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内耳エネルギー不全の病態解析に基づいた突発性難聴の新規治療法開発

平成１６～１８年度　総合研究報告書

主任研究者　松永　達雄

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主任研究者　　松永　達雄　　国立病院機構東京医療センター臨床研究センター
聴覚障害研究室長

研究要旨

突発性難聴は比較的発症頻度の高い難聴であるが、その病態は不明であり、発症後１ヶ月を過ぎた難聴には治療法がない。最終的な病態としては内耳循環障害による急性内耳エネルギー不全が指摘されている。本研究の目的は、我々の施設で化学的アプローチを用いて世界で初めて開発した急性内耳エネルギー不全の突発性難聴モデル動物を用いて、その聴覚・平衡障害の病態を分子レベルから解明し、有効な治療法を開発することである。

本研究では、突発性難聴モデル動物を中心に、聴覚、組織、組織内 ATP 量、アポトーシスと再生と炎症、小胞体ストレス関連分子/シャペロン分子/サイトカインの発現と機能、蝸牛外側壁 DNA マイクロアレイ解析、平衡機能障害と前庭組織、カスパーゼ阻害薬の予防・治療効果、骨髄間葉系幹細胞（MSC）移植による治療効果、蝸牛線維細胞と MSC との相互作用、蝸牛線維細胞の発生分化様式を検討した。

本研究結果より以下のことが明らかになった。突発性難聴モデル動物の難聴では蝸牛線維細胞の障害が主たる病変であった。これはカリウムイオンのリサイクル障害が本難聴の主たる病態であることを示唆する。蝸牛線維細胞にアポトーシスが生じ、その周囲の線維細胞で細胞増殖が認められ、それぞれ難聴発症と回復に関与していると考えられた。線維細胞障害に小胞体ストレス関連分子の関与を確認した。HSP70 および IL6 の顕著な発現増強が防御機構としての作用を有すると推測した。カスパーゼ阻害薬の予防的あるいは治療的投与は、いずれも組織障害と難聴の顕著な抑制効果を認めた。内耳に移植された MSC は、傷害部位へ生着し、聴覚改善を有意に促進した。MSC からの因子は未分化様蝸牛線維細胞の増殖を有意に促進し、蝸牛線維細胞からの因子は MSC の線維細胞への分化を誘導した。発生において各タイプ線維細胞の共通前駆細胞の存在が示唆された。

分担研究者　藤井　正人
国立病院機構東京医療センター
臨床研究センター聴覚平衡覚研究部長
分担研究者　小川　郁
慶応義塾大学医学部耳鼻咽喉科学教室
教授

A. 研究目的

突発性難聴は国内で年間約 25000 人発症する比較的発症頻度の高い難聴である。その病態は不明であり、高度の内耳性難聴が持続あるいは徐々に回復するが、発症後１ヶ月を過ぎて回復しない場合には治療法がない。また、平衡障害を合併する頻度も高い。原因は不明であるが、内耳循環障害とウイルス感染が多いと考えられており、ウイルス感染の場合でも蝸牛内毛細血管の内皮細胞腫張から結果的に内耳循環障害を生じる可能性が指摘されている。突発性難聴の有効な治療法がない理由として、直接患者の内耳を採取できないこと、動物モデルがないため病態の研究ができなかった点が挙げられる。我々の施設では化学的に内耳エネルギーの欠乏を起こすことにより世界で初めて急性内耳エネルギー不全による突発性難聴モデル動物を開発した（図 1)。本研究の目的は、この動物モデルを用いて、これまで不明であった急性内耳エネルギー不全の聴覚・平衡障害の病態を分子レベルから初めて解明し、その結果に基づいて有効な治療法を新規に開発することである。

B. 研究方法

急性内耳エネルギー不全の動物モデルを用いて以下の研究を実施した。詳細は平成 16-18 年の各年度の総括・分担研究報告書に記した。

1. 急性内耳エネルギー不全の程度と聴覚の経時的測定
2. 組織障害の経時的観察
3. bioluminescent assay による組織内 ATP 量の定量
4. 線維細胞アポトーシスとその後の再生の TUNEL 法と BrdU 免疫染色による同定
5. 小胞体ストレス関連分子/シャペロン分子/神経栄養因子についての RT-PCR による発現の網羅的スクリーニング、候補分子のリアルタイム PCR による mRNA 定量、 Western blotting による活性化型蛋白発現の同定、免疫組織化学による局在。蝸牛での小胞体ストレス誘導による聴覚障害の病態解明。
6. 蝸牛外側壁における DNA マイクロアレイによる遺伝子発現の網羅的かつ経時的発現の解析
7. 蝸牛外側壁での炎症系細胞およびサイトカインファミリーの経時的局在あるいは発現の変化
8. 平衡障害の病態を解明するための眼振、カロリック刺激に対する反応、姿勢および歩行、前庭内耳の組織像の解析
9. 蝸牛におけるカスパーゼ活性化の検討とカスパーゼ阻害薬の腹腔内投与による予 防あるいは経静脈投与による治療の検討
10. ラット骨髄間葉系幹細胞の内耳への移植による難聴治療効果および組織像の検討
11. 幼弱および成熟のマウスとラットの蝸牛外側壁線維細胞の分散培養法確立
12. 成熟マウス蝸牛の外側壁線維細胞と骨髄間葉系幹細胞との共培養による相互

の分化および増殖を促進する細胞分泌因子の探索

13. 発生における蝸牛外側壁線維細胞の分化様式の免疫組織学的検討。
14. 蝸牛感覚上皮の発生・再生に働く分子機構の解明

（倫理面への配慮）

本研究では、動物実験を行なうため、「ヘルシンキ宣言」、「大学等における動物実験について」、「国立病院機構東京医療センター動物実験指針」を遵守して進める。本研究は、事前に国立病院機構東京医療センター動物実験委員会の審査、承認による研究の適正性の確保を得て実施された。

C. 研究結果

研究方法の項目に対応して以下に記した。詳細は平成16-18年の各年度の総括・分担研究報告書に記した。

1. ミトコンドリア阻害薬の濃度により永続性難聴モデルと可逆性難聴モデルが作成された（図1）
2. 蝸牛線維細胞がまず障害され、その後血管条の萎縮と外有毛細胞および支持細胞の変性が認められた（図2）。この結果は 急性内耳エネルギー不全による難聴はカリウムイオンのリサイクル障害が主たる病態であることを示唆する（図3）
3. 永続性高度難聴の蝸牛では ATP 量が傷害 1 日後と7日後に正常蝸牛に比して約50%低下が認められた。
4. 外側壁とラセン板縁に限局性のアポトーシスが起こり、さらにその周囲の線維細胞で急激な細胞増殖による再生が認められた。
5. 蝸牛外側壁において小胞体ストレス関連分子の発現増強が認められ（図4）、さらに実験的に内耳に小胞体ストレスを誘導することで難聴発症を確認した。可逆性難聴モデルでは HSP70 の一過性発現増強が認められ蝸牛の保護因子である可能性が示唆された。
6. マイクロアレイ解析で炎症関連因子、アポトーシス関連因子、蛋白質分解酵素、酸化還元酵素などの遺伝子発現に動きが認められた。
7. IL6 の顕著な一過性の発現増強が認められ蝸牛の防御に関係する可能性が示唆された（図5）。
8. 高度の平衡機能障害および前庭有毛細胞障害を認めた。
9. 傷害部位における活性型カスパーゼの発現を認め、予防的カスパーゼ阻害薬投与により組織障害と難聴の顕著な抑制効果を認めた（図6）。さらに治療的カスパーゼ阻害薬投与でも有意な難聴の抑制効果を認めた。
10. 骨髄間葉系幹細胞（MSC）の内耳への移植により、傷害部位への生着と目的とする細胞の分化マーカー発現、聴覚の有意な改善が認められた。
11. 蝸牛線維細胞は 10%血清存在下で各タイプの線維細胞が増殖した。
12. 骨髄間葉系幹細胞（MSC）からの因子

