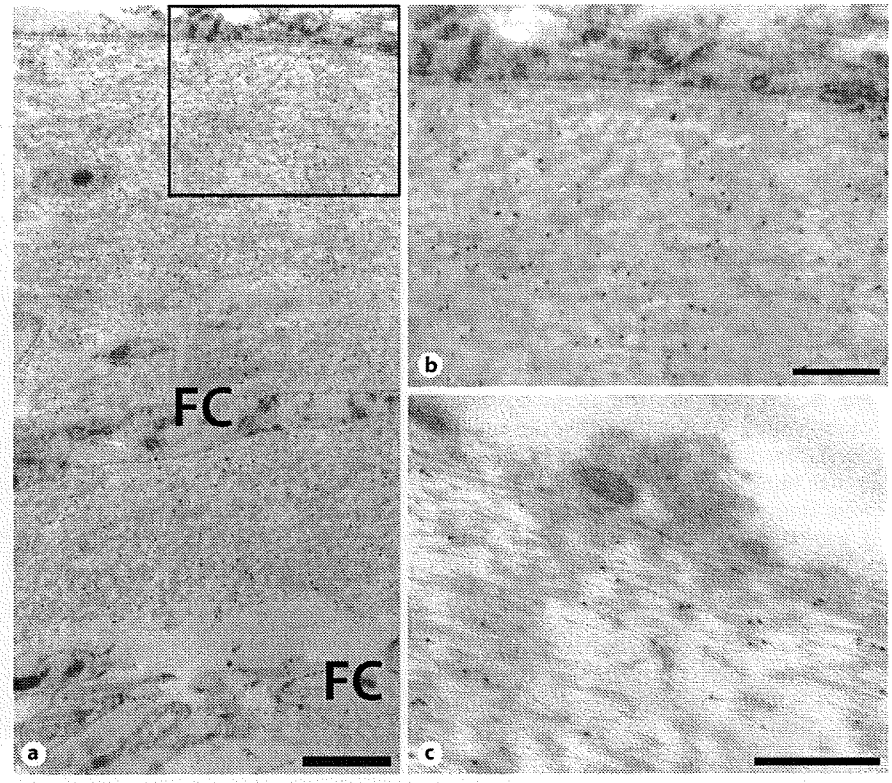

Fig. 1. Immuno-electronmicroscopy for cochlin and type II collagen expression in the spiral limbus. **a** Cochlin: gold particles in the fibrous substance in the spiral limbus. Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 0.5 μm. **c** Type II collagen: gold particles were seen in the fibrous substance in the spiral limbus. Bar = 0.5 μm. FC = Fibrocytes.

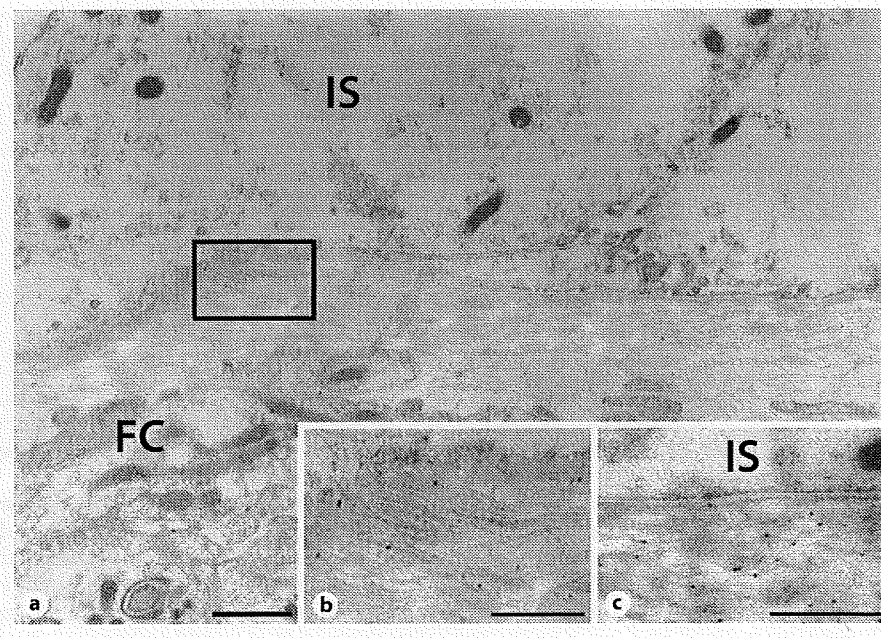

Fig. 2. Immuno-electronmicroscopy for cochlin and type II collagen expression beneath the inner sulcus cells. **a** Cochlin: immunolabeling of cochlin was seen in the fibrous substance beneath the inner sulcus cells. Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 0.5 μm. **c** Type II collagen: gold particles were seen in the fibrous substance beneath the inner sulcus cells. Bar = 0.5 μm. FC = Fibrocytes; IS = inner sulcus cells.

