II. 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
中川隆之、伊藤壽一	第２章　生体シグナル因子の利用．１．細胞増殖因子	田畑泰彦	MOOK１３号 臨床再生誘導治療２００９ 患者までとどいている再生誘導治療	メディカルドゥ	大阪	2009	
坂本達則	内耳障害に対する細胞移植治療	山中伸弥，中内啓光	再生医療へ進む最先端の幹細胞研究	羊土社	東京	2008	774-779

雑誌

著者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Inaoka T, Nakagawa T, Kikkawa YS, Tabata Y, Ono K, Yoshida M, Tsubouchi H, Ido A, Ito J	Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs	Acta Otolaryngol	2009 Feb 13:1-5. [Epub ahead of print]		
Ogita H, Nakagawa T, Lee KY, Inaoka T, Okano T, Kikkawa YS, Sakamoto T, Ito J	Surgical invasiveness of cell transplantation into the guinea pig cochlear modiolus	ORL J Otorhinolaryngol Relat Spec	71(1)	32-9	2009
Hiraumi H, Nakagawa T, Ito J	Efficiency of a transtympanic approach to the round window membrane using a microendoscope	Eur Arch Otorhinolaryngol	266(3)	367-71	2009
Matsumoto M, Nakagawa T, Kojima K, Sakamoto T, Fujiyama F, Ito J	Potential of embryonic stem cell-derived neurons for synapse formation with auditory hair cells	J Neurosci Res	86(14)	3075-85	2008
Hori R, Nakagawa T, Sugimoto Y, Sakamoto T, Yamamoto N, Hamaguchi K, Ito J	Prostaglandin E receptor subtype EP4 agonist protects auditory hair cells against noise-induced trauma	Neuroscience	in-print		
Kada S, Nakagawa T, Ito J	A mouse model for degeneration of the spiral ligament	J Assoc Res Otolaryngol	2009 Feb 11. [Epub ahead of print]		
Nakagawa T, Ito J	Local drug delivery to inner ear for treatment of hearing loss	Current Drug Therapy 3: 143-147, 2008	3	143-147	2008
中川隆之	内耳疾患の治療をめざして一基礎研究の最前線　薬物の経正円窓投与	日本耳鼻咽喉科学会会報	111	655-663	2008

III. 研究成果の刊行物 別刷

8. 内耳障害に対する細胞移植治療

坂本達則

聴覚と平衡機能を司る内耳は，哺乳類ではきわめて限られた再生能力しかもたず，これが内耳障害の回復が困難である原因と考えられていたが，これを克服するために，次々に明らかになってきた内耳発生に関する情報を用いたさまざまなアプローチが試みられている．幹細胞から有毛細胞を誘導することは原理的には可能であることが示されたが，有毛細胞に対する移植治療を行うには移植細胞にも移植方法にも今後の改良が待たれる．ラセン神経節細胞の幹細胞治療については，幹細胞由来神経細胞の移植によって聴覚機能が改善することがわかり，臨床応用が期待される．

はじめに

聴覚と平衡機能を司る内耳は，感覚受容器として働く有毛細胞※1を中心に機能している．聴覚では20Hz～20kHz，平衡覚では数Hzの振動をその頂側にみられる不動毛で受容した有毛細胞は，ラセン神経や前庭神経を介して中枢に刺激を伝達する（図1）．内耳障害は，加齢・強大音への曝露（音響障害）・薬物（アミノグリコシド系抗生剤，抗腫瘍薬，一部の利尿薬など）・遺伝子異常などさまざまな原因で生じるが，組織学的には主として有毛細胞や神経細胞などの障害であることが知られている．有毛細胞は，いったん傷害されるとほとんど再生することがなく，それが内耳障害の回復が非常に困難である原因と考えられていた．しかし，鳥類において，内耳障害後の有毛細胞が再生することが明らかになり[1)2)]，さらに，有毛細胞が再生した後にトリが新たに鳴き声を覚える[3)]ことから，組織学的に再生するだけではなく，聴覚としても機能回復することも明らかになり，哺乳類においても内耳障害に対する再生治療の可能性が模索されるようになった．これまで，細胞移植や遺伝子導入，薬物導入など，さまざまな方法が試みられてきたが，本稿では背景として内耳の発生と内耳幹細胞について述べた後，有毛細胞とラセン神経節細胞※2の細胞移植治療の現状について取り上げる．

[キーワード]
内耳障害，有毛細胞，支持細胞，ラセン神経節細胞，内耳発生，内耳幹細胞，細胞移植

1 内耳発生と内耳幹細胞

1）内耳発生

内耳は，初期体節期（マウスで発生8日頃）に後脳の外側の皮膚外胚葉に形成される耳プラコードを起源

※1 有毛細胞
内耳の蝸牛および前庭の感覚上皮に存在する感覚受容細胞．頂側に存在する聴毛が振動に応じて変位することにより，脱分極し，神経伝達物質を放出する．

Cell transplantation therapy for inner ear damage
Tatsunori Sakamoto：Department of Otolaryngology, Head and Neck Surgery, Kyoto University Graduate School of Medicine（京都大学大学院医学研究科耳鼻咽喉科・頭頸部外科）

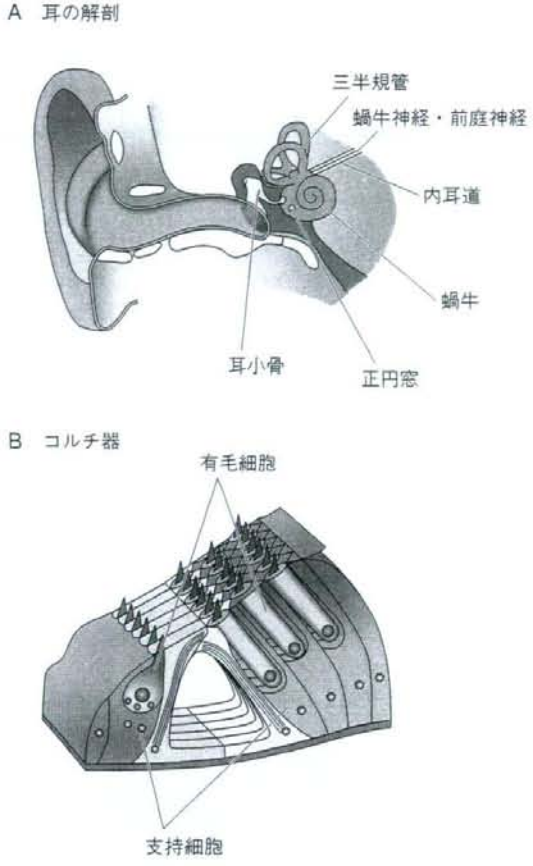

図1 内耳
Kelley et al.：Nature Rev. Neurosci., 7：837-849. 2006より改変

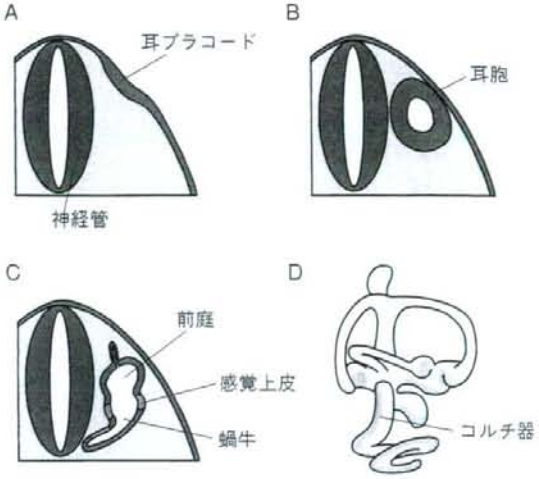

図2 内耳の発生

とし，陥凹して皮膚から切り離されて耳胞となり，複雑な形態形成を経て内耳を形成する（図2）．この間に起こる細胞レベルでの運命決定について明らかになってきたのはごく最近のことである．耳胞の壁の一部はNotch情報伝達系を介して感覚上皮として決定を受ける．その中の前駆細胞からはラセン神経節・前庭神経節の神経細胞が分化し，$p27^{Kip1}$やpRbのような細胞増殖制御因子の発現によって細胞増殖が停止したあと[4]は，再びNotchシグナルによって有毛細胞と支持細胞※3が交互に存在するように分化する[5]（図3）．

