内耳DDS

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感音難聴の治療はあまり選択肢もなく，困難なことが多い．その背景には全身投与した薬剤の内耳での利用率が低いことや，蝸牛内での障害部位を非侵襲的に診断する方法がないことなどの問題がある．内耳，特に蝸牛へのDDSは局所投与に始まっていくつかの方法が試みられ開発されようとしているが，なかでも正円窓膜上に置いた徐放製剤を用いて蝸牛に薬物を投与する方法は，臨床的にみて現実的な方法である．我々は急性感音難聴ステロイド無効例に対して，ハイドロゲルを用いたインスリン様細胞成長因子1投与による難聴治療効果をみる臨床試験を実施している．また，リドカイン徐放PLGAパーティクルを用いた耳鳴治療の臨床試験を計画中である．

KEY WORDS
◇感音難聴
◇耳鳴
◇正円窓
◇ゼラチンハイドロゲル
◇PLGAマイクロパーティクル

はじめに

聴力障害を理由に身体障害者認定を受けている人は，わが国に36万人いるとされているが，この方々の難聴は「耳もとでどなり声で話してもコミュニケーションに不自由がある」という非常に高度な両側性の難聴である．ここまで高度ではなくても，実際に聴力障害で不自由を感じている人は600万人いるともいわれている．この難聴に対して，現在までに提供できている治療は限られており，約30年前に本格的に始まった人工内耳以外には，新しい治療も開発が進んでいない．また，耳鳴は難聴に伴う代表的な症状の1つである．米国では人口約3億人のうち5,000万人以上が耳鳴を感じており，そのうち1,200万人が激しい耳鳴に対する治療を必要としているといわれているが，これに対する現実的な治療はない．しかし，難聴に対しても耳鳴に対しても，治療の可能性のある薬剤が全くないわけではない．本稿では，内耳治療の問題点から内耳ドラッグデリバリーシステム(DDS)を用いた臨床応用までを解説する．

聴覚の仕組みと難聴の原因

音は，空気の疎密波として外耳道を経由して鼓膜を振動させる(**図1A**)．中耳腔にある耳小骨が振動を蝸牛に伝達し，蝸牛内では基底板を振動させる(**図1B**)．蝸牛内の前庭階と鼓室階を満たす外リンパ液は通常の細胞外液と同様の組成であるが，中央階を満たす内リンパ液は高カリウム・低ナトリウムという細胞内液に近い組成になっている．基底板の上にはコルチ器があり，振動に伴って有毛細胞の聴毛が偏位することで物理的にイオンチャネルが開き，中央階に豊富なカリウムイオンが細胞内に流入することがきっかけとなって有毛細胞が脱分極し，神経伝達物質が放出され，聴神経とのシナプスを介して脳幹，大脳へと伝えられる(**図1C**)．

この経路のどこが傷害されても難聴になり得るが，中耳・外耳の障害による難聴は伝音難聴と呼ばれ，手術などの治療が可能な場合も多い．内耳よりも中枢側の難聴が感音難聴である．

現在の感音難聴治療と問題点

感音難聴に対する治療の選択肢は，決して広いとはいえない．急性感音難聴の最も代表的な疾患である突発性難聴の治療としては急性期にステロイド大量療法を行うことが一般的であるが，これを行なっても反応しない症例が約3分の1存在するといわれている．こ

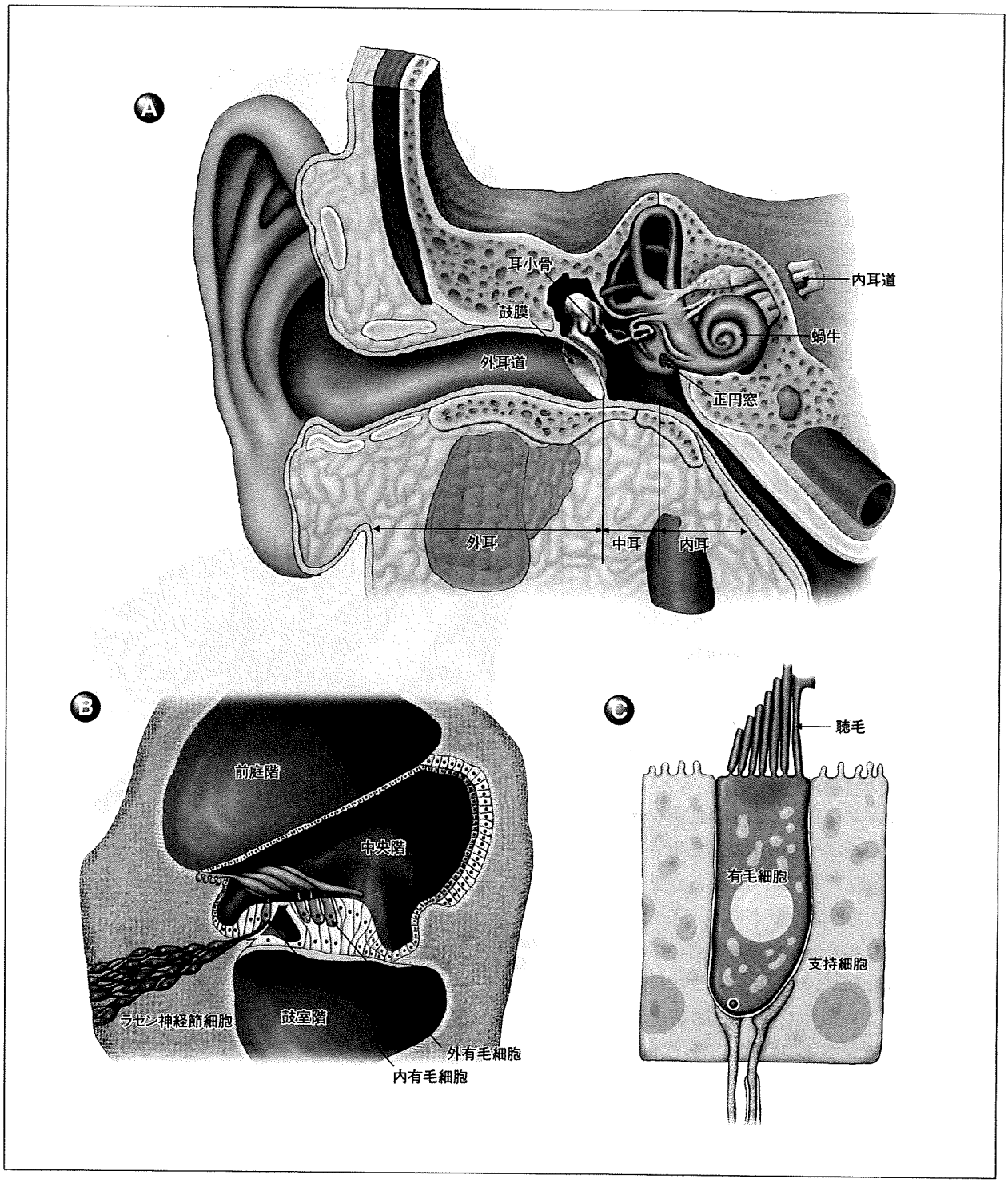


図 1．内耳・蝸牛

A：外耳・中耳・内耳の解剖．B：蝸牛の断面．C：有毛細胞．詳細は本文参照．

れらに対して高気圧酸素療法やプロスタグランジンE1製剤，ステロイドの鼓室内投与，ビタミン製剤などの使用が試みられてきたが，いまだに一定の評価を得たものはない．これには幾つかの要因が考えられるが，1つは内耳血流の問題である．内耳は前下小脳動脈(AICA)の枝である迷路動脈の血流支配を受けており，蝸牛にはその分枝が分布する(**図2**)．蝸牛血流量はげっ歯類で心拍出量の10,000分の1，ヒトでは1,000,000分の1と極めて少ない[1]うえに，脳の血液脳関門と同様の血液蝸牛関門(blood-cochlear barrier)と呼ばれる障壁が存在して薬剤の内耳への到達を阻んでいるため[2]，全身投与した薬剤の蝸牛での利用率は限られている．もう1つの問題は病態が確定できないという点である．突発性難聴はその背景にウイルス感染，虚血，出血などがあると考えられており，障害部位として有毛細胞，ラセン神経節細胞，血管条といった蝸牛内の様々な部位が想定されている．当然病態に応じて異なる治療法が存在するはずであるが，現在これを非侵襲的に診断する技術が無い．

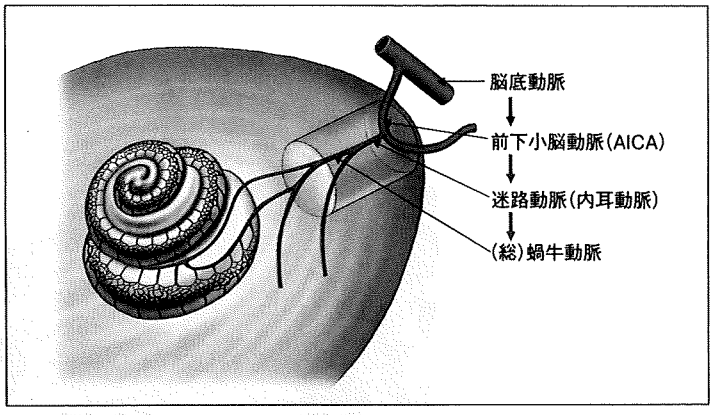


図2．蝸牛の血流

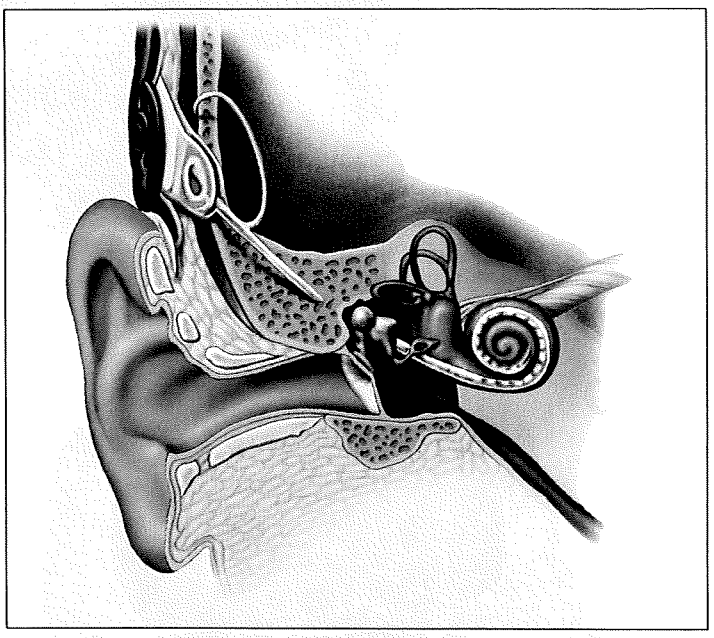


図3．人工内耳
側頭部皮下に置いたレシーバーから伸びた電極は蝸牛の内部に挿入され，直接ラセン神経節細胞を刺激する．

通常，難聴がかなり強くても慢性感音難聴の場合は対症療法として補聴器を用いるが，難聴が両側性で特に高度になった場合には人工内耳という選択肢がある．人工内耳は，蝸牛内に埋め込んだ電極を用いてラセン神経節細胞(聴覚の一次ニューロン)を直接刺激するというものである(**図3**)．デバイスや音声情報処理の改善によって性能が向上し，言語聴取についてはある程度の成績を出せるようになってきたとはいうものの，音楽がわからないなど，音質はいまだ補聴器を超えるほどではない．人工内耳の機構上，ラセン神経節細胞が存在しない，あるいは高度に変性した症例では効果がない．挿入時に蝸牛の破壊を伴うため，高度難聴にしか適応できないこと，保険適応とはいうもののデバイスが高価であること，全身麻酔による手術が必要であること，術後の調整(マッピング)が継続的に必要であること，ランニングコストを含めた費用の問題などがある．

内耳をターゲットとしたDDS

1．経中耳(経正円窓膜)投与

全身投与した薬物が内耳に十分に到達するなら臨床的には簡便で望ましい方法であるが，前述したように全身投与した薬剤の内耳での利用率は低い．これに対して，局所投与は比較的古くから用いられてきた方法である．点耳は外耳道に直接薬剤を投与するもので，外耳炎や鼓膜炎に対しては直接の効果があり，鼓膜穿孔がある場合には中耳にも薬剤を直接投与することができる方法であるが，内耳への効果は一定しない．鼓室内注入は鼓膜麻酔あるいは無麻酔で鼓膜を穿刺して薬剤を中耳腔に注入するもので，手技も容易なのでよく用いられている(**図4A**)．蝸牛は内耳骨包という緻密な骨に包まれていて外部からのアクセスが難しいが，正円窓と呼ばれる部分だけは中耳腔と膜(正円窓膜)で隔てられており，中耳に投与した薬剤はここを通って内耳に到達すると考えられている(**図1A**)[2]．しかし，投与した薬剤の大部分は嚥下に伴って耳管を通じて咽頭へ排出されるため，投与効率は維持できない．

正円窓膜に着目し，ここに直接持続的に薬剤を投与するという方法がいくつか考按されている．MicroWick™(**図4B**)は，鼓膜上に置いたチューブを通して正円窓膜上にポリ酢酸ビニル製のスポンジ状のスティックを留置する．ここにステロイドなどの点耳することで薬剤は正円窓膜に到達するが，スティックの留置が意外に難しく，あまり用いられていない．正円窓膜上にカテーテルを用いて持続投与するものとしてRWmicroCath (DURECT社，**図4C**)があるが，本格的な中耳手術に相当する留置術と抜去術が必要になる．最近ドイツでRWmicroCathを用いた急性感音難聴に対するステロイド局所投与の臨床試験が行なわれたが[3]，このカテーテルは販売中止になっていて，現在は使用できない．

正円窓膜上に徐放製剤を留置する方法は，より現実的である．我々は，臨床応用へのハードルが低い材料を用いた徐放製剤の作成を試みてきた．ゼラチンは食品として，あるいは医薬品の剤形保持などで広く用いられている．このゼラチンを架橋してポリマー化したものがゼラチンハイドロゲルで，親水性が高く，等電点を操作することで様々な電荷を持つ物質と結合することができる生体吸収性ポリマーである[4]．ゼラチンハイドロゲルの分解に伴って生体内で内包した薬剤を徐放すると考えられている．すでに脛骨高位骨切り術後の骨再生[5]や下肢の虚血[6]に対する線維芽細胞増殖因子2(FGF-2)投与の臨床試験などで，ヒトでの安全性も確かめられている．ゼラチンハイドロゲルを用いた動物の蝸牛への薬物投与として，脳由来神経栄養因子(BDNF)[7]，インスリン様細胞成長因子-1 (IGF-1)[8)-10]，肝由来細胞成長因子(HGF)[11]などが報告されている．我々が行なっているヒトでの臨床試験については後に述べる．疎水性の物質に対しては，ポリ乳酸(PLA)やポリグリコール酸(PGA)，乳酸・グリコール酸共重合体(PLGA)などの生体吸収性ポリマーによるパーティクルを用いることができる．これらの材料はすでに吸収性縫合糸や吸収性組織補強剤として広く臨床で用いられていて，安全性の問題は少ない．動物の蝸牛に投与したときのパーティクルの動態についてはすでに報告がある[12]．臨床応用については後に述べる．

2．蝸牛内への局所投与，人工内耳を用いた内耳DDS

蝸牛内に直接注入する方法は内耳障害が必発であるため，通常は用いられない．しかし，人工内耳は蝸牛内に電極を直接挿入するため，薬物投与に利用する良い機会である．また，人工内耳は残存する蝸牛神経節細胞に依存し，人工内耳電極と神経節細胞の間が導体である必要があることから，これらの細胞を生存させることや蝸牛内の線維化・骨化を抑制することは目的にかなっている．BDNF[13]や神経栄養因子-3 (NT-3)[14)15)]，グリア由来神経栄養因子(GDNF)[16)17)]，HGF[11]などがこの目的での使用が想定されている．浸透圧ポンプを接続して薬物を投与するチャネルを持つ人工内

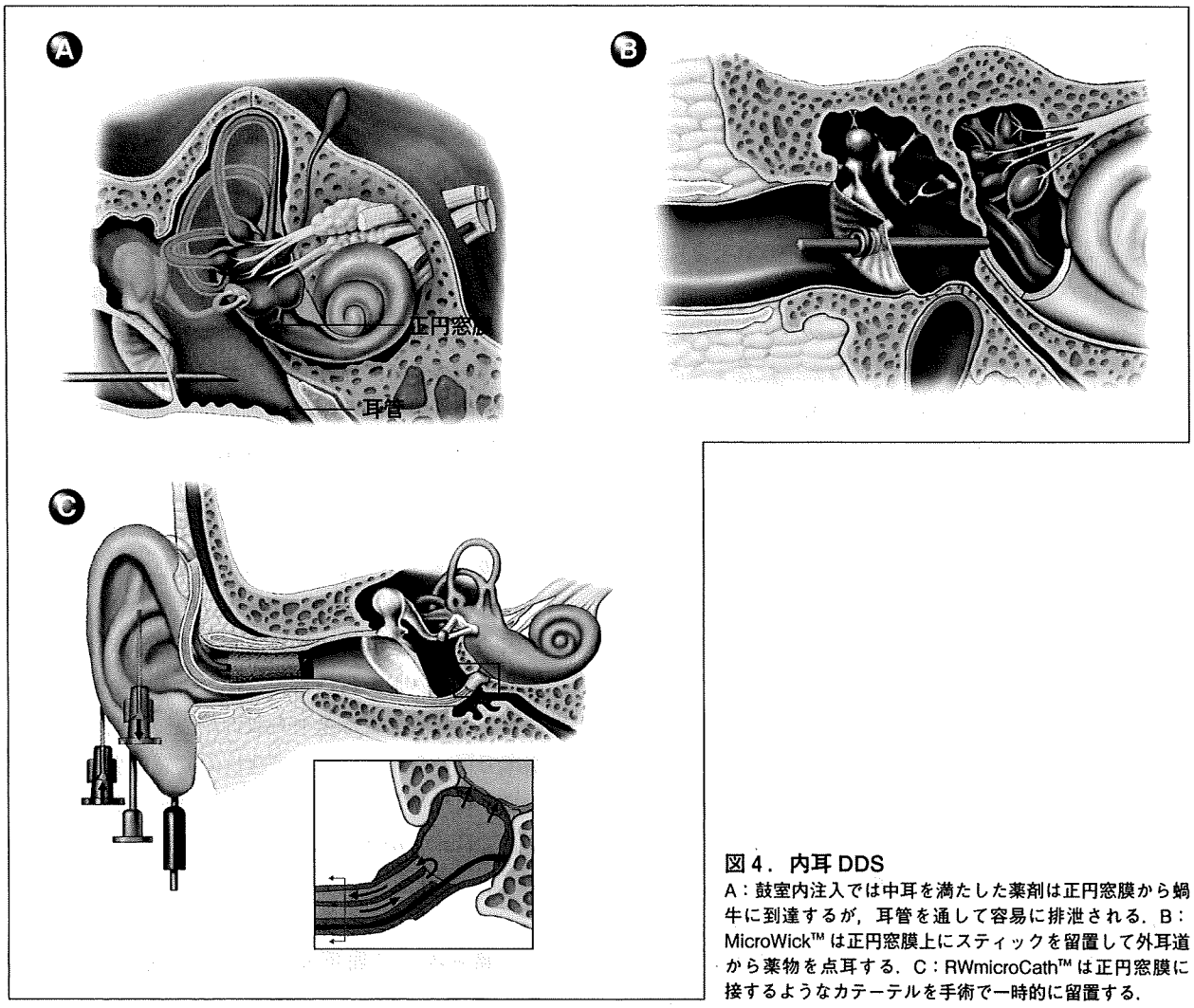


図4．内耳DDS
A：鼓室内注入では中耳を満たした薬剤は正円窓膜から蝸牛に到達するが，耳管を通して容易に排泄される．B：MicroWick™は正円窓膜上にスティックを留置して外耳道から薬物を点耳する．C：RWmicroCath™は正円窓膜に接するようなカテーテルを手術で一時的に留置する．

耳電極は，動物用のものは既に開発されている[18)19)]．また，電極表面に徐放製剤を付着させて蝸牛内で徐放させる方法も検討されている[20)21)]が，近い将来の臨床応用が望まれる．

IGF-1徐放ハイドロゲルによる難聴治療

IGF-1はインスリンと類似の配列を持つポリペプチドで，リコンビナント製剤(rhIGF-1)が成長ホルモン(GH)欠損症や不応症による成長障害の治療薬として市販されている(メカセルミン，ソマゾン®：アステラス製薬)．内耳関連では発生[22)]や発達[23)]に重要な役割があり，この遺伝子の異常はマウス[24)]でもヒト[25)]でも難聴を伴うことが知られている．rhIGF-1を含浸させたゼラチンハイドロゲルがrhIGF-1を徐放することを利用して，騒音難聴による内耳障害に対して

rhIGF-1を蝸牛に持続投与したところ，有毛細胞保護効果と聴力改善がみられた(ラット[10]，モルモット[9])．これらを背景に，我々は急性感音難聴のステロイド無効例に対するIGF-1徐放ハイドロゲルによるフェーズⅠ/Ⅱ臨床試験を行なっている．

対象症例は，突発性難聴を含む急性感音難聴の標準治療であるステロイド全身投与を行なって無効であった成人で，発症後30日以内，他治療を受けていないなどの条件を満たすものである．鼓膜切開を行ない(**図5A**)，細径中耳内視鏡で正円窓窩・正円窓膜を確認し(**図5B**)，ここにrhIGF-1を含浸させたゼラチンハイドロゲルを留置する．目標症例数25例に対し，20例に投与を完了し(平成21年4月末現在)，プロトコル治療と関連する重篤な有害事象はなく，一部の症例では聴力の改善を認めている(論文投稿中)．

本臨床試験は，ヒトにおいて内耳に成長因子を投与する初めての試みである．rhIGF-1の内耳治療における効果を評価するだけではなく，今後の同様の内耳DDSの臨床試験を行なう場合のテンプレートとなり得るという点でも意義が大きい．

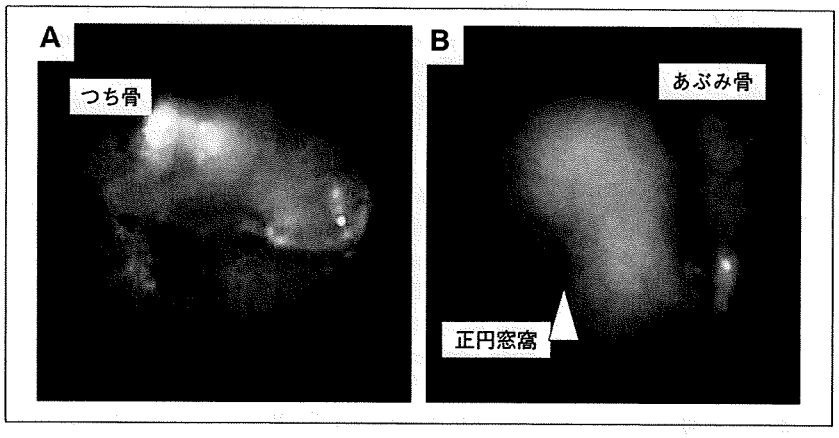
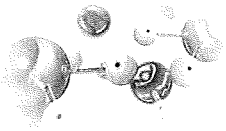


図5．正円窓へのアプローチ
細径内視鏡を用いて，鼓膜切開開窓部(A)から鼓室内を観察すると，あぶみ骨や正円窓窩が確認できる(B)．

リドカイン徐放パーティクルによる耳鳴治療

耳鳴は，体外の明らかな音源なしに知覚される音と定義され，非常にありふれていて，我慢ならない自覚症状である．難聴とともに出現することが多いとされているためこの治療をまず考慮するが，多くの場合に問題となる感音難聴で前述のように急性期にしか積極的な治療手段が無く，それも無効な症例もあり，結局耳鳴が遷延することになる症例も多い．慢性的な耳鳴の場合，不眠や抑うつ状態を引き起こし，病院ショッピングや悪徳業者による詐欺など様々な社会問題とも関連することになり得るが，耳鳴を抑制する現実的な治療法はない．そのなかで，局所麻酔薬リドカインに耳鳴抑制効果があることは経験的に知られていた．全身投与での有効性は二重盲検でも示されているもの[26][27]，持続時間が数時間以内と非常に短く，めまいや不整脈などの副作用があり得るために一般的な治療とはなっていない．また局所投与(鼓室内投与)でも高い有効性が示されているが[28][29]，内耳麻酔による非常に強いめまいが発生することから，治療として受け入れがたいものであった．我々は，内耳DDSを用いることで経正円窓膜的に濃度を制御して持続的に投与できると考え，リドカイン含有PLGAマイクロパーティクルを作成して*in vitro*での徐放性を確認，モルモットの正円窓膜上にパーティクルを留置してモルモットの蝸牛内でリドカインが数日にわたって検出できること，眼振が起きないことなどを確認した(larryngoscope)(in print)．耳鳴は自覚症状であるため動物実験は容易ではないが，行動モデルを用いた実験系が報告されており[30][31]，これらを用いた効果



の確認をするとともに，慢性耳鳴症例を対象とした臨床試験を準備中である．

内耳DDSの将来

内耳DDSは，研究面でも臨床面でもようやく動き始めたところである．例えば，正円窓膜を経由した内耳DDSにおいて決定的なファクターである正円窓膜の透過性についての評価はまだ十分ではなく，これを向上させることで投与効率の向上が期待できる．従来型のPLGAナノパーティクルでもパーティクル自体が正円窓膜を透過することがわかっているが[12]，NANOEAR (http://www. nanoear. org/)というヨーロッパ諸国を中心としたプロジェクトでは，様々に修飾したナノパーティクルの使用を試みている．また，全身投与による内耳ターゲティングにはこれからの技術的なブレイクスルーが望まれるが，我々はステルス型ナノパーティクルによるステロイド投与について検討中である(投稿準備中)．

内耳DDSを用いて何をするかという点は，今後更に重要になる．いったん失われた聴力を回復させることが難しい根本的な理由の1つは，いったん失われた内耳有毛細胞が再生しないことである．発生学研究の中でも内耳の発生は現在ホットな領域であり，これを応用した内耳再生治療が現実のものとして近づいている．例えば，Notchシグナルの阻害薬であるγセクレターゼ阻害薬を内耳に投与すると新たな有毛細胞が発生することや[32-34]，bHLH型転写因子Atoh1を強制発現することで傷害された有毛細胞が再生することなどが報告されている[35]．例えば，これらを内耳DDSