Table I. CTP expression in perilymph and CSF.

Sample	Total	CTP positive	CTP negative
Perilymph	65	60	5
Stapedectomy	36	34	2
Cochlear implant	29	26	3
CSF	60	0	60

any sign of inflammation or infection. The MEL in 54 of 55 non-PLF cases was negative for the CTP detection test, i.e. the specificity of the test was found to be 98.2%. To further elucidate the limitations of this test, we analysed the MEL collected from patients with middle ear infections, which can give a false positive result. The MEL in 43 of 46 cases with chronic suppurative otitis media or middle ear cholesteatoma was negative for CTP. The specificity of the CTP detection test decreases to 93.5% when applied to infected ears [7]. The high protein concentration of the thick pus present with infection was the most likely cause. In the present study we studied a non-infected ear with BOR syndrome, so the specificity is thought to be the former.

Results

CTP expression in perilymph and CSF (Tables I and II, Fig. 2)

In all, 34 perilymph samples from 36 stapedectomy and 26 samples from 29 cochlear implant patients were positive for CTP. In total, 60 of 65 perilymph samples were positive for CTP. However, CTP was not detected in any of the 60 CSF samples.

Analysis of profuse fluid leakage from cochleostomy site (Table III, Fig. 3)

As a control, MEL was taken from the middle ear before cochleostomy. The MEL described here contains middle ear mucosal secretions and other substances normally expressed in the middle ear cavity. These substances may cause false positive reactions to the antibody. The MEL taken before the fenestration of cochlea was negative for CTP.

Immediately after the fenestration of the cochlea, fluid leaked excessively from the cochleostomy site. The leakage collected at 0 min showed a CTP signal above the high-level standard signal and at 0.5–3 min

Table II. Western blot analysis of CTP expression in perilymph and CSF.

Lane	Sample	Amount of sample per lane	Result
(a) Perilymph and MEL			
1	High-level standard	rhCTP 0.27 ng	+
2	Low-level standard	rhCTP 0.13 ng	–
3	Case A: perilymph stapedectomy	2 µl	+
4	Case A: MEL before stapedectomy	16 µl of MEL	–
5	Case B: perilymph cochleostomy	2 µl	+
6	Blank	...	...
7	Case B: MEL before cochleostomy	16 µl of MEL	–
8	Case C: perilymph stapedectomy	2 µl	–
9	Case C: MEL before stapedectomy	16 µl of MEL	–
10	Perilymph (positive control)	1 µl	+
(b) CSF			
1	High-level standard	rhCTP 0.27 ng	+
2	Low-level standard	rhCTP 0.13 ng	–
3	CSF	10 µl	–
4	CSF	10 µl	–
5	CSF	10 µl	–
6	CSF	10 µl	–
7	CSF	10 µl	–
8	CSF	10 µl	–

MEL, middle ear lavage.

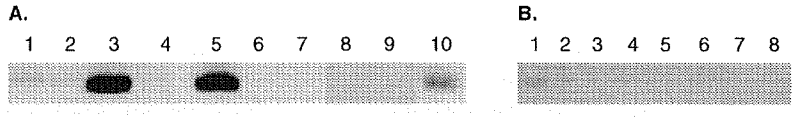

Figure 2. Western blot analysis of CTP expression in perilymph and CSF. The expression of CTP was analysed by Western blot using the anti-CTP antibody. CTP expression (16 kDa) was only detected in the perilymph (cases A and B), not in the CSF. The perilymph sample from case C was negative for CTP. Further details are shown in Table II.

showed a negative result, with a faint signal below the high-level standard signal, and the signal disappeared at 6 min and thereafter.

Discussion

In the present study we have further tested the specific expression of CTP in the perilymph. Sixty of 65 perilymph samples were positive for CTP. However, CTP was not detected in any of the 60 CSF samples. In the previous study, we tested 20 perilymph and 20 CSF samples [6], and the results showed that CTP was detected in all the perilymph samples and was negative in all the CSF samples. Therefore, the sum total is that 80 of 85 perilymph samples were positive for CTP and all 80 SCF samples were negative for CTP. These results further confirm that CTP is a perilymph-specific protein.

CTP was not detected in five of the perilymph samples, and this may be attributed to the low CTP protein concentrations because of dilution by blood and seepage in the surgical field. Alternatively, especially in the three CTP-negative cases of cochlear implantation with profound deafness, abnormal cochlin isoform processing might have resulted in an undetectable level of CTP production due to mutations in COCH or related genes. No genetic testing to confirm this theory has been performed in these cases as yet.

Table III. Results of CTP detection test by Western blot of the leakage from cochleostomy.

Lane	Sample	Amount of sample per lane	Result
1	High-level standard	rhCTP 0.27 ng	+
2	Low-level standard	rhCTP 0.13 ng	–
3	Pre-cochleostomy	16 µl of MEL	–
4	Leakage at 0 min	2 µl of fluid	+
5	Leakage at 0.5 min	2 µl of fluid	–
6	Leakage at 1 min	2 µl of fluid	–
7	Leakage at 2 min	2 µl of fluid	–
8	Leakage at 3 min	2 µl of fluid	–
9	Leakage at 6 min	2 µl of fluid	–
10	Perilymph (control)	2 µl of fluid	+

Note that leakage collected at 25, 35 and 45 min was negative for CTP (data not shown). MEL, middle ear lavage.

Using CTP as a marker to detect perilymph, we tested the nature of the profuse leakage from cochleostomy in an anomalous cochlea case with BOR syndrome. The fluid that leaked at the beginning of the cochleostomy was proved to contain CTP, i.e. perilymph, and the CTP detection signals gradually disappeared as time elapsed. Even though the CTP signal was below the high-level standard signal and was evaluated as negative by standardization, faint CTP signals were detected from 0.5 to 3 min (Fig. 3). The total volume of leakage was approximately 10 ml over 3 min. Since the volume of the human perilymph is estimated to be 150 µl by MRI [11], we consider the perilymph to have been washed out from the cochlea immediately after the leakage started. The faint signals observed here might be derived from the perilymph pooled in the middle ear and mastoid cavity.

Perilymph is thought to be derived from both CSF and the vascular supply of blood plasma [12]. Protein analysis revealed the perilymph to be different from blood plasma and CSF, supporting the dual origin theory [13,14]. The average protein concentration is 40 mg/dl in the CSF and 200 mg/dl in the perilymph of human samples, and recent proteomic analysis of mouse samples revealed a 2.8 times higher amount of protein in the perilymph. The exclusive expression of CTP in the perilymph presented in this study also shows that these three human body fluids are discrete in nature.

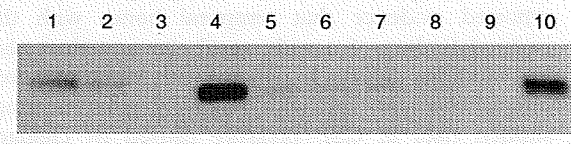

Figure 3. The results of the CTP detection test by Western blotting of the leakage from cochleostomy. MEL obtained before the fenestration of the cochlea was negative for CTP (lane 3). The leakage collected at 0 min showed a CTP signal above the high-level standard signal (lane 4) and the samples collected at 0.5–3 min showed negative results with a faint signal below the high-level standard signal, and the signal disappeared at 6 min and thereafter. Further details are shown in Table III.