その後の聴毛の形成に関わる細胞骨格とモーターに関連する分子[6]や，有毛細胞が整然と再配置されるときに平面内細胞極性を司る分子群が機能していること[7]なども明らかになってきた．その後の成熟した内耳では細胞増殖は起こらない[8]．

2）内耳幹細胞は存在するか

成熟した内耳における細胞増殖がみられないことや，ラットの内耳有毛細胞障害後，細胞の修復機構によって少数の有毛細胞の再生がみられるが細胞増殖による細胞の補充は起こらない[9]ことは，内耳に幹細胞が存在しないことを示唆すると考えられていた．しかし，最近になって，ラットの蝸牛由来の細胞を*in vitro*で培養して，細胞増殖を介して新生有毛細胞が生じたとする報告[10]や，マウスの前庭感覚上皮から増殖してsphereを形成し，三胚葉に分化し得る多分化能をもつ細胞が得られたという報告[11]がなされ，内耳にも幹細胞が存在すると考えられるようになってきた．しかし，内耳由来の幹細胞の分離が生後直後から週を重ねるごとに急激に困難になる[12]ことから，組織幹細胞としての内耳幹細胞は生後急激に消失すると考えられる．発生段階の耳胞由来の幹細胞の分離も試みら

※2 ラセン神経節細胞
双極性の神経細胞で，末梢では有毛細胞とシナプスを形成して有毛細胞からの刺激を受容し，中枢では脳幹の蝸牛神経核に伝達する．

※3 支持細胞
有毛細胞の周囲に存在する細胞で，有毛細胞と共通の前駆細胞から発生する．

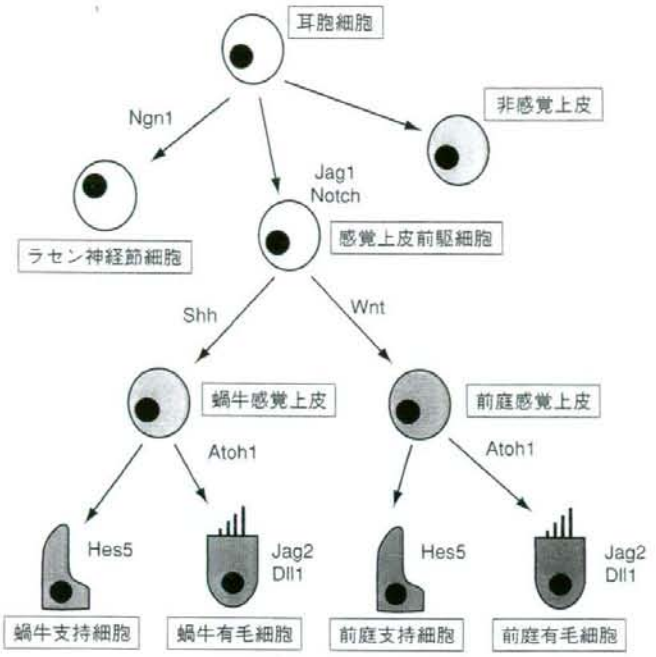

図3　内耳細胞の運命決定
Kelley et al.：Nature Rev. Neurosci., 7：837-849, 2006より改変

れている．齧歯類の耳胞由来の幹細胞分離の試みのほか[13) 14)]，ヒト胎児蝸牛からも未分化な感覚上皮に相当する細胞が得られ，ここから有毛細胞を分化させることができる[15)]．これらが上述した内耳細胞の運命決定のどの段階に相当するかははっきりしていない．NestinやMusashi1を用いた組織学的な検討では耳における幹細胞の位置を指摘し得ていないが，有毛細胞と支持細胞の前駆細胞の存在や，$p27^{Kip1}$・Prox1など，前駆細胞と支持細胞の共通のマーカーが多数あることなどから，内耳幹細胞は支持細胞の中にあると考えられている[16)]．

2 有毛細胞の幹細胞治療

内耳障害で最も象徴的に傷害される細胞は有毛細胞であり，これを標的とした新しい治療が模索されている．その1つが，幹細胞から有毛細胞を作製して移植するというものである．

1）幹細胞から内耳有毛細胞の分化誘導は可能か？

さまざまな幹細胞を用いて，内耳有毛細胞を作製することが試みられてきた．Liら[11)]は，マウスES細胞から胚様体を形成し，EGF，bFGF，IGF-1存在下に付着細胞を選択して分化させることによって，MyoⅦaやParvalbuminなどの有毛細胞のマーカーを発現し，さらに発生中のニワトリの耳胞に移植すると耳胞に取り込まれて聴毛を有するまでに分化したとしている．ほかにも，ラットの神経幹細胞を有毛細胞に分化誘導する試み[17)]やヒトES細胞から分化させる方法[18)]なども報告されている．これらの報告は，有毛細胞のような高度に分化した細胞でも原理的には幹細胞から分化誘導することが可能であることを示している．しかし，細胞移植の材料として用いるほど多量に，安定して得られるというほどの制御を得られているわけではない．また，誘導された有毛細胞が機能することを確認するためには，聴毛の動きに対する膜の脱分極や細胞内カルシウム濃度の上昇がみたいところであるが，そこまで成熟した有毛細胞が得られているわけではない．さらに，有毛細胞には，蝸牛の内有毛細胞・外有毛細胞，前庭のⅠ型・Ⅱ型の4種類があり，それぞれ役割が異なるものであるが，そもそもこれらの発生学上の差違についての研究はほとんど進んでおらず，上記の方法で誘導された有毛細胞がどれに相当するかもわかっていない．有毛細胞の分化発生機構について，前節に述べたような詳細が明らかになってきたのはむしろ上記の報告以降であり，今後，これらの新しい知識を用いることで，有毛細胞の*in vitro*での誘導という面での新たな展開がある可能性がある．

2）有毛細胞の移植治療は可能か

内耳有毛細胞が得られたとして，それを移植することによる難聴の治療はできるのであろうか．内耳有毛細胞は，細胞自体が高度に分化した機能細胞であるだけでなく，整然と配列することも聴覚に必須であり，そのような複雑な構築をもった配列を移植によって再現することは，細胞を作製することよりもさらに困難が予想される．これまで，内耳に幹細胞を導入するさまざまな方法が報告されており[19)～22)]，内耳のさまざまな部分に細胞の導入が可能であることは示されているが，多数の有毛細胞が望ましい配列で感覚上皮に挿入されるというところまで制御できるわけではない．あるいは，有毛細胞として移植するよりも，支持細胞を作製・移植して有毛細胞への分化転換を図るとか，これらの前駆細胞を移植して感覚上皮を形成させるというのも，今後試すべき方法であろう．障害を与えた内耳に有毛細胞発生のキーになる遺伝子である *Atoh1* をウイルスベクターを用いて導入することで整然とした配列をもった有毛細胞が発生したという報告もあり[23)]，有毛細胞発生機構あるいは分化誘導から得られた情報をもとに，遺伝子導入や阻害剤の導入を行って有毛細胞の自発的再生を促すという考え方は，細胞移植と並行して，あるいは組み合わせて考えていかなければならないと考えている．

3 ラセン神経節細胞の幹細胞治療

聴覚の一次感覚ニューロンであるラセン神経節細胞は，内耳障害において第一義的に傷害されるほか，有毛細胞の障害に引き続いて二次的に失われることも知られている．現時点で高度感音難聴に対する唯一の治療方法である人工内耳は，蝸牛に電極を挿入し，外部のマイクで拾った音に応じて蝸牛内電極でラセン神経節細胞を直接刺激することで聴覚を得るという人工臓器である（図4）．保険適応にもなっており，すでに日常の診療で用いられているが，ラセン神経節の障害が高度になると人工内耳の効果が低下すると考えられており，ラセン神経節が再生できるならその意義は大きい．

1）幹細胞移植によるラセン神経節細胞の再生

神経細胞は幹細胞から比較的容易に作製することのできる細胞であるが，ラセン神経節細胞に対する移植には，ES細胞・神経幹細胞・骨髄由来間葉系細胞など由来する未分化な神経細胞や内耳由来の不死化神経細胞株[24)]などさまざまな細胞が用いられている．実験動物でラセン神経節に移植する方法として，3つの方法が主に用いられている．①蝸牛壁あるいは半規管から内腔（外リンパ腔）に注入する方法[19)]，②蝸牛壁からラセン神経節を容れているローゼンタール管※4を直接穿刺して注入する方法[25) 26)]，③内耳道から聴神経に沿って逆行性に注入する方法[27)]である（図5）．①は手技的に容易であり，汎用されていたが，最近では②のように目的とする場所に直接注入するより確実な方法が好まれる．②は蝸牛の破壊を伴うのに対し，③は蝸牛を温存できるが，内耳道からローゼンタール管へ到達する細胞数は限られている．

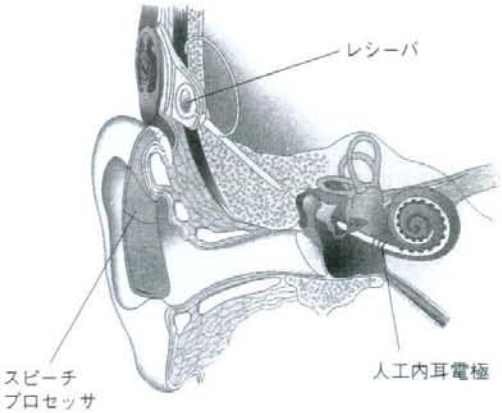

図4　人工内耳
http://corporate.cochlear.com/Corp/Press/186.asp より改変

2）移植幹細胞は内耳で機能するか

われわれはさまざまな試みを行ってきた中で，齧歯類と霊長類（サル）において，ES細胞由来の未熟な神経細胞を内耳障害を与えたラセン神経節に移植することで，いったん高度に悪化した電気聴性脳幹反応（eABR）※5が改善するというデータを得ている（投稿準備中）．いかにしてこのような機能回復が起こるのであろうか．ラセン神経節細胞はグルタミン酸作動性の双極性ニューロンである．われわれを含めて，グ

※4　ローゼンタール管
蝸牛軸のうち最もコルチ器に近い部分で，ラセン神経節の細胞体が存在する．

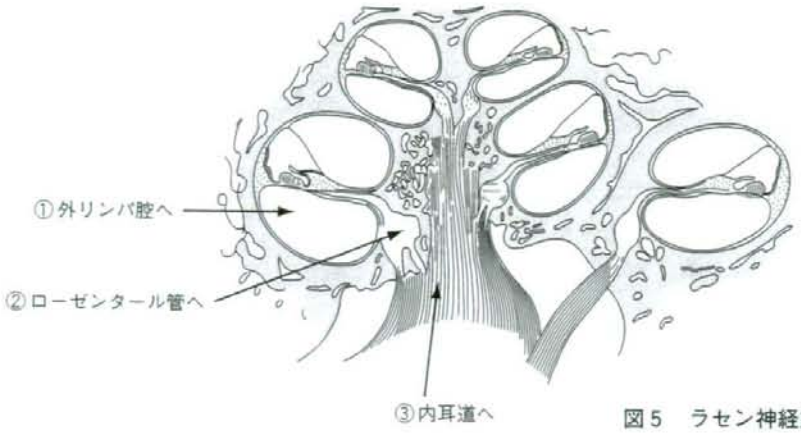

図5　ラセン神経節への移植経路

ルタミン酸作動性ニューロンを特異的に誘導した幹細胞由来神経を移植したという報告はないが，少なくともES細胞由来の神経細胞にグルタミン酸受容体が存在することは知られている[28]．また，ローゼンタール管に移植された神経細胞が有毛細胞基底部へ，あるいは内耳道を中枢の方向へと線維を伸張すること[25]，ES細胞由来の神経細胞が培養コルチ器の有毛細胞とシナプスを形成することが示されている[29][30]．以上から考えると，移植神経細胞が有毛細胞と脳幹の蝸牛神経核をつなぐ神経回路を再構成し，機能しているという可能性がある．

もう1つの可能性としては，移植した細胞が分泌する神経栄養因子が障害を受けたラセン神経節細胞を保護する[31]ということがあり得る．NT-3やBDNFなどの神経栄養因子が障害を受けたラセン神経節細胞の生存を助けることはよく知られており[32]，移植した細胞がこれらを分泌することで，残存したラセン神経節細胞を生存させるというものである．この効果のみを目的とするならば，移植する細胞は幹細胞である必要はなく，BDNFを発現する線維細胞を蝸牛に移植するというcell-gene therapyという方法がすでに報告されている[33]．

※5　電気聴性脳幹反応（eABR）

聴性脳幹反応（ABR）は音刺激に対する聴神経・脳幹聴覚路の神経活動電位を記録したものであるが，eABRは音刺激の代わりに蝸牛に挿入した電極からの電気刺激に対する活動電位を記録したもの．有毛細胞の機能に依存せずに聴神経よりも中枢の神経活動を評価できる．

どちらにしても，幹細胞移植による機能回復がみられるという点での意義は大きい．このラセン神経節の再生を人工内耳と組み合わせることを想定すれば，すでに人工内耳挿入術は通常行われている手術であり，細胞移植はその延長で行える．その結果として相乗的な機能回復が期待できるので，ヒトにおける臨床応用に向けての現実性は高いと考えられる．