（培養上清添加）は未分化様蝸牛線維細胞の増殖を有意に促進した（図 7）。一方、蝸牛線維細胞からの因子（培養上清添加）は骨髄間葉系幹細胞（MSC）の線維細胞への分化を効率よく誘導した（図 8）。

13. 発生における各タイプ別の線維細胞マーカーの発現パターンより、各タイプに分化する共通前駆細胞の存在が示唆された。

14. 生後 1 日から 14 日齢にかけての蝸牛上皮中の前駆胞数低下と同時期に遺伝子転写が負に調節される遺伝子 Pou3f3/Brn1 を蝸牛上皮中に見出した。本遺伝子は感覚細胞へ分化転換能をもつ支持細胞特異的に発現しており、支持細胞機能との関連が示唆された。

図 1　突発性難聴モデル動物を開発

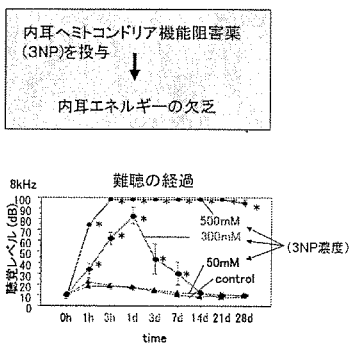


図 2　最初に蝸牛線維細胞が傷害

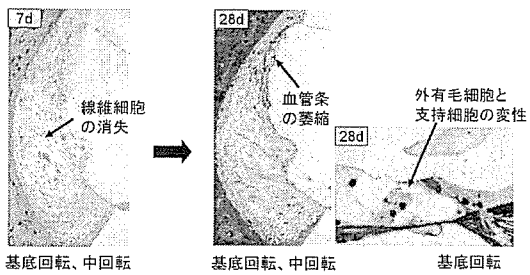


図 3　K^+イオンのリサイクル障害による難聴

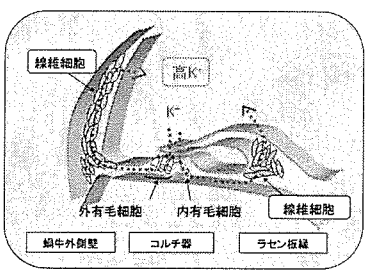


図 4　アポトーシスに小胞体ストレスが関与

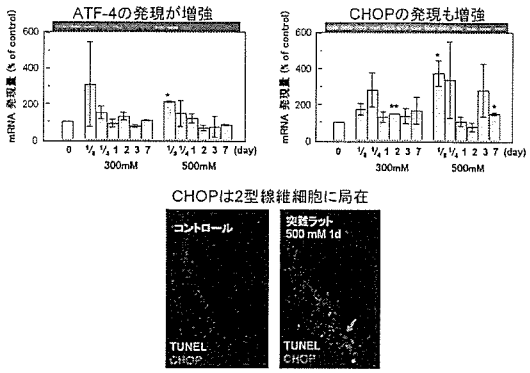


図 5　突難ラットの病態に炎症反応が関与

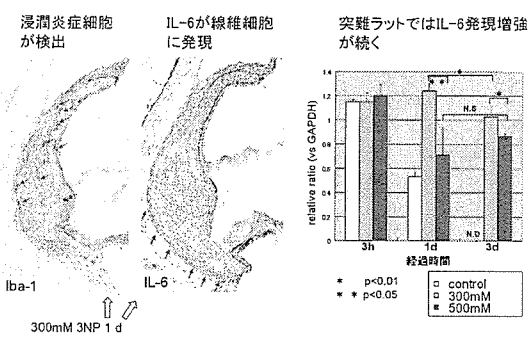


図 6　カスパーゼ阻害薬は難聴回復を促進

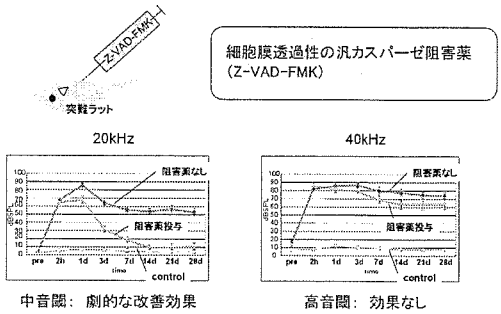


図 7

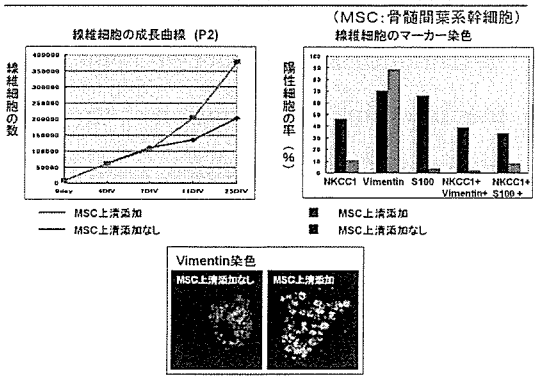
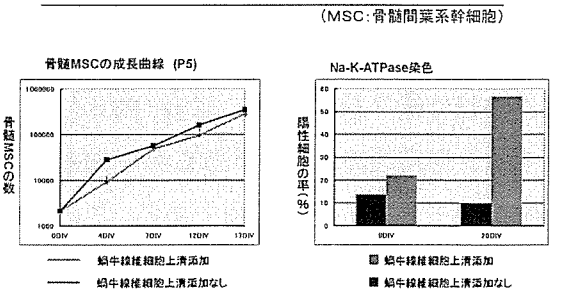


図 8



D. 考察

これまで急性内耳エネルギー不全の動物を長期生存させて解析することができなかったため、その分子病態は国際的な関心であるにも関わらず不明であった。今回、本病態の解析のために従来にない動物モデルを、分子レベルの研究情報と試薬が揃っているラットで開発できたことにより、分子メカニズムの解明を、世界に先駆けて大きく進めることができた。

さらに解明された病態に基づく新規治療を、本モデル動物で検討することにより、これまでステロイド投与以外に治療法がなかった突発性難聴に対して、急性期および慢性期に有効性を期待できる治療法を開発することができた。そして蝸牛線維細胞の発生あるいは再生という新しい観点からの研究から、新規治療の効果をより高めるために役立つ基礎的メカニズムを解明し、臨床応用への可能性を高めた。

急性内耳エネルギー不全による障害の分子メカニズムには、脳梗塞、心筋梗塞などの内耳以外の臓器の急性エネルギー不全による障害と多くの共通点が認められたことより、脳梗塞、心筋梗塞などに適応のある薬剤を、突発性難聴にも適応拡大して使用することで、効果的治療が可能な場合があると考えられた。