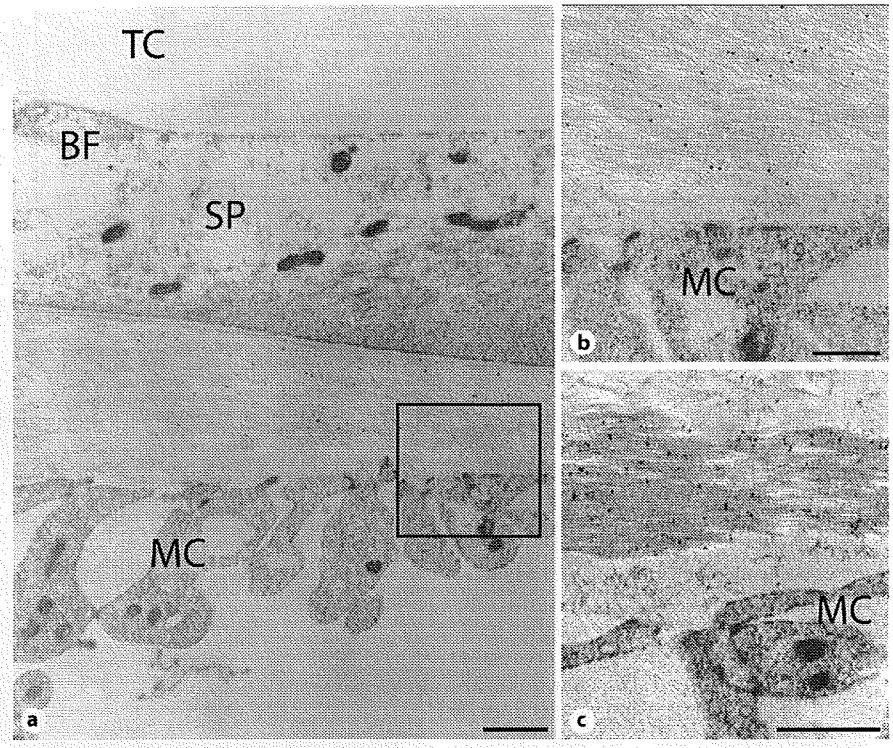

Fig. 3. Immuno-electronmicroscopy for cochlin and type II collagen expression in the basilar membrane. **a** Cochlin: the fibrillar bundles in the basilar membrane were immunoreactive for cochlin. Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 0.5 μm. **c** Type II collagen: gold particles were seen in the fibrillar bundles in the basilar membrane. Bar = 0.5 μm. TC = Tunnel of Corti; BF = tunnel basilar fiber; SP = supporting cells of the sensory cells; MC = mesothelial cells.

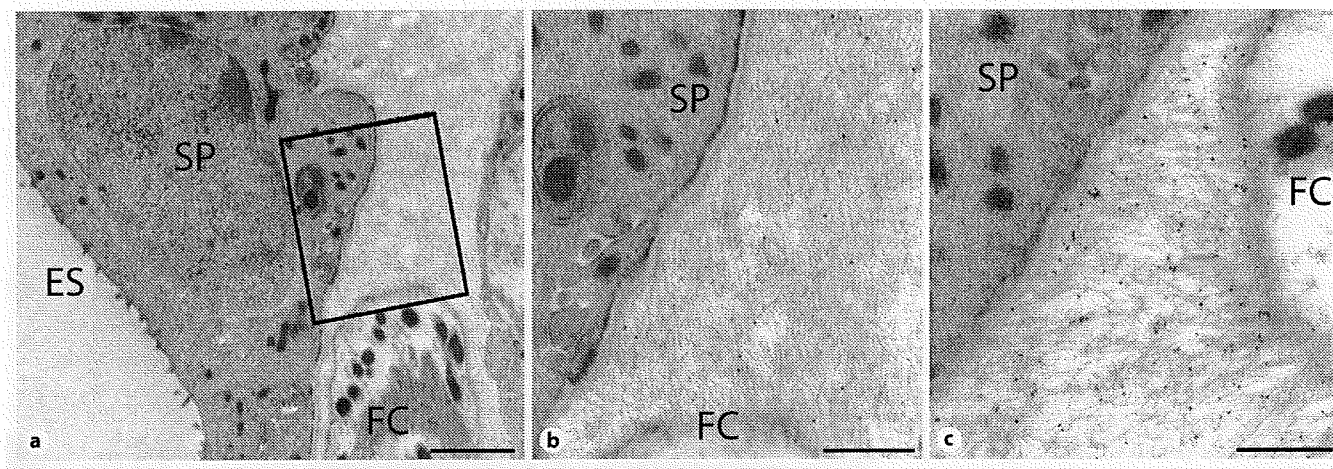

Fig. 4. Immuno-electronmicroscopy for cochlin and type II collagen expression beneath the epithelial cells of the spiral prominence. **a** Cochlin: gold particles in the fibrous substance beneath the epithelial cells of the spiral prominence. Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 0.5 μm. **c** Type II collagen: gold particles were seen beneath the epithelial cells of the spiral prominence. Bar = 0.5 μm. SP = Epithelial cells of the spiral prominence; FC = fibrocytes; ES = endolymphatic space.

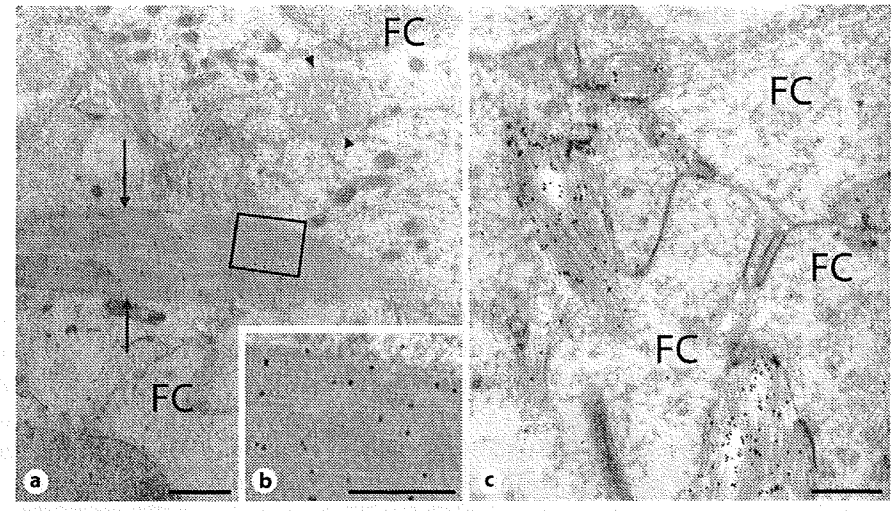

Fig. 5. Immuno-electronmicroscopy for cochlin and type II collagen expression in the spiral ligament. **a** Cochlin: gold particles were also observed in the banded bundles, which have a parallel array in the area of the fibrocytes in the spiral ligament. Parallel cut of the fibrous bundles (arrows). Vertical cut of the fibrous bundles (arrow heads). Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 0.5 μm. **c** Type II collagen: gold particles were seen on the fibrillar bundles in the spiral ligament. Bar = 0.5 μm. FC = Fibrocytes.

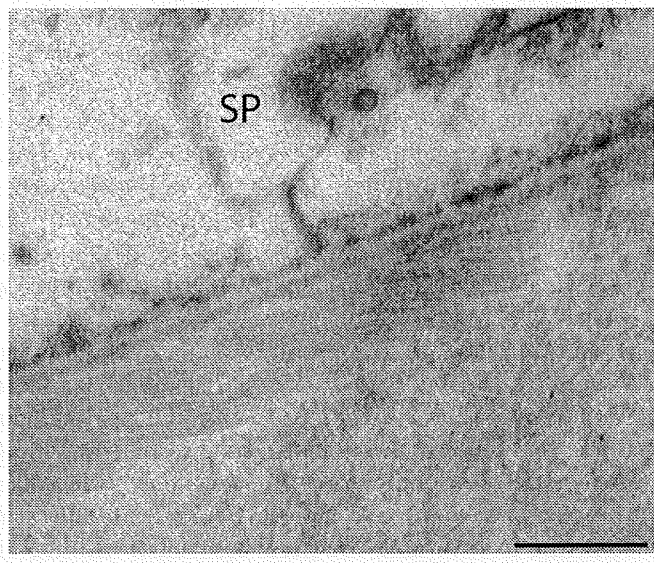

Fig. 6. Control. No gold particles were observed in the fibrillar substance in the basilar membrane. SP = Supporting cells of the sensory cells. Bar = 0.5 μm.

Spiral Ligament

Immunolabeling of cochlin and type II collagen was observed in the fibrous bundles in the spiral ligament (fig. 5a–c).

Control

When pre-immune IgG was used as a negative control, the fibrillar substance of the basilar membrane exhibited no immunoreactivity (fig. 6).

Discussion

Immunoreactivity for cochlin and type II collagen was observed in the same ECM areas of the cochlear duct, particularly in the following sites: the fibrous substance in the spiral limbus, beneath the inner sulcus cells, in the basilar membrane, beneath the epithelial cells of the spiral prominence and in the spiral ligament. The present study is the first report to demonstrate the localization of cochlin in these structures at the ultrastructural level. Localization of cochlin in the basilar membrane was not detected in our previous studies using the same anti-cochlin antibody at the light microscopic level [Robertson et al., 2006; Shindo et al., 2008]. This might be due to the different tissue processing and staining in the applications of light and electron microscopy. Interestingly, another antibody, which recognizes a different peptide of cochlin (residues 337–355) than the one targeted here (residues 163–181), was reactive to cochlin in this area under light microscopy [Kommareddi et al., 2007].

Our findings for type II collagen at the basilar membrane and the spiral ligament are consistent with previous ultrastructural studies [Dreiling et al., 2002; Kaname et al., 1994]. At the electron microscopic level, we previously localized cochlin and type II collagen on the fibrous structures beneath the epithelial cells and supporting cells in the rat semicircular canal [Mizuta et al., 2008]. Cochlin appears to localize with type II collagen in the fibrous structures in the ECM of the cochlea as well as in the vestibule. Nagy et al. [2008] recently demonstrated that the second vWFA-like domain of cochlin has an affinity for type II collagen. This report supports the

hypothesis that these two proteins interact with each other.

Several types of collagen have been detected in the ECM of the inner ear [Yoo et al., 1988; Slepecky et al., 1992; Usami et al., 2008]. Of these subtypes, type II collagen is responsible for the fibrous structure and appears to play a critical role in maintaining structural stability in the cochlea and vestibule [Slepecky et al., 1992]. Localization of these two proteins in the same fibrous substance of the ECM in the cochlear duct indicates that cochlin may play a role in the structural homeostasis of the cochlea by cross-linking to the fibrillar type II collagen bundles.

Kommareddi et al. [2007] showed that a prominent 64-kDa band of cochlin co-immunoprecipitated with choline transporter-like protein 2 (CTL2). CTL2 is a multitransmembrane protein expressed on inner ear supporting cells that was discovered as a target of antibody-induced hearing loss [Nair et al., 2004]. The present findings, together with the fact that cochlin co-immunoprecipitates with CTL2, indicate that these proteins may interact with other proteins. Indeed, cochlin and type II collagen have also been implicated as a disease-causing antigen in autoimmune hearing loss in humans [Baek et al., 2006; Yoo et al., 1984]. The association of these three proteins may therefore have very interesting implications with regard to DFNA9 pathogenesis.

In DFNA9 pathogenesis, Robertson et al. [2006] hypothesized that mutated cochlin accumulates acellular eosinophilic deposits and that this accumulation leads to degeneration of other cochlin-associated proteins. Our findings suggested that type II collagen is one of the candidates of the cochlin-associated proteins. To clarify this hypothesis, it will be interesting to analyze chronologically the ultrastructural pathology and immunohistochemistry of a mutant mouse model of DFNA9 which exhibited progressive age-related hearing loss [Robertson et al., 2008].

The late-onset progressive sensorineural hearing loss in the DFNA9 ear also suggests a relationship between cochlin and presbycusis, an impairment of hearing characteristic of elderly individuals [de Kok et al., 1999]. Robertson et al. [2008] suggested that *COCH* might play important roles in presbycusis, and that cochlin is a major target antigen for autoimmune sensorineural hearing loss. The histopathologic correlates of age-related hearing loss suggest several categories for this type of auditory impairment: sensory-neural, strial, cochlear-conductive, mixed, and indeterminate [Schuknecht and Gacek, 1993]. In these categories, cochlear-conductive hearing loss may be related to pathologic change in the basilar membrane. Several studies have shown that thickening of the basilar membrane in aged animals may underlie presbycusis [Ishii et al., 1994; Shimada et al., 1998]. The basilar membrane is responsible for the mechano-electrical transduction exhibited by sensory cells, which enables them to absorb stress and withstand traveling waves. This explains why changes in these mechanical properties can cause hearing impairment. Buckiova et al. [2006] have shown a reduction in type II collagen immunoreactivity at the light microscopic level in the spiral ligament, but not in the basilar membrane, of aged Fischer 344 rats (an animal model of strial presbycusis). However, the reduction of type II collagen in the spiral ligament led us to consider that degeneration of type II collagen might occur in the basilar membrane of the aging ear. The pathology of the DFNA9-affected ear includes degeneration of the basilar membrane [Khetarpal et al., 1991; Khetarpal, 1993]. At present, however, the relationship between cochlin and the age-related pathology of the inner ear remains unknown. For thin structures such as the basilar membrane, ultrastructural analysis can reveal more detailed pathology than light microscopy, and may be a better tool for determining age-related pathology. Accordingly, in the near future we plan to conduct an ultrastructural analysis of age-related changes in type II collagen and cochlin in the basilar membrane as well as in the spiral ligament.

