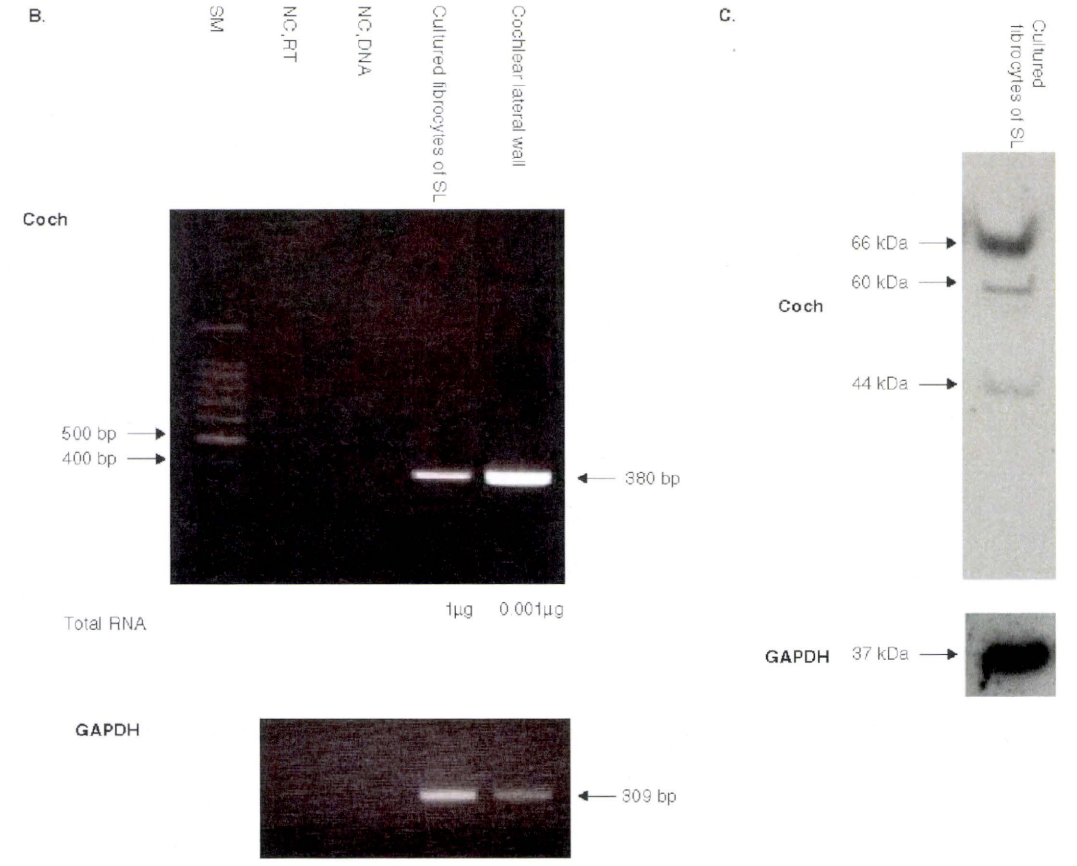

Figure 2. (*Continued*). (A) Cultured fibrocytes were immunopositive to caldesmon (a) and S-100 protein (b) and negative to IgG (c) and Na^+-K^+-ATPase (d). Bar – 50 μm. The immunoreactivity to these antibodies bears the characteristics of type I fibrocytes of the SL (spiral ligament). (B) RT-PCR of total RNA from the cultured fibrocytes (1 μg) and the cochlear lateral wall (0.001 μg). *GAPDH* was used as loading control. *Coch* mRNA (380 bp PCR product) was amplified. NC RT-, NC cDNA-; negative control without AMV reverse transcriptase XL or template cDNA, respectively. (C) Cochlin isoforms detected in the cultured fibrocytes. Immunoreactive proteins with sizes of 66, 60, and 44 kDa were detected. *GAPDH* was used as an internal standard.

Table I. *Coch* sequence identity (%) of the nucleotides and amino acids, a comparison of the guinea pig, human, Bovine, mouse and rat.

