOBP was still observed in the rootlet of hair bundles (Fig. 2b). All surviving hair cells exhibited espin expression in hair bundles in a similar distribution to normal hair cells (Fig. 2e). In utricles treated with 1.0 μg/ml IGF-1, the hair bundles of surviving hair cells exhibited a similar distribution of TRIOBP (Fig. 2c) to utricles immediately after dissection or after gentamicin exposure. The

Fig. 2



Expression of trio-binding protein (TRIOBP) and espin in hair bundles of hair cells and FM1-43 labeling of functional hair cells. Green fluorescence in (a–f) shows F-actin labeling with phalloidin. TROBP expression (red fluorescence in a–c) was found in the rootlet of hair bundles in explants immediately after dissection (Normal), incubation with 0.5 mM gentamicin for 24 h (GM), and following additional 48-h culture with 1.0 μg/ml insulin-like growth factor 1 (IGF-1). Espin (red fluorescence in d–f) colocalized with F-actin in hair bundle in each condition. In normal (g) and IGF-1 treated explants (i), FM1-43 labeling (red fluorescence in g–i) was identified, whereas virtually no labeling was found in explants immediately after incubation with GM (h). Scale bar in (a) represents 10 μm for (a–c), that in (d) represents 10 μm for (d–f), and that in (g) represents 10 μm for (g–i).

expression of espin in hair bundles was also observed in all surviving hair cells (Fig. 2f). These findings show that surviving hair cells treated with IGF-1 maintain the expression of TRIOBP and espin in hair bundles during the culture period after gentamicin incubation, suggesting that IGF-1 contributes to the protection of vestibular hair cell functionality.

FM1-43 dye is frequently used to evaluate MET channels in hair cells, and clearly labeled the hair cells in normal utricles in this study (Fig. 2g). In contrast, utricles immediately after gentamicin exposure showed no FM1-43 labeling in surviving hair cells (Fig. 2h), which might have been because of the blockage of MET channels by gentamicin [16,17]. Following the additional 48-h culture with 1.0 μg/ml IGF-1, the FM1-43 labeling in surviving hair cells recovered (Fig. 2i). Together with TRIOBP and espin immunohistochemistry findings, these results suggest that the surviving hair cells that were rescued by IGF-1 application retained their functionality.

Discussion

IGF-1 is a mitogenic peptide that plays essential roles in the regulation of the growth and development of the inner ear [9]. Earlier, we showed the efficacy of local IGF-1 application on the round-window membrane using gelatin hydrogels for the protection of auditory hair cells against damage induced by noise [12,18] or ischemia reperfusion [19] in animal models. On the basis of these findings, we performed a prospective clinical trial, which indicated the clinical efficacy of local IGF-1 application using gelatin hydrogels for sudden sensorineural hearing loss that was resistant to systemic glucocorticoid treatment [20].

Local IGF-1 treatment could be effective for peripheral vestibular diseases that involve hair cell loss. Unlike cochlear hair cells, mammalian vestibular hair cells have the capacity for regeneration [1–5], and IGF-1 could contribute to this [7,8], and to the protection of vestibular hair cells [11,21]. The IGF-1 concentrations used in this study were adjusted to match the concentrations in the perilymph that can be achieved using local IGF-1 application onto the round-window membrane with gelatin hydrogels [12]. In addition, IGF-1 application was initiated after gentamicin intoxication in this study to reflect clinical situations.

Quantitative analyses of the numbers of surviving hair cells showed that 24-h exposure to 0.5 mM gentamicin caused extensive loss of hair cells in explant cultures of neonate mouse utricles. An additional 48-h culture with neither gentamicin nor IGF-1 resulted in extended hair cell loss in utricle cultures. However, IGF-1 application at a concentration of 0.1 or 1.0 μg/ml efficiently rescued hair cells from such postexposure effects of gentamicin. Notably, 1.0 μg/ml IGF-1 largely inhibited hair-cell loss because of these effects, suggesting that local IGF-1 application using gelatin hydrogels could reasonably be expected to protect vestibular hair cells *in vivo*.

In contrast, no significant increase in hair cell numbers was induced by IGF-1 application in this study, indicating that few or no hair cells were newly produced in utricle explants during the observation period. Recently, Kawamoto *et al.* [5] investigated the capacity of mouse utricles for hair cell regeneration after gentamicin treatment *in vivo*, and found that new hair cells appeared 3 weeks after gentamicin treatment. Taura *et al.* [4] showed the emergence of new hair bundles 13 days after gentamicin intoxication in explant cultures of rat utricles. The culture period used in this study might be too short for evaluating the regeneration of hair bundles.

This study examined the functionality of surviving hair cells. Morphologically, the hair bundles in surviving hair cells well maintained the expression patterns of TRIOBP and espin. In addition, FM1-43 labeling was also observed in utricles that were treated with IGF-1, indicating the maintenance of hair cell function. These findings strongly suggest that hair cells rescued by IGF-1 treatment retain their functionality.

Conclusion

These findings suggest that IGF-1 has the ability to protect vestibular hair cells against aminoglycoside toxicity at concentrations that are close to in-vivo conditions, even after ototoxic insults. This encourages us to investigate protective effects of IGF-1 for vestibular hair cells *in vivo*.

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