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Ⅳ. 研究成果の刊行物・別刷

Acta Oto-Laryngologica, 2010; Early Online, 1 7

informa
healthcare

ORIGINAL ARTICLE

CTP (Cochlin-tomoprotein) detection in the profuse fluid leakage (gusher) from cochleostomy

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Abstract

Conclusions: By testing 125 samples, we confirmed that Cochlin-tomoprotein (CTP) is present in the perilymph, not in cerebrospinal fluid (CSF). Perilymph and CSF exist in two distinct compartments, even in the case of a malformed inner ear with a bony defect in the lamina cribrosa, as described here. Cochleostomy might have suddenly decreased the perilymph pressure, allowing the influx of CSF into the inner ear resulting in profuse fluid leakage, first perilymph then CSF. *Objectives:* The first purpose of this study was to further confirm the specificity of the perilymph-specific protein CTP that we reported recently. Secondly, we assessed the nature of the fluid leakage from the cochleostomy using the CTP detection test. *Methods:* A standardized CTP detection test was performed on 65 perilymph and 60 CSF samples. Samples of profuse fluid leakage collected from cochleostomy during cochlear implantation surgery of one patient with branchio-oto-renal (BOR) syndrome were also tested by the CTP detection test. *Results:* CTP was detected in 60 of 65 perilymph samples but not in any of the CSF samples. The leaked fluid was shown to contain CTP, i.e. perilymph, at the outset, and then the CTP detection signals gradually disappeared as time elapsed.

Keywords: *Perilymph, CSF, cochlear implant, COCH gene, Cochlin isoform, branchio-oto-renal syndrome, sensorineural hearing loss*

Introduction

Normally the cerebrospinal fluid (CSF) in the subarachnoid space extends laterally into the internal auditory canal (IAC) as far as the lateral fundus, where it is separated from the perilymph by the bony plate of the lamina cribrosa, the nerves that pass through the lamina cribrosa and the spiral ganglion. Profuse leakage of perilymph and CSF is the result of an abnormal bony defect in the lamina cribrosa, rather than enlargement of the cochlear aqueduct. In some congenitally dysplastic ears there is a deficiency in this barrier, allowing direct confluence between the CSF and perilymph [1,2].

In a large series of cochlear implants, the incidence of this profuse leakage of perilymph and CSF, i.e. a 'gusher', was approximately 1% of all cases. The term gusher is widely used in the literature to describe egress of clear fluid from the cochleostomy site. However, the term is variably applied and bears no explicit relation to the amount of fluid leaking, time elapsed until the flow ceases, pressure of the fluid column or origin of the fluid. The fluid may be CSF, perilymph, or a mixture of the two [3]. Even though the current level of diagnostic imaging technology is considerably advanced, at present it is unclear whether the perilymph and CSF spaces are separate and function as two distinct compartments or not. The fragile membranous tissue at the fundus of the IAC can be perforated after the fenestration of the cochlea due to a pressure decrease in the perilymphatic space resulting from the cochleostomy.

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(Received 28 September 2009; accepted 19 November 2009)

ISSN 0001-6489 print/ISSN 1651-2251 online © 2010 Informa UK Ltd. (Informa Healthcare, Taylor & Francis AS)
DOI: 10.3109/00016480903508910

Alternatively, these liquid compartments may be mixed preoperatively because of a defect in the lamina cribrosa. The confluence of these two fluids could be one of the causes of the deterioration of hearing [2].

Recently, we reported the features of Cochlin-tomoprotein (CTP), a novel perilymph-specific protein. By proteomic analysis of inner ear proteins, we found certain unique properties of the cochlin isoforms. We detected three cochlin isoforms, p63s, p44s and p40s, in the inner ear tissue, and a short 16 kDa isoform, named Cochlin-tomoprotein (CTP), in the perilymph [4,5]. Since COCH gene/cochlin is known to be highly specific to the inner ear, we examined CTP expression in body fluid. CTP was selectively expressed in the perilymph of all 20 perilymph samples tested, but not in 77 samples of the body fluids, i.e. serum (28 samples), CSF (20 samples) or saliva (29 samples) [6]. Using CTP as a biomarker, we have established a standardized CTP detection test for the diagnosis of perilymphatic fistula (PLF), using a spiked standard of recombinant human (rh)CTP in Western blotting [7].