In conclusion, the present study suggests that cochlin could cross-link to type II collagen fibers in the spiral limbus, beneath the inner sulcus cells, the basilar membrane, the spiral prominence and the spiral ligament in the cochlear duct, and that it is responsible for the structural integrity of this organ, in particular by enabling the structure to withstand the stress associated with traveling waves. Further studies, however, will be needed in order to determine the nature of the interaction between cochlin and type II collagen.

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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

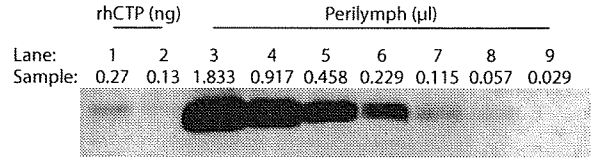
In order to evaluate the specificity of the CTP detection test, we examined MEL from non-PLF cases. In this study, we defined 'non-PLF' as those cases with otosclerosis (which had undergone stapedectomy), profound deafness (cochlear implant surgery) or conductive hearing loss (exploratory tympanotomy). 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.

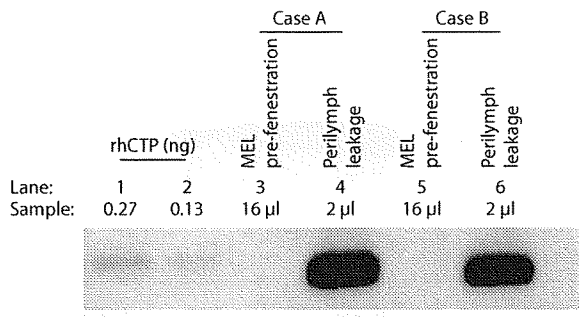
Detection Limit

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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Cochlin-Tomoprotein: A Novel Perilymph-Specific Protein and a Potential Marker for the Diagnosis of Perilymphatic Fistula

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Key Words
COCH gene · Cochlin isoform · Cochlin tomoprotein · Hearing loss · Vertigo

Abstract
Background: Perilymphatic fistula (PLF) is an abnormal connection between the inner and middle ear. A procedure for obtaining definite proof of a PLF remains elusive, and methods of diagnosis remain controversial. To date, there is no clinically relevant biochemical marker for perilymph leakage. Using proteomic analysis of inner ear proteins, we have previously found unique properties of cochlin, encoded by the *COCH* gene. We detected 3 cochlin isoforms (p63s, p44s and p40s) in the inner ear tissue and a short 16-kDa isoform of cochlin-tomoprotein (CTP) in the perilymph. Since cochlin was found to be highly specific to the inner ear, we speculated that CTP might also be specific to the perilymph. The aim of this study was to determine whether CTP, a novel perilymph-specific protein, could be used as a marker for the diagnosis of PLF. ***Methods:*** By Western blotting, we investigated the specificity of CTP expression in a range of body fluids that included perilymph, serum, saliva and cerebrospinal fluid. To elucidate the detection limit of CTP, serially diluted recombinant human (rh)CTP as well as human perilymph was tested. ***Results:*** CTP was selectively expressed in all 20 perilymph samples tested, but not in 77 samples of the other body fluids. The detection limit of rhCTP was 0.27 ng or 0.022 μl of perilymph per well on Western blot analysis. ***Conclusion:*** The results strongly suggest that CTP can be a specific marker of perilymph leakage. Moreover, CTP has the potential to be a biochemical marker that allows a definitive diagnosis of the etiology of PLF-related hearing loss and vestibular disorders.

Introduction

Perilymphatic fistula (PLF) is defined as abnormal connections between the fluid (perilymph)-filled space of the inner ear and the air-filled space of the middle ear.

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PLF appear in the disrupted tissues of the round or oval window and in fractured bony labyrinth or minor fissures that occur after head trauma or barotrauma or after chronic inflammation. They have also been reported to develop spontaneously [Jackler and Brackmann, 2005]. The primary manifestations of perilymph fistulization are sudden or progressive fluctuating sensorineural hearing loss and vertigo. Other symptoms that may be present include tinnitus, disequilibrium and aural fullness. However, the symptoms are not characteristic, especially in cases that have no history of trauma, and so PLF might be confused with idiopathic sudden sensorineural hearing loss, Menière's disease or vestibular neuronitis [Fitzgerald, 2001; Maitland, 2001].