It has been reported that there is communication between the labyrinthine perilymph and the CSF space. Histological study revealed that the cochlear modiolus is highly porous [14]. The porous structure in the surface of the modiolus allows communication between perilymph and the perivascular and perineural space in the modiolus. A recent MRI study in humans using intratympanic injection of gadolinium diethylenetriaminepentaacetic acid revealed the permeability of the modiolus [15]. In terms of pathology, this communication is important as a potential route for the spread of infection and subarachnoid haemorrhage. In addition, an extremely wide communication channel can result in a gusher during cochlear implantation [2,3]. In evaluating the pathology of an anomalous inner ear, it is helpful to check for two possible pathological conditions, i.e. whether a congenital defect of the bony barrier to CSF at the lateral end of the IAC caused CSF leakage into the perilymphatic space preoperatively, or whether a sudden decrease of perilymphatic pressure induced by the cochleostomy resulted in the rupture of the weak boundary of these two spaces and thereby caused CSF influx. As discussed above, the CSF and perilymph are different body fluids, not only based on the protein constituents, but also other characteristics, such as their electrolyte concentrations and pressure [8,12–14]. The potassium gradient from the CSF, perilymph and endolymph is 2.8, 10.7 and 144.2 (mEq/l), respectively, on average in human samples [16–18]. Mixture of these two fluids abruptly changes the homeostasis of the inner ear and may cause functional disturbances such as hearing loss.

In a review of congenital malformations of the cochlea by Graham et al. [2], a large defect in the IAC fundus was found to be one of the causes of the profound deafness, and gradual or intermittent mixture of these two fluids resulted in fluctuations and progressive hearing loss. The pulsatile perilymph often found at cochleostomy would be more compatible with a small direct communication between CSF and perilymph, of the kind found in the Mondini and common cavity deformities. Lemmerling et al. [19] reported evidence that temporal bones with the isolated finding of a wide vestibular aqueduct also had modiolar defects. In patients with Mondini deformities who start life with relatively good hearing, sudden rises in CSF pressure caused by changes in posture or in intra-abdominal and/or thoracic pressure can result in fluctuation and deterioration in the auditory threshold.

We have tested samples of profuse fluid leakage from only one patient and further study will be necessary to understand the pathology of this disease entity. In the case of cochlear implantation, it would be interesting to record the presence of a gusher at the time of cochleostomy, thus providing evidence for the increased pressure of the perilymph and the temporal CTP detection test result reported in this study.

Conclusion

This report has confirmed that CTP is exclusively expressed in the perilymph. Furthermore, the CTP detection test revealed the nature of the profuse leakage from cochleostomy in an anomalous cochlea of a case with BOR syndrome. The initial egress of CTP-positive fluid (perilymph) changed to CTP-negative CSF as time elapsed, indicating that the membranous boundary between these two spaces had ruptured intraoperatively. We have previously reported CTP as a specific diagnostic marker of perilymph leakage. This marker will help shed light on the mechanism of perilymph production and the pathology of anomalous cochlea.

Acknowledgments

We thank Dr S. Iwasaki, Hamamatu Redcross Hospital, Japan, for a critical review of the manuscript. This study was supported by Health and Labor Sciences Research Grants in Japan (Research on Measures for Intractable Diseases, Researches on Sensory and Communicative Disorders), a grant from the Ministry of Education, Culture, Sports, Science and Technology.

Declaration of interest: All authors hereby state that they have received no financial support and have no conflicts of interest that might bias their work.

References

[1] Jackler RK, Hwang PH. Enlargement of the cochlear aqueduct: fact or fiction? Otolaryngol Head Neck Surg 1993;109:14–25.

[2] Graham JM, Phelps PD, Michaels L. Congenital malformation of the ear and cochlear implantation in children: review and temporal bone report of common cavity. J Laryngol Otol Suppl 2000;25:1–14.

[3] Papsin BC. Cochlear implantation in children with anomalous cochleovestibular anatomy. Laryngoscope 2005;115 (1 Pt 2 Suppl 106):1–26.

[4] Ikezono T, Omori A, Ichinose S, Pawankar R, Watanabe A, Yagi T. Identification of the protein product of the Coch gene – hereditary deafness gene – as the major component of bovine inner ear protein. Biochim Biophys Acta 2001;1535:258–65.

[5] Ikezono T, Shindo S, Li L, Omori A, Ichinose S, Watanabe A, et al. Identification of a novel Cochlin isoform

in the perilymph: insights to Cochlin function and the pathogenesis of DFNA9. Biochem Biophys Res Commun 2004;314:440–6.

[6] Ikezono T, Shindo S, Sekiguchi S, Hanprasertpong C, Li L, Pawankar R, et al. Cochlin-tomoprotein (CTP), a novel perilymph-specific protein and a potential marker for the diagnosis of perilymphatic fistula. Audiol Neurootol 2009;14:338–44.

[7] Ikezono T, Shindo S, Sekiguchi S, Morizane T, Pawankar R, Watanabe A, et al. The performance of cochlin-tomoprotein detection test in the diagnosis of perilymphatic fistula. Audiol Neurootol 2009;15:168–74.

[8] Thalmann I, Kohut RI, Ryu J, Comegys TH, Senarita M, Thalmann R. Protein profile of human perilymph: in search of markers for the diagnosis of perilymph fistula and other inner ear disease. Otolaryngol Head Neck Surg 1994;111:273–80.

[9] Kochhar A, Fischer SM, Kimberling WJ, Smith RJ. Branchio-oto-renal syndrome. Am J Med Genet 2007;143A:1671–8.

[10] Propst EJ, Blaser S, Gordon KA, Harrison RV, Papsin BC. Temporal bone findings on computed tomography imaging in branchio-oto-renal syndrome. Laryngoscope 2005;115: 1855–62.

[11] Buckingham RA, Valvassori GE. Inner ear fluid volumes and the resolving power of magnetic resonance imaging: can it differentiate endolymphatic structures? Ann Otol Rhinol Laryngol 2001;110:113–17.

[12] Zou J, Pyykkö I, Counter SA, Klason T, Bretlau P, Bjelke B. In vivo observation of dynamic perilymph formation using 4.7 T MRI with gadolinium as a tracer. Acta Otolaryngol 2003;123:910–15.

[13] Hara A, Salt AN, Thalmann R. Perilymph composition in scala tympani of the cochlea: influence of cerebrospinal fluid. Hear Res 1989;42:265–71.

[14] Rask-Andersen H, Schrott-Fischer A, Pfaller K, Glueckert R. Perilymph/modiolar communication routes in the human cochlea. Ear Hear 2006;27:457–65.

[15] Naganawa S, Satake H, Iwano S, Sone M, Nakashima T. Communication between cochlear perilymph and cerebrospinal fluid through the cochlear modiolus visualized after intratympanic administration of Gd-DTPA. Radiat Med 2008;26:597–602.

[16] Schielke GP, Betz AL. 1992. Electrolyte transport. In: Bradbury MWB, editor. Physiology and pharmacology of the blood-brain barrier. Heidelberg: Springer-Verlag. p 221–43.

[17] Anniko M, Wróblewski R. Ionic environment of cochlear hair cells. Hear Res 1986;22:279–93.

[18] Swan EE, Peppi M, Chen Z, Green KM, Evans JE, McKenna MJ, et al. Proteomics analysis of perilymph and cerebrospinal fluid in mouse. Laryngoscope 2009;119: 953–8.

[19] Lemmerling MM, Mancuso AA, Antonelli PJ, Kubilis PS. Normal modiolus: CT appearance in patients with a large vestibular aqueduct. Radiology 1997;204:213–19.

