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厚生労働科学研究費補助金

感覚器障害研究事業

内耳プロテオーム解析を応用した外リンパ瘻の新たな診断法の開発・治療指針の作成

平成１９　（２００７）年度　総括研究報告書

主任研究者　池園　哲郎

平成２０（２００８）年　3月

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外リンパ瘻の新たな診断法の開発・治療指針の作成

主任研究者　池園哲郎　日本医科大学准教授

研究要旨

１．研究目的

難聴・平衡障害の原因疾患「外リンパ瘻」は迅速に手術治療を行うことで劇的な治療効果が得られる疾患である。本研究の目的は外リンパ瘻の確定診断法を開発して、早期に確定診断し、治癒率の向上をもたらすことにある。

２．研究方法

背景：内耳性難聴・めまいの原因疾患である外リンパ瘻の症状、検査所見は多彩でありその他の突発性難聴やメニエール病と鑑別が容易ではない。確定診断法が確立しておらず、治癒率の低下をもたらしている。我々は内耳プロテオーム解析を行い、外リンパ瘻の生化学的確定診断マーカーＣＴＰを発見し国内・国際特許出願した。

方法：[1] 検査システムのクオリティーコントロール ウェスタンブロット法による検査システムの至適条件を設定し、クオリティーコントロール（精度管理）を確立する。

[2] 外リンパ瘻診断の精度（diagnostic performance）　ＣＴＰ検出法による外リンパ瘻の診断精度を、STARD statement（STAndards for the Reporting of Diagnostic accuracy studies）に準拠して評価する。

[3] 臨床症例の検討　下記分類に従い検討する。1.頭部外傷性　2.中耳外傷性　3.特発性（いわゆる鼻かみ型）4. 小児変動性難聴　5. 医原性　6. 真珠腫性中耳炎内耳瘻孔　7. 慢性・遅発性外リンパ瘻

[4] POCT(Point of care testing;臨床の現場での検査)の開発。　ELISA法の基礎研究を行う。

３．研究結果及び考察

[1] 検査システムのクオリティーコントロール　標品（リコンビナント蛋白）の検出感度下限濃度a（0.27 ng/ well）及びその1/2濃度bの点の2点を毎回検査サンプルと共に泳動し、精度管理した。希釈外リンパの検出限界は平均0.022μl/well であった。

[2] 外リンパ瘻診断の精度　本検査は下記のように非常に良好な診断精度を有すると評価された。

感受性　91.5%（95%CI , 83.5 - 99.5%）　　特異性　98.2%（95%CI, 96.4 - 100.0%）

[3] 臨床症例の検討　前記分類に従って、臨床例の検討を行った。それぞれの疾患カテゴリーで、診断・治療両面にわたり新知見が得られた。（内容略）

[4] POCT(Point of care testing;臨床の現場での検査)の開発。ELISA法を開発中である。本方法は、cut off値の設定により明確な判定基準が設定できることから、検査者の熟練度が低くても施行可能であり、本検査の広い普及が可能となる。

４．結論

総括　本検査は世界で初めて外リンパ瘻の生化学的確定診断を可能にするものであり、国内外の臨床家、基礎研究者から多くの注目を集めている。今後、本研究成果を広め、国内外でマルチセンタースタディーを行う予定である。外リンパ瘻は的確な診断で大きな治療効果が期待できる疾患である。耳鼻咽喉科・神経耳科の診断が患者のQOL、後遺症の有無を大きく左右する。本研究事業の成果を広く普及させることで大きな具体的な効果が望める。

分担研究者

渡邉　淳　日本医科大学第２生化学

（日本医科大学付属病院）

助手

１．研究目的

難聴・平衡障害の原因疾患「外リンパ瘻」は迅速に手術治療を行うことで劇的な治療効果が得られる疾患である。本研究の目的は外リンパ瘻の新たな確定診断法を開発して、早期に確定診断し、治癒率の向上をもたらすことにある。

２．研究方法

背景：内耳性難聴・めまいの原因疾患である外リンパ瘻の症状、検査所見は多彩でありその他の突発性難聴やメニエール病と鑑別が容易ではない。確定診断法が確立しておらず、治癒率の低下をもたらしている。我々は内耳プロテオーム解析を行い、外リンパ瘻の生化学的確定診断マーカーＣＴＰを発見し国内・国際特許出願した。

方法：

［１］確実にＣＴＰを検出するためには、高い検出感度、特異度が求められる。数種類のポリクローナル（polyAb）を作成し、ウェスタンブロット法による検査システムの至適条件を設定し、クオリティーコントロール（精度管理）を確立する。

[2] 外リンパ瘻の診断精度（diagnostic performance）

ＣＴＰ検出法による外リンパ瘻の診断精度を、診断に関する研究のガイドラインであるSTARD statement（STAndards for the Reporting of Diagnostic accuracy studies）に準拠して評価する。

[3] このシステムを用いて臨床症例の検討を行う。対象疾患は、１．従来の診断基準による、特発性外リンパ瘻疑い例、２．耳かきによる外傷性鼓膜穿孔、３．側頭骨骨折、４．真珠腫性中耳炎内耳瘻孔、５．小児変動性難聴、6. 医原性等等の術中サンプルを採取してＣＴＰの存在を判定する。

[4] 新規に抗体を作成し、POCT(Point of care testing;臨床の現場での検査)のためにイミュノクロマト法、ELISA法の基礎研究を行う。

（倫理面の配慮）検体提供者に対しては試料採取前に研究の趣旨を説明し検体採取による不利益や危険性の無いことを説明、理解を得たうえで同意を得る。本学倫理委員会承認済みである。動物実験は、日本医科大学動物実験倫理委員会の審査を経て許可を受けて行う。動物実験にあたっては、倫理的な動物実験を遂行し、動物の愛護的扱いに留意する。

３．結果

[1] 現在の検査システムのクオリティーコントロール

標品の検出感度下限濃度a（0.27ng/ well）及びその1/2濃度bの点の2点を毎回検査サンプルと共に泳動し、aが陽性、bが陰性となった検査を精度管理上問題なしと判断して採用した。この方法で良好な再現性が得られ検査の精度管理が可能になった。この方法で得られた希釈外リンパの検出限界は平均0.022μl/well であった。

[2] 外リンパ瘻の診断精度（diagnostic performance）

外リンパ瘻では診断のゴールドスタンダードが確立しておらず、本検査法のような新規診断法の診断精度を正しく評価することは容易ではない。そこで、我々は下記のようにPLF確実例、non-PLF例を定義して、CTP検査を施行した。診断精度の評価に必要な症例数は統計学的（power analysis）に算出した。

	PLF確定例 （アブミ骨手術　人工内耳）	non-PLF例（PLFである可能性が極めてゼロに近い症例） （アブミ・蝸牛開窓前、伝音難聴の試験的鼓室開放）
CTP陽性	43	1

陰性　　4　　54

感受性　91.5% (95%CI , 83.5 - 99.5%)

特異性　98.2% (95%CI, 96.4 - 100.0%)