おわりに

内耳障害に対する幹細胞を中心とした細胞移植治療に関連するいくつかのトピックスについて説明した．有毛細胞に対する細胞移植は，移植細胞の準備という点でも移植による組織の再構築という点でも現時点ではまだ模索中であるが，発生学からの情報が急速に集まりつつあるので今後の展開が望まれる．ラセン神経節に対する細胞移植は臨床応用の実現により近い段階にある．現在はES細胞を用いて実験動物における機能回復を得た段階であるが，今後は脂肪幹細胞やiPS細胞などの自己由来幹細胞へと移行していくことになるであろう．

有毛細胞，ラセン神経節・前庭神経節と並んで内耳障害の原因と考えられている部位にラセン靱帯・血管条がある．薬物や遺伝子異常によるこれらの部位の障害が知られているが，幹細胞治療をここに応用しようという試みも最近報告された[34]．内耳のような複雑な構築をもった組織を幹細胞を用いて治療することは決して簡単ではないが，今後も再生医療による内耳障害治療の実現に向けてさまざまなアプローチで取り組んでいく必要がある．

本稿執筆にあたり，ご指導，ご監修いただいた京都大学大学院医学研究科耳鼻咽喉科・頭頸部外科伊藤壽一教授に深謝いたします．

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坂本達則：1995年京都大学医学部卒業．2004年京都大学大学院医学研究科修了後，理化学研究所発生・再生科学総合研究センターリサーチアソシエイト，'06年より京都大学大学院医学研究科耳鼻咽喉科・頭頸部外科助教．ES細胞の分化誘導と内耳発生についての研究を行い，医学博士取得．現在，再生医学を用いた内耳障害の治療に向けての研究を行っている．

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Surgical Invasiveness of Cell Transplantation into the Guinea Pig Cochlear Modiolus

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Key Words
Cell transfer · Surgery · Regeneration · Spiral ganglion neuron · Cochlear function

Abstract
Objective: Previous studies have demonstrated the potential of cell transplantation for regeneration of spiral ganglion neurons (SGNs). However, the effect of surgical invasion on host cochleae has yet to be evaluated. The present study investigated the efficiency and invasiveness of our surgical procedure using a fine glass pipette for injections into the cochlear modiolus. ***Methods:*** We examined the survival of transplanted embryonic stem cell-derived neurons in the cochlear modiolus of guinea pigs. Surgical invasiveness was assessed by measurements of electrically evoked auditory brainstem responses (eABRs) and SGN densities after an injection of 5 μl of saline into the cochlear modiolus. ***Results:*** All of the transplanted animals exhibited localization of transplanted cells in the cochlear modiolus. No significant alterations in the eABR thresholds or SGN densities were found following surgery. ***Conclusion:*** These findings indicate that our procedure is a viable method for testing the potential of transplants for SGN replacement.

Introduction

The mammalian inner ear was originally believed to have no regenerative ability [1]. Therefore, in order to try and induce the regeneration of inner ear cells, novel strategies including cell and/or gene therapy have been investigated. Several studies have shown the efficacy of cell transplantation for the replacement of spiral ganglion neurons (SGNs), auditory primary neurons [2–8]. However, surgical procedures for cell transplantation involve a risk of damaging the host's auditory system. It is therefore crucial to determine the level of surgical invasiveness and evaluate the efficacy of cell transplantation on functionality, although as of the present, these issues have yet to be well documented.

We previously reported that transplantation of embryonic stem (ES) cell-derived neurons into the cochlear modiolus rescued impaired auditory function in guinea pigs [9]. In this previous study, we used a 30-gauge needle for the injection of cell suspensions into the cochlear modiolus. More recently, we have revised our surgical procedure in order to reduce the surgical invasiveness to the host SGNs. In the revised procedure, we use a fine glass pipette to introduce the cell suspensions into the cochlear modiolus of guinea pigs. In the present study, we investigated the efficiency of our revised procedure, and

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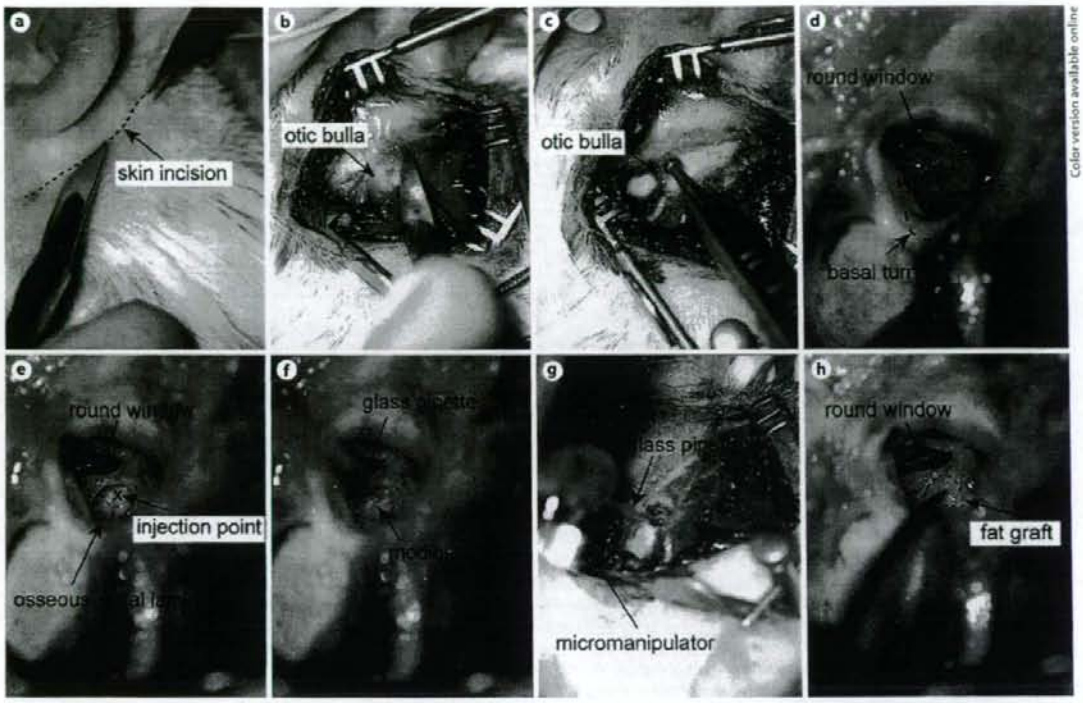

Fig. 1. Surgical procedure for cell transplantation into the cochlear modiolus. **a** Skin incision is made in the retroauricular region. **b** An otic bulla is exposed. **c** A small hole on the bulla is made using a drill. **d** The round window and the basal turn of the cochlea are observed through an opening on the bulla. An interrupted line indicates the location of the basal turn. **e** Cochleostomy is performed on the basal portion of the cochlea corresponding to the location of the scala tympani. A dotted line indicates the location of the osseous spiral lamina. A cross indicates the injection point. **f**, **g** A glass pipette is inserted into the cochlear modiolus using a micromanipulator. **h** The cochleostomy site is closed with a fat graft (dotted line).

evaluated the functional and histological damage to the host SGNs. To evaluate cell transplantation efficiency, we examined the survival of the transplanted ES cell-derived neurons in the cochlear modiolus of guinea pigs. The level of surgical invasiveness was estimated by measuring electrically evoked auditory brainstem responses (eABRs) and SGN densities in Rosenthal's canal following an injection of 5 μl of saline.