PLF was first proposed as a clinical entity more than a century ago, yet it remains a topic of controversy, especially regarding the occurrence of spontaneous PLF. It is also known that although several potential pathways exist between the perilymphatic space and the middle ear, actual leaking of fluid can be difficult or impossible to prove. The conventional gold standard of PLF detection is the intraoperative visualization of perilymph leakage, which ostensibly confirms the existence of PLF. If the patient does not have PLF, leakage will not be detected. However, the surgical procedure itself invites seepage and bleeding, which accumulates in the concave-shaped round and oval window niches, and this could be misinterpreted as perilymph leakage [Nomura, 1994]. 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, 1999].

This has led to a series of research efforts to identify an endogenous marker of perilymph [Bassiouny et al., 1992; Thalmann et al., 1994; Olaf et al., 2005a, 2005b] or exogenous substances such as intrathecal fluorescein [Gehrking et al., 2002], which might be used to diagnose PLF. Although beta2-transferrin was thought to be a marker, a more recent study showed that, because of the relative amount of serum and perilymph in a mixed sample, electrophoretic separation of the transferrin variant might not be diagnostic [Rauch, 2000]. To date, there is no clinically relevant biochemical marker for perilymph leakage.

Previously, by proteomic analysis of inner ear proteins we found unique properties of cochlin, encoded by the *COCH* gene, mutated in DFNA9, in hereditary hearing loss [Robertson et al., 1998, 2006]. We detected 3 cochlin isoforms (p63s, p44s and p40s) in the inner ear tissue and a short 16-kDa isoform, cochlin-tomoprotein (CTP), in the perilymph [Ikezono et al., 2001, 2004]. An analysis of the isoform structure suggested that the short 16-kDa isoform can be produced by proteolytic cleavage of full length cochlin, and our recent study on splicing variants of cochlin mRNA confirmed this (submitted elsewhere). Therefore we named it 'cochlin-tomoprotein' (*tomo* meaning 'cut' in Greek). Since cochlin was found to be highly specific to the inner ear, we speculated that CTP might also be specific to the perilymph.

CTP was detected in all 20 perilymph samples. By contrast, CTP was not detected in any of the 77 body fluid samples of serum, cerebrospinal fluid (CSF) and saliva. Here, we describe the specificity of CTP expression in perilymph, and discuss the future clinical application of CTP as a diagnostic marker of PLF. CTP has the potential to be a biochemical marker to allow a definitive diagnosis of the etiology of PLF-related hearing loss and vestibular disorders.

Methods

Collection and Processing of Body Fluid Samples

For the assessment of the specificity of CTP expression in body fluids, we collected perilymph during translabyrinthine vestibular schwannoma surgery, stapedectomy for otosclerosis or cochleostomy for cochlear implant surgery. We collected serum and saliva from normal controls. CSF was purchased from Biotech (Valley Center, Calif., USA). The CSF had been collected from consenting donors at an FDA licensed and registered facility. No adverse events were observed during sample collection. The samples were centrifuged at 1250 *g* for 1 min and the supernatants were frozen and stored at –80°C until use. All patients gave their full informed consent and the study was approved by the ethics committee of Nippon Medical School.

Analysis of CTP Expression by Western Blot Analysis

Two micrograms of perilymph, serum, saliva or CSF were mixed with 5 μl of sample buffer (0.188 M Tris buffer, 2.39 mM SDS, 30% glycerol and 15% 2-mercaptoethanol) after normalization per average protein concentration (perilymph 200 mg/dl, plasma 7000 mg/dl, CSF 40 mg/dl and saliva 100 mg/dl) [Thalmann et al., 1994; Mata et al., 2004], then analyzed by Western blot.

For Western blot analysis, the rabbit polyclonal anti-CTP antibody (formerly anti-LCCL-C Ab) was prepared as previously described (fig.1) [Ikezono et al., 2004]. In brief, 14-mer peptide (LSRWSASFTVTKGK) corresponding to residues 114–127 in the LCCL domain was used to generate antibody. We added cysteine residues to the C termini of the peptides to permit coupling of the peptides to KLH as a carrier protein for immunization. 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

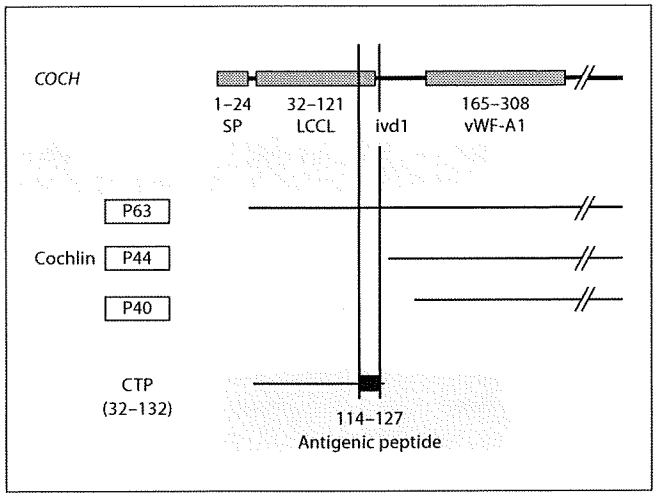

Fig. 1. Representation of the *COCH* gene, cochlin and CTP. The top line denotes the deduced amino acid sequence of human *COCH*, showing the positions of the signal peptide (SP), the Limulus factor C, cochlin and the late gestation lung protein Lgl1 domain (LCCL), the intervening domains 1 (ivd1) and the von Willebrand factor type A-like domains 1 (vWF-A1). The middle lines depict the cochlin isoforms, p63s, p44s and p40s, expressed in inner ear tissue. The bottom line depicts the cochlin-tomoprotein (CTP) isoform expressed in the perilymph. The black bar indicates the location of the antigenic peptide and vertical lines represent the alignment of antigenic peptide and cochlin isoforms. Numbers are the corresponding amino acid sequence of human cochlin. 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] for amino acid residues 32–132 was used to measure the detection limit of hrCTP.

Table 1. Specificity of CTP expression in body fluids

Sample	Total	CTP positive	CTP negative
Perilymph			
Vestibular Schwannoma surgery	9	9	0
Stapedectomy	9	9	0
Cochlear implant	2	2	0
Total	20	20	0
Body fluids			
Serum	28	0	28
CSF	20	0	20
Saliva	29	0	29
Total	77	0	77

was confirmed by dot blot analysis and a peptide absorption test (data not shown).

Samples were loaded onto 15% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked overnight at 4°C in 5% skim milk and 0.2% polyoxyethylenesorbitan (Tween-20) dissolved in PBS (pH 7.5). Membranes were then incubated in PBS containing 1% skim 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, Tokyo, Japan) 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; GE Healthcare, Amersham, UK) and then analyzed by an LAS-3000 image analyzer (Fuji Film, Tokyo, Japan).

Detection Limit of the Recombinant Human (rh)CTP by Western Blot

The recombinant human (rh)CTP was produced to measure the detection limit of 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] for the positions 101–403 of the cDNA, corresponding to amino acid residues 32–132 (fig. 1), was amplified by PCR from a human expressed sequence tag clone, Image ID 27789 (Kurabo, Japan). According to the manufacturer's protocol, rhCTP was produced at a final concentration of 0.17 mg/ml using pCR/T7/TOPO/TA expression kits (Invitrogen, Tokyo, Japan). Serially diluted rhCTP were tested for assessment of the detection limit. Serially diluted perilymph samples were also tested to establish the detection limit of CTP.

Results

CTP Expression in Body Fluid Samples

Perilymph from 9 vestibular schwannoma surgery, 9 stapedectomy and 2 cochlear implant patients were all positive for CTP (table 1, fig. 2). However, CTP was not detected in any of the 77 body fluid samples (28 serum, 20 CSF and 29 saliva). These results show that CTP is a perilymph specific protein.

Detection Limit of the Recombinant Human (rh)CTP by Western Blot

The molecular weight of rhCTP exactly matched that of native CTP (16 kDa) on Western blot (fig. 2c). This rhCTP is suitable for future use as a spiked standard when we test the clinical samples by Western blot. The detection limit of the rhCTP was between 0.27 and 0.14 ng/well (fig. 3). Two serially diluted perilymph samples were tested to show the detection limit. The average minimum detection limit of CTP from perilymph was 0.022 μl peri-

lymph/well (data not shown). This detection limit could be good for the clinical use of CTP as a diagnostic marker of PLF.

Discussion

PLF has some proven etiologies, and these must be considered in the appropriate settings (e.g. osseous labyrinth fracture, blast explosion, middle ear trauma, ear surgery such as post-stapedectomy) [Shea, 1963; Schuknecht, 1969; Strohm, 1986; Jackler et al., 1987; Fitzgerald, 1996; DePalma et al., 2005; Jackler and Brackmann, 2005]. A PLF should be considered in pediatric patients with recurrent meningitis, and middle ear exploration should be pursued [Jackler et al., 1987; Reilly, 1989]. PLF without perilymph leakage has also been established as a clinical entity, as seen in superior canal dehiscence syndrome or semicircular canal fistula caused by cholesteatoma [Minor, 2003], which can be diagnosed by high resolution CT scan. Other etiologies have also been proposed to cause PLF (which require detection of perilymph leakage detection to establish a diagnosis), such as a traumatic or barotraumatic event resulting in disruption of the membranes of the round and/or oval window(s) or leakage from minor fissures [Kohut et al., 1986; Goodhill, 1971; Klingmann et al., 2007].