陽性尤度比　50.2

[3] 現行の LCCL３抗体を用いたウェスタンブロット法によるＣＴＰ検出法の検査結果

上記診断精度を確立したCTP検査法を用いて臨床例の検討を加えた結果を以下に記す。

■外リンパ瘻診断における留意事項

CTP検査は発症早期の方が陽性に出やすく、瘻孔が自然閉鎖すると陰性になる。また、本検査は特異性が高いのが特徴で、検査結果がＣＴＰ陽性の場合、間違いなく外リンパ瘻と言ええるが、結果が陰性だった場合下記の可能性が考えられる。

１．漏出なし　　外リンパ瘻ではない

２．瘻孔が自然治癒した　外リンパ瘻

３．間欠的漏出であった　外リンパ瘻

４．漏出外リンパが微量で検出できず

外リンパ瘻

上記を念頭に置いた上で、各カテゴリーにおける検査結果のまとめと、症例の検討、治療方針の概略を記す。

臨床の現場で遭遇する外リンパ瘻の分類

1. 頭部外傷性
2. 中耳外傷性
3. 特発性（いわゆる鼻かみ型）
4. 小児変動性難聴
5. 医原性
6. 真珠腫性中耳炎内耳瘻孔

7. 慢性　遅発性外リンパ瘻

頭部外傷性

検査結果と症例の検討

難聴の程度、頭部外傷の程度様々だが約３割の症例で陽性であった。骨折が全くないのに聾の２名はともにCTP陽性だった。外傷後の難聴が軽度でも陽性の症例もあり、頭部外傷では外リンパ瘻になりやすいと言える。

当院の人工内耳手術症例（両側高度難聴）の病歴を調べてみると、30例中２例が交通外傷（全身打撲、頭部打撲）後の失聴であった。受傷当時には担当医師から原因不明で治療法が無いと説明されていた。外傷性難聴の原因として外リンパ瘻があることは、北米では有名であるが本邦では、ほとんど認知されていないと思われる。

頭部外傷性外リンパ瘻治療方針

頭部外傷後の難聴では外リンパ瘻を積極的に疑い治療すべきである。CTで異常が無いからと言って、外傷と関連が無い、治療法が無いと思ってはいけない。内耳窓閉鎖手術の治療効果については今後の検討課題である。内耳に骨折があると、将来的に同部位が3rd Mobile Window (Minor LB)となる可能性があり、骨折部位の修復は積極的に行う意義があろう。

2. 鼓膜・中耳外傷　　５／１４人　陽性

検査結果と症例の検討

本邦では耳かきが日常習慣的に行われており、中耳（鼓膜、耳小骨）外傷の最も多い原因となっている。当然ながら、アブミ骨に耳かきがあたるアブミ外傷が外リンパ瘻をきたしやすい。しかしアブミ付近に鼓膜外傷があっても、外リンパ瘻をきたしているとは限らず、CTP検査で外リンパ漏出をきたしたのか、それとも単にアブ

ミ骨に外力が加わっただけなのか判定できる。外リンパ漏出をきたさなくても、外力が加わっただけで難聴をきたすことは以前より鼓室形成術における合併症などで広く知られており、この場合の原因としては音響外傷、内耳への直接の影響が考えられる。

アブミ骨直達外傷に関して、臨床上役立つ新知見を列記する。

急性例；聴力が正常で、めまいを主訴とするCaseが存在する。また進行性に聴力が悪化しない症例でも、めまいの増悪が病態の進行を敏感に反映する。

慢性化する場合；漏出が続いていてもゆっくりとした漏出なら聾にならない。過去に報告されている通り、長時間放置すればいずれ聾になることが予想される。

アブミ骨輪状靭帯損傷・陥入　治療方針

外リンパ漏出の持続があるか否か、すなわちCTP陽性かどうかが決め手となる。

急性例　アブミ骨を整復すべきか否か、迷う症例がある。すなわち輪状靭帯周囲組織が修復されて自然治癒する場合があるからである。受傷後、外リンパの漏出が持続していれば迷わず手術する。

慢性例　外リンパ漏出が続くと、数年の経過で聾になることが予想される。聾になる前にこれを診断して手術を行う必要がある。外傷部位の結合組織が修復され、漏出が停止していれば保存的に治療することも可能である。ただし、ここが3rd Mobile Windowとなっていれば慢性めまいの原因となる。

3．特発性外リンパ瘻

検査結果と症例の検討

本邦の厚労省急性高度難聴研究班　診断基準に基づいて診断された症例である。

病歴上、誘因が一切無い真の特発性外リンパ瘻も存在した。この症例は、水様性耳鳴、聴力・眼振の変動から外リンパ瘻が疑われ内耳窓閉鎖術を行った。突発性難聴と診断されている症例の中に、外リンパ瘻が含まれているのは間違いないことが、初めて科学的に証明されたと言える。北米では特発性外リンパ瘻が疾患として認められておらず、未だに議論の対象となっている。本邦の厚労省急性高度難聴研究班が診断基準を作成した当初の目的は、突発性難聴の鑑別診断として外リンパ瘻を診断し治療しようということであったが、その目的を側面から支持する結果となった。

特発性外リンパ瘻の発症リスクが高いのは、外気・水圧変動と高度炎症である。具体的には、飛行機搭乗、スキューバダイビング、そして高度炎症を伴う好酸球性中耳炎であった。中耳炎罹患中、もしくは、飛行機を乗り継ぐ間に中耳炎（航空性中耳炎）を発症し、そのまま旅行を続けることで外リンパ瘻が発症した症例があった。また、両側好酸球性中耳炎による高度慢性中耳炎症がある症例で、鼻かみにより突然聾なった症例もあった。

特発性外リンパ瘻治療方針

今後早期治療と聴力改善の関係、特発性外リンパ瘻でCTP陽性と確定診断された症例の詳細な解析を行うことで、治療方針を検討する予定である。

4．小児変動性難聴の中にも外リンパ瘻が存在した。中耳の微細な奇形に伴って外リンパ瘻が発症し、変動性・進行性難聴を呈する可能性があることは以前からピッツバーグ小児病院ブルーストーンが提唱していた。本研究で、この疾患が実際に存在することが証明された。

5.医原性外リンパ瘻

耳鼻咽喉科診療で行われる耳管通気治療による医原性外リンパ瘻と思われる症例を3例経験した。病歴上、通気直後から発症した３症例で、高度難聴となり補聴器を装用した症例、すでに数年前に聾となっており人工内耳埋め込み術を行った症例、進行性の高度難聴となり内耳窓閉鎖手術でほぼ正常まで改善した症例である。病歴上通気治療が原因になっていると考えられる。

6. 真珠腫性中耳炎内耳瘻孔

中耳真珠腫合併症の内耳瘻孔は5-10%の頻度で存在する。瘻孔の深達度は本疾患の治療を行う上で術後合併症の発症を左右する非常に重要なポイントである。Milewskiは瘻孔の大きさと深度によってこれを３度に分類した。１度は骨迷路が破壊されても内耳膜が保たれている、２度は内耳膜が開放され(a)外リンパ漏出なく膜迷路は正常、(b)外リンパ漏出があり膜迷路の障害がある、３度は膜迷路の破壊がある、である。実際の術中所見を観察してもその深達度を正確に判定することは難しく、１度なのか２度なのか、実際に漏出があるのかないのか、正確に判定することは困難である。なぜなら瘻孔局所を操作して正確な判定を行おうとすればそれがすなわち内耳膜損傷、内耳障害をもたらすからである。我々は、外リンパ特異的蛋白CTPにより外リンパ漏出の有無を判定し、内耳瘻孔の深達度診断の参考にすることができた。術中所見で瘻孔が大きく骨欠損が明らかでも、内耳膜が保たれており、外リンパ漏出は見られなかった症例がある一方で、骨欠損が小さくても内耳膜が欠損し、外リンパが漏出していた症例があった。