	Protein				
cDNA (CDS)	Homo sapiens	Bos taurus	Cavia porcellus	Mus musculus	Rattus norvegicus
Homo sapiens		94.3%	95.0%	93.6%	92.9%
Bos taurus	92.1%		93.3%	92.9%	92.4%
Cavia porcellus	91.6%	89.5%		93.3%	93.6%
Mus musculus	89.0%	87.6%	87.4%		98.2%
Rattus norvegicus	88.1%	87.2%	86.9%	94.3%	

human, Bovine, mouse, and rat, respectively. This high degree of homology, which is maintained among the species examined and distributed throughout all domains, including LCCL, vWF-1, and vWF-2, suggests that *Coch* is important for inner ear function. Interestingly, the highest homology was observed between the guinea pig and human isoforms. In the present study, the guinea pig Cochlin isoforms are 63,

44, and 40kDa in size, which were identical to the bovine and human inner ear isoforms, showing the Cochlin isoform to be well preserved in mammals.

Because the function of the *Coch* gene is unknown as yet, study of the gene expression pattern in vitro is necessary. Exogenous expression of Cochlin in 293T cells, COS7 cells, and NIH3T3 cells resulted in production of the full-length (60 kDa) polypeptide in cell lysates, and two polypeptides of 60 and 50 kDa that were secreted into the media were attributed to proteolytic processing of the protein [19]. In HeLa cells, the full-length protein (63 kDa) was detected in cell lysate and 69 kDa protein was detected in the medium [20]. This isoform pattern found in gene transfection experiments is very different from that found in the human and bovine samples. This indicates that the proper processing of Cochlin, such as enzymatic cleavage, may only occur in the case of native gene expression and/or the unique extracellular environment of the inner ear.

Therefore, a native *Coch* gene expression study was performed on the primary cell culture of SL fibrocytes. The immunoreactivity of the cultured fibrocytes in this study has representative type I fibrocyte characteristics, i.e. a positive reaction for caldesmon and the S-100 protein, and negative for Na^+-K^+-ATPase [17]. We detected the expression of *Coch* mRNA using RT-PCR and real-time PCR analysis in cultured fibrocytes. The level of *Coch* mRNA expression in the cultured fibrocytes was unexpectedly very small compared with that of the cochlear lateral wall, suggesting that *Coch* gene expression is dependent on a specific tissue micro-environment. We sought to identify the culture condition that would up-regulate *Coch* gene expression, so we cultured cells in several conditions under several kinds of cytokine stimulations. However, none of these modified conditions altered *Coch* mRNA or Cochlin expression in the cultured fibrocytes, which confirmed the necessity and importance of the extracellular micro-environment of the inner ear in vivo for *Coch* gene expression.

In addition to the level of mRNA expression, the isoform formation was different in vivo and in vitro. Proteins of 66, 60, and 44 kDa were detected in cultured fibrocytes, which were different from the isoforms expressed in vivo, i.e. the sizes of 63, 44, and 40 kDa. The origin of Cochlin isoforms has been attributed to a variety of mechanisms, including multiple transcription, glycosylation, and enzymatic protein processing [5,13,19,20]. Our results suggest that the mechanisms that contribute to the proper Cochlin isoform formation are tightly controlled in vivo.

In conclusion, we have cloned guinea pig *Coch* cDNA, the sequence for which is conserved in mammals, and the sequence information will be of value for future molecular studies combined with physiological recordings and surgical manipulation, taking advantage of the anatomic advantages of the guinea pig. *Coch* mRNA is expressed in cultured fibrocytes in vitro at a very low level, and isoform formation is different between cultured fibrocytes and the cochlear lateral wall in vivo, probably due to the lack of the proper cellular micro-environment. These results provide insight into *Coch* gene expression and its regulation related to isoform formation.

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Ultrastructural Localization of Cochlin in the Rat Cochlear Duct

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Key Words
Hereditary hearing impairment · DFNA9 · *COCH* gene · Inner ear · Immunogold method

Abstract
Cochlin, a product of the *COCH* gene, is a major constituent of the inner ear extracellular matrix. Type II collagen, a protein that contributes to structural stability, is also a component of this extracellular matrix. In this study, using the postembedding immunogold method, we demonstrate the localization of cochlin and type II collagen in the cochlear duct at the ultrastructural level. The immunolabeling of cochlin was observed in the fibrillar substance in the spiral limbus, beneath the inner sulcus cells, and in the basilar membrane, the spiral prominence and the spiral ligament. Immunolabeling of type II collagen was observed in the same fibrillar substance in the extracellular matrix of the cochlear duct. This localization of cochlin is consistent with the expected localization of type II collagen. The localization of cochlin and type II collagen indicates the important roles played by these proteins in the hearing process.