There is no established diagnostic test with enough sensitivity and specificity to identify the presence or absence of perilymph leakage. This has made it difficult to establish criteria to determine when surgical exploration might be indicated. Additionally, there are no universally accepted criteria to confirm diagnosis at surgery; the determination of perilymph leakage is still a subjective decision of the surgeon. The presence of clear fluid in the middle ear at the time of surgery may represent perilymph or may be CSF, serum, seepage, or local anesthetic. No reliable and accurate test is currently available to distinguish these fluids from one another [Nomura, 1994].

The *COCH* gene mutated in DFNA9, an autosomal dominant hereditary sensorineural hearing loss and vestibular disorder, encodes cochlin. Eleven missense mutations and 1 in-frame deletion have been reported (http://webh01.ua.ac.be/hhh). By immunohistochemistry on the DFNA9 temporal bone sections, we have shown cochlin staining of the characteristic cochlear and vestibular acellular eosinophilic deposits, indicating aggregation of cochlin in the same structures in which it is normally expressed [Robertson et al., 2006].

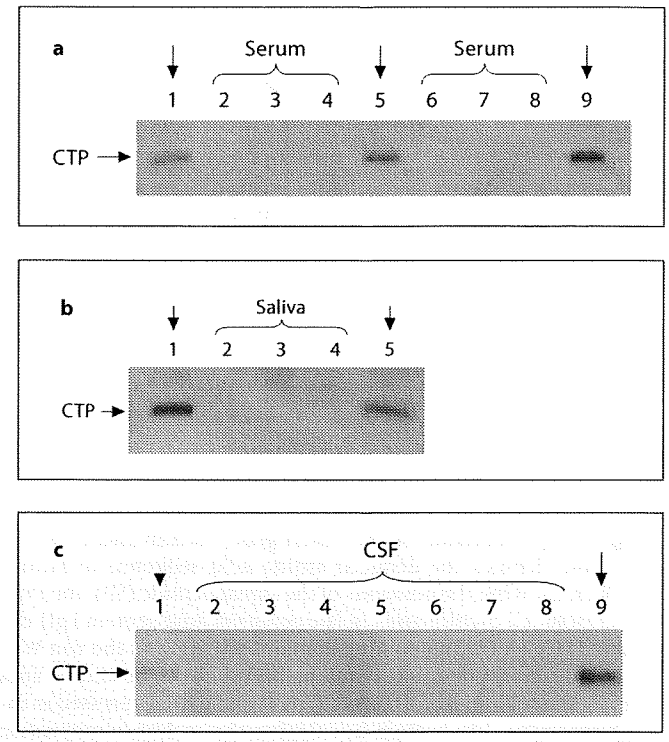

Fig. 2. Western blot analysis for CTP expression in body fluids. The expression of CTP in various body fluids was analyzed by Western blot using the anti-CTP antibody. Two micrograms of perilymph, serum, saliva or CSF was mixed with 5 μl of sample buffer after normalization per protein concentration (perilymph 200 mg/dl, plasma 7000 mg/dl, CSF 40 mg/dl, saliva 100 mg/dl). CTP expression (16 kDa) was detected in the perilymph, but not in the serum, CSF or saliva. CTP was detected only in perilymph samples. **a** Lanes 1, 5 and 9 (arrows) contain perilymph, all others contain serum. **b** Lanes 1 and 5 (arrows) contain perilymph, all others contain saliva. **c** Lane 1 (arrowhead) contains rhCTP (0.27 ng), lane 9 (arrow) contains perilymph, and all others contain CSF. The molecular weight of rhCTP exactly matched that of native CTP (16 kDa) on Western blot.

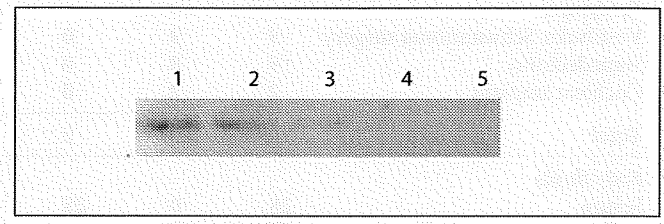

Fig. 3. Detection limit of the rhCTP by Western blot. rhCTP was produced using pCR/T7/TOPO/TA expression kits. Serially diluted rhCTP were tested for assessing the detection limit. The detection limit of the rhCTP was between 0.27 and 0.14 ng/well. Amount of protein per lane: lane 1 = 1.08 ng; lane 2 = 0.54 ng; lane 3 = 0.27 ng; lane 4 = 0.14 ng; lane 5 = 0.07 ng.

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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