深達度の正確な判定は、真珠腫による内耳機能障害のメカニズムの解明、術後の機能障害の予測、予防、治療など様々な分野に重要な示唆を与えると思われた。

7. 慢性・遅発性症例が存在することが判明した。

①頭部外傷後、３ヶ月後に感音難聴が悪化し、CTPが陽性であった症例。

②小児期の内耳骨折が原因で、一側聾となり、24歳でめまいが発症し、遅発性内リンパ水腫と診断されていた。30歳時に当科で骨折線を骨パテで埋め、内耳（外リンパ）瘻孔を塞ぐことでめまいが完治した。CTPは陰性であり、外リンパ漏出が停止した状態で3rd mobile window となっていたと考えられる。

③交通外傷後、低音障害型難聴とめまい発作を反復し、メニエール病と診断されていた症例は内耳窓閉鎖手術で完治した。CTPは陽性であった。

[4] 今年度作成したIBL-ポリクローナル抗体の品質を検討

昨年度、様々な工夫をこらして作成した抗ペプチドポリクローナル抗体（IBL-B2, IBL-C）は、本検査開始当時使用していたLCCL３抗体と比べて特異性・感受性ともに大きく改善した。この抗体を用いてイミュノクロマト法開発に着手した。イミュノクロマト法では変性処理を加えないNative CTPを検出するが、基礎研究の結果、本方法は実用化には適さない事が判明した。現在、部分的な変性処理を加えたCTPを検出するELISA法を開発中である。本方法は、cut off値の設定により明確な判定基準が設定できることから検査者の熟練度が低くても施行可能であり、本検査の広い普及が可能となる。

４．考察　（研究成果の評価）

１）達成度について

ウエスタンブロット法で、臨床サンプルの検討を開始した。今年度は１５０検体を検査（国内24病院）さらに韓国の検体を検査し、北米小児病院と共同研究が開始され

た。検査依頼があった。

当初計画ではＨ１９年度より一般の病院からの検体受付を開始する計画であったが、計画を前倒しして、Ｈ１８年度すでに一般病院からの検体受けを開始した。現在その結果に基づいて治療指針を作製中である。

また、検査の迅速化、のためELISA法の基礎研究を行っている。

２）研究成果の学術的意義について

我々は外リンパ瘻の診断技術の開発ならびに診断マーカーＣＴＰの基礎研究を同時に進行させている。　今まで不可能であった外リンパ瘻の確定診断が世界で初めて可能になり、その医学的意義は非常に高い。厚生省特定疾患急性高度難聴調査研究班が定めた外リンパ瘻診断基準に基づき診断された「特発性外リンパ瘻」症例からＣＴＰが検出されたことで、従来その存在を疑問視する意見も根強かった特発性外リンパ瘻症例の存在を改めて生化学的に確定診断した。また、以前は難聴の発症機序が特定できなかった様々な症例からもＣＴＰが検出され、その手術時期、手術適応の判断に大きく寄与する事ができる。

今まで原因のはっきりしなかった外傷性難聴、小児の変動性感音難聴、突発性難聴、メニエール病などに外リンパ瘻が含まれている事を明らかにした。さらに、めまいのみで難聴が無い外リンパ瘻も存在し、今までの常識を覆す結果だった。さらに、耳管通気治療による医原性外リンパ瘻と思われる3症例（病歴上、通気直後から発症した症例であり2/3症例がCTP陽性）を経験した。これらの結果は、難聴診断治療のEBM確立に多大なる貢献をすると期待されている。

いままで主観的な判断に頼っていた外リンパ瘻診断を客観的に診断できればマルチセンタースタディーが可能になり、新たな診断・治療指針作成が可能になる。治癒率の向上はきたるべき超高齢化社会におけるコミュニケーション障害の予防に貢献する。

３）研究成果の行政的意義について

外リンパ瘻は手術により完治が望める疾患である。本検査の普及により早期診断・治療が可能になり、治癒率が大きく改善し、保険医療の適正な運用をもたらす。後遺障害に悩む症例が減り、健康で労働可能な成人数を増加させる。

難聴・めまい疾患の診断・治療のEBM確立に多大なる貢献をする。その結果、適切な医療が施行され無駄な投薬、通院、入院が減り、医療費の適正使用を推進する。

４）その他特記すべき事項について

平成18年度、厚生労働省感覚器障害研究事業　尤も実り多い研究に選ばれた。

５．結論（まとめ）

本検査は世界で初めて外リンパ瘻の生化学的確定診断を可能にするものであり、国内外の臨床家、基礎研究者から多くの注目を集めている。今後、本研究成果を広め、国内外でマルチセンタースタディーを行う予定である。

外リンパ瘻は的確な診断で大きな治療効果が期待できる疾患である。耳鼻咽喉科・神経耳科の診断が患者のQOL、後遺症の有無を大きく左右する。本研究事業の成果を広く普及させることで大きな具体的な効果が望める。

Ｆ．健康危険情報

特になし

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H．知的財産権の出願状況

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別紙４

研究成果の刊行に関する一覧表

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著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版年	ページ
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Mizuta K, Ikezono T, Iwasaki S, Araia M, Hashimotoa Y, Pawankar R, Watanabe T, Shindob S, Mineta H.	Ultrastructural co-localization of cochlin and type II collagen in the rat semicircular canal.	Neurosci Lett.	21;434(1)	p. 104-7.	2008
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Shindo S, Ikezono T, Ishizaki M, Sekiguchi S, Mizuta K, Li L, Takumida M, Pawankar R, Yagi T.	Spatiotemporal expression of cochlin in the inner ear of rats during postnatal development.	Neurosci Lett.	444	148-152	2008

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Spatiotemporal expression of Cochlin in the inner ear of rats during postnatal development

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ABSTRACT

Cochlin (encoded by *COCH*) constitutes 70% of non-collagenous protein in the inner ear, and the expression of cochlin is highly specific to the inner ear. Eleven missense mutation and one in-frame deletion have been reported in the *COCH* gene, causing hereditary progressive sensorineural hearing loss and vestibular dysfunction, DFNA9. These data imply that cochlin should bear an essential and crucial role in the inner ear function. However, the role of cochlin has not been fully clarified. We have investigated the spatiotemporal expression of cochlin in the inner ear of rats during postnatal development to better understand the functional role of cochlin. By immunohistochemistry, cochlin expression was faint in the cochlea and vestibule on the 6th day after birth (DAB6). At DAB70, strong expression of cochlin was detected in the spiral limbus and spiral ligament within the cochlea, and in the stromata of the maculae of otolithic organs and crista ampullaris within the vestibule. Immunoreactivity for cochlin increased during the postnatal development. Western blot analysis also showed an increase in the expression of cochlin isoforms. Furthermore, the dominant isoform of cochlin expressed changed from p63s to p40s between DAB24 and DAB70. These results suggest that the expression of cochlin may be related to the maturation of inner ear function, and the change in isoforms of cochlin expressed will provide important insight into the understanding of both cochlin function and formation of cochlin isoforms. This is the first to report about the spatiotemporal expression of cochlin in the developing rat inner ear.

The protein product of *COCH* gene, cochlin [8,9,13,14,16,19,18], is an extracellular matrix protein, and a major constituent of the inner ear, comprising 70% of the non-collagenous inner ear protein [7], and the expression of cochlin is highly specific to the inner ear [14]. Eleven missense mutation and one in-frame deletion have been reported in the *COCH* gene, causing hereditary progressive sensorineural hearing loss and vestibular dysfunction, DFNA9 [2–6,17,23,26]. In two-dimensional gel electrophoresis, cochlin in the inner ear tissue is composed of 16 different protein spots, with charge and size heterogeneity, and is classified into three groups (p63s, p44s, p40s) [7]. In addition, a short isoform of cochlin named cochlin-tomoprotein (CTP) was identified in the periylmph [9]. The deduced amino acid sequence of full-length cochlin (p63s) is a mosaic molecule consisting of a secretion signal peptide followed by two different types of domain, a Limulus factor C, cochlin and late gestation lung protein (LCCL) module and two von Willebrand factor A (vWF-A)-like domains, which are also found in combination with other motifs in proteins with diverse function [21]. p63s has an LCCL module and two vWF-A like domains, whereas p40s and p44s lack the LCCL module [7]. Interestingly, CTP, a 16-kDa short isoform that contains only an LCCL module without any vWF-A like domains, is found in the perilymph [9] (Fig. 1). Motif analysis of the *COCH* gene has suggested that cochlin may have a role in host defense through antibody-independent innate immunity (via its LCCL module) and may be critical for the highly structured architecture of the sensory organ (via its vWF-A like domains) [25]. These data imply that cochlin should play an essential and crucial role in the inner ear function. However, the functional role of cochlin has not been fully clarified. To better understand the function of cochlin, we have focused on the spatiotemporal expression

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of cochlin. Knowing when, where, and to what extent a protein is expressed in the developing inner ear will provide important clues to protein function. Here we demonstrate the spatial and temporal expression of cochlin in the rat inner ear during postnatal development using a cochlin-specific antibody. This is the first to report about the spatiotemporal expression of cochlin in the developing inner ear using immunohistochemistry and Western blot analysis.

Wistar rats were used at day after birth (DAB) 6 and 70 ($n = 5$ for each group) for immunohistochemistry and at DAB13, 17, 20, 24, and 70 ($n = 6$ for each group) for Western blot analysis. Each rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) according to the ethical regulations for treatment of animals. In this study, we used the anti-vWF-A1 antibody that recognizes all three cochlin isoforms expressed in the inner ear tissue, and this antibody is referred to here as the anti-cochlin antibody (Fig. 1). The generation of this antibody has previously been described [9,18]. In brief, a 19-amino-acid (KADIAFLIDGSFNIGQRRF) peptide corresponding to residues 163–181 in the vWF-A1 domain was used to generate the antibody. The specificity of this antibody for the corresponding antigenic peptide was confirmed by dot blot analysis and a peptide absorption test (data not shown).

For immunohistochemistry, rats were sacrificed after intracardiac perfusion with cold saline, followed by 4% paraformaldehyde fixative. The temporal bones were excised and fixed for 2–3 h. Temporal bones were then decalcified in phosphate-buffered saline (PBS, pH 7.4) with 5% EDTA at −4 °C for 4–7 days. Temporal bones were embedded in paraffin, and serial sections of 3–4 μm thickness were cut using a microtome (Rotationsmikrotom, Leika, Nussloch, Germany). The immunoperoxidase method was used for the detection of cochlin using the Vectastain ABC kit (Vector laboratories, Burlingham, CA, USA). Paraffin sections were deparaffinized, and the sections were then treated with 0.3% H_2O_2 in methanol in order to block the endogenous peroxidase activity. After treatment with 10% normal goat serum, the sections were incubated with 1:1000 dilution of the anti-cochlin antibody overnight at 4 °C. The sections were then incubated for 60 min with a 1:500 dilution of the biotinylated goat anti-rabbit IgG antibody (E0432, Dako, Denmark) and then treated with the streptavidin-biotin-peroxidase complex (Vector laboratories, Burlingham, CA, USA). After rinsing in PBS, the reaction was developed with the substrate 3,3′-diaminobenzidine, rinsed twice in distilled water, and counterstained with Mayer's hematoxylin. Preimmune serum was used instead of the primary antibody as negative control.

For Western blot analysis, membranous cochlear and vestibular labyrinths were dissected from the rat inner ear. Protein lysates were prepared by a solubilization mixture containing 0.5% SDS and protease inhibitors (Complete mini EDTA (−), Boehringer-Mannheim, Mannheim, Germany) in 10 ml of PBS. We homogenized inner ear tissues using a mortar and pestle. The homogenate was centrifuged at 1000 × g for 15 min and the supernatant was stored at −70 °C until use. Protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). 0.1 μg of total inner ear protein was loaded onto a 10% SDS-polyacrylamide gel. Prior to loading, samples were diluted with 0.188 M Tris buffer to a total volume of 10 μl and then mixed with 5 μl of sample buffer (0.188 M Tris buffer, 2.39 mM SDS, 30% glycerol, and 15% of 2-mercaptoethanol). The samples were heated to 98 °C for 5 min and then loaded into each lane of the gel. Electrophoresis was performed on gels with 10% polyacrylamide (PAG mini Daiichi, Daiichi Pure Chemicals, Japan) in running buffer (25 mM Tris, 192 mM glycine, 1 g/l SDS, pH 8.3) at 20 mA for 2 h. The separated proteins were electrophoretically transferred onto a PVDF membrane (Immobilon-PSQ, Millipore, USA) using an Atto HorizBlot semi-dry transfer unit with a discontinuous buffer system, as recommended by the manufacturer (ATTO, Japan). Non-specific binding was blocked by incubating the membranes overnight at 4 °C in 5% skimmed milk and 0.2% polyoxyethylenesorbitan (Tween-20) dissolved in PBS. Membranes were then incubated in PBS containing 1% skimmed milk and 0.1% Tween-20 for 2 h at room temperature with the primary anti-cochlin antibody diluted at 1:2000. After being washed in 0.05% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with Horse Radish Peroxidase-labeled goat anti-rabbit IgG (P0448, Dako, Denmark) diluted at 1:5000 in the same buffer used for the primary antibody reaction. The membranes were washed again and the reaction was developed with a chemiluminescence reaction kit (ECL advance, GE Healthcare, England). The level of protein expression of each isoform was measured by a densitometer LAS-3000 (Fuji Film, Japan), and the relative amount of each was calculated relative to the expression of DAB70 taken as 100. For normalization, samples were also analyzed using an anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (IMGENEX, USA) as the primary antibody.

At DAB6, cochlin expression was not detected in the cochlea except for weak immunoreactivity in the spiral limbus (Fig. 2a). At DAB70, cochlin was highly expressed in the spiral ligament, the spiral limbus, the cells lining Rosenthal's canal and the channels of the osseous spiral lamina (Fig. 2b). In contrast, the organ of Corti, stria vascularis, Reissner's membrane and spiral ganglion cells were all negative for cochlin staining.