In the present study, we further analysed the specificity of CTP expression in body fluids, testing 65 perilymph and 60 CSF samples. The results confirm that CTP is present in the perilymph, but not in the CSF. Perilymph and CSF are distinguished by the presence or absence of a particular protein, CTP. Then we tested the samples of the profuse fluid leakage collected temporally from one case of cochleostomy during cochlear implantation surgery. The leaked fluid at the beginning of the cochleostomy contained CTP, i.e. perilymph, and the CTP signal gradually disappeared as time elapsed. These results indicate that even in the malformed inner ear of the case presented here, with a defect in the bony barrier at the fundus, the perilymph and CSF fluid spaces function as two separate compartments. Cochleostomy might have suddenly decreased the perilymph pressure and allowed CSF influx into the inner ear, resulting in profuse fluid leakage (perilymph then CSF) from the cochleostomy site. This is the first report of a temporal analysis of the CTP protein in gusher fluid.

Material and methods

Analysis of CTP expression in CSF and perilymph: collection and processing of body fluid samples

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

Samples of perilymph or CSF (4 µg) were mixed with 5 µl of sample buffer (150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.3% bromophenol blue, 300 mM DTT) after normalization per average protein concentration (perilymph 200 mg/dl, CSF 40 mg/dl) [8], then analysed by Western blot.

Analysis of CTP expression in the profuse fluid leakage from cochleostomy

We performed cochlear implantation in a 50-year-old male patient who was diagnosed as having branchio-oto-renal (BOR) syndrome (without renal manifestation) based upon the diagnostic criteria [9]. The patient started to use a hearing aid at the age of 8 years, and his hearing had progressively deteriorated. At the time of his first visit to our office, he had profound bilateral sensorineural hearing loss, right preauricular sinus and a slightly malformed left auricle. The tympanic membranes showed no anatomical abnormalities. High resolution computed tomography (HRCT) of the temporal bones (Fig. 1) presented findings typically seen in BOR cases; hypoplastic apical turn of the cochlea, a funnel-shaped IAC. The fundus of the IAC was wide, the lamina cribrosa was hypoplastic and no bony structure was seen [10]. Echogram and HRCT revealed no renal malformation. An 18-year-old daughter of the patient had bilateral profound sensorineural hearing loss and a history of surgical removal of bilateral cervical fistulas. She had similar HRCT findings. The patient's father also had bilateral progressive hearing loss.

During cochlear implantation in this case, profuse fluid leakage from the cochleostomy (gusher) was observed. We collected the leakage samples in a temporal manner at the following time points: 0 (immediately after fenestration), 0.5, 2, 3, 6, 15, 25, 35 and 40 min. It is the policy of this department to wait until the flow of the leakage weakens and then seal the cochleostomy with fascia and fibrin glue to avoid lumbar drainage. As a control, middle ear lavage (MEL), i.e. lavaging the middle ear cavity three to four times with the same bolus of 0.3 ml of saline and recovering the fluid, was

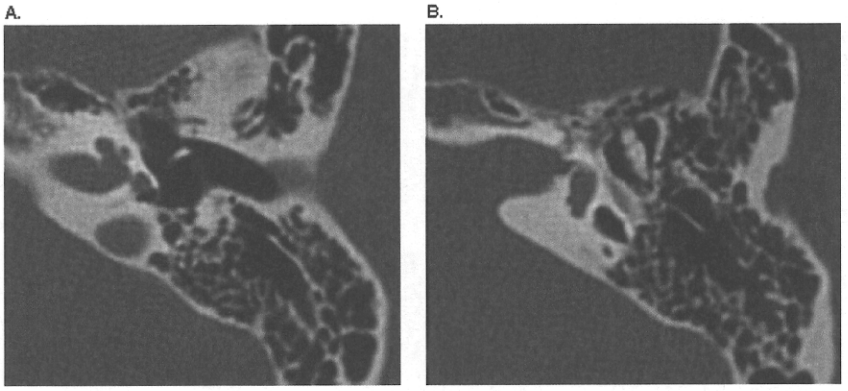

Figure 1. HRCT of the right ear in the axial plane. Both ears had findings typically seen in BOR cases: (a) the hypoplastic apical turn of the cochlea, the fundus of the internal auditory canal was wide, the lamina cribrosa was hypoplastic and no bony structure was seen; (b) the funnel-shaped internal auditory canal (IAC).

taken from the middle ear before cochleostomy. MEL (16 µl) was mixed with 8 µl of 3× concentrated sample buffer (150 mM Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 0.3% bromophenol blue, 300 mM DTT) for Western blot analysis.