Acta Oto-Laryngologica
AUTHOR'S QUERY SHEET

Author(s): Tetsuo Ikezono

Article title: **Molecular cloning of the *Coch* gene of guinea pig inner ear and its expression analysis in cultured fibrocytes of the spiral ligament**

Article no: 449794

Dear Author,

Some questions have arisen during the preparation of your manuscript for typesetting. Please consider each of the following points below and make any corrections required in the proofs.

Please do not give answers to the questions on *this* sheet. All corrections should be made directly in the printed proofs.

AQ1: Please insert a declaration of interest statement
AQ2: Please check the author names and affiliations carefully.
AQ3: We have inserted a running head. Please approve or provide an alternative.

end

Acta Oto-Laryngologica, 2009; 00: 000–000

informa
healthcare

ORIGINAL ARTICLE

Molecular cloning of the *Coch* gene of guinea pig inner ear and its expression analysis in cultured fibrocytes of the spiral ligament

LISHU LI[1], TETSUO IKEZONO[1], KUWON SEKINE[1], SUSUMU SHINDO[1], TOMOHIRO MATSUMURA[2], RUBY PAWANKAR[1], ISSEI ICHIMIYA[3] & TOSHIAKI YAGI[1]

[1]*Department of Otorhinolaryngology,* [2]*Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo and* [3]*Ichimiya Clinic, Oita, Japan*

Abstract

Conclusions: We have cloned guinea pig *Coch* cDNA and the sequence information will be useful for future molecular study combined with physiological experiments. Proper *Coch* gene expression appears to be dependent on the unique extracellular micro-environment of the inner ear in vivo. These results provide insight into the *Coch* gene expression and its regulation. *Objective:* To characterize the guinea pig *Coch* gene, we performed molecular cloning and expression analysis in the inner ear and cultured fibrocytes of the spiral ligament. *Methods:* The *Coch* cDNA was isolated using RACE. Cochlin isofoms were studied by Western blot using three different types of mammalian inner ear. The cochlear fibrocytes were cultured and characterized by immunostaining. *Coch* mRNA expression in the fibrocytes was investigated and the influence of cytokine stimulation was evaluated. *Results:* The full-length 1991 bp *Coch* cDNA that encodes a 553 amino acid protein was isolated. The sequence had significant homology with other mammals, and the sizes of the Cochlin isoforms were identical. In the cultured fibrocytes, *Coch* mRNA was expressed in a very small amount and the isoform production was different, compared with the results in vivo. Cytokine stimulation did not alter the level of mRNA expression or isoform formation.

Keywords: *Cochlin, cytokine, culture*

Introduction

The *Coch* gene, which is mutated in DFNA9, an autosomal dominant hereditary sensorineural hearing loss and vestibular disorder, was initially isolated by organ-specific and subtractive approaches [1]. Sequence analysis of individuals with DFNA9 had previously demonstrated the existence of 11 missense mutations and 1 in-frame deletion in *COCH*. Cochlin contains multiple domains, including a signal peptide, an LCCL (Limulus factor C, Cochlin, late gestation lung protein, also called factor C homology) domain of unknown function, a first intervening domain (Ivd1), and 2 von Willebrand factor A (vWFA) homology domains, separated by a second intervening (Ivd2) domain [2,3]. We previously showed that the protein product of the *Coch* gene constitutes 70% of the non-collagenous inner ear proteins and the Cochlin isoforms can be classified into four groups, namely p63, p44, and p40 in the inner ear, and a short isoform, Cochlin-tomoprotein (CTP), in the perilymph [4,5]. The expression of full-length Cochlin p63 is specific to the inner ear [6], and CTP is specifically and exclusively expressed in the perilymph [7]. Recently, we reported the molecular mechanisms that regulate the perilymph specific expression of CTP [8]. The clinical significance of the specific expression of Cochlin in the inner ear is that CTP

Correspondence: Tetsuo Ikezono, Department of Otorhinolaryngology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. E-mail: ctp16kdaplf@nms.ac.jp

(Received 5 October 2009; accepted 8 November 2009)

ISSN 0001-6489 print/ISSN 1651-2251 online
DOI: 10.3109/00016480903493766

can be considered a specific biochemical marker for the diagnosis of perilymphatic fistula [7]. Cochlin has also been suggested as a target antigen for inner ear-specific autoimmune disorders [9].

Cochlin is an extracellular matrix protein. Motif analysis and a recent electron microscopic study have revealed that Cochlin is a secretory protein that binds to type II collagen [10]. The expression is substantially confined to the fibrocytic regions of the sulcus, spiral prominence, vessels, spiral limbus, and spiral ligament (SL) [11,12], but not the stria vascularis [13]. SL fibrocytes reportedly play important roles in cochlear physiology, including potassium recycling, hydration maintenance in the endolymph, inflammation, and glutamine metabolism [14,15]. SL fibroctyes have a key role in the elucidation of *Coch* gene function and the pathophysiology of deafness.

The characteristics of the *Coch* gene expression in the inner ear and the *Coch* cDNA sequence have been reported in bovine, human, mouse, and rat species, but not in the guinea pig. The guinea pig offers a potentially viable model with good anatomical perspective for auditory studies and is the most frequently used animal in otologic studies [16].

In this study, we report the guinea pig full-length *Coch* cDNA sequence, as well as *Coch* gene expression in the inner ear and in cultured fibrocytes of the SL in vitro. The data show that proper Cochlin expression requires the cellular micro-environment of the inner ear, and provide important insight into the understanding of *Coch* gene expression and isoform production.

Material and methods

Tissue preparation

Bovine temporal bones were purchased from a slaughterhouse and guinea pig (6–8-week-old females weighing 400 g) temporal bones were removed under deep anesthesia with sodium pentobarbital (Nembutal, 50 mg/kg, i.p.). We approached the inner ear via the internal auditory canal and dissected the cochlear membranous labyrinthine tissues from bovine or guinea pig temporal bones. We collected human membranous labyrinthine tissue from the posterior and lateral semicircular canals during surgery performed to remove an acoustic neuroma by a translabyrinthine approach. Patients gave their full informed consent and the study was approved by the ethics committee of Nippon Medical School. This study was approved by the Animal Experimentation Ethics Committee (no. 17-091, approved on March 20, 2006) of Nippon Medical School. Samples were frozen and stored at –80°C until use.

Identification of full-length Coch cDNA

RACE (rapid amplification of cDNA ends) was used to obtain full-length sequences. Gene-specific primers were designed based on the mouse *Coch* sequence. A PrimeSTAR RT-PCR kit (Takara, Tokyo, Japan) was used for the 3' RACE. First-strand cDNA synthesis reaction was performed using 200 ng total RNA and Oligo dT-3 sites Adaptor Primer. PCR on the cDNA (10 ng) was done with the 3 sites Adaptor Primer (5'-CTGATCTAGAGGTACCGGATCC-3' and gene-specific forward primer, *Coch* F1 (5'-TGCTCTCTTGAGGAATTCTCTGTGT-3') corresponding to nucleotides 234–258 over 35 amplification cycles (98°C for 10 s, 55°C for 5 s, and 72°C for 3 min). For the 5' RACE, a 5' RACE System (Invitrogen, Carlsbad, CA, USA) was used. First-strand cDNA synthesis was performed using the gene-specific reverse primer *Coch* R1 (5'-CCTGAAGAACTGGGGATGGTT-3') corresponding to nucleotides 990–1010 to give the *Coch* R1 cDNA, which was then tailed with terminal transferase and dCTP to create an abridged primer-binding site [oligo (dC)] on the 3' end of the cDNA. The target cDNA was amplified by nested PCR. The first round PCR was performed with the gene-specific reverse primer *Coch* R2 (5'-GAAAGCATCTGAGACTGGATGCC-3') corresponding to nucleotides 396–418 and Abridged Anchor Primer (AAP; 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3', I; inosine). The second round of PCR was performed using the gene-specific reverse primer *Coch* R3 (5'-ACTGGATGCCGTTGGCATCTAC-3') corresponding to nucleotides 384–405 and Abridged Universal Amplification Primer (AUAP; 5'-GGCCACGCGTCGACTAGTAC-3'). The PCR conditions described above were as follows: denaturation at 94°C for 2 min, 35 amplification cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min) and a final extension at 72°C for 7 min.