Materials and Methods

Animals

A total of 12 Hartley strain guinea pigs weighing 350–400 g were purchased from Japan SLC Inc. (Hamamatsu, Japan). All of the animals had otoscopically normal tympanic membranes and normal hearing, as determined by tone-burst ABR. The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all of the experimental protocols. Animal care was carried out under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Surgical Procedure

The animals were anesthetized with an intramuscular injection of ketamine (75 mg/kg) and xylazine (9 mg/kg), and body temperature was maintained at 37 ± 1°C using a heating pad. We used a conventional retroauricular approach in the lateral recumbent position (fig. 1a). After exposure of the otic bulla (fig. 1b), a small hole was made on the otic bulla to expose the round window niche and the basal turn of the cochlea (fig. 1c, d). After fixation of the head position using clay and surgical tape, cochleostomy was performed

on the basal portion of the cochlea to visualize the cochlear modiolus through the scala tympani (fig. 1e). A fine glass pipette was made with a Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, Calif., USA), with the tip cut with a fine blade to make an outer diameter of approximately 100 μm. A micromanipulator was used to insert the glass pipette, which was connected to a microsyringe (Hamilton, Reno, Nev., USA), into the cochlear modiolus of the basal portion of the cochlea (fig. 1f, g). After removal of the perilymph with filter paper, we directly inserted the glass pipette into the bony wall of the cochlear modiolus from a point slightly basal to the junction of the osseous spiral lamina and the modiolus (fig. 1f). In this particular region, a glass pipette is capable of penetrating the bony wall of the cochlear modiolus due to the thickness of the bony wall. Subsequently, we infused 5 μl of the substrate through the glass pipette using a microinfusion pump set at a rate of 1 μl/min. The glass pipette was removed 1 min after stopping the infusion. Finally, the cochleostomy site was closed with a fat graft and then covered with fibrin glue (fig. 1h).

Efficiency of the Cell Transplantation Procedure

We estimated the transplant survival of mouse ES cell-derived neural progenitors in the cochlear modiolus using histological analysis. Mouse G4-2 ES cells (provided by H. Niwa of Riken CDB, Kobe, Japan) that were derived from the E14tg2ab ES cell line and which carried the EGFP gene driven by the CAG promoter were used as the transplant source. Neural induction of ES cells was performed by stromal cell-inducing activity [10] before transplantation. Briefly, undifferentiated ES cells were cultured on a feeder layer of PA6 stromal cells (RCB1127; Riken Cell Bank, Tsukuba, Japan) in Glasgow's modified Eagle's medium (GMEM; Invitrogen, Carlsbad, Calif., USA) supplemented with 5% knockout serum replacement (Invitrogen), 1 mM pyruvate (Sigma, St. Louis, Mo., USA), 0.1 mM nonessential amino acids (Invitrogen), and 0.2 mM 2-mercaptoethanol (Wako, Osaka, Japan). Colonies that formed on the PA6 monolayer after 6 days of culture were isolated and prepared as suspensions of 10^4 cells/μl GMEM.

In 4 guinea pigs, 5 μl of the cell suspensions were injected into the cochlear modiolus in each animal using the procedure described above. At 1 week after cell transplantation, the otic bullae of the experimental animals were opened under ketamine and xylazine anesthesia, and then 4% paraformaldehyde in 0.01 M phosphate-buffered saline at pH 7.4 was perfused into the perilymph from the round window. The animals were deeply anesthetized with a lethal dose of ketamine and xylazine that was perfused intracardially with physiological saline, followed by infusion of the same fixative. The temporal bones were then collected and immersed in the same fixative for 4 h at 4°C. Specimens (10 μm thick) were prepared using a cryostat after decalcification with 0.1 M ethylenediaminetetraacetic acid in phosphate-buffered saline for 3 weeks at 4°C. From each cochlea, three mid-modiolus sections were stained with 2 μg/ml 4′,6-diamino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, Oreg., USA), and then viewed with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan). Settlement of transplants was determined by the existence of both EGFP- and DAPI-positive cells in Rosenthal's canal or the cochlear modiolus.

Functional Estimation of Surgical Invasiveness

We injected 5 μl of physiological saline into the modiolus of the left cochlea of each of 8 guinea pigs. Functional damage was evaluated by determining eABR thresholds immediately before and after surgical treatment, and on days 1 and 14 after surgical treatment. eABR measurements were performed in a sound-attenuated and electrically shielded room. One platinum/iridium ball electrode (Nihon Koden, Tokyo, Japan) was inserted into the scala tympani through the cochleostomy site and was then placed approximately 1.5 mm deep from the cochleostomy site. Another electrode was fixed to the temporal bone where it served as an extracochlear ground for monopolar stimulation (fig. 2a).

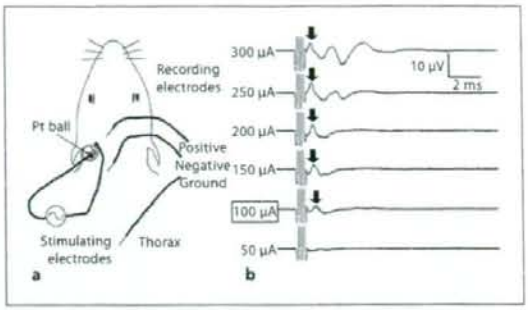

Fig. 2. Measurement of eABRs. **a** A platinum ball electrode is inserted into the scala tympani in the basal portion of the cochlea. Another wire serves as extracochlear ground for monopolar stimulation, and is fixed to the temporal bone. For recording, three electrodes are used (positive; vertex, negative; neck, ground; thorax). **b** The threshold is defined as the smallest current amplitude required to evoke a response within a latency of 4 ms after stimulus onset. Arrows indicate the first positive wave after stimulus onset. The threshold of this sample is 100 μA.

Biphasic current pulses were generated under computer control using a real-time processor (Tucker-Davis Technologies, Alachua, Fla., USA). Stimuli were altered in 50-μA steps, with the stimulus current levels calibrated by measuring the voltage with an oscilloscope from 1,000 to 10,000 Ω. The responses against electrical stimuli were recorded separately using stainless steel needle electrodes (fig. 2a, vertex positive, neck negative, thorax ground). The electrical stimulus consisted of charge-balanced biphasic current pulses, which occurred 50 times per second. The scalp-recorded response was amplified by 3×10^3 and band-pass-filtered (200–1,500 Hz). The filter output was fed to an analog-to-digital converter and sampled for 10 ms following stimulus onset. For each recording, 500 responses were averaged and then stored for subsequent analysis. Two sets of recordings were made at each current level, and the current amplitude was reduced to levels below threshold. The thresholds for eABRs were determined as described previously [11–15]. The threshold was defined as the smallest current amplitude that was required to evoke a response with a latency of 4 ms following the stimulus onset for both responses (fig. 2b).

Histological Estimation of Surgical Invasiveness

After saline injection into the cochlear modiolus, the temporal bones were collected on days 1 (n = 4) and 14 (n = 4). Right co-

Fig. 3. Locations of transplanted cells in the cochlea. EGFP-expressing cells (green; light gray in the printed version) are found in the cochlear modiolus and Rosenthal's canal from the basal to the middle portion of cochleae (**a**, **b**, arrows). One specimen exhibits settlement of transplanted cells in the scala vestibuli (**c**, arrowhead). Blue fluorescence (rendered dark gray in the printed version) shows nuclear staining with DAPI.