Introduction

Cochlin, a product of the *COCH* gene, is associated with an autosomal dominant sensorineural hearing loss referred to as DFNA9. The symptoms of DFNA9 include not only hearing loss but also vestibular disorders [Bom et al., 1999; de Kok et al., 1999; Fransen et al., 1999; Grabski et al., 2003; Khetarpal, 1993; Manolis et al., 1996; Robertson et al., 1998; Usami et al., 2003; Verhagen et al., 1989, 1992]. On light microscopic analysis, Khetarpal et al. [1991] and Khetarpal [1993] reported severe degeneration of the cochlea and vestibule in association with the deposition of an acidophilic ground substance in the spiral ligament, spiral limbus, spiral lamina and basilar membrane of DFNA9-affected ears. Robertson et al. [2006] also reported the loss of cellularity and the accumulation of an abundant homogeneous acellular eosinophilic deposit in the cochlea and vestibule of DFNA9-affected ears. They suggested that these extracellularly deposited aggregates contain mutated cochlin, and that this mutated cochlin alters the interactions between cochlin and other cochlin-associated proteins. Khetarpal [2000] compared the normal spiral ligament with that in DFNA9-affected ears at the ultrastructural level, and noted the absence of major fibrillar type II collagen bundles. We speculated that the proteins interacting with cochlin might include type II collagen and have previously re-

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ported that these two extracellular matrix (ECM) proteins coexist in the same fibrillar substance in the subepithelial area of the semicircular canal [Mizuta et al., 2008]. This localization suggests that cochlin plays a role in structural homeostasis of the vestibule. At present, however, the exact role of cochlin, which accounts for 70% of the inner ear proteins [Ikezono et al., 2001], remains incompletely understood.

Some clues that may help to elucidate the role of cochlin have, nevertheless, been reported. Since the sensorineural hearing loss observed in DFNA9 has a late onset and progresses slowly, the *COCH* gene has been implicated in this age-related hearing impairment [de Kok et al., 1999]. On the other hand, cochlin and type II collagen have been implicated in autoimmune hearing loss in humans [Baek et al., 2006; Yoo et al., 1984]. Further, Ikezono et al. [2009] recently reported that a short 16-kDa cochlin isoform (cochlin-tomoprotein) is specific to the perilymph and that this isoform could function as a diagnostic marker of perilymphatic fistula, which is related to hearing loss and vestibular disorder. Thus, we considered that it would be important to investigate the localization of cochlin in the cochlea, and in the present study we accordingly expanded our immuno-electromicroanalysis of cochlin to the cochlea. The role of cochlin in the cochlea, particularly in the basilar membrane, is also briefly discussed.

Materials and Methods

Antibodies

Cochlin has von Willebrand factor type A (vWFA)-like domains [Robertson et al., 2003]. We used a rabbit polyclonal antibody that recognizes all three cochlin isoforms. This was raised against the vWFA-like domain 1 of cochlin and has been previously described by Ikezono et al. [2004]. Briefly, a 19-mer (KADIAFLIDGSFNIGQRRF) peptide corresponding to residues 163–181 in the vWFA-like domain 1 was used to generate antibodies. The specificity of these antibodies for the corresponding antigenic peptide was confirmed by dot blot analysis and a peptide absorption test (data not shown). Antibodies against type II collagen (Chemicon International acquired by Millipore, Billerica, Mass., USA) were purchased commercially.

Tissue Processing

Wistar rats (body weight, 100–200 g) were anesthetized with pentobarbital (50 mg/kg body weight, i.p.) according to our institution's ethical regulations for the treatment of animals. A fixative of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was perfused from the left ventricle, and the temporal bones were then isolated and immediately immersed in the same fixative. Thereafter, the cochleae were dissected under a stereomicroscope and further fixed for 2 h at 4°C.

The preparation of samples for embedding in Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, Pa., USA) was performed according to a previously published procedure [Mizuta et al., 2008]. Ultra-thin sections were cut using an ultramicrotome and mounted onto nickel grids (400 mesh).

Immunogold Labeling

The grid-mounted sections were immersed in a droplet (25 μl) of 1% bovine serum albumin (BSA; Sigma, St. Louis, Mo., USA) in phosphate-buffered saline (PBS, 0.9% NaCl in 6.7 mM phosphate buffer, pH 7.2) for 1 h at room temperature, then incubated overnight at 4°C in a droplet of the optimal concentrations of rabbit antibodies against either cochlin (3.3 μg/ml in BSA/PBS) or type II collagen (4 μg/ml in BSA/PBS). After rinsing in PBS, the sections were incubated in colloidal gold-conjugated goat anti-rabbit IgG secondary antibody (diameter, 15 nm; BB International, Cardiff, UK) at 1:50 dilution in BSA/PBS for 1 h at room temperature. Subsequently, the sections were washed with PBS and distilled water, and counterstained with uranyl acetate for 3 min and lead citrate for 30 s. These sections were then observed under a JEOL JEM-1220 electron microscope. As a negative control, pre-immune rabbit IgG (4 μg/ml in BSA/PBS) was used instead of the primary antibody.

This study protocol was approved by the Hamamatsu University School of Medicine Animal Use Committee.

Results

Immunoreactivity for cochlin and type II collagen was observed in the spiral limbus, beneath the inner sulcus cells and the basilar membrane, beneath the epithelial cells of the spiral prominence and the spiral ligament.

Spiral Limbus

Immunolabeling of cochlin and type II collagen was observed in the fibrous area of the spiral limbus (fig. 1a–c).

Inner Sulcus Cells

Immunolabeling of cochlin and type II collagen was observed in the fibrous substance beneath the inner sulcus cells (fig. 2a–c).

Basilar Membrane

The structures that exhibited immunoreactivity for cochlin and type II collagen were the fibrous bundles in the basilar membrane (fig. 3a–c).

Spiral Prominence

The stained fibrils for cochlin and type II collagen beneath the epithelial cells of the spiral prominence were observed to be widely scattered and without orientation (fig. 4a–c).

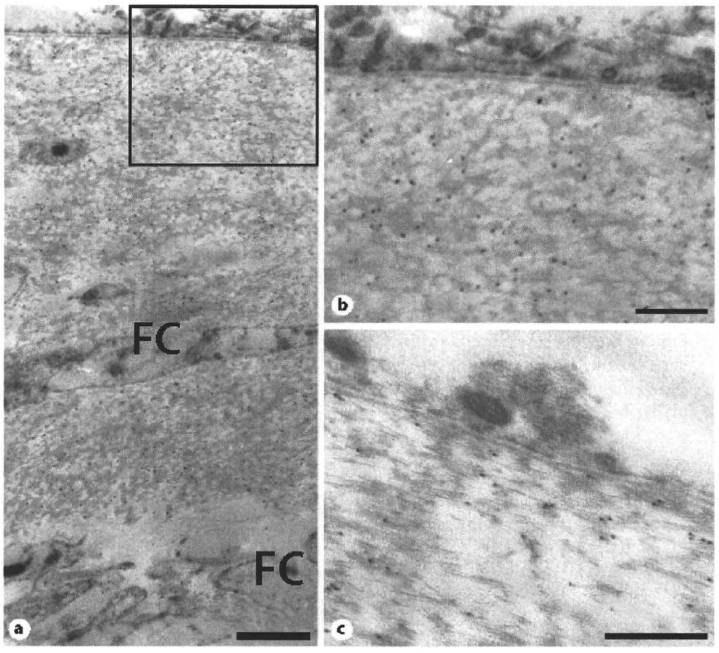

Fig. 1. Immuno-electronmicroscopy for cochlin and type II collagen expression in the spiral limbus. **a** Cochlin: gold particles in the fibrous substance in the spiral limbus. Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 0.5 μm. **c** Type II collagen: gold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

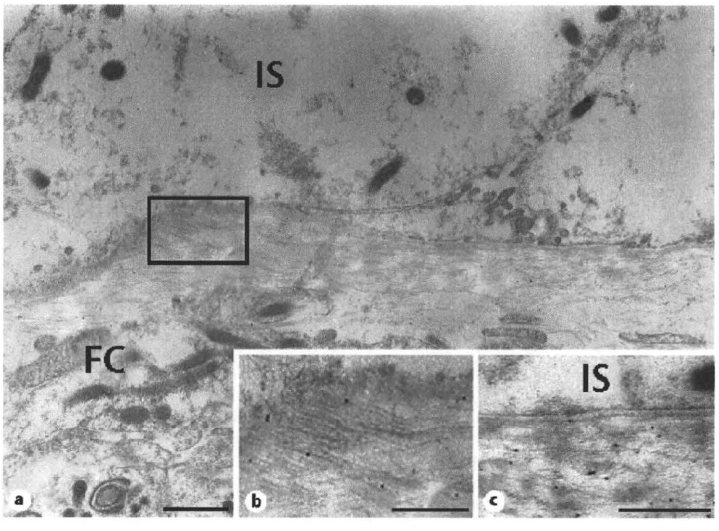

Fig. 2. Immuno-electronmicroscopy for cochlin and type II collagen expression beneath the inner sulcus cells. **a** Cochlin: immunolabeling of cochlin was seen in the fibrous substance beneath the inner sulcus cells. Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 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.

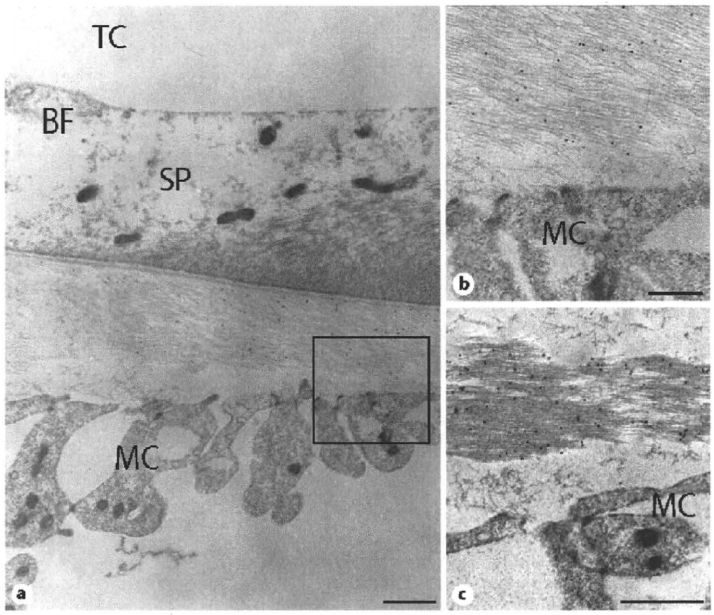

Fig. 3. Immuno-electronmicroscopy for cochlin and type II collagen expression in the basilar membrane. **a** Cochlin: the fibrillar bundles in the basilar membrane were immunoreactive for cochlin. Bar = 1 μm. **b** Cochlin: higher magnification of the open square area in **a**. Bar = 0.5 μm. **c** Type 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.

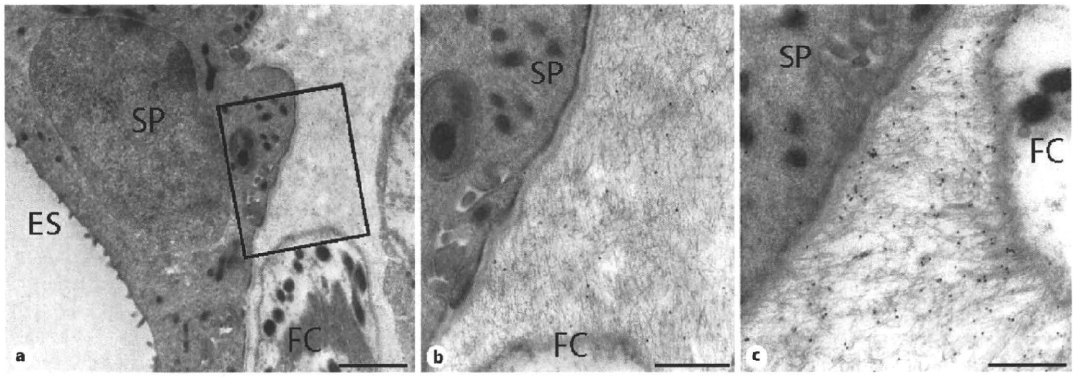

Fig. 4. Immuno-electronmicroscopy for cochlin and type II collagen expression beneath the epithelial cells of the spiral prominence. **a** Cochlin: gold particles in the fibrous substance beneath the epithelial cells of the spiral prominence. Bar = 1 μm. **b** Cochlin: higher magnification of the open 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.