従来、感音難聴では感覚受容細胞である有毛細胞が主として障害されると考えられてきたが、急性内耳エネルギー不全による障害では内耳イオン恒常性の維持機構を担う蝸牛線維細胞が主として障害された。蝸牛線維細胞の障害は、急性内耳エネルギー不全による突発性難聴のみでなく、メニエール病、騒音性難聴、老人性難聴などとの関連が近年多数報告されており、その研究ニーズが高まっている。

蝸牛線維細胞は増殖能を有し、かつ様々な蛋白質の分泌や発現を介して周囲の細胞に多様な影響を与える点で、有毛細胞と大きく異なる性質を有する。このため研究のアプローチも有毛細胞の研究とは大きく異なる。これまで感音難聴の研究は有毛細胞に集中していたが、今後は蝸牛線維細胞の研究にも重点を置くべきと考える。

今回我々は、急性内耳エネルギー不全に対する新規治療法を開発したが、今後この治療法を臨床の場で応用する際には、どのような感音難聴で蝸牛線維細胞の障害（内耳リンパのイオン恒常性の障害）があるかを知るための検査方法がない点が問題である。このため今後の臨床的研究として、蝸牛線維細胞の障害により血中に流出する微量の本細胞特異的蛋白質を検出する方法の開発、あるいは内耳リンパのイオン組成の異常を検出できる画像診断の開発が必要である。

E. 結論

虚血などの原因による突発性難聴の病態を解明し、新規治療法を開発するために、急性内耳エネルギー不全のモデル動物を用いた研究を行った。この結果、これまで不明であった障害と回復のメカニズムを、臓器レベルから分子レベルまで解明することができた。さらに病態に基づいた新規治療を本動物モデルに実施してその有効性を証明することができた。その効果を高めるための基礎的研究を推進して、臨床応用への可能性を高めることができた。

F. 研究発表

1 論文発表

Hoya N, et al. A novel animal model of acute cochlear mitochondrial dysfunction: Model of cochlear mitochondrial dysfunction, Neuroreport 15,1597-1600,2004.

Okamoto Y, et al., Permanent threshold shift caused by acute cochlear mitochondrial dysfunction is primarily mediated by degeneration of the lateralwall of the cochlea, Audiol Neurootol,10,220-233,2005.

Matsunaga T, Kamiya K, Okamoto Y, Hoya N, Mizutari K, Fujinami Y, Fujii M., Degeneration and regeneration of cochlear fibrocytes mediate hearing loss and its recovery in a model of acute cochlear mitochondrial dysfunction, In Proceedings of the Fifth International Symposium. Meniere's disease & Inner Ear Homeostasis Disorders. David J Lim, editor. House Ear Institute. Los Angels, California, USA. 250-251, 2005.

神崎仁、松永達雄、突発性難聴-最近の話題、日医雑誌 134,1504-1508,2005.

神崎仁、佐藤美奈子、松永達雄、熊埜御堂浩、神崎晶、小川郁、突発性難聴の可逆性について、Audiology Japan 49,782-788,2006.

松永達雄、メニエール病と蝸牛線維細胞障害、Equilibrium Res. 65（2）129, 2006

2. 学会発表

Kamiya K, Hoya N, Okamoto Y, Fujinami Y, Komatsuzaki R, Kusano R,

Nakagawa S, Fujii M, Matsunaga T, Regeneration of cochlear fibrocyte leads to Hearing Recovery in a rat model of acute cochlear mitochondrial dysfunction, 5 th Molecular Biology of Hearing & Deafness, September 30 – October 3, 2004, Bethesda, Maryland, USA

Mizutari K, Kamiya K, Fujinami Y, Nakagawa S, Fujii M, Matsunaga T., Inhibition of caspases promotes recovery of hearing in a rat model of acute cochlear mitochondrial dysfunction, The forty second workshop on inner ear biology, September 18-20, 2005, Tubingen, Germany

Kamiya K, Hoya N, Okamoto Y, Fujinami R, Komatsuzaki R, Kusano R, Satoh H, Fujii M, Matsunaga T., Mesenchymal stem cell transplantation targeting cochlear fibrocytes accelerates the hearing recovery in a rat model of acute mitochondrial dysfunction, The forty second workshop on inner ear biology, September 18-20, 2005, Tubingen, Germany

Matsunaga T, Kamiya K, Okamoto Y, Hoya N, Mizutari K, Fujinami Y, Fujii M., Degeneration and regeneration of cochlear fibrocytes mediate hearing loss and its recovery in a model of acute cochlear mitochondrial dysfunction. The fifth international symposium. Meniere's disease & inner ear homeostasis disorders, April 2-5, 2005, Los Angels, California, USA

Fujioka M, Fujinami Y, Hosoya M, Matsunaga T , Okano HJ , Ogawa K, Okano H., Inflammation and immune response in lateral wall of damaged cochleae, The second Shanghai International Conference on Physiological Biophysics –audition and vision–, November 3-7, 2006, Shanghai, China

Matsunaga T, Kamiya K, Fujinami Y, Fujii M, Kouike H, Sun G, Komatsuzaki R, Kusano R, Repair of injured cochlear lateral wall by mesenchymal stem cell transplantation, The second Shanghai International Conference on Physiological Biophysics –audition and vision–, November 3-7, 2006,Shanghai, China

Mizutari K, Fujioka M, Fujii M, Ogawa K, Matsunaga T., Acute inner ear Energy Failure Causes Vestibular Hair Cell Damage and Balance Disorder, Thirtieth ARO Midwinter Meeting, February 10-15, 2007, Denver, Colorado,

USA

岡本康秀、小川郁、松永達雄、内耳急性エネルギー不全による不可逆性閾値上昇(PTS)モデルの長期経過(組織学的変化を中心に)、第14回日本耳科学会、2004年10月20日、大阪

神谷和作、保谷則之、岡本康秀、新田清一、中川進、藤井正人、松永達雄、内耳ミトコンドリア機能障害による難聴モデルラットの作成とその難聴の病態解析、第51回日本実験動物学会総会、2004年5月20-22日、長崎

松永達雄、保谷則之、岡本康秀、増田圭奈子、小沢宏之、新田清一、鈴木隆史、水足邦雄、神谷和作、藤井正人、急性内耳ミトコンドリア障害の病態解析と治療法開発のためのラット難聴モデルの作成、第105回日本耳鼻咽喉科学会総会、2004年5月13-15日、広島

水足邦雄、岡本康秀、小川郁、藤井正人、松永達雄、急性内耳エネルギー不全による平衡機能障害と有毛細胞特異的変化、第64回日本めまい平衡医学会総会、2005年11月24-25日、東京

Fujinami Y, Kamiya K, Fujii M, Matsunaga T., Expression of GADD153/CHOP in the lateral wall of the cochlea following acute mitochondrial dysfunction、第78回日本生化学会大会、2005年10月19-22日、神戸

新田清一、松永達雄、岡本康秀、神谷和作、水足邦雄、滝口洋一郎、藤波義明、南修司郎、藤井正人、小川郁、内耳急性エネルギー不全における一過性閾値上昇モデルにおける内耳保護分子の発現、第15回日本耳科学会総会学術講演会、2005年10月20-22日、大阪

水足邦雄、神谷和作、藤井正人、松永達雄、急性エネルギー不全ラットにおけるカスパーゼ阻害薬による難聴阻止効果、第15回日本耳科学会総会学術講演会、2005年10月20-22日、大阪

神谷和作、藤波義明、藤井正人、松永達雄、内耳エネルギー不全ラットにおける蝸牛線維細胞再生および骨髄間葉系幹細胞移植、第15回日本耳科学会総会学術講演会、2005年10月20-22日、大阪

藤波義明、神谷和作、藤井正人、松永達雄、急性内耳エネルギー不全による蝸牛外側壁での小胞体ストレス関連因子GADD153/CHOPの発現増強、第15回日本耳科学会総会学術講演会、2005年10月20-22日、大阪

藤岡正人、岡本康秀、新田清一、岡野James洋尚、神崎晶、松永達雄、岡野栄之、小川郁、内耳エネルギー不全モデル蝸牛外側壁における炎症性サイトカインの発現に関

する検討、第 15 回日本耳科学会総会学術講演会、2005 年 10 月 20-22 日、大阪

松永達雄、内耳液恒常性の障害に対する再生治療、第 1 回感覚器シンポジウム「内耳再生医療に向けて－基礎研究から治療戦略へ－」、2006 年 3 月 24 日、東京

務台英樹、藤井正人、松永達雄、転写因子 Pou3f3/Brn1 の内耳発達における発現解析、第 16 回日本耳科学会総会学術講演会、2006 年 10 月 19-21 日、青森

水足邦雄、藤岡正人、藤井正人、小川郁、松永達雄、急性内耳エネルギー不全による平衡機能障害と有毛細胞の微細構造変化、第 16 回日本耳科学会総会学術講演会、2006 年 10 月 19-21 日、青森

孫コウイ、藤井正人、松永達雄、骨髄間葉系幹細胞の蝸牛線維細胞への影響　に関する研究、第 16 回日本耳科学会総会学術講演会、2006 年 10 月 19-21 日、青森

藤波義明、水足邦雄、藤井正人、松永達雄、内耳局所的な急性小胞体ストレスによる難聴動物モデルの開発、第 16 回日本耳科学会総会学術講演会、2006 年 10 月 19-21 日、青森

瀧口洋一郎、松永達雄、水足邦雄、藤波義明、藤井正人、小川郁、急性内耳エネルギー不全による永久的聴力閾値上昇に対するアポトーシス阻害剤の聴力改善効果、第 16 回日本耳科学会総会学術講演会、2006 年 10 月 19-21 日、青森

藤岡正人、藤波義明、水足邦雄、岡本康秀、岡野 James 洋尚、小川郁、岡野栄之、松永達雄、蝸牛外側壁において細胞内呼吸障害に続発する炎症反応・免疫応答の検討、第 16 回日本耳科学会総会学術講演会、2006 年 10 月 19-21 日、青森

務台英樹、孫コウイ、藤井正人、松永達雄、蝸牛外側壁線維細胞の生後発達に伴うタイプ特異的マーカーの出現、第 51 回日本聴覚医学学会総会、2006 年 9 月 28-29 日、山形

藤波義明、神谷和作、水足邦雄、中川進、長嶋玲子、小松崎理絵、草野律子、松永達雄、急性内耳小胞体ストレスによる難聴モデル動物の開発と病態、日本薬学会 第 127 年会、2007 年 3 月 28-30 日、富山

松永達雄、内耳の再生治療　第 2 回感覚器シンポジウム、シンポジウム I 感覚器医学最近の進歩　2007 年 2 月 24 日、東京

G.　知的財産権の出願・登録状況

1.　特許取得

なし

2.　実用新案登録

なし

3.　その他

特許出願中（国際特許）：1 件

IL-6 アンタゴニストを有効成分として

含有する内耳障害治療剤

PCT/JP2005/006202

2005 年（H17 年）3 月 24 日

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研究成果の刊行に関する一覧表

書籍

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	出版地	出版年	ページ
Matsunaga T, Kamiya K, Okamoto Y, Hoya N, Mizutari K, Fujinami Y, Fujii M	Degeneration and regeneration of cochlear fibrocytes mediate hearing loss and its recovery in a model of acute cochlear mitochondrial dysfunction	David J Lim	Proceedings of the Fifth International Symposium. Meniere's disease & Inner Ear Homeostasis Disorders	House Ear Institute	Los Angels, California, USA	2005	250-251

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
Hoya N, Okamoto K, Kamiya K, Fujii M, Matsunaga T	A novel animal model of acute cochlear mitochondrial dysfunction: Model of cochlear mitochondrial dysfunction	Neuroreport	15(10)	1597-1600	2004
Okamoto Y, Hoya N, Kamiya K, Fujii M, Ogawa K, Matsunaga T	Permanent threshold shift caused by acute cochlear mitochondrial dysfunction is primarily mediated by degeneration of the lateralwall of the cochlea	Audiol Neurootol	10(4)	220-233	2005
神崎仁、松永達雄	突発性難聴-最近の話題	日医雑誌	134(8)	1504-1508	2005

神崎仁、 佐藤美奈子、 松永達雄、 熊埜御堂浩、 神崎晶、 小川郁	突発性難聴の可逆性について	Audiology Japan	49(6)	782-788	2006
松永達雄	メニエール病と蝸牛線維細胞障害	Equilibrium Res.	65(2)	129	2006

Degeneration and Regeneration of Cochlear Fibrocytes Mediate Hearing Loss and its Recovery in a Model of Acute Cochlear Mitochondrial Dysfunction

Tatsuo Matsunaga*, Kazusaku Kamiya, Kunio Mizutari, Yoshiaki Fujinami, Yasuhide Okamoto, Noriyuki Hoya, Masato Fujii

National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan.
*Corresponding author: matsunagatatsuo@kankakuki.go.jp

Mutations in mitochondrial DNA cause both syndromic and nonsyndromic hearing loss by impairment of mitochondrial function, i.e. generation of cellular energy. We have recently conducted a detailed audiological evaluation on a large number of subjects with nonyndromic hearing loss due to A1555G mitochondrial DNA mutation and showed that the damage is restricted to the cochlea with no or little involvement of the spiral ganglion.[1,2] Cochlear energy failure is also likely to play important roles in some types of acute sensorineural hearing loss such as ischemia- or noise-induced hearing loss. Recently, we have developed a novel rat model of acute mitochondrial dysfunction using a mitochondrial toxin 3-nitropropionic acid (3-NP) [3] as a model for acute cochlear energy failure.[4,5] Depending on the amount of 3-NP, both permanent threshold shift (PTS) and temporary threshold shift (TTS) were induced in this model. In comparison with the previous models of cochlear energy failure, the present model has several unique features. First, the TTS model demonstrates the gradual recovery of hearing over a few weeks from profound hearing loss, which is similar to the clinical course frequently observed in patients with sudden deafness. Second, our model can survive for several months or longer after 3-NP exposure because of the relatively mild surgical stress by local administration of 3-NP to the oval window. This feature enables us to observe the progress of hearing loss and its recovery for a relatively long duration and to perform various analyses on the cochlea at different time points after the onset of hearing loss. In this study, we aimed to reveal the structural basis of the hearing loss in this model.

Materials and Methods

Sprague-Dawley rats (8~10 weeks, 180~220 g) were used. 3-NP was administered into the round window niche of the left cochlea. Auditory brainstem response (ABR) was recorded before surgery and 2h, 1d, 3d, 7d, 14d, 21d, 28d, 35d and 42d after surgery. Pure tone bursts of 8, 20 and 40 kHz (0.2 ms rise/fall time and 1 ms flat segment) were generated. The right cochlea was surgically destroyed to avoid cross hearing during ABR recording. Light microscopic and transmission electron microscopic analysis on Epon-embedded sections as well as light microscopic analysis on paraffin-embedded sections stained with HE were conducted at various time points after 3-NP administration in both PTS and TTS models.

Results

In the PTS model, light microscopic analyses revealed that there was a basal-to-apical gradient in the extent of cochlear damage, and the structural changes were most evident in the lateral wall in the middle and basal turns with lesser changes in the organ of Corti and modiolus. Fibrocytes of the spiral ligament are divided into four cell types based on structural features, immunostaining patterns and general location.[6] By light and electron microscopic analyses, we detected marked and progressive degeneration in type 2 and type 4 fibrocytes in the spiral ligament, and moderate and temporary degeneration in the marginal cells and intermediate cells in the stria vascularis, starting as early as 3h after 3-NP administration. In the TTS model, light microscopic analysis revealed drastic and focal apoptosis showing chromatin condensation and apoptotic bodies in the lateral wall fibrocytes and the spiral limbus fibrocytes at 3d after 3-NP administration. However, prominent morphological changes were not observed in the organ of Corti and spiral ganglion. By light and electron microscopic analyses in the rats showing moderate (~40db) hearing impairment, clearly demarcated areas of cells exhibiting the signs of apoptosis such as cell shrinkage and chromatin condensation around the nuclear periphery was observed in the lateral wall fibrocytes and spiral limbus fibrocytes. In rats with severe (~60db) hearing impairment, areas of cell loss were identified in the lateral wall at 3d after administration. After that time point, the density of normal fibrocytes within the lateral wall gradually increased. At 42d after administration, the density of fibrocytes in the lateral wall appeared almost normal.

Discussion

In the mammalian cochlea, ATP-dependent potassium recycling pathways have been well known as the essential mechanism for normal hearing.[7] These potassium ion transport systems generate endocochlear potential and high potassium concentrations in the endolymph, both of which are essential for the transduction of sound by hair cells.[8] Cochlear fibrocytes in the lateral wall play a critical role in this system. They transport K^+ into the endolymph and keep K^+ concentration high via Na^+/K^+-ATPase and gap junctions. The present study indicated that administration of the mitochondrial toxin 3-NP induced degeneration of the cochlear fibrocytes, which may lead to abnormal K^+ transport and, thereby, decrease of K^+ concentration in endolymph and loss of endocochlear potential. Thus, our data indicate that deterioration of hearing due to acute cochlear mitochondrial dysfunction is primarily mediated by focal and drastic degeneration of the cochlear fibryocytes. Furthermore, possible regeneration of the cochlear fibrocytes in the TTS model supports the hypothesis that regeneration of the cochlear fibrocytes is the essential process in the recovery of hearing loss due to acute cochlear mitochondrial dysfunction. Because a long observation period is possible following exposure to 3-NP using the present model, further study of the molecular mechanism underlying the functional and morphological changes observed in this model may lead to the discovery of a novel therapeutic strategy.

Conclusions

These results suggest that deterioration of hearing due to acute

cochlear mitochondrial dysfunction is primarily mediated by degeneration of the cochlear fibryocytes in both TTS and PTS models and regeneration of these fibrocytes is the essential process in the recovery of hearing in the TTS model.

References

1. Matsunaga T, Kumanomido H, Shiroma M, et al. Deafness due to A1555G mitochondrial mutation without use of aminoglycoside. Laryngoscope 2004; 114(6): 1085-1091.
2. Matsunaga T, Kumanomido H, Shiroma M, et al. Audiological features and mitochondrial DNA sequence in a large family carrying mitochondrial A1555G mutation without use of aminoglycoside. Ann Otol Rhinol Laryngol 2005; 114(2): 153-160.
3. Alston TA, Mela I, Bright HJ. 3-Nitropropionate, the toxic substance of Indigofera, is a suicide inactivator of succinate dehydrogenase. Proc Natl Acad Sci USA 1977; 74(9): 3767-3771.
4. Hoya N, Okamoto Y, Kamiya K, et al. A novel animal model of acute cochlear mitochondrial dysfunction: Model of cochlear mitochondrial dysfunction. Neuroreport 2004; 15(10): 1597-1600.
5. Okamoto Y, Hoya N, Kamiya K, et al. Permanent threshold shift caused by acute cochlear mitochondrial dysfunction is primarily mediated by degeneration of the lateral wall of the cochlea. Audiol Neurootol 2005; 10(4): 220-233.
6. Spicer SS, Schulte BA. The fine structure of spiral ligament cells relates to ion return to the stria and varies with place-frequency. Hear Res 1996; 100(1-2): 80-100.
7. Salt AN. Dynamics of the inner ear fluids. In: Jahn AF, Santos-Sacchi J, editors. Physiology of the Ear. 2nd ed. San Diego: Singular, Thompson Learning; 2001. p. 333-55.
8. Santos-Sacchi J. Cochlear physiology. In: Jahn AF, Santos-Sacchi J, editors. Physiology of the Ear. 2nd ed. San Diego: Singular, Thompson Learning; 2001. p. 357-91.

A novel animal model of acute cochlear mitochondrial dysfunction

Noriyuki Hoya, Yasuhide Okamoto, Kazusaku Kamiya, Masato Fujii and Tatsuo Matsunaga[CA]

Laboratory of Auditory Disorders, National Institute of Sensory Organs, National Tokyo Medical Center, 2-5-1 Higashigaoka, Meguro-ku, Tokyo 152-8902, Japan

[CA]Corresponding Author: matsunagatatsuo@kankakuki.go.jp

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Acute mitochondrial dysfunction in the cochlea is likely to result in hearing loss as a consequence of local energy shortage, similar to ischemia- or noise-induced hearing loss. To establish an animal model of acute cochlear mitochondrial dysfunction, we applied a mitochondrial toxin, 3-nitropropionic acid (3-NP) in the rat cochlea. Rats treated with 500 mM 3-NP exhibited permanent threshold shifts in acoustic brainstem response while the same volume of 300 mM 3-NP caused temporary threshold shifts. Histological examination in the permanent threshold shift model revealed severe degeneration of fibrocytes within spiral ligament and spiral limbus, indicating these cells are vulnerable to acute mitochondrial dysfunction. This model represents a novel tool for investigating the pathophysiology of acute cochlear mitochondrial dysfunction. *NeuroReport* 15:1597–1600 © 2004 Lippincott Williams & Wilkins.

Key words: Cochlea; Fibrocytes; Lateral wall; Mitochondria; 3-Nitropropionic acid; Potassium recycling; Temporary threshold shift

INTRODUCTION

Hearing loss is one of the most prevalent features of mitochondrial diseases [1]. The fact that mitochondrial DNA mutations frequently lead to hearing loss suggests that cochlear cells are strongly dependent upon mitochondrial function [2]. Because the majority of mitochondrial diseases are characterized by a deficiency of enzymes in the electron transport chain [3–5], a resultant reduction of ATP production may underlie the pathogenesis of sensorineural hearing loss that accompanies these diseases. In addition, mitochondrial dysfunction may mediate the pathogenesis of ischemia- and noise-induced hearing loss since decreased blood supply or excessive stimulation might cause local energy shortages [6]. Thus, mitochondrial dysfunction may underlie the pathogenesis of several types of sensorineural hearing loss related to energy failure.