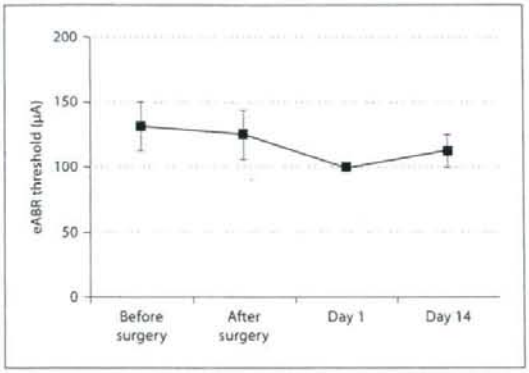

Fig. 4. eABR thresholds immediately before and after surgical treatment, and on days 1 and 14 after surgical treatment. The y-axis shows the mean of eABR thresholds. There are no significant differences in eABR thresholds among experimental groups. Bars represent standard errors.

chleae, which underwent no surgical treatments, were used as controls. After eABR recordings, cochlear specimens were collected and prepared as cryostat sections using a similar procedure as for the transplanted ES cell-derived neurons. Sections were stained with hematoxylin and eosin (HE). SGN counting in Rosenthal's canals was performed in accordance with a method that has previously been described [13–15]. SGN numbers were determined in the basal, mid-basal or second turn from 5 randomly selected mid-modiolar sections of the cochleae that underwent surgery, and from the contralateral cochleae that received no surgical treatment. The cross-sectional areas of Rosenthal's canals were measured using Image/J software (http://www.rsb.info.nist.gov/ij). SGN densities were then calculated by dividing the number of SGNs by the area. This value was used to reduce the variance caused by differences in the cutting directions among the cochlear specimens.

We also evaluated cell infiltration into the cochlea on days 1 and 14 after surgery. The mean numbers of nucleated cells in the scala tympani, scala vestibuli and scala media were determined in the basal, mid-basal and second turns from 5 randomly selected mid-modiolar cochlear sections.

Statistics

The overall effect on the eABR threshold shifts during the surgical procedure was examined by one-way factorial analysis of variance (ANOVA). The values of SGN densities in Rosenthal's canals that were obtained on days 1 or 14 after surgical treatment were compared with those of the contralateral cochleae by an unpaired t test. A p value less than 0.05 was considered statistically significant. All data are presented as the mean ± standard error.

Results

Efficiency of Cell Transplantation

We examined the survival and the location of engrafted ES cell-derived neurons in order to determine the efficiency of cell transplantation with our surgical procedure. All of the cochlear specimens exhibited settlement of the EGFP-expressing cells in Rosenthal's canals or the modioli in the basal portions of the cochleae, indicating that our transplantation procedure using a fine glass pipette was efficient for introducing transplants into the cochlear modiolus. EGFP-positive cells were also identified in the middle portion of the cochlear modiolus in 2 out of 4 experimental animals (fig. 3a, b). EGFP-positive

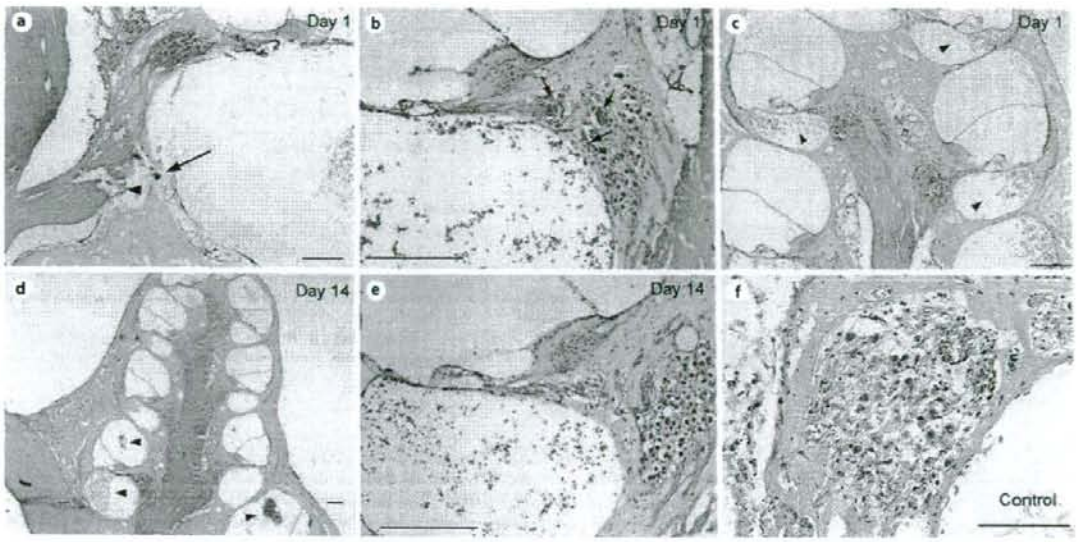

Fig. 5. Histological damage in a cochlea due to surgical procedures. **a** At the injection site, bone fracture of the cochlear modiolus (arrow) and injury of nerve fibers (arrowhead) is found on day 1 after surgery. **b** In the mid-basal portion of the cochlea on day 1 after surgery, red blood cells are observed in Rosenthal's canal (arrows). **c** From the second to the apical turn of the cochlea, no degenerative changes in the organ of Corti and spiral ganglions are identified, although a number of red blood cells are found in the scala tympani (arrowheads). **d** Cell infiltration (arrowheads) is observed in the scala tympani and scala vestibuli of the basal portion of the cochlea on day 14 after surgery. **e** In the basal turn of the cochlea on day 14 after surgery, the organ of Corti and the spiral ganglion exhibited normal morphology, despite cell infiltration in the scala tympani. **f** Spiral ganglion in the basal turn of the control cochlea. Bars = 20 μm.

cells were also observed in the scala vestibuli in 1 of the experimental animals (fig. 3c), indicating leakage of injected transplants from the cochlear modiolus.

Functional Damage due to Surgical Procedures

To evaluate the functional damage to SGNs and nerve fibers, we measured the eABRs, which are frequently used for evaluation of SGN function [11–15]. The mean values of the eABR thresholds immediately before and after surgery were 131.3 ± 18.6 and 125.0 ± 18.9 μA, respectively. On days 1 and 14 after surgery, the mean values were 100.0 ± 0.0 and 112.5 ± 12.5 μA, respectively. There were no significant differences in the eABR thresholds among the experimental groups (fig. 4), indicating that our procedure caused no severe functional damage of the SGNs.