At DAB6, immunoreactivity for cochlin was not detected in the crista ampullaris (Fig. 2d). At DAB70, strong immunoreactivity for cochlin was detected in the stroma of the crista and in the area

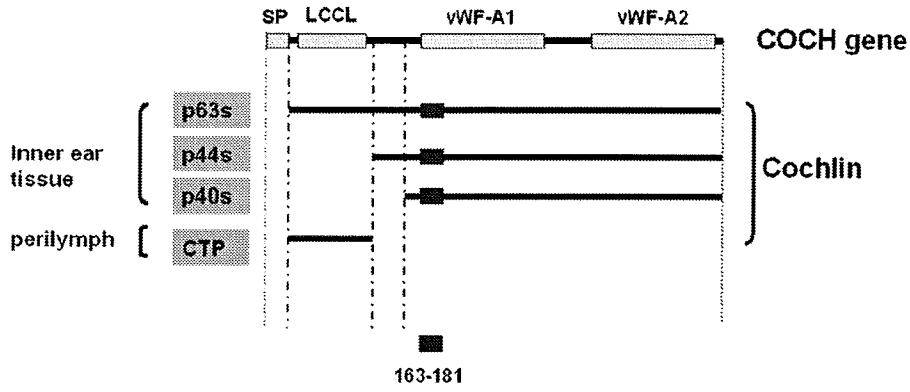

Fig. 1. Schematic representation of the deduced amino acid sequence of human *COCH* gene, which encodes the protein cochlin, shows a predicted signal peptide (SP), followed by a region in limulus factor C, cochlin and late gestation lung protein Lgl1 (LCCL) domain, and two vWFA-like domains (vWFA1 and vWFA2). The cochlin antibody used in this study was made against a small peptide in the N-terminus of the vWFA1 domain (black bold bar; amino acid residues 163–181), shown in the figure. This antibody recognizes all three isoforms of cochlin in the inner ear tissue (p63s, p44s, and p40s), and does not recognize cochlin-tomoprotein (CTP) in the perilymph.

beneath the planum semilunatum, dark cells and neurosensory epithelium. In contrast, apical surface of the sensory epithelia, including the cupula, subcupular space, sensory cells, transitional cells, dark cells, and supporting cells were negative for cochlin staining (Fig. 2e).

At DAB6, cochlin expression was not detected in the otolithic organs (Fig. 2g). At DAB70, strong expression of cochlin was detected in the stromata of all maculae, the lining of the membranous labyrinth and the channels in the bony labyrinth. In contrast, the apical surface of sensory epithelia, including the otoconia, gelatin layer, submembranous space, sensory cells, and supporting cells lacked cochlin staining (Fig. 2h).

We examined the expression of the cochlin isoforms by Western blot analysis of the inner ear during postnatal development of the rat (Figs. 3 and 4). We performed three independent experiments. Representative data of three different experiments are shown in Fig. 3, and the average relative expression of each isoform is as shown in Fig. 4. At DAB13, no immunoreactivity for cochlin was detected. The expression of cochlin gradually increased from DAB17 to DAB24. We also observed changes in the expression pattern of each isoform. The level of expression of p63s was the highest among all three isoforms between DAB17 and DAB24. At DAB70, by contrast, the expression of p63s had decreased and p40s was the most dominant isoform. The expression of p44s was lower than that of the other isoforms throughout the developmental process.

Mutations in the *COCH* gene have been shown to correlate with DFNA9, an autosomal dominant nonsydromic hearing disorder that causes sensorineural deafness and vertigo [11,15,22,20]. Sequence analysis of individuals with DFNA9 has demonstrated the existence of eleven missense mutations and one in-frame deletion in *COCH* [2–6,17,23,26]. The onset of symptoms is relatively late and eventually leads to profound deafness and vestibular failure. Vestibular disorder may occur and progressive hearing loss can show fluctuations and asymmetry.

We have conducted a series of studies elucidating the different cochlin isoforms and the pathogenesis of DFNA9. In the previous studies describing the cochlin isoform structure in the inner ear, we used 4 different antibodies [9]. In the present work we used the anti-cochlin antibody (previously named anti-vWF-A1 antibody), which showed the best performance in the immunohistochemical analysis of cochlin expression in the rat inner ear during postnatal development. Studying gene expression during the postnatal development of the rat inner ear provides certain advantages, because functional maturation of the inner ear occurs during the postnatal period. In the present studies, we used the DAB70 rat as an adult as in other studies published elsewhere [1,24]. As a result, we found a marked change in cochlin expression in the connective tissues of the inner ear during postnatal development (Fig. 2). These results suggest cochlin might play an important role in maturation of the inner ear, and areas of the inner ear that expressed cochlin also contain various proteins that are thought to play an important role of the inner ear function [12,16,21]. Among these proteins, we interest in the correlation between cochlin and type II collagen. Our recent immunohistochemical study of cochlin and type II collagen in the rat semicircular canal using electron microscopy has shown that the co-localization of these proteins in the same fibrillar substance [16]. These results indicate that cochlin may play a role in the structural homeostasis of the inner ear acting in concert with the fibrillar type II collagen bundles.

We used Western blot analysis for a temporal study of cochlin expression during development. Cochlin expression was not detected at DAB13, but gradually increased from DAB17 to DAB24, when the total amount of cochlin reached adult levels (Figs. 3 and 4). These results indicate that temporal expression of

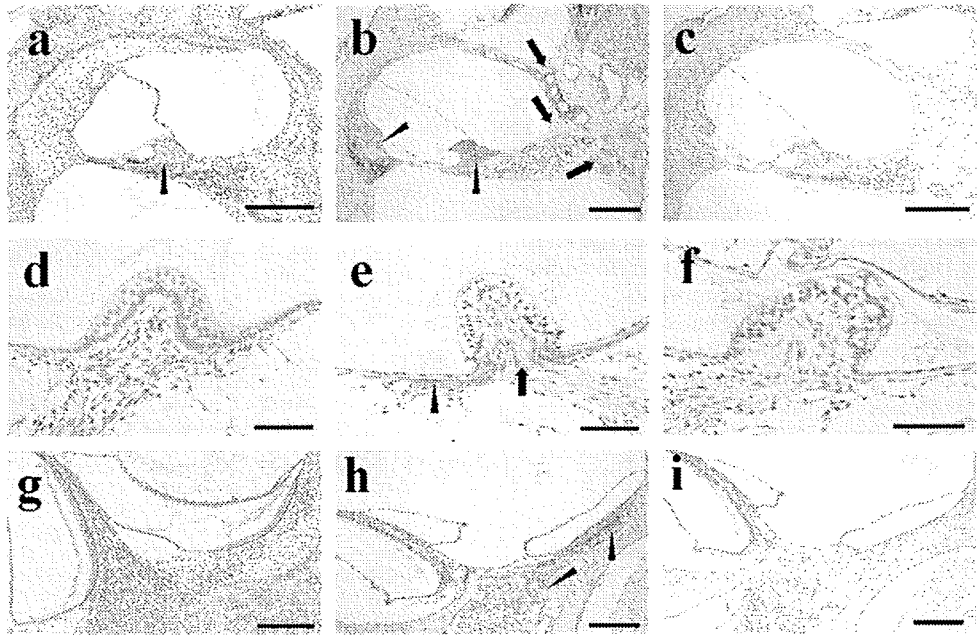

Fig. 2. (a–c) Representative immunohistochemistry for cochlin expression in the developing rat cochlea. (a) At 6th day after birth (DAB6), immunoreactivity for cochlin was faint in the spiral limbus (arrow head). (b) At DAB70, immunoreactivity for cochlin was strong in the area of the spiral limbus (arrow head), spiral ligament (arrow head), cells lining Rosenthal's canal and perivascular rings around blood vessels in the modiolus (arrow). (c) Negative control of DAB70 did not show immunoreactivity for cochlin in any of these structures. Scale bars in (a)–(c) = 200 μm. (d–f) Representative immunohistochemistry for cochlin expression in the developing rat crista ampullaris of the horizontal semicircular canal. (d) At DAB6, no immunoreactivity for cochlin was detected. (e) At DAB70, immunoreactivity for cochlin was strong in the stroma of the crista (arrow), the area beneath the planum semilunatum and the dark cells (arrow head). (f) Negative control of DAB70 did not show immunoreactivity for cochlin in any of these structures. Scale bars in (d)–(f) = 50 μm. (g–i) Representative immunohistochemistry for cochlin expression in the developing rat maculae. (g) At DAB6, no immunoreactivity for cochlin was detected. (h) At DAB70, immunoreactivity for cochlin was strong in the stromata of all maculae and the channels in the bony labyrinth (arrow head). (i) Negative control of DAB70 did not show immunoreactivity for cochlin in any of these structures. Scale bars in (g)–(i) = 200 μm.

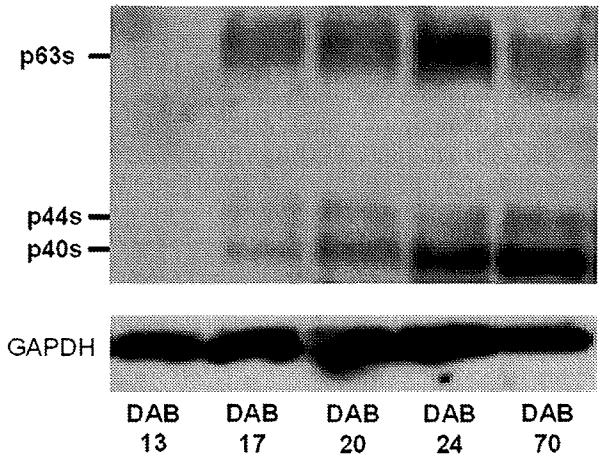

Fig. 3. Representative image of Western blot analysis of the rat inner ear homogenates from DAB13 to DAB70 ($n=6$ for each group; 0.1 μg per lane). p63s was not expressed at DAB13, but its expression gradually increased up to DAB24. At DAB70, however, expression of p63s was lower than at DAB24. p44s was weakly expressed from DAB17 to DAB70. p40s was not expressed at DAB13, but its expression gradually increased up to DAB70.

cochlin in the inner ear is similar to that of other proteins expressed in the fibrocytes of inner ear, such as connexin 26 [24], Na-K ATPase [27], and GLAST [10]. In the rat, temporal expression of these three proteins shows a sigmoidal time course with a rapid increase during DAB10 to DAB20 when the auditory function, as measured by electrophysiological methods, reaches that of the adult. These results indicate that cochlin might interact with other functional proteins in the inner ear, and might play an important role in maturation of the inner ear. Furthermore, Western blot analysis suggests that expression of the cochlin isoforms has unique characteristics during postnatal development. The expression of p63s was the highest, and the total amount of cochlin gradually increased until DAB24. From DAB24 to DAB70, there were no marked changes in the total amount of cochlin, but the dominant isoform of cochlin shifted from p63s to p40s (Figs. 3 and 4). Because the morphology and function of the inner ear reach adult levels by DAB24 in the rat, the isoform shift between DAB24 and DAB70 may provide important information for our understanding of cochlin function. One possibility is that the full-length cochlin (p63s) may be metabolized to form shorter isoforms (p40s, p44s and CTP); thus, the relative amount of the p63s isoform would become smaller. In addition, the LCCL module-containing isoform in the inner ear, full-length cochlin (p63s), may play a critical role in the early stage of postnatal development, whereas the shorter isoforms, p40s and p44s, may serve as extracellular matrix protein in the mature inner ear (Fig. 1). We are now performing further studies to clarify the transcriptional and post-translational modification of cochlin expression during development of the inner ear.

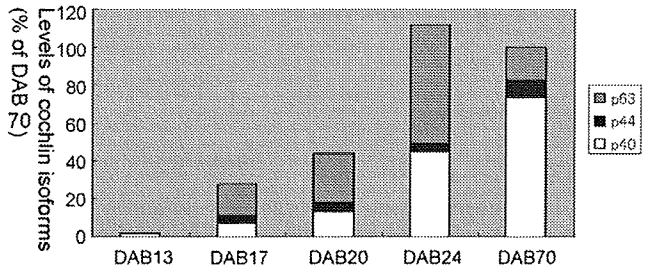

Fig. 4. Levels of expression of the cochlin isoforms in the rat inner ear during postnatal development. We performed 3 independent experiments. The level of expression of each isoform was measured by a densitometer relative to the expression at DAB70 taken as 100%. The data are an average of 3 independent experiments except for DAB20, where the average data of 2 experiments are given.

Acknowledgements

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Ultrastructural co-localization of cochlin and type II collagen in the rat semicircular canal

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Abstract

Cochlin and type II collagen are major constituents of the inner ear extracellular matrix. To investigate the morphological relation of cochlin and type II collagen in the rat semicircular canal, immuno-electronmicroscopic analysis was performed using the post-embedding immunogold method. Immunolabeling for cochlin was detected in the fibrillar substance underlying the supporting epithelium of the sensory cells and beneath the epithelial cells facing the endolymph in the semicircular canals. Immunolabeling for type II collagen was observed in the same fibrillar substance in the subepithelial area. The co-localization of cochlin and type II collagen in the fibrillar substance in the subepithelial area indicate that cochlin may play a role in the structural homeostasis of the vestibule acting in concert with the fibrillar type II collagen bundles.

Keywords: Hereditary hearing impairment; DFNA9; *COCH*; Vestibular organ; Immunogold method

A number of genetic loci for familial syndromic or non-syndromic sensorineural hearing losses have been mapped [22]. Mutations of the *COCH* gene, which encodes cochlin, are associated with an autosomal dominant progressive sensorineural hearing loss and vestibular disorders including Meniere's disease like symptoms at the DFNA9 locus [3,6,7,15,16,19,23,24]. Cochlin has von Willebrand factor type A (vWFA) like domains which are present in a variety of secreted proteins, both soluble and insoluble, are involved in the production of extracellular matrix and have been shown to bind fibrillar collagens, glycoproteins and proteoglycans [4,5]. Although, the function of cochlin is not yet known, our recent studies demonstrated immunoreactivity for cochlin in the type II collagen rich areas of the vestibular organ [10]. Type II collagen is also an extracellular matrix material in the acellular structures and subepithelial connective tissue of the inner ear [21]. The interaction between cochlin and type II collagen has been suggested in histopathological findings of DFNA9 [14]. To precisely examine the morphological relation of cochlin and type II collagen, in the present study we performed immuno-electronmicroscopic evaluation of cochlin and type II collagen using the immunogold method.

Rabbit polyclonal antibody used in this study was raised against vWFA like domain 1 of cochlin and has been previously described [11]. Briefly, a 19-mer (KADIAFLIDGSFNIGQRRF) peptide corresponding to residues 163–181 in the vWF-A1 domain was used to generate antibody. This sequence is present in all three cochlin isoforms and is completely homologous with the sequences in both human and mouse proteins, as deduced from the sequence of the corresponding genes and the sequence of bovine cochlin. This antibody recognizes all the three cochlin isoforms in the inner ear. The specificity of this antibody for the corresponding antigenic peptide was confirmed by dot blot analysis and a peptide absorption test (data not shown). Antibody against type II collagen (Millipore, Billerica, MA) was purchased commercially.

Wistar rats (100–200 g body weight) were anesthetized with pentobarbital (50 mg/kg body weight, i.p.) according to the ethical regulations for treatment of animals. A fixative of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate

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buffer (PB, pH 7.4) was perfused from the left ventricle, and then the temporal bones were isolated. The temporal bones were immediately immersed in the same fixative. Thereafter, the vestibular organs were dissected under a stereomicroscope and further fixed for 2 h at 4 °C. The specimens were immersed into 50 mM NH_4Cl in 0.1 M PB for 2 h and were washed in 0.1 M PB overnight at 4 °C. Embedding in Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, PA) was done in accordance with the manufacturer's instruction with slight modification. Briefly, the specimens were dehydrated in a graded series of ethanol as follows: 30% ethanol for 30 min at 4 °C; 50% ethanol for 1 h at −20 °C; 70% ethanol for 1 h at −20 °C; 100% ethanol for 1 h at −20 °C. The specimens were then infiltrated with Lowicryl K4M at −20 °C; with 100% ethanol:Lowicryl K4M (1:1) for 1 h; with 100% ethanol:Lowicryl K4M (1:2) for 1 h; with 100% Lowicryl K4M for 1 h and with 100% Lowicryl K4M overnight. The tissue subsequently was embedded in Lowicryl K4M and polymerized by ultraviolet light irradiation for 24 h at −35 °C followed by 48 h at room temperature. Ultrathin sections were cut using an ultra-microtome and mounted onto nickel grids (400 mesh).

The grid with sections was immersed in a droplet (25 μl) of 1% bovine serum albumin (BSA; Sigma, St. Louis) 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 in a droplet of the optimal concentrations of rabbit antibodies against cochlin (3.3 μg/ml in BSA/PBS) or type II collagen (4 μg/ml in BSA/PBS), overnight at 4 °C. After rinsing in PBS, the sections were incubated in colloidal gold conjugated goat anti-rabbit IgG secondary antibody (15 nm in diameter, 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 counter stained with uranyl acetate for 3 min, and lead citrate for 30 s. These sections were then observed under a JEOL JEM-1220 electron microscope. Pre-immune rabbit IgG (4 μg/ml in BSA/PBS) was used instead of the primary as negative control.

Immunoreactivity for cochlin was seen in the fibrillar substance in the region beneath the sensory cells (Fig. 1) and subepithelial area in the crista ampullaris (Fig. 2a). Immunoreactivity was detected in the banded bundles which have a parallel array in the area of fibrocytes (Fig. 2b). No immunoreactivity was detected in any epithelial cells or fibrocytes of the crista ampullaris.

Immunoreactivity for Type II collagen was detected on the fibrillar substance in the subepithelial area of the crista ampullaris (Fig. 3). Immunoreactivity for cochlin and type II collagen was seen in the same fibrillar substance (Fig. 2 with Fig. 3, respectively).

Negative control using pre-immune IgG did not exhibit any immunoreactivity on the fibrillar substance of the crista ampullaris (Fig. 4).

The present study is the first to report the localization of cochlin, ultrastructurally. Here, we found that cochlin was co-localized with type II collagen in the fibrillar substance in the extracellular area of crista ampullaris. Temporal bone studies on DFNA9-affected subjects have revealed a unique histopathology showing loss of cellularity and aggregation of abundant homogeneous acellular eosinophilic deposits in the cochlear and vestibular labyrinths [13,15]. Recently, Robertson et al. conducted immunohistochemichal study of the DFNA9-affected inner ear, and found eosinophilic deposits in the collapsed ampulla that stained with the anti-cochlin antibody (the same antibody as used in the present study). Proteomic analysis of the same subject detected cochlin as one of the primary proteins [17]. This study showed that the eosinophilic substance was mutated cochlin itself and was not another component of the inner ear, a cochlin-interacting protein, or some other downstream effect of the *COCH* mutations. This work strongly supports the hypothesis of DFNA9 pathogenesis that mutated cochlin accumulates in the inner ear, present as acellular eosinophilic deposits, and this accumulation leads to neuroepithelial and neural degeneration in the inner ear.

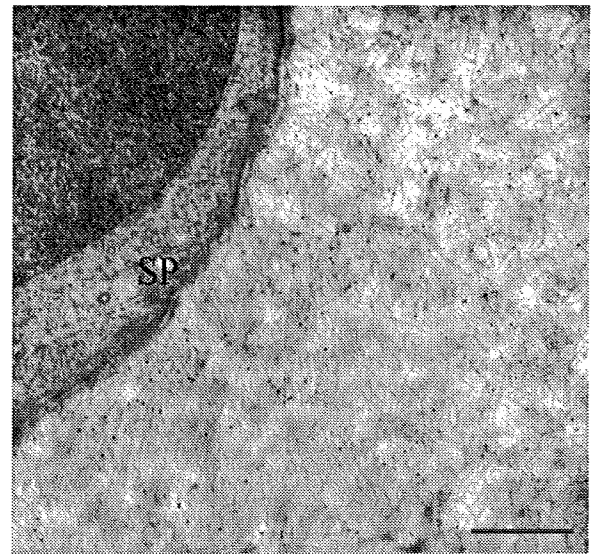

Fig. 1. Immuno-electronmicroscopy for cochlin expression in the inner ear. Immunolabeling for cochlin was seen on the fibrillar substance beneath the supporting cells in the crista ampullaris. SP = supporting cell of the sensory cells; bar = 1 μm.

A latticework of type II collagen fibers compose the extracellular matrix of cartilage with retaining of proteoglycan aggregate [9]. Co-localization of cochlin and type II collagen suggests that cochlin may also bind to the type II collagen to form an intact structure in the inner ear. An investigation of DFNA9 affected inner ear using electron microscopy has shown the absence of major fibrillar type II collagen [14] in the spiral ligament where cochlin is expressed abundantly in normal subjects [20]. The disturbance of the correct interaction between cochlin and type II collagen is one of the downstream effects of cochlin mutation, and results in loss of type II collagen fibers.

In DFNA9 affected inner ear, a thickening of the ampullary wall with abnormal eosinophilic substance is one of the striking findings in the vestibular organ. The thin layer under the epithelial cells of ampullary wall contains both type II collagen [12] and cochlin [10], indicating that the abnormal interactions between mutated cochlin and type II collagen may have contributed to the marked thickening of the wall. Interestingly, an

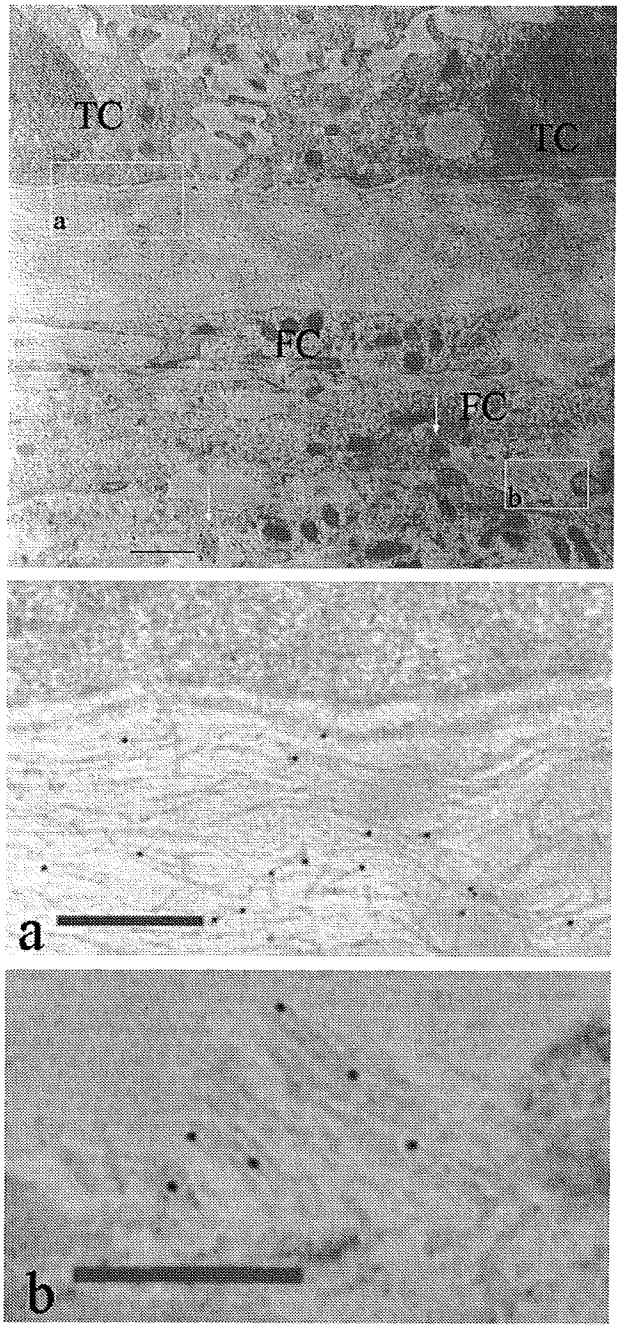

Fig. 2. Immuno-electronmicroscopy for cochlin expression in the inner ear. Immunolabeling for cochlin was seen on the fibrillar substance in the region beneath the transitional cells and on the banded bundles in fibrocytes area (arrows). Gold particles cannot be detected in any epithelial cells or fibrocytes of the crista ampullaris. TC = transitional cell; FC = fibrocyte; bar = 1 μm. Higher magnification of the open square (a) area in this figure. Gold particles labeled the fibrillar substance. Bar = 0.5 μm. Higher magnification of the open square (b) area in this figure. Gold particles were also seen in the banded bundles which have a parallel array in the area of fibrocytes. Bar = 0.5 μm.

increase in the levels of cochlin with a parallel decrease in type II collagen has been reported in the trabecular meshwork in human glaucoma as well as in an animal model of glaucoma (DBA/2J mice) [1,2]. The precise pathogenesis or genetic abnormalities in DBA/2J mice is not yet known, but DBA/2J mice also exhibit very early progressive sensorineural hearing loss [25], suggesting a relationship between auditory function and cochlin/type II collagen interactions.

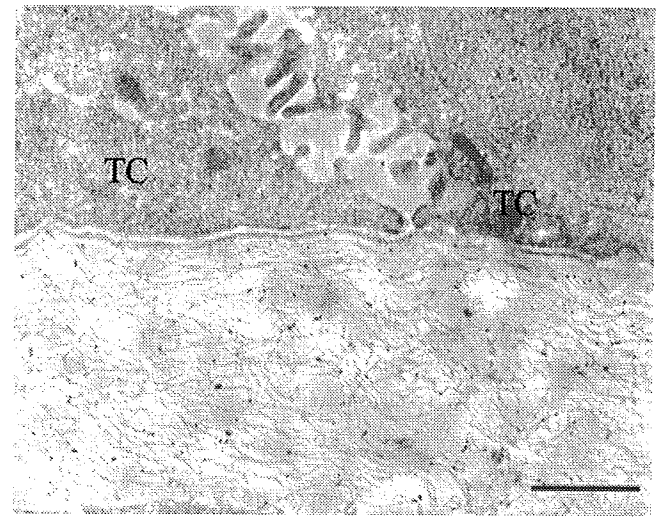

Fig. 3. Immuno-electronmicroscopy for type II collagen expression in the inner ear. Immunolabeling for type II collagen was seen on the fibrillar substance in the area of subepithelial cells in the semicircular canal. TC = transitional cell; bar = 1 μm.

In our previous study at the light microscopic level, cochlin staining was seen in the crista ampullaris of the semicircular canals and the stromata of the maculae of otolithic organs [10]. These areas also exhibited high expression of *COCH* mRNA, in particular in the fibrocytes underlying the sensory epithelium [20]. The current study indicates an absence of cochlin from fibrocytes and a presence of cochlin on the extracellular matrix, showing that cochlin is a secretory protein, which is in concert with the previous assumption. From a motif analysis, cochlin has two tandem von Willebrand factor A-like domains which are present in majority of extracellular matrix proteins [4,5]. Transient transfection of *COCH* gene into 293T or Hela cells reveled that cochlin is secreted out into the culture medium [8,18].

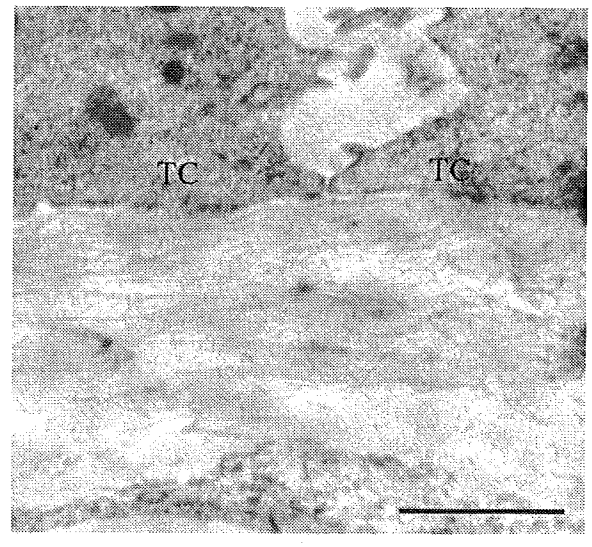

Fig. 4. Control. No gold particle was seen on the fibrillar substance in the region beneath the transitional cells. TC = transitional cell; bar = 1 μm.