The mitochondrial toxin 3-nitropropionic acid (3-NP) is an irreversible inhibitor of succinate dehydrogenase, and blocks complex II of the mitochondrial electron transport chain [7]. Systemic administration of 3-NP produces selective striatal legion damage and clinical features of Huntington's disease [8–10]. The effect of this agent in the cochlea can be regarded as insufficiency of cellular energy in the cochlea. In the present study, we investigated the effect of local application of 3-NP in rat to establish an animal model of acute mitochondrial dysfunction in the cochlea.

MATERIALS AND METHODS

Animals and surgery: Twenty-seven female Sprague–Dawley rats weighing 180–220 g were anesthetized with pentobarbital (30–40 mg/kg, i.p.) and an incision was made following local administration of lidocaine (1%). The left otic bulla was opened using the retroauricular approach. The end of PE 10 tubing (Becton Dickinson, NJ, USA) was drawn to a fine tip in a flame and gently inserted into the round window niche. 3-NP (Sigma, St. Louis, MO, USA) dissolved in saline (pH 7.4) was administered at 500 mM, 300 mM and 50 mM for 2 min at a rate of 1.5 µl/min with a syringe pump. Saline was used as a control. A tiny piece of gelatin was put on the niche and the wound was closed. The right cochlea was surgically destroyed to avoid cross hearing during auditory brainstem response (ABR) recording. Experimental procedures reported in this study were approved by the Institutional Animal Care and Use Committee of The National Tokyo Medical Center.

ABR recording: ABR was recorded before surgery and 1 and 3 h and 1, 3, 7, 14, 21 and 28 days after surgery using Scope waveform storing and stimulus control software and the PowerLab data acquisition and analysis system (PowerLab2/20, AD Instruments, Castle Hill, Australia). EEG recordings were acquired using the Digital Bioamp extracellular amplifier system (BAL-1, Tucker-Davis Technologies, FL, USA). Sound stimuli were produced by a coupler type speaker (ES1spc, Bio Research Center, Nagoya, Japan) inserted into the ear canal. Pure tone bursts of 8, 12, 16 and 20 kHz (0.2 ms rise/fall time and 1 ms flat segment) were generated and the amplitude specified by a real-time processor and programmable attenuator (RP2.1 and PA5, Tucker-Davis Technologies, FL, USA). Sound levels were calibrated using a sound level meter (NL32, RION, Tokyo,

Japan) and the maximum output level at each frequency was 93, 96, 80, 79 dB at 8, 12, 16, 20 kHz, respectively. For recording, the animals were anesthetized with pentobarbital before stainless steel needle electrodes were placed ventrolateral to the bilateral ears. Waveforms of 512 stimuli at a frequency of 9 Hz were averaged and the visual detection threshold was determined with increment or decrement of sound pressure level by 5 dB steps. The effect of 3-NP on the ABR threshold was analyzed by comparing the threshold in the presence of 3-NP to the control using two-way repeated measures ANOVA, followed by a multiple comparison procedure (Student-Newman-Keuls method). The significance level for all statistical procedures was set at $p < 0.05$.

Histological analysis: Fourteen days after administration, control rats and the experimental rats treated with 50 mM or 500 mM 3-NP were deeply anesthetized with pentobarbital and transcardially perfused with 100 ml 0.01 M phosphate buffered saline (PBS) followed by 50 ml fixative consisting of freshly depolymerized 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M PBS. Left temporal bones were removed and immediately placed in the fixative. Openings were made at the round window, oval window and the apex of the cochlea. After immersion in the same fixative overnight, cochleae were decalcified in 0.1 M EDTA with 5% sucrose for 6 days, rinsed in 0.1 M PBS overnight, and post-fixed in 1% osmium tetroxide for 150 min. They were then dehydrated in a graded ethanol series, embedded in Epon 812 resin, sectioned in a horizontal plane parallel to the modiolus at 12.5 μm, and stained with toluidine blue for light microscopic examination.

RESULTS

The ABR thresholds for all frequencies tested were significantly elevated 1 h after administration of either 500 mM or 300 mM 3-NP while the administration of 50 mM 3-NP or physiological saline did not result in a detectable threshold shift (Fig. 1). In the 500 mM group, ABR thresholds exceeded the maximum recording limit for all frequencies tested 3 h after administration and persisted for at least 28 days. In the 300 mM group, the ABR thresholds started to improve at all frequencies tested after 3 days and recovered to levels that were not significantly elevated at 8, 12 and 16 kHz in 14 days; however, they remained significantly elevated at 20 kHz even after 28 days. The average hearing improvement was 59, 66, 65 and 73 dB at 20, 16, 12 and 8 kHz, respectively.

In rats treated with 500 mM 3-NP, all structures in the apical turn and the spiral ganglion neurons in the modiolus were well preserved 14 days after administration. In the middle turn, the fibrocytes in the spiral ligament and spiral limbus exhibited substantial degenerative changes (Fig. 2c, Fig. 3c). In the spiral ligament, most types of fibrocytes were degenerated. Loss of type II fibrocytes was apparent around the spiral prominence and root cells appeared to have shrunk. Type IV fibrocytes in the basilar crest were conspicuously degenerated. Obvious changes were not detected within the stria vascularis by light microscopy. In the spiral limbus, loss of stellate fibrocytes of the limbic central zone was prominent while interdental cells in the same zone were normal. Degeneration of supralimbal fibrocytes was also detected with marked swelling of supralimbal epithelium. In contrast, hair cells, supporting cells and nerve fibers in the organ of Corti were well preserved (Fig. 3c). In the basal turn, fibrocytes in the spiral ligament and spiral limbus showed severe degeneration while cells in the organ of Corti and the stria vascularis showed only slight degeneration. In rats treated with control or 50 mM 3-NP, the cochlea showed a normal structure in all turns 14 days after administration (Fig. 2a,b, Fig. 3a,b).