Histological Damage due to Surgical Procedures

HE staining of mid-modiolar sections revealed actual points of injection in the cochlear modiolus. In all specimens, the injected points were located in the cochlear modiolus of the basal turn from a point slightly inferior to Rosenthal's canal (fig. 5a). On day 1 after surgery, infiltration of nucleated cells in the modiolus and injury in the nerve fibers was observed in the injected site (fig. 5a). From the basal to the second turn, a number of red blood cells were found in the scala tympani, scala vestibuli and scala media. Red blood cells were also found in Rosenthal's canal of the basal and mid-basal portions of cochleae (fig. 5b). However, organs of Corti and SGNs were well preserved in these portions of cochleae. In the apical portion of cochleae, no apparent histological damage was identified, although cell infiltration into the scala tympani and scala vestibuli was observed (fig. 5c). On day 14, red blood cells in the cochlear fluid space decreased (fig. 5d); however, cell infiltration in the scala tympani of the basal and mid-basal portions were still observed (fig. 5d, e). Remarkable degeneration of SGNs was not observed in the cochleae on day 14 in comparison with normal cochleae (fig. 5f). We quantified the histological damage to SGNs

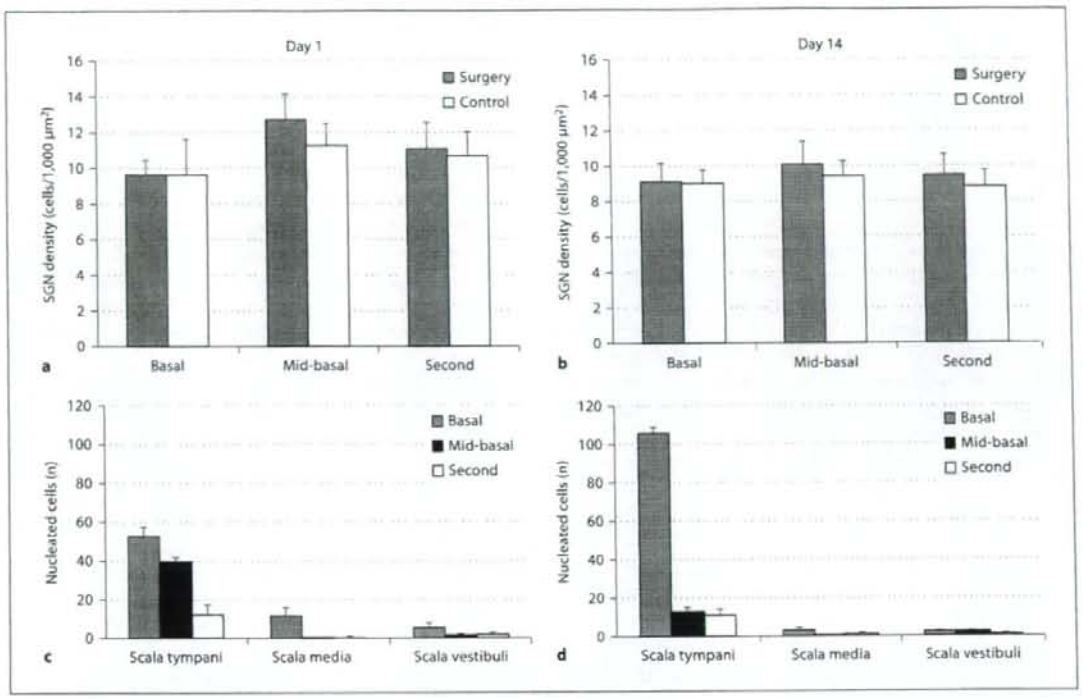

Fig. 6. Mean density of SGNs (**a**, day 1; **b**, day 14) and mean number of nucleated cells (**c**, day 1; **d**, day 14) in the basal, mid-basal and second turns of cochleae. No significant differences in the density of SGNs (SGN density) are found in each turn of cochleae between specimens after surgery and control specimens (**a**, **b**; unpaired t test). Bars show standard errors.

by measuring SGN densities in Rosenthal's canals. There were no significant differences in the SGN densities in every portion between the cochleae with surgical treatment and the cochleae without surgical treatment on days 1 or 14 after surgery (fig. 6a, b). We also quantified the number of nucleated cells in the scala tympani, scala media and scala vestibuli. A number of nucleated cells were found in the basal and mid-basal turns of the cochleae, notably in the scala tympani, on day 1 after surgery (fig. 6c). On day 14, there was a trend for the numbers of nucleated cells to decrease in the mid-basal and second turn of cochleae, while in the scala tympani of the basal turn, an increase in the number of nucleated cells was observed (fig. 6d). These findings indicate that our surgical procedure causes no significant loss of SGNs, although certain injury in the injected points and consecutive cell infiltration occurred in the basal turn of cochleae.

Discussion

The primary aim of this study was to establish a minimally invasive procedure for cell transplantation into the cochlear modiolus, which would allow for successful introduction of the transplants into the cochlear modiolus. Previously, Brown [16] established a method for recording single efferent and afferent auditory nerve cells. In this method, after cochleostomy in the basal turn of the cochlea, a small opening on the osseous spiral lamina is made using a fine insect pin followed by insertion of a fine glass electrode through the opening. This method is actually a very low invasive and efficient procedure for accessing the auditory nerves from the scala tympani. The aim of the present study was to establish a method for cell transplantation into the cochlear modioli of guinea pigs by introducing cell spheres through a glass pi-

pette. To achieve this primary aim, it is necessary to use glass pipettes with larger tips. However, the comparatively thick glass pipettes that were used in the present study were too large for insertion into the osseous spiral lamina. Therefore, we inserted a glass pipette into the modiolus of the basal portion of the cochlea.

The present findings demonstrated that there was no significant elevation of eABR thresholds after surgery, indicating that no severe functional damage of the SGNs due to surgical procedures had occurred. Measurements of eABR thresholds have frequently been used for evaluation of SGN function in animal experiments [9, 11–15]. In such experiments, animal models for severe SGN degeneration are used. Although eABR thresholds are actually effective for evaluating severe SGN degeneration, it has been reported that the eABR is of limited value when it comes to detecting only minimal SGN loss [17]. Therefore, our present findings for eABRs did not indicate that there was a complete preservation of host SGNs. We recorded eABRs immediately before and after surgery, and on days 1 and 14 after surgery in order to evaluate acute and chronic effects of our surgical procedure on ABR thresholds. The results demonstrated that there were no significant alterations in eABR thresholds between the time points for which the data were collected, indicating that the surgical procedure itself and the inflammatory responses that follow surgery do not affect eABR thresholds.

Histological analysis of the SGNs also revealed that there was no significant loss of SGNs due to our surgical procedure, which supports our present findings for eABR measurements. Although there was no significant decrease in SGNs after surgery, infiltration of nucleated cells and the presence of bleeding were found in the cochleae, mainly in the scala tympani. On day 1, nucleated cells were predominantly found in the basal and mid-basal portions of cochleae, while on day 14, the distribution of nucleated cells was limited in the basal portion of cochleae. However, the number of nucleated cells in the basal portion of cochleae on day 14 increased from that on day 1. These findings indicate that immediate inflammatory responses due to surgery occur in the basal and mid-basal portions of cochleae, and chronic inflammation continues in the basal portion until day 14. However, such inflammatory responses caused no significant SGN degeneration.

In our previous studies [9, 18], we used a 30-gauge needle for introducing transplants into the cochlear modiolus, which resulted in efficient settlement of the transplants in the cochlear modiolus. In the present study, in order to reduce the surgical invasiveness to host SGNs, a glass pipette was used for the injection of substrates into the cochlear modiolus instead of a needle. The outer diameter of a 30-gauge needle is approximately 500 μm, while that of a glass pipette is only 100 μm, which is likely to reduce the extent of surgical damage. Although the use of a glass pipette could result in an insufficient introduction of ES cell-derived neurons into the cochlear modiolus, the present histological findings demonstrated that there was an efficient settlement of transplanted cells in the cochlear modiolus. We therefore believe that the use of a glass pipette is an efficient and safe method for the introduction of cells into the cochlear modiolus of guinea pigs.