Anti-Cochlin antibody, gel electrophoresis, and Western blot analysis

Generation of the anti-Cochlin antibody, gel electrophoresis, and Western blot analysis were performed as described previously [5,6]. A 19-mer peptide (KADIAFLIDGSFNIGQRRF) corresponding to residues 163–181 in the vWF-A1 domain was used as an antigen to generate rabbit polyclonal anti-Cochlin antibody. The specificity of the antibody was confirmed by dot-blot analysis (data not shown). We homogenized the inner ear tissues from human, bovine, and guinea pig, and cultured fibrocytes (after

washing with PBS) in a solubilization mixture containing 0.5% SDS and protease inhibitors. The homogenate was centrifuged and the protein concentration of the supernatant was measured with the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

Inner ear tissues (10 μg of protein) and cultured fibrocytes (5 μg) were resolved by electrophoresis using 15% polyacrylamide gels (ReadyGel, BioRad, Hercules, CA, USA) and transferred onto nitrocellulose membranes (BioRad). The dilution factor conditions of the primary and secondary antibodies detection method were as follows. Inner ear tissues: anti-Cochlin antibody diluted at 1:2000, HRP-labeled goat anti-rabbit IgG diluted at 1:1000, detected by 3,3′-diaminobenzidine (DAB). Cultured fibrocytes: anti-Cochlin antibody diluted at 1:4000, HRP-labeled goat anti-rabbit IgG diluted at 1:50 000, detected by chemiluminescence reaction kit (ECL Advance, Amersham).

SL fibrocyte culture

Culture of guinea pig SL fibrocytes was carried out as described previously [15,17], with minor modifications. Briefly, the cochlear lateral wall (SL and stria vascularis) was dissected and the fragments were cultured on petri dishes. Culture medium consisting of MEM-α supplemented with 10% fetal calf serum (FCS), 1× antibiotic-antimycotic, and 1% ITS-G supplement (Gibco BRL) was used and maintained in an incubator (37°C; 5% CO_2, 95% air). For cytokine treatment, medium was changed to the experimental conditions. Cells were treated with the following cytokines at 50 ng/ml: LIF (Chemicon, Temecula, CA, USA) and IFN-γ, BDNF, NT3, TGF-β3, LIF + BDNF, LIF + TGF-β3, and LIF + NT3 (R&D Systems, Minneapolis, MN, USA) [18]. Cells were treated with cytokine for 3 or 8 h for mRNA analysis and 24 or 48 h for protein analysis. Cultures with 80% confluence were used throughout the experiments. Cell proliferation and viability were assessed by a Cell counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) and trypan blue dye exclusion according to the manufacturer's protocol.

Immunocytochemistry of cultured SL fibrocytes

Secondary cultures were subcultured onto four-well chamber slides (Nalge Nunc, Roskilde, Denmark), fixed in 10% phosphate-buffered formalin for 10 min, and dehydrated. The slides were exposed to a blocking buffer (Dako, Carpinteria, CA, USA), and then incubated overnight with mouse anti-caldesmon (Sigma, St Louis, MO, USA), mouse anti-Na^+-K^+-ATPase (Sigma), rabbit anti-S-100 protein antibody (Sigma). The avidin-biotin complex (ABC) method (ABC Elite kit, Vector Laboratories, Burlingame, CA, USA) was used to detect the bound primary antibodies. The primary antibodies were replaced with the normal serum of each species to confirm the specificity of the staining.

RT-PCR and real-time PCR

Total RNAs from the cochlear lateral wall and cultured fibrocytes were prepared using an RNeasy mini kit (Qiagen, Hilden, Germany) and reverse-transcribed using AMV Reverse Transcriptase XL (Takara, Tokyo, Japan) with random primers for 10 min at 30°C, 30 min at 42°C, and 5 min at 99°C. PCR was carried out using Takara Ex Taq Hs (Takara, Tokyo, Japan) with the following protocol: a hot start at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and an extension at 72°C for 1 min. A gene-specific forward primer, *Coch* F1, and a reverse primer, *Coch* R4 (5'-CGGCGCTGCCCAATATT-3'), corresponding to nucleotides 597–613, were used. As a negative control (NC), duplicate cDNA templates were prepared as above without either the reverse transcriptase or template cDNA and were used in equivalent PCR reactions. The PCR product was sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Applied Biosystems). As a loading control, *GAPDH* cDNA was amplified using the forward primer (5'-TCCCTCAAGATTGTCAGCAA-3') and reverse primer (5'-AGATCCACAACGGATACATT-3'). For TaqMan real-time PCR, the reaction mixture was prepared using TaqMan Universal PCR Master Mix with predesigned and prelabeled TaqMan PCR primer and probe sets for human *COCH* (Hs00187937_m1) or Rodent *GAPDH* (VIC). Real-time PCR was performed using an ABI PRISM 310 Sequence Detection System (Applied Biosystems). The expression levels of *Coch* were standardized to *GAPDH* mRNA and the amount of mRNA in the cochlear lateral wall is referred to as 100. The thermal cycling was performed as follows: 1 cycle at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. These data are the means ± SD of four separate experiments measured in duplicate.

Results

Identification of guinea pig Coch cDNA

The guinea pig *Coch* cDNA was successfully isolated by RACE. After removing the overlapping sequence,

the guinea pig *Coch* cDNA was obtained and submitted to GenBank with the accession no. EU881087 (Fig. 1A).

Our data showed that the guinea pig *Coch* cDNA was 1991 bp and contained an open reading frame (ORF) of 1659 bp, flanked by stretches of 5 bp and 327 bp at the 5'- and 3'-untranslated regions, respectively. The complete cDNA sequences and predicted protein sequence were detected. The initiation Met was assigned to the first ATG codon, and the stop codon (TAA) with one putative polyadenylation signal site was recognized in the 3' untranslated region, which were followed by a short polyA tail.

Sequence analysis based on GenBank search revealed that the guinea pig *Coch* nucleotides and deduced amino acids had significant sequence identity with the human (nt 91.6%, aa 95.0%) and mouse (nt 87.4%, aa 93.3%) and rat (nt 86.9%, aa 93.6%) (Table I). This high degree of homology is conserved in these mammals throughout the whole sequence, including the LCCL, vWF-1, and vWF-2 domains (Fig. 1B).

Western blot analysis of Cochlin isoform expression in the mammalian inner ear

In the human, bovine, and guinea pig inner ear, a set of immunoreactive proteins with sizes of 63, 44, and 40 kDa was detected by Western blotting (Fig. 1C). There were no marked differences in the size and quantity of these Cochlin isoforms among these species.

Culture and immunocytochemistry of SL fibrocytes

The first signs of growth in the cochlear lateral wall explants were observed within 96 h of dissection. Cell expansion was observed initially as spindle-shaped cells extending from the ends of the explant. The morphological characteristics of the secondary cultures were consistent with those of fibrocytes. These spindle-shaped cells possessed both large nuclei and cytoplasm. Those cells were positive for caldesmon and S-100 protein, and were negative for Na^+-K^+-ATPase (Fig. 2A). Caldesmon staining in the cultured fibrocytes was limited to the cytoplasm, and S-100 protein staining was observed in both the nuclei and cytoplasm. The immunoreactivity of these antibodies is representative of the characteristics of the type I fibrocytes in the SL [15,17].

Coch mRNA and Cochlin expression in cultured fibrocytes

We analyzed the expression of *Coch* mRNA in the cultured fibrocytes by RT-PCR using 1 μg of total RNA from the cultured fibrocytes, and 0.001 μg from the cochlear lateral wall. *GAPDH* was used as loading control. *Coch* mRNA (380 bp PCR product) was amplified. A 380 bp cDNA was amplified in both the cultured fibrocytes and cochlear lateral wall (Fig. 2B), and the PCR product was directly sequenced and confirmed as *Coch*. *GAPDH* cDNA was detected in both samples.

Then, we quantified the expression of *Coch* mRNA in cultured fibrocytes and the cochlear lateral wall using TaqMan real-time RT-PCR. *Coch* values were normalized to *GAPDH*, and expressed as a percentage of the value of the cochlear lateral wall. We detected a very low level of mRNA in the cultured fibrocytes ($2.66 \pm 0.49 \times 10^{-3}$%). The negative control without reverse transcriptase or template cDNA did not display any signal on the RT-PCR or real-time PCR.

A set of immunoreactive proteins detected by the anti-Cochlin antibodies with sizes of 66, 60, and 44 kDa was detected in the cultured fibrocytes (Fig. 2C).

Expression of Coch mRNA and Cochlin in cultured fibrocytes after cytokine treatment

Previous studies showed that the cytokines we used (LIF, IFN-γ, BDNF, NT3, TGF-β3, LIF + BDNF, LIF + TGF-β3 or LIF + NT3) have maximal survival-promoting effects on inner ear cells in vitro when used at 50 ng/ml [18], hence this concentration was used in the present study. The amounts of *Coch* mRNA and Cochlin isoforms were determined by real-time PCR and Western blot analysis in cultured fibrocytes in the cytokine-treated cells. Treatment with cytokine (LIF, IFN-γ, BDNF, NT3, TGF-β3, LIF + BDNF, LIF + TGF-β3 or LIF + NT3) did not alter the amount of *Coch* mRNA or Cochlin isoform expression in cultured fibrocytes as compared to the untreated controls (data not shown). The proliferation and viability of the cells did not change after cytokine treatment.

Discussion

In recent years, due to the technological advances of molecular biology, many experimental animal models have been reported, mainly using the mouse or rat. However, a guinea pig animal model is preferable for otologic research, because it affords a potentially

A.

```
        10   20   30   40   50   60   70   80   90  100  110  120

ACACCATGCCTGCAGCCTGGATGCCAGTCCTCCGCCTCGGTGTCTCGCTGCTGCTGCTGCTGCTGCCGCCGGGGCCCGCGGGCGGCGACGGAGCCGTTCCC
ATTGCTATCACATGCTTTA
  M P A A W M P V L R L G V S L L L L L L P P G P A G G D G A V P I A I T C F T

       130  140  150  160  170  180  190  200  210  220  230  240

CCAGAGGCTTGGACATCAGGAAGGAGAAAGCAGATGTCCTCTGCCCAGCAGGCTGCCCTCTTGAGGAATTCTCTGTGTTTGGGAACATAGTTTATGCTTCCGTGTCCAG
CATATGTGGCG
  R G L D I R K E K A D V L C P A G C P L E E F S V F G N I V Y A S V S S I C G A

       250  260  270  280  290  300  310  320  330  340  350  360

CGGCAGTGCACAGGGGAGTAATCAGCATCTCAGGGGGACCTGTGCGAGTGTATAGCCTACCCGGTCGAGAAAACTATTCCTCAGTGGATGCCAATGGCATCCAGTCTC
AAACCCTTGCCA
  A V H R G V I S I S G G P V R V Y S L P G R E N Y S S V D A N G I Q S Q T L A R

       370  380  390  400  410  420  430  440  450  460  470  480

GATGGTCTGCTTCTTTCACAGTAACTAAAGGCAAAAGTAGTACCCAAGAAGCCACGGGACAGGCAGTGTCCACGGCGCGTCCACCAACAGGGAAACGACTAAAGAAAAC
ACCTGAGAAGA
  W S A S F T V T K G K S S T Q E A T G Q A V S T A R P P T G K R L K K T P E K K

       490  500  510  520  530  540  550  560  570  580  590  600

AAACTGGCAATAAAGACTGTAAAGCAGATATTGCATTTCTAATTGATGGAAGCTTTAATATTGGGCAACGCCGCTTTAATTTACAGAAGAATTTTGTTG

AAACTGGCAATAAAGACTGTAAAGCAGATATTGCATTTCTAATTGATGGAAGCTTTAATATTGGGCAACGCCGCTTTAATTTACAGAAGAATTTTGTTGGGAAGGTGGCGC
TGATGTTGG
  T G N K D C K A D I A F L I D G S F N I G Q R R F N L Q K N F V G K V A L M L G

       610  620  630  640  650  660  670  680  690  700  710  720
```

Figure 1. (*Continued*).

GAATTGGAACAGAAGGACCACATGTTGGCCTTGTTCAAGCCAGTGAACATCCCAAAATAGAATTTTACTTGAAAAACTTCACATCAGCCAAAGATGTTTTGTTTGCCATAA
AGGAAGTAG

I G T E G P H V G L V Q A S E H P K I E F Y L K N F T S A K D V L F A I K E V G

730 740 750 760 770 780 7 90 800 810 820 830 840

GTTTCAGAGGGGGTAATTCCAATACAGGAAAAGCTTTGAAGCACACGGCTCAGAAATTCTTCACAGCAGATACTGGAATGAGAAAAGGGATCCCCAAAGTGGTGGTGGT
ATTTATTGATG

F R G G N S N T G K A L K H T A Q K F F T A D T G M R K G I P K V V V V F I D G

850 860 870 880 890 900 910 920 930 940 950 960

GCTGGCCATCTGATGACATAGAGGAAGCAGGAATTGTGGCCAGAGAGTTTGGAGTCAATGTATTTATAGTTTCTGTAGCCAAGCCAATCCCTGAAGAACTGGGCATGGTT
CAGGATGTTG

W P S D D I E E A G I V A R E F G V N V F I V S V A K P I P E E L G M V Q D V A

970 980 990 1000 1010 1020 1030 1040 1050 1060 1070 1080

CATTTGTTGACAAGGCTGTCTGTCGGAATAACGGCTTCTTCTCTTACCACATGCCCAACTGGTTCGGCACCACAAAATATGTAAAGCCTCTGGTACAGAAGCTCTGCTCT
CATGAGCAAA

F V D K A V C R N N G F F S Y H M P N W F G T T K Y V K P L V Q K L C S H E Q M

TGATGTGCAGCAAGACCTGTTATAACTCAGTGAACATTGCCTTCCTGATTGACGGCTCCAGCAGTGTTGGGGATAGTAATTTCCGCCTCATGCTTGAATTTGTTTCCAACA
TAGCCAAGA

M C S K T C Y N S V N I A F L I D G S S S V G D S N F R L M L E F V S N I A K T

1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320

CTTTTGAAATCTCAGACATTGGTGCCAAGATAGCTGCTGTGCAGTTCACTTACGACCAACGCACAGAGTTCAGTTTCACTGACTACAGCACTAAGGAGAATGTCCTAGCC
GTTATCAGAA

F E I S D I G A K I A A V Q F T Y D Q R T E F S F T D Y S T K E N V L A V I R S

1330 1340 1350 1360 1370 1380 1390 1400 1410 1420 1430 1440

Figure 1. (*Continued*).