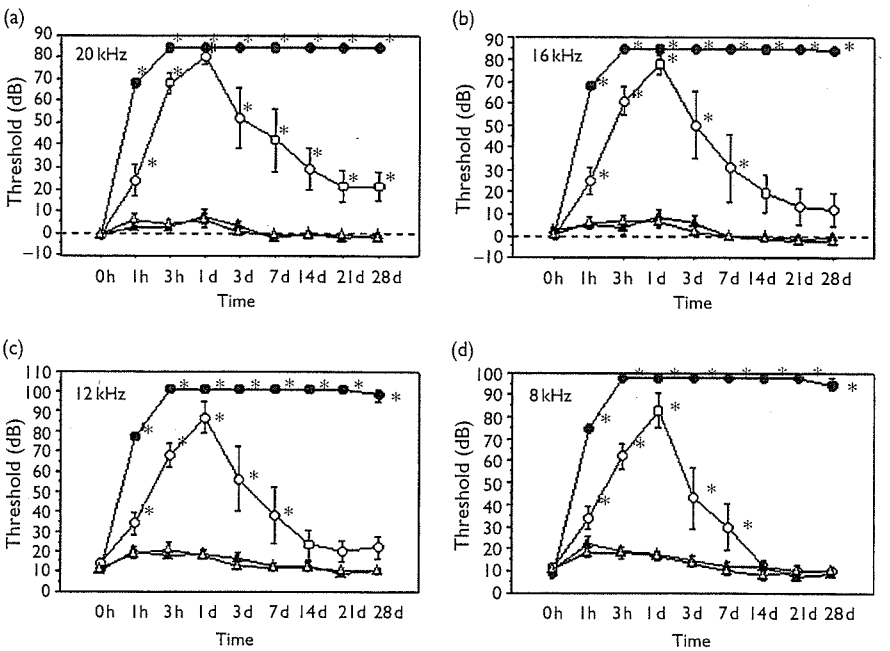

Fig. 1. The ABR threshold (dB SPL) before and after local administration of 3-NP (mean ± s.e.) at each frequency tested: (**a**) 20 kHz, (**b**) 16 kHz, (**c**) 12 kHz, (**d**) 8 kHz. Filled circles, open circles, filled triangles and open triangles indicate 500 mM (n=5), 300 mM (n=5), 50 mM (n=6), 3-NP and control groups (n=6), respectively. *$p < 0.05$ *vs* controls (Student-Newman-Keuls multiple comparison procedure).

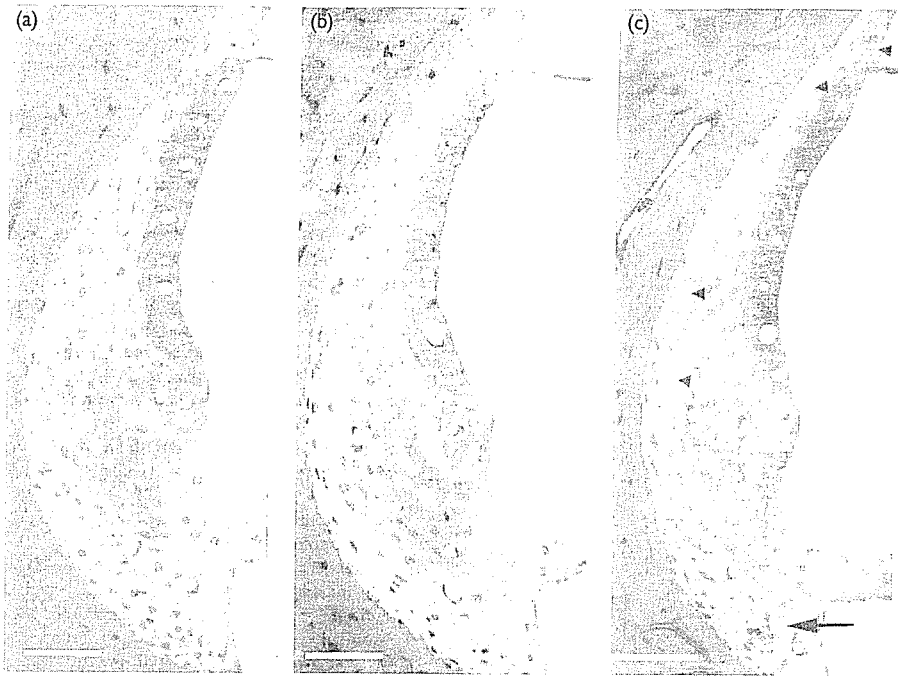

Fig. 2. Histological findings of the lateral wall in the cochlear middle turn 14 days after administration of control saline (**a**), 50 mM 3-NP (**b**), or 500 mM 3-NP (**c**). A normal structure was observed in the lateral wall of the control ear (a) and in that of the experimental ear treated with 50 mM 3-NP (b). (**c**) Upon administration of 500 mM 3-NP, loss and degeneration of fibrocytes were evident throughout the spiral ligament (black arrowheads). Loss of type II fibrocytes was prominent around the spiral prominence (white arrow), and root cells appeared shrunken (white arrowhead). Atrophy of type IV fibrocytes within the basilar crest was detected (black arrow). The *stria vascularis* was structurally well preserved. Toluidine blue stain. Bars=50 μm.

DISCUSSION

The present study demonstrates that cochlear mitochondrial dysfunction caused by administration of 3-NP results in hearing loss in a concentration-dependent manner, and the degree of hearing loss induced by different concentrations of 3-NP was well correlated with the extent of histological abnormality. Both the function and structure of the cochlea were normal in the rats treated with control saline or 50 mM 3-NP, indicating that the rat cochlea is tolerant to the effect of 50 mM 3-NP administration. However, administration of 300 mM 3-NP and 500 mM 3-NP induced temporary threshold shifts and permanent threshold shifts, respectively. Within the cochlea treated with 500 mM 3-NP, cellular damage was most prominent in the basal turn, intermediate in the middle turn, and minimal in the apical turn. Because 3-NP was applied from the round window and diffused in the perilymph, the difference in the damage by turns was most likely due to the different levels of exposure to 3-NP. Extreme susceptibility of cochlear fibrocytes to mitochondrial dysfunction is striking because the organ of Corti and/or stria vascularis are generally considered to be more susceptible to insults such as ischemia/hypoxia and noise and are essential for normal hearing [11,12].

The role of fibrocytes in the pathophysiology of sensorineural hearing loss is not fully understood. However, a number of recent studies have shown that degeneration of the cochlear fibrocytes could be a primary cause for some kinds of hearing loss. In a mouse model of aging, loss of fibrocytes within the spiral ligament was detected as the first morphological sign of cochlear degeneration [13], although another study using a gerbil model reported that these changes were secondary to dysfunction of the stria vascularis [14]. Extreme vulnerability of fibrocytes to noise insult was also reported in the mouse spiral ligament and spiral limbus [15]. Further study by the same group also revealed temporarily decreased endocochlear potential in the same experiment with a permanent threshold shift, suggesting reversible dysfunction of the lateral wall fibrocytes [16]. Furthermore, a mouse model of DFN3 nonsyndromic deafness exhibiting severe hearing loss had altered cochlear fibrocytes and decreased endocochlear potential [17].

Consistent with these reports, the degeneration of cochlear fibrocytes observed in this study might be the main cause for hearing loss in our experiments because the changes in the organ of Corti and the stria vascularis were minimal as observed by light microscopy. These cochlear fibrocytes are thought to play important roles in maintaining high K^+ concentrations in the endolymph by recycling K^+ from the perilymph [18,19]. The possible mechanism for ABR threshold elevation may be decreased K^+ concentration in the endolymph and resultant loss of endolymphatic potential. The slow recovery of ABR threshold observed in rats treated with 300 mM 3-NP suggests that these fibrocytes may regenerate as previously reported [20] and restore organ function. Since the possibility of slow hearing recovery is one of the remarkable clinical features in patients with acute sensorineural hearing loss, mitochondrial dysfunction in the cochlea may be one of the pathological pathways of such hearing impairment.

CONCLUSION

The present animal model of acute cochlear mitochondrial dysfunction exhibited either a permanent threshold shift, a temporary threshold shift, or normal hearing depending on

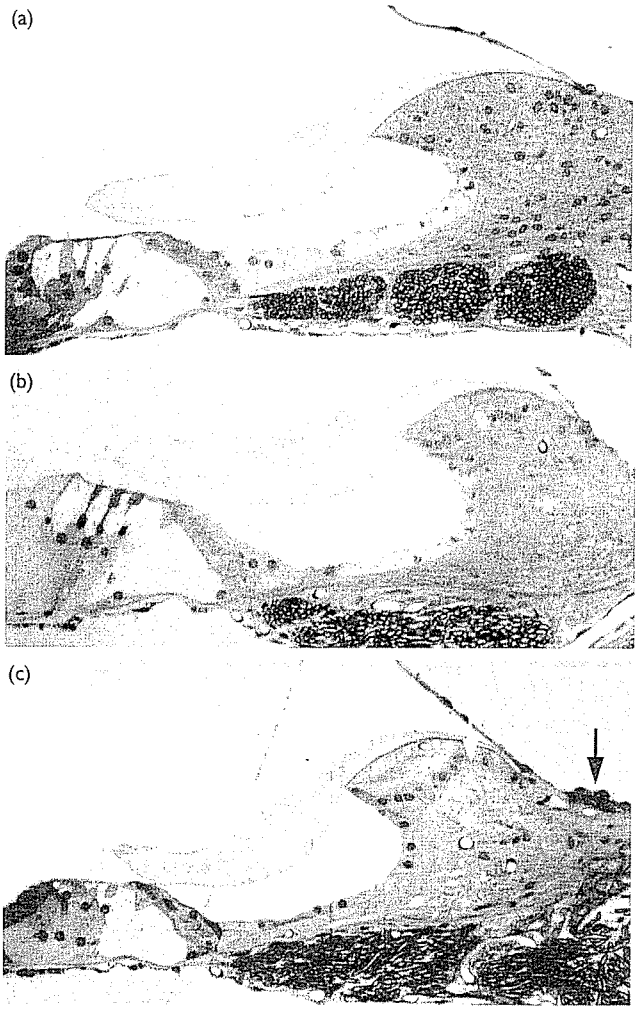

Fig. 3. Histological findings of the organ of Corti and spiral limbus in the cochlear middle turn 14 days after administration of control saline (**a**), 50 mM 3-NP (**b**), or 500 mM 3-NP (**c**). A normal structure was observed in the organ of Corti and spiral limbus of the control ear (a) and in those of the experimental ear treated with 50 mM 3-NP (b). (c) Upon administration of 500 mM 3-NP, loss of stellate fibrocytes was prominent in the limbic central zone (white arrow) while interdental cells appeared normal. Swelling of supralimbal fibrocytes (black arrow) was prominent. The organ of Corti was structurally well preserved. Toluidine blue stain. Bars=50 μm.

the amount of 3-NP administered into the cochlea, and these functional changes were well correlated with the histological changes in the cochlea. This model revealed the extreme vulnerability of cochlear fibrocytes to mitochondrial dysfunction and is likely to contribute to further understanding of the pathophysiology of acute sensorineural hearing loss.

REFERENCES

1. Gold M and Rapin I. Non-Mendelian mitochondrial inheritance as a cause of progressive genetic sensorineural hearing loss. *Int J Pediatr Otolaryngol* 1994; **30**:91–104.
2. Hutchin TP and Cortopassi GA. Mitochondrial defects and hearing loss. *Cell Mol Life Sci* 2000; **57**:1927–1937.
3. Goto Y, Horai S, Matsuoka T, Koga Y, Nihei K, Kobayashi M *et al.* Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutation. *Neurology* 1992; **42**:545–550.
4. Wallace DC, Zheng XX, Lott MT, Shoffner JM, Hodge JA, Kelley RI *et al.* Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. *Cell* 1988; **55**:601–610.
5. Degoul F, Nelson I, Lestienne P, Francois D, Romero N, Duboc D *et al.* Deletions of mitochondrial DNA in Kearns-Sayre syndrome and ocular myopathies: genetic, biochemical and morphological studies. *J Neurol Sci* 1991; **101**:168–177.
6. Seidman MD, Quirk WS and Shirwany NA. Mechanisms of alterations in the microcirculation of the cochlea. *Ann NY Acad Sci* 1999; **884**:226–232.
7. Alston TA, Mela I and Bright HJ. 3-Nitropropionate, the toxic substance of Indigofera, is a suicide inactivator of succinate dehydrogenase. *Proc Natl Acad Sci USA* 1977; **74**:3767–3771.
8. Hamilton BF and Gould DH. Nature and distribution of brain lesions in rats intoxicated with 3-nitropropionic acid: a type of hypoxic (energy deficient) brain damage. *Acta Neuropathol (Berl)* 1987; **72**:286–297.
9. Brouillet E, Hantraye P, Ferrante RJ, Dolan R, Leroy-Willig A, Kowall NW *et al.* Chronic mitochondrial energy impairment produces selective striatal degeneration and abnormal choreiform movements in primates. *Proc Natl Acad Sci USA* 1995; **92**:7105–7109.
10. Sato S, Gobbel GT, Honkaniemi J, Li Y, Kondo T, Murakami K *et al.* Apoptosis in the striatum of rats following intraperitoneal injection of 3-nitropropionic acid. *Brain Res* 1997; **745**:343–347.
11. Shulman JB, Lambert PR and Goodhill V. Acoustic Trauma- and noise-induced hearing loss. In: Canalis RF and Lambert PR (eds). *The Ear. Comprehensive Otology.* Philadelphia: Lippincott Williams & Wilkins; 2000, pp. 773–783.
12. Martini A and Silvano P. Disorders of the inner ear in adults. In: Luxon LM, Furman JM, Martini A and Stephens D (eds). *Text Book of Audiological Medicine. Clinical Aspects of Hearing and Balance.* London: Martin Dunitz; 2003, pp. 451–475.
13. Hequembourg S and Liberman MC. Spiral ligament pathology: a major aspect of age-related cochlear degeneration in C57BL/6 mice. *J Assoc Res Otolaryngol* 2001; **2**:118–129.
14. Spicer SS and Schulte BA. Spiral ligament pathology in quiet-aged gerbils. *Hear Res* 2002; **172**:172–185.
15. Wang Y, Hirose K and Liberman MC. Dynamics of noise-induced cellular injury and repair in the mouse cochlea. *J Assoc Res Otolaryngol* 2002; **3**:248–268.
16. Hirose K and Liberman MC. Lateral wall histopathology and endocochlear potential in the noise-damaged mouse cochlea. *J Assoc Res Otolaryngol* 2003; **4**:339–352.
17. Minowa O, Ikeda K, Sugitani Y, Oshima T, Nakai S, Katori Y *et al.* Altered cochlear fibrocytes in a mouse model of DFN3 nonsyndromic deafness. *Science* 1999; **285**:1408–1411.
18. Schulte BA and Steel KP. Expression of alpha and beta subunit isoforms of Na,K-ATPase in the mouse inner ear and changes with mutations at the Wv or Sld loci. *Hear Res* 1994; **78**:65–76.
19. Spicer SS and Schulte BA. Evidence for a medial K^+ recycling pathway from inner hair cells. *Hear Res* 1998; **118**:1–12.
20. Roberson DW and Rubel EW. Cell division in the gerbil cochlea after acoustic trauma. *Am J Otol* 1994; **15**:28–34.

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