Several previous studies have documented the secretion of trophic factors from transplanted stem cells in the cochlea [19–21]. Culture media can also include various trophic factors, and thus, an injection of cell suspensions or culture media has the potential to protect SGNs against surgical invasiveness. Therefore, to evaluate surgery-related functional and histological damage to host SGNs, instead of cell suspensions, we injected saline into the cochlear modiolus.

In conclusion, our present findings demonstrate that a surgical procedure that uses a glass pipette can efficiently introduce transplants into the cochlear modiolus with no significant alteration in eABR thresholds and in SGN densities in normal adult guinea pigs. Our revised procedure for cell transplantation into the cochlear modiolus can be used to investigate the potential of various transplants for functional regeneration of SGNs in the guinea pig.

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OTOLOGY

Efficiency of a transtympanic approach to the round window membrane using a microendoscope

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Abstract There has been increasing interest in cochlear drug delivery through the round window membrane (RWM). However, placing drugs on the RWM is difficult because of anatomical barriers. We examined the efficacy of a microendoscope for a transtympanic approach to the RWM. We evaluated the visibility of the RWM using four approaches: transtympanic microendoscopic, transtympanic microscopic, transmastoid microendoscopic, and transmastoid microscopic in ten human temporal bones. For the transtympanic approach, we made a fenestration (2 × 1 mm) in the postero-inferior quadrant of the tympanic membrane. For the transmastoid approach, conventional posterior hypotympanotomy was performed. The transtympanic microendoscopic approach enabled visualization of the RWM in all specimens, whereas the transtympanic microscopic approach only permitted visualization in three specimens. Through the transmastoid approach, the RWM was visible in all specimens using either a microendoscope or a microscope. The transtympanic microendoscopic approach can be utilized for cochlear drug delivery through the RWM.

Keywords Microendoscope · Round window membrane · Cochlea · Drug delivery

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in industrial countries. Systemic administration of steroids has been widely used for the treatment of acute profound hearing loss [1]; however there are limitations in their clinical efficacy [2]. At present, therapeutic strategies are limited to hearing aids and cochlear implants for patients with chronic SNHL. Based on this background, basic investigations have elucidated several agents that are effective for the treatment of SNHL. However, the problem of how to deliver drugs to the inner ear has been a considerable obstacle to the development of treatments for SNHL. The blood-inner ear barrier prevents the transportation of serum drugs to the inner ear, and the blood flow to the inner ear is very limited.

Drug transduction through the round window membrane (RWM) is one option for delivering drugs into the inner ear. Continuous infusion of RWM with an osmotic pump and microcatheter has been reported as an effective and safe approach [3]. However, it requires surgery and the invasion cannot be overlooked. Recently, new local drug application procedures using biodegradable substances are gaining interest [4, 5]. The inner ear is one of the targets for local drug administration using biodegradable gelatin hydrogels [6, 7]. In this drug delivery system, positively charged proteins or peptides are electrostatically trapped in negatively charged gelatin polymer chains. As the gelatin polymer chains degrade, proteins or peptides are released from the hydrogel. The released protein is conveyed through the RWM into the inner ear via a concentration gradient. Therefore, close contact of biodegradable hydrogels with the RWM is critical for efficient drug delivery to inner ear fluids.

The RWM is situated perpendicular to the tympanic membrane and deep in the round window niche. In some cases, a false membrane covers the RWM. For safe and certain drug administration, hydrogels containing drugs should be placed on the RWM under direct visualization. Use of a

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microendoscope is an effective method for visualization of the RWM [8]. It is equipped with a working channel, which can be used in drug administration. However, the potential of microendoscopes for placing substrates on the RWM has not been evaluated, and it is important to clarify the prevalence of subjects in whom the RWM is microendoscopically visible. In the present study, we examined the potential of a specially modified microendoscope for a transtympanic approach to the RWM using human temporal bones.

Materials and methods

Ten formalin-fixed temporal bones with no middle or inner ear diseases were obtained from six individuals (aged from 68 to 76 years at death, five male, and one female). A microendoscope (0.9 mm in outer diameter, 50 mm in length; FiberTech, Tokyo, Japan) was specially modified in the fit angle for observation of the RWM through the tympanic membrane. The tip is curved 15° (Fig. 1). The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter).

We used four different approaches to observe the RWM as follows: (1) transtympanic microendoscopic, (2) transtympanic microscopic, (3) transmastoid microendoscopic, and (4) transmastoid microscopic. For the transtympanic approach, a small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife (Fig. 2). The microendoscope was inserted into the middle ear through this fenestration and set to provide the best view of the RWM. For observation with a microscope, the fenestration edge in the tympanic membrane was gently pushed with a curved needle to obtain the best access to the RWM. For transmastoid approaches, canal-wall up complete mastoidectomy and posterior hypotympanotomy were performed under conventional microscopy (Leica M300, Leica Microsystems, Wetzlar, Germany). The bones covering the middle cranial fossa dura, the posterior fossa dura, and the sigmoid sinus were drilled to be as thin as possible. The bony wall of the external auditory canal was preserved. The facial nerve and chorda tympani nerve were skeletonized and the facial recess was opened as large as possible (Fig. 2).

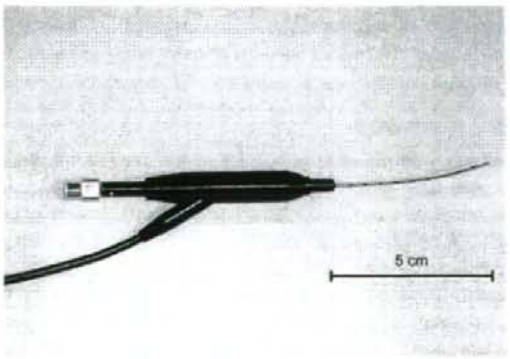

Fig. 1 A microendoscope specially modified for better visualization of the RWM. The outer diameter is 0.9 mm and the length is 50 mm. The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter)

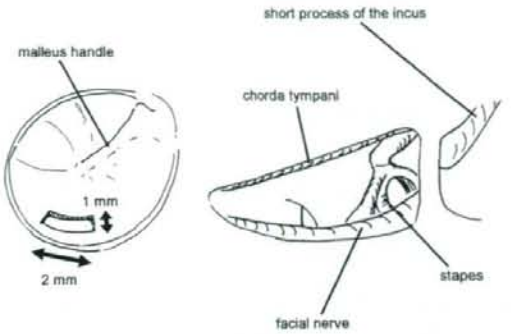

Fig. 2 A small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife. Posterior hypotympanotomy was made as large as possible. In all specimens, the facial nerve and chorda tympani were skeletonized

The RWM was observed through a posterior hypotympanotomy with a microendoscope or a microscope. Surgical procedures were performed by one author (Harukazu Hiraumi). The view of the RWM and surrounding structures using the four approaches was video-captured. Frames showing best view of the RWM were converted into still images, and the area of the RWM was measured using image-processing program, ImageJ. An angled hook (1.0 mm sharp tip) was used as a reference. Total area of the RWM was measured after drilling the round window niche. The visibility of the RWM was calculated and graded into three classes: Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. In three samples, the round window niche was covered with false membranes. In these cases, the false membranes were removed with a curved needle under microscopic view via posterior hypotympanotomy.

Results

A microendoscope was smoothly inserted into the middle ear cavity and the incuidostapedial joint was observed easily in all the specimens. The percentage of the area of the