Standardized CTP detection test by Western blot

Samples were tested by the standardized CTP detection test [6,7], with minor modifications. For Western blot analysis, the rabbit polyclonal anti-CTP antibody (formerly anti-LCCL-C Ab) was prepared as described previously. In brief, a 14-mer peptide (LSRWSASFTVTKGK) corresponding to residues 114–127 in the LCCL domain was used as an antigenic peptide to generate antibodies.

Samples were loaded onto 15% polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked overnight at 4°C in 5% skim milk and 0.2% polyoxyethylenesorbitan (Tween-20) dissolved in PBS (pH 7.5). Membranes were then incubated in PBS containing 1% skim milk and 0.1% Tween-20 for 2 h at room temperature with the primary antibody (anti-CTP antibody) diluted to 1:2000. After washing with 0.05% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with horseradish peroxidase-labelled goat anti-rabbit IgG antibody (Dako, Tokyo, Japan) diluted to 1:10 000 in the same buffer as 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 analysed with an image analyser LAS-3000 (Fuji Film, Tokyo, Japan). Tests were performed and analysed 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 qualitatively (positive or negative) by the presence or absence of the anti-CTP antibody reactive protein, with a molecular weight that exactly matched the molecular weight of native CTP (16 kDa) on Western blot.

rhCTP was used as a spiked standard on the Western blot. A putative CTP sequence predicted from our previous study, the 101–403 positions of the cDNA corresponding to amino acid residues 32–132, was amplified by PCR from a human expressed sequence tag clone, IMAGE ID 27789 (Kurabo, Japan). rhCTP was produced using pCR/T7/TOPO/TA expression kits (Invitrogen). To establish a clinical test for the diagnosis of perilymph leakage and avoid test variability, we standardized the CTP detection test using high (0.27 ng) and low (0.13 ng) spiked standard levels of rhCTP on Western blot. When the intensity of the band in the samples tested was below the high-level standard signal, the result was considered to be negative. The low spiked standard was used to estimate the protein transfer efficiency.

Diagnostic performance of the CTP detection test

To evaluate the specificity of the CTP detection test in the clinical setting, we previously reported the test results of samples from non-PLF cases. In that study, we defined non-PLF as those cases with otosclerosis (that had undergone stapedectomy), profound deafness (cochlear implant surgery), or conductive hearing loss (exploratory tympanotomy), without

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.

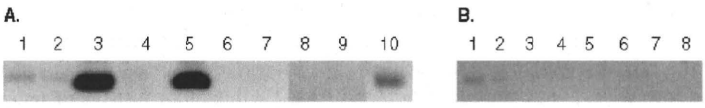

Figure 2. Western blot analysis of CTP expression in perilymph and CSF. The expression of CTP was analysed by Western blot using the anti-CTP antibody. CTP expression (16 kDa) was only detected in the perilymph (cases A and B), not in the CSF. The perilymph sample from case C was negative for CTP. Further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

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.

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_2 middle ear lavage.

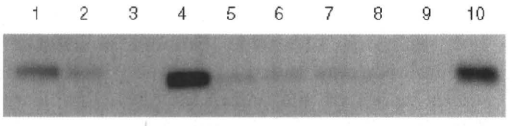

Figure 3. The results of the CTP detection test by Western blotting of the leakage from cochleostomy. MEL obtained before the fenestration of the cochlea was negative for CTP (lane 3). The leakage collected at 0 min showed a CTP signal above the high-level standard signal (lane 4) and the samples collected 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.

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Acta Oto-Laryngologica, 2010; 130: 868 880

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ORIGINAL ARTICLE

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

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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 isoforms were studied by Western blot using three different types of mammalian inner ear. The cochlear fibrocytes were cultured and characterized by immunostaining. *Coch* gene 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

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(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 Gene Bank 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%), Bovine (nt 89.5%, aa 93.3%), 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

730 740 750 760 770 780 790 800 810 820 830 840

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*).

GCATCGGCTATATGAGTGGAGGAACAGCTACTGGTGATGCCATTTCCTTTACTGTTAGAAATGTGTTTGGTCCTGTGAGGGATAGTCCCAATAAAAACTTCCTGGTGATCA
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*).

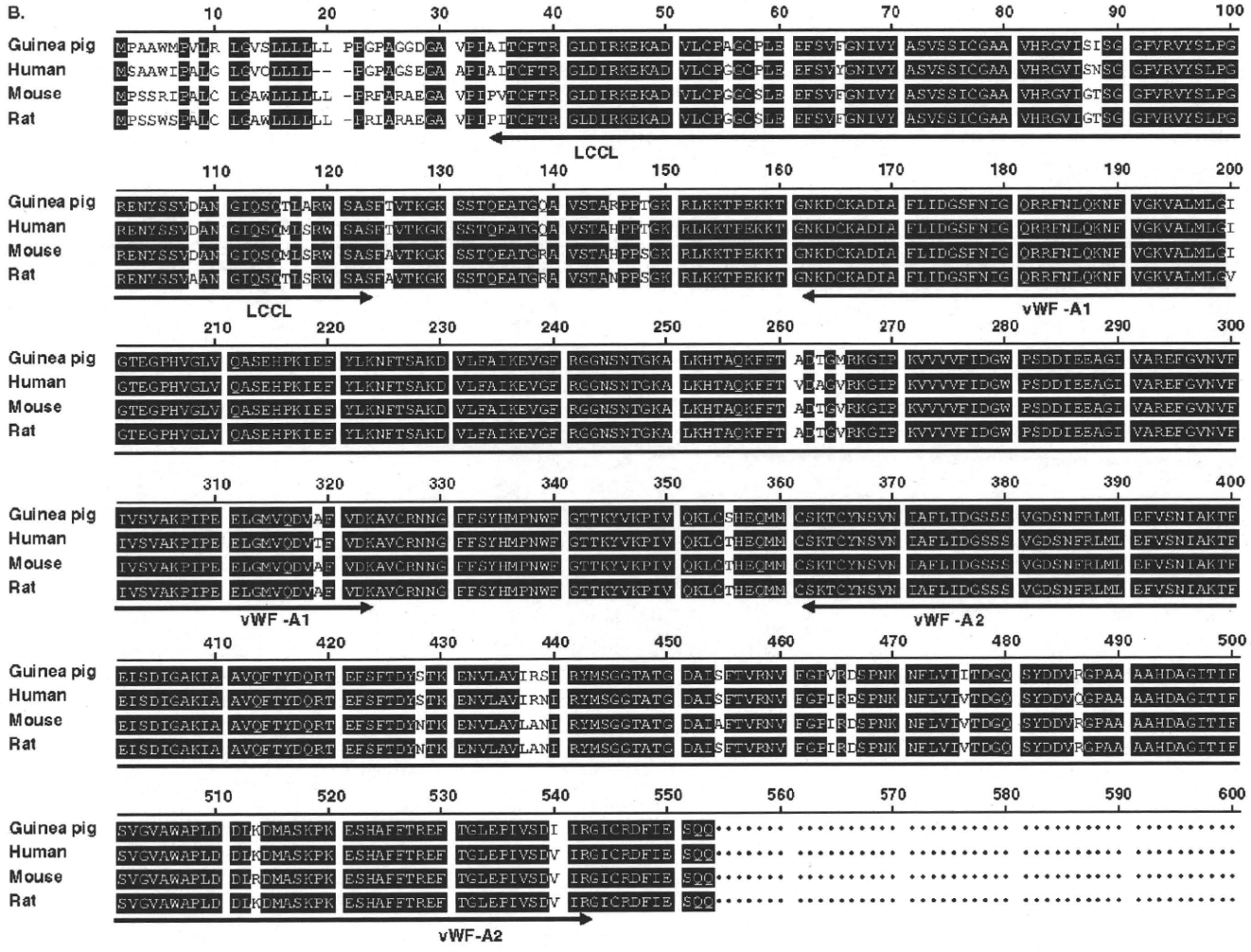

Figure 1. *(Continued).*

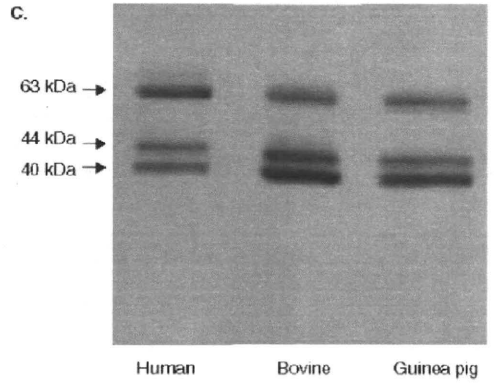

Figure 1. *(Continued)*. (A) The full-length cDNA and deduced amino acid sequence of guinea pig *Coch*. Number of nucleotides, as labeled above; start codon (ATG), in bold type; stop codon (TAA), shaded in bold; polyadenylation signal AATAAA, boxed; primers used for RT-PCR are underlined in bold. (B) 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.

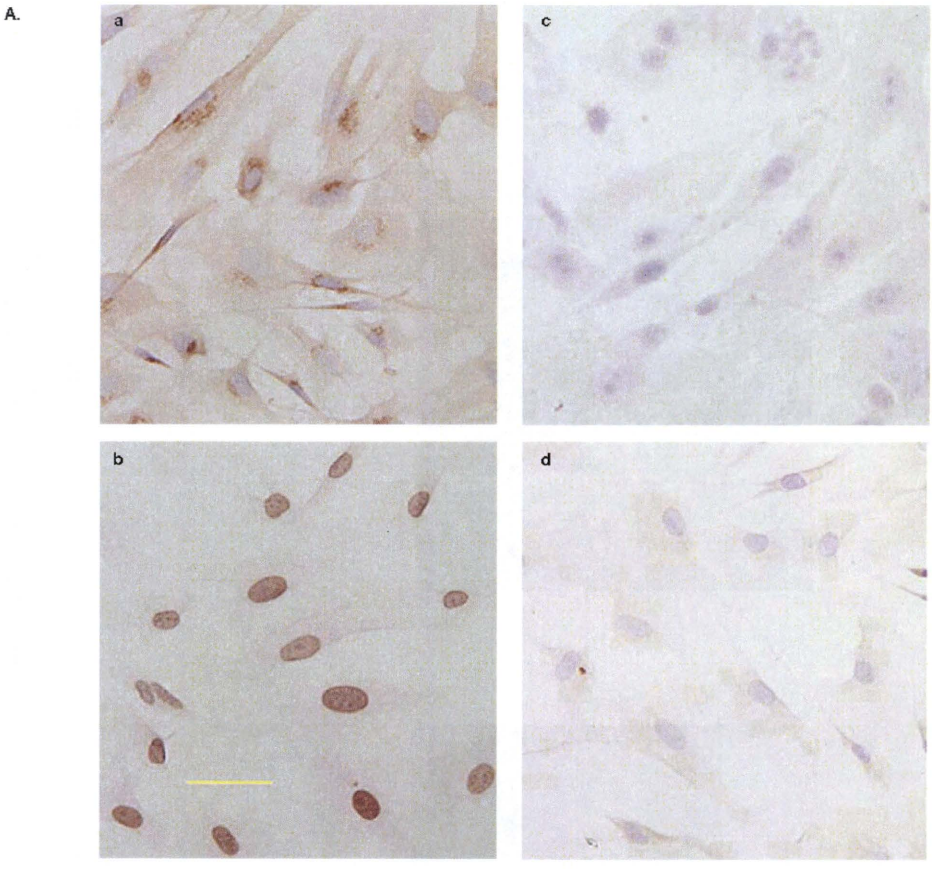

Figure 2. *(Continued).*

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%, 93.3%, and 93.6% identity with the