GCATCCGCTATATGAGTGGAGGAACAGCTACTGGTGATGCCATTTCCTTTACTGTTAGAAATGTGTTTGGTCCTGTGAGGGATAGTCCCAATAAAAACTTCCTGGTGATCA
TCACTGACG

I R Y M S G G T A T G D A I S F T V R N V F G P V R D S P N K N F L V I I T D G

1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560

GGCAGTCCTATGATGATGTCCGAGGCCCTGCTGCTGCAGCACATGATGCAGGTATCACCATTTTCTCTGTAGGTGTGGCTTGGGCACCTCTGGATGACCTGAAAGATAT
GGCCTCTAAAC

Q S Y D D V R G P A A A A H D A G I T I F S V G V A W A P L D D L K D M A S K P

1570 1580 1590 1600 1610 1620 1630 1640 1650 1660 1670 1680

CAAAGGAATCACATGCGTTCTTCACAAGAGAGTTCACAGGACTAGAACCAATCGTTTCTGATATCATTAGAGGCATTTGTAGAGATTTCTTAGAATCCCAACAATAATGGT
GGTATTTTG

K E S H A F F T R E F T G L E P I V S D I I R G I C R D F L E S Q Q

1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800

ATAACCAAAAAGTGCAAGATTTAATGTATAAATTGTATTCTTATGATAGTGAAATACCATAGCATACTAGGATCAGATACATTAAACATGTCAACAGCTATTTTAAGCAAATA
AACATTC

1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920

ATTTAAAATTACTACCTTCTAGTTACAACTTAGACTTTTGACTGAGGCTTCATAACCTTAGCCCTTAGAAATCCAAGAAAGATGATCATGTTTTAAAACCTTAAAAGTTCTAA
TATACCT

1930 1940 1950 1960 1970 1980 1990 2000

ATAAAATGTACAGATTTGCAAGTGCCACAGCTCAATAAAAGAATCTGATACTTACAACAAAAAAAAAAAAA

Figure 1. (*Continued*).

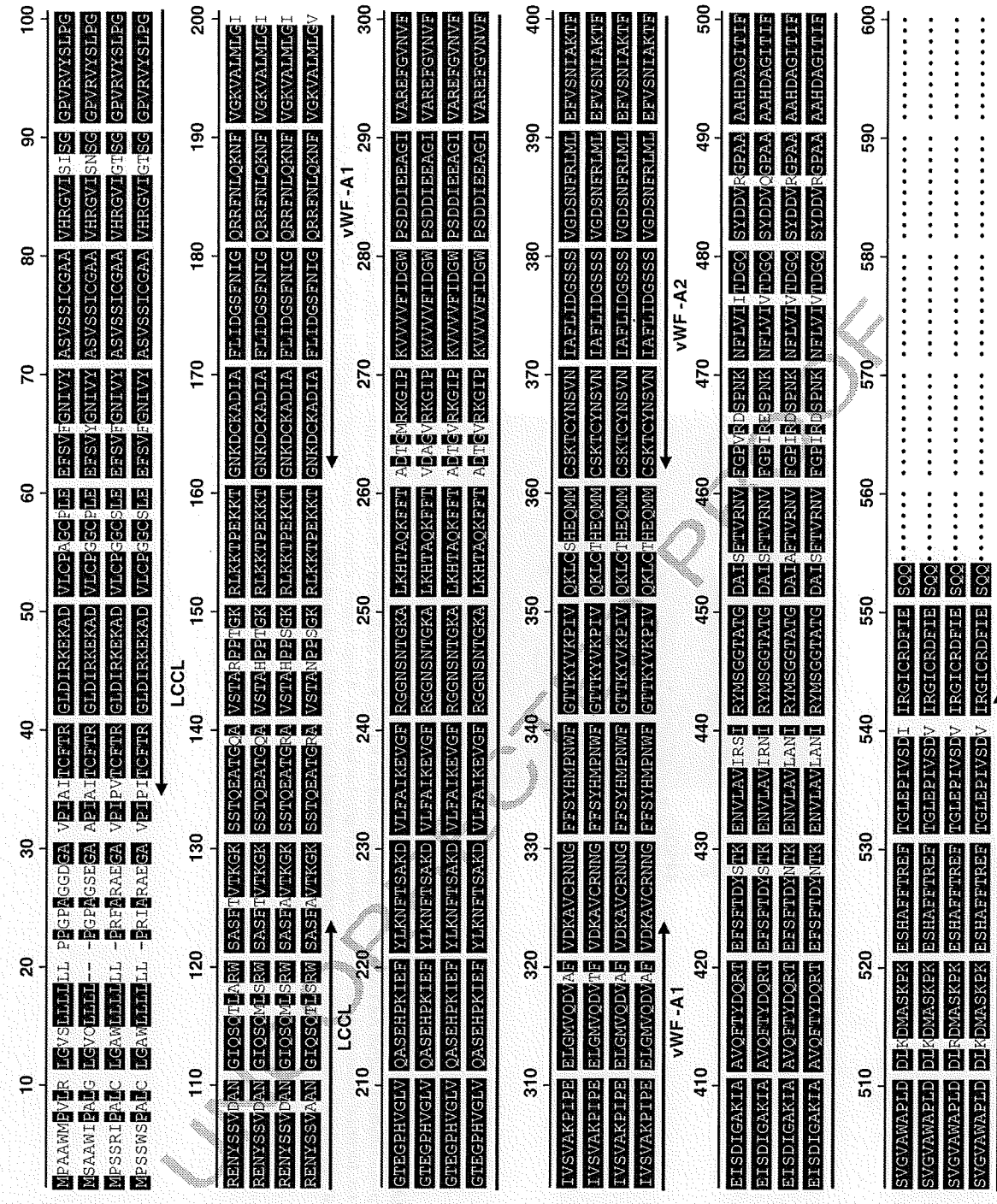

Figure 1. (*Continued*).

Figure 1. (*Continued*). (A) The full-length cDNA and deduced amino acid sequence of guinea pig *Coch*. Number of nucleotides, as labeled above; start codon (ATG), in bold type; stop codon (TAA), shaded in bold; polyadenylation signal AATAAA, boxed; primers used for RT-PCR are underlined in bold. (B) Multiple sequence alignment of *Coch* amino acid sequences from guinea pig, human, mouse, and rat. Identical amino acids are shown in white with gray background. A high degree of homology is conserved in these species. (C) Cochlin isoforms detected in the inner ear of human, bovine, and guinea pig species. The anti-Cochlin antibody detected isoforms p63, p44, and p40.

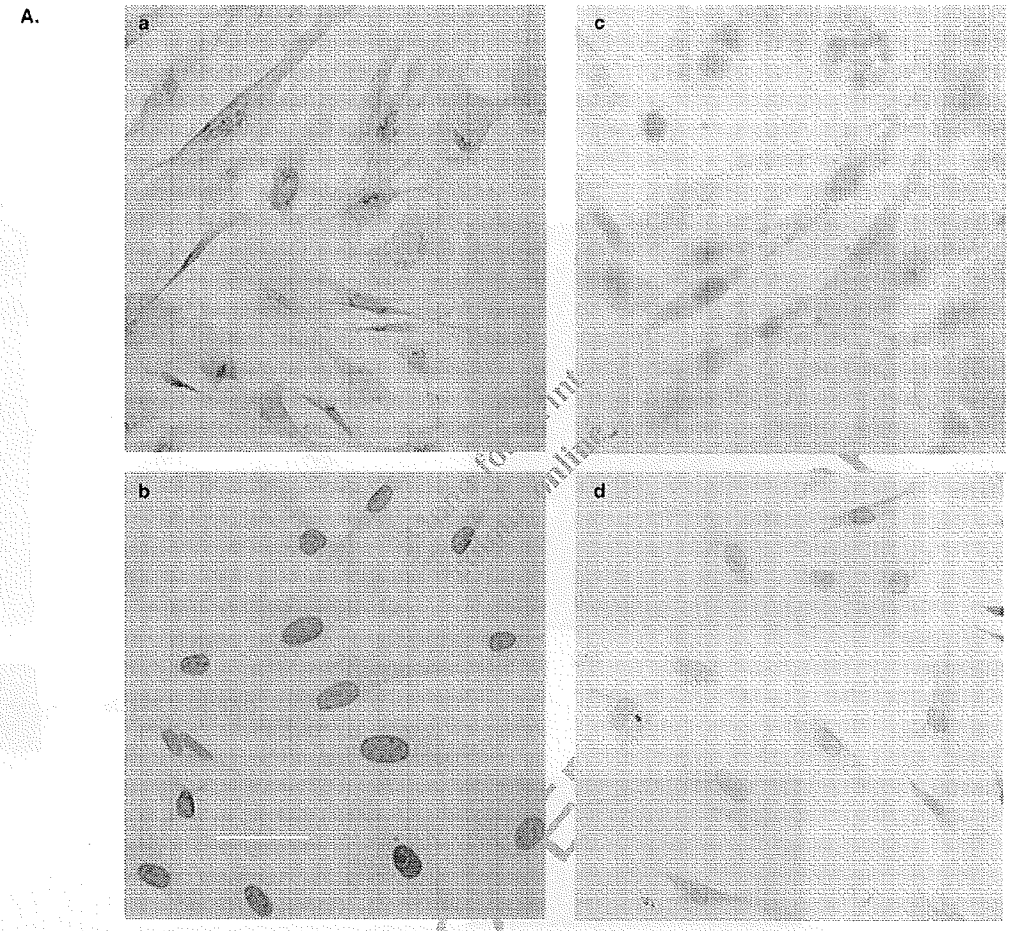

Figure 2. *(Continued)*.

viable model of anatomical perspective for auditory studies, as not only can this animal be readily deafened, but vestibular function can be manipulated by precise surgical manipulation. The size of the eardrum and middle ear is relatively large and the postauricular surgical approach to the middle ear is not complicated by the presence of major blood vessels or muscles. The petrous bone (otic capsule) is easily entered and dissected away, without drilling, to expose the inner ear structures [16]. The guinea pig inner ear is also suitable for cell culture, and various types of cells have been cultured, including epithelial cells of the endolymphatic sac, supporting cells of the vestibular sensory epithelia, or cochlear sensory epithelial cells, as well as fibrocytes of SL [15,17].

This is the first report of the cloning and characterization of the guinea pig *Coch* cDNA from the inner ear by 5' and 3' RACE. The nucleotide sequence obtained was submitted to the GenBank database under accession no. EU881087. The cDNA had 1991 bp nucleotides and contained a 5' untranslated sequence of 5 bp, a 3' untranslated sequence of 327 bp, and an ORF of 1659 bp coding for 553 amino acids. The first initiation Met is preceded by an in-frame stop codon. It should be noted, however, that the alignment of the RACE products resulted in a cDNA with only 5 bp sequence of the 5' end of the putative start codon, whereas human and mouse *Coch* cDNA have 56 bp and 68 bp, respectively, in the 5' region of their start codons. This indicates that the 5' RACE reaction might not have been entirely successful in fully mapping the guinea pig *Coch*, but it is not clear if this was due to variability in the RACE reaction or to a difficulty inherent in the guinea pig template, such as the formation of secondary structures.

Sequence analysis revealed that the deduced amino acid sequence of the guinea pig sequence displays 95.0%, 93.3%, and 93.6% identity with the human,

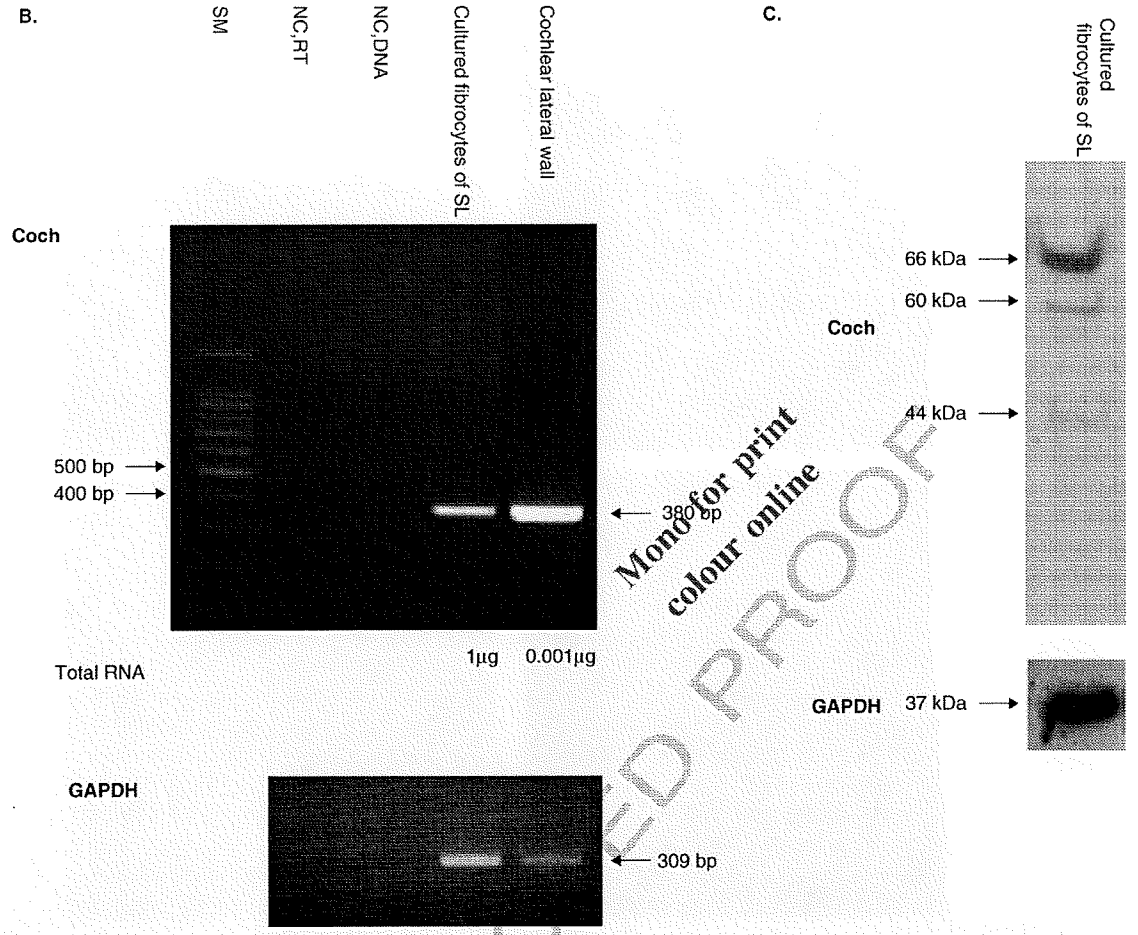

Figure 2. (*Continued*). (A) Cultured fibrocytes were immunopositive to caldesmon (a) and S-100 protein (b) and negative to IgG (c) and Na^+-K^+-ATPase (d). Bar = 50 μm. The immunoreactivity to these antibodies bears the characteristics of type I fibrocytes of the SL (spiral ligament). (B) RT-PCR of 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,cNDA-, 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, 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%	

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.

Acknowledgments

AQ1

This study was supported by Health and Labor Sciences Research Grants in Japan (Research on Measures for Intractable Diseases, Researches on Sensory and Communicative Disorders), a grant from the Ministry of Education, Culture, Sports, Science and Technology.

References

[1] Robertson NG, Khetarpal U, Gutiérrez-Espeleta GA, Bieber FR, Morton CC. Isolation of novel and known genes from a human fetal cochlear cDNA library using subtractive hybridization and differential screening. Genomics 1994;23:42–50.

[2] Robertson NG, Lu L, Heller S, Merchant SN, Eavey RD, McKenna M, et al. Mutations in a novel cochlear gene cause DFNA9, a human nonsyndromic deafness with vestibular dysfunction. Nat Genet 1998;20:299–303.

[3] Trexler M, Bányai L, Patthy L. The LCCL module. Eur J Biochem 2000;267:5751–7.

[4] Ikezono T, Omori A, Ichinose S, Pawankar R, Watanabe A, Yagi T. Identification of the protein product of the Coch gene (hereditary deafness gene) as the major component of bovine inner ear protein. Biochim Biophys Acta 2001;1535:258–65.

[5] Ikezono T, Shindo S, Li L, Omori A, Ichinose S, Watanabe A, et al. Identification of a novel Cochlin isoform in the perilymph: insights to Cochlin function and the pathogenesis of DFNA9. Biochem Biophys Res Commun 2004;314:440–6.

[6] Li L, Ikezono T, Watanabe A, Shindo S, Pawankar R, Yagi T. Expression of full-length Cochlin p63s is inner ear specific. Auris Nasus Larynx 2005;32:219–23.

[7] Ikezono T, Shindo S, Sekiguchi S, Hanprasertpong C, Li L, Pawankar R, et al. Cochlin-tomoprotein, a novel perilymph-specific protein and a potential marker for the diagnosis of perilymphatic fistula. Audiol Neurotol 2009;14:338–44.

[8] Sekine K, Ikezono T, Matsumura T, Shindo S, Watanabe A, Li L, et al. Expression of cochlin mRNA splice variants in the inner ear. Audiol Neurootol 2009;15:88–96.

[9] Solares CA, Edling AE, Johnson JM, Baek MJ, Hirose K, Hughes GB, et al. Murine autoimmune hearing loss mediated by CD4+ T cells specific for inner ear peptides. J Clin Invest 2004;113:1210–17.

[10] Mizuta K, Ikezono T, Iwasaki S, Arai M, Hashimoto Y, Pawankar R, et al. Ultrastructural co-localization of cochlin and type II collagen in the rat semicircular canal. Neurosci Lett 2008;434:104–7.

[11] Shindo S, Ikezono T, Ishizaki M, Sekiguchi S, Mizuta K, Li L, et al. Spatiotemporal expression of Cochlin in the inner ear of rats during postnatal development. Neurosci Lett 2008;444:148–52.

[12] Ikezono T, Shindo S, Ishizaki, Li L, Tomiyama S, Takumida M, et al. Expression of Cochlin in the vestibular organ of rats. ORL J Otorhinolaryngol Relat Spec 2005; 67:252–8.

[13] Kommareddi PK, Nair TS, Raphael Y, Telian SA, Kim AH, Arts HA, et al. Cochlin isoforms and their interaction with CTL2 (SLC44A2) in the inner ear. J Assoc Res Otolaryngol 2007;8:435–46.

[14] Kikuchi T, Adams JC, Miyabe Y, So E, Kobayashi T. Potassium ion recycling pathway via gap junction systems in the mammalian cochlea and its interruption in hereditary nonsyndromic deafness. Med Electron Microsc 2000; 33:51–6.

[15] Ichimiya I, Yoshida K, Hirano T, Suzuki M, Mogi G. Significance of spiral ligament fibrocytes with cochlear inflammation. Int J Pediatr Otorhinolaryngol 2000;56:45–51.

[16] Goksu N, Haziroglu R, Kemaloglu Y, Karademir N, Bayramoglu I, Akyildiz N. Anatomy of the guinea pig temporal bone. Ann Otol Rhinol Laryngol 1992;101:699–704.

[17] Suko T, Ichimiya I, Yoshida K, Suzuki M, Mogi G. Classification and culture of spiral ligament fibrocytes from mice. Hear Res 2000;140:137–44.

[18] Marzella PL, Gillespie LN, Clark GM, Bartlett PF, Kilpatrick TJ. The neurotrophins act synergistically with LIF and members of the TGF-beta superfamily to promote the survival of spiral ganglia neurons in vitro. Hear Res 1999;138:73–80.

[19] Robertson NG, Hamaker SA, Patriub V, Aster JC, Morton CC. Subcellular localisation, secretion, and post-translational processing of normal Cochlin, and of mutants causing the sensorineural deafness and vestibular disorder, DFNA9. J Med Genet 2003;40:479–86.

[20] Grabski R, Szul T, Sasaki T, Timpl R, Mayne R, Hicks B, et al. Mutations in Coch that result in non-syndromic autosomal dominant deafness (DFNA9) affect matrix deposition of Cochlin. Hum Genet 2003;113:406–16.

Original Paper

Audiology & Neurotology

Audiol Neurotol 2010;15:247–253
DOI: 10.1159/000256664

Received: March 26, 2009
Accepted after revision: September 10, 2009
Published online: November 10, 2009

Ultrastructural Localization of Cochlin in the Rat Cochlear Duct

Seiji Hosokawa[a] Kunihiro Mizuta[a] Hiroshi Nakanishi[a] Yasuyuki Hashimoto[a]
Maki Arai[a] Hiroyuki Mineta[a] Susumu Shindo[b] Tetsuo Ikezono[b]

[a]Department of Otolaryngology, Hamamatsu University School of Medicine, Hamamatsu, and
[b]Department of Otolaryngology, Nippon Medical School, Tokyo, Japan

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-

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1420–3030/10/0154–0247$26.00/0

Accessible online at:
www.karger.com/aud

Seiji Hosokawa, MD
Department of Otolaryngology, Hamamatsu University School of Medicine
1-20-1 Handayama, Higashi-ku
Hamamatsu 431-3192 (Japan)
Tel. +81 53 435 2252, Fax +81 53 435 2253, E-Mail seijih@hama-med.ac.jp

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, preimmune 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).