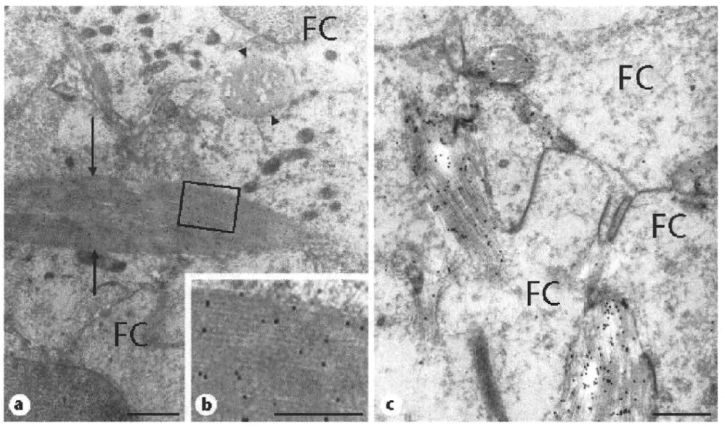

Fig. 5. Immuno-electronmicroscopy for cochlin and type II collagen expression in the spiral ligament. **a** Cochlin: gold particles were also observed in the banded bundles, which have a parallel array in the area of the fibrocytes in the spiral ligament. Parallel cut of the fibrous bundles (arrows). 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.

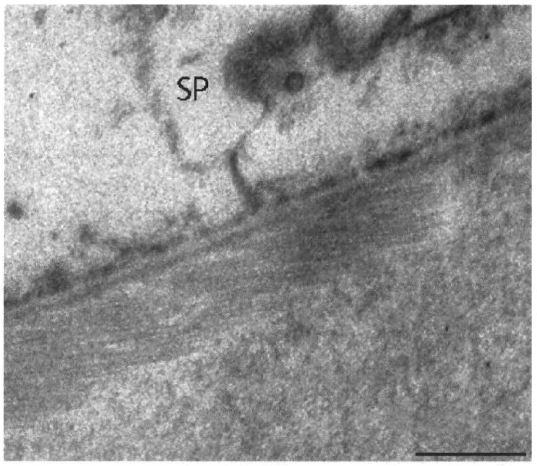

Fig. 6. Control. No gold particles were observed in the fibrillar substance in the basilar membrane. SP = Supporting cells of the sensory cells. Bar = 0.5 μm.

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

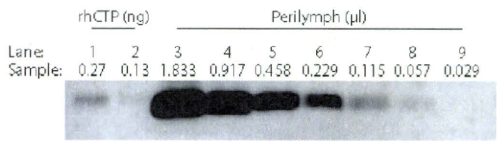
In order to evaluate the specificity of the CTP detection test, we examined MEL from non-PLF cases. In this study, we defined 'non-PLF' as those cases with otosclerosis (which had undergone stapedectomy), profound deafness (cochlear implant surgery) or conductive hearing loss (exploratory tympanotomy).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

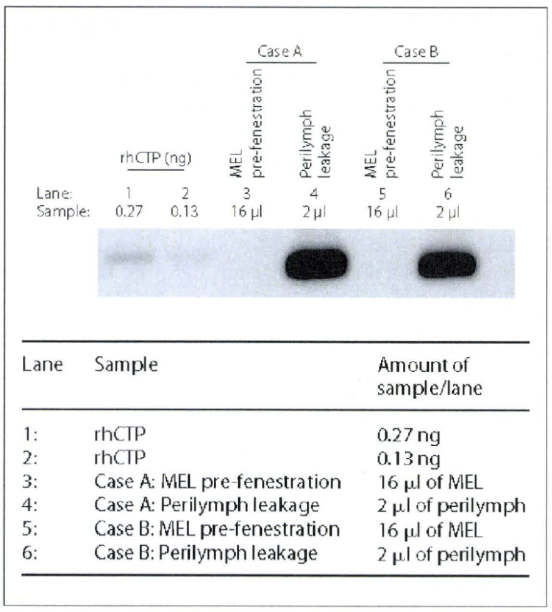
Detection Limit

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 × 24/22 = 0.176 μl; perilymph in the total MEL: 0.176 × 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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JOHNS
特集●耳鼻咽喉・頭頸部画像アトラス●

耳・側頭骨

外リンパ瘻

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Susumu SHINDO　Tetsuo IKEZONO

● Key Words ●画像診断，外リンパ瘻，迷路気腫，HRCT ●

Ⅰ．疾患の概説

外リンパ瘻とは外リンパが中耳腔へ漏出し，聴覚障害や平衡障害をきたす疾患とされる。外リンパ瘻の診断は，症状や聴力，眼振などの検査によりその存在を疑い，手術や内視鏡により漏出を確認することが一般的である。そのため厚生省の外リンパ瘻診断基準案には画像診断の項目がない。しかしながら画像検査だけで外リンパ瘻の確定診断ができたり，強く疑う所見が得られる症例も稀ながら存在する。

Ⅱ．推奨される画像検査

画像検査のみで外リンパ瘻の確定診断ができる病態に，迷路気腫(pneumolabyrinth)がある[1~3]。その他，外リンパ瘻を強く疑う画像所見に，アブミ骨の前庭内陥入や内耳に及ぶ側頭骨骨折などが挙げられる。いずれの病態にも高分解能CT(HRCT)が最も診断に優れている検査法であることから，まずHRCTを行うことが推奨される。

Ⅲ．画像における一般的な特徴

迷路気腫が最も生じやすいのは，耳かきなどがアブミ骨に当たることで生じる，アブミ骨外傷性外リンパ瘻においてである。このため，迷路気腫では前庭内にair densityを認めることが多い(図1-a)。しかし，外力の形態や外力の大きさによっては，蝸牛内にair densityを認めることもある(図1-b)。その他，内耳にair densityが認められなくても，アブミ骨が前庭内に陥入した所見や内耳に及ぶ骨折線が認められた場合，外リンパ瘻が生じた可能性が高い。

Ⅳ．臨床に結びつく重要な画像所見

外傷性外リンパ瘻の臨床上最も重要なポイントは，アブミ骨の前庭内陥入を合併しているかどう

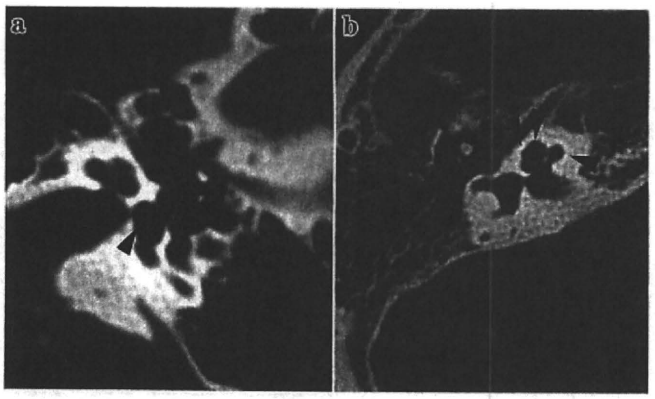

図1　迷路気腫のHRCT像（水平断）
a：アブミ骨外傷性外リンパ瘻症例。前庭内にair density（►）を認める。
b：急性中耳炎に合併した外リンパ瘻症例。蝸牛内に複数のair density（►）を認める。

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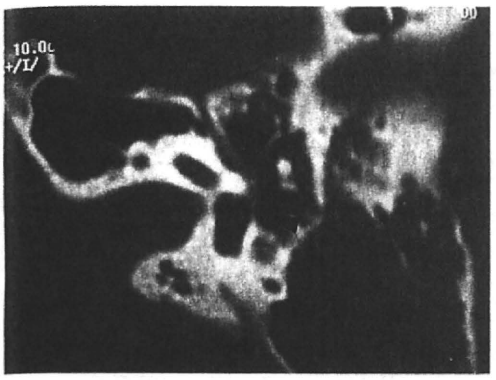

図 2　図 1-a と同じ症例の HRCT 像（水平断）
アブミ骨（►）が深く前庭内に陥入している所見が認められる。

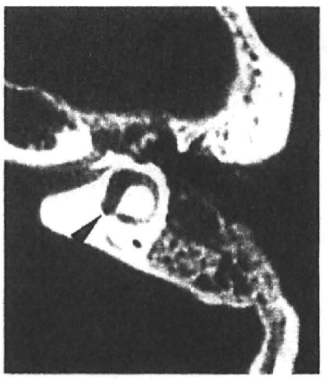

図 3　正常内耳（真珠腫術後耳）におけるアンダーシュート・アーチファクトの例
一部 air density に見える箇所がある（►）。

かである（図 2）。アブミ骨陥入を合併した外リンパ瘻では，保存的治療，手術いずれの治療法を選択しても，難聴が増悪する可能性がある病態であることを患者に話しておく必要がある[2,3]。

V．画像検査において鑑別すべき疾患

HRCT では，正常内耳でも迷路気腫があるように見えることがある（図 3）。これはアンダーシュート・アーティファクトと呼ばれ，錐体骨のような厚い骨や後頭蓋骨に隣接した軟部組織や内耳を撮影した際に生じることが多い。

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ORIGINAL ARTICLE

Subjective visual vertical in patients with ear surgery

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Abstract

Conclusion: Dysequilibrium is one of the most important side effects of ear surgery. The subjective visual vertical can be used as a good indicator for the evaluation of otolithic function in patients with ear surgery. *Objective:* To investigate the influence of various types of ear surgery on the otolithic organs. *Methods:* Seventy-one patients underwent ear surgery. Subjective visual vertical (SVV) test was performed before and after ear surgery. We investigated the directional changes of SVV before and after the ear surgery. *Results:* The postoperative SVV of two patients who underwent translabyrinthine removal of vestibular schwannoma shifted toward the operated side, but following other surgical procedures the SVV tended to shift toward the healthy side.

Keywords: *Utricidus, subjective visual horizontal, SVH, vestibular, tympanoplasty, otoliths*

Introduction

It is known that the perception of vertical not only depends on visual information but is also affected by the head position relative to the direction of gravity. Measurement of the subjective visual vertical (SVV) is used clinically as a method to assess the degree of otolithic dysfunction, primary vestibular nerves, and central graviceptive pathways [1]. Significant tilts of the SVV have been described in patients with peripheral vestibular disorders and patients who have undergone labyrinthectomy, as well as in patients with brainstem and cerebellar lesions [2 6]. Patients with various unilateral vestibular lesions, including vestibular neurectomy and labyrinthectomy, consistently tilt the SVV towards the side of the lesion [2 6].

Tribukait and Bergenius [7] reported that patients with stapedotomy showed significant deviations of the subjective visual horizontal (SVH) toward the healthy ear and it contrasts with the consistent deviation of the SVV toward the affected ear in acute vestibular lesions such as vestibular neurectomy and labyrinthectomy [2 6]. The goals of this study were to measure the SVV and to define the influence of the otolithic functions before and after the various types of ear surgery.

Material and methods

We investigated SVV in 71 patients with unilateral ear disease. They underwent various types of ear surgery in our hospital from 2006 to 2008. There were 38 women and 33 men; their mean age was 44 years, ranging from 5 to 81 years. The operated side was the right ear in 32 patients and the left in 39. All surgery was carried out under general anesthesia. Four surgeons performed ear operations on a total of 71 patients. Surgical procedures were as follows: tympanoplasty in 48 patients, cochlear implants in 11, stapes surgery in 4, exploratory tympanotomy in 3, translabyrinthine removal of vestibular schwannoma in 2, canal plugging in 1, partial removal of temporal bone in 1, and removal of external ear osteoma in 1 (Table I). Types of tympanoplasty were type I, 11 cases; type III, 26 cases; type IV, 8 cases; and first-stage operation, 3 cases. A surgical bur was used for all surgical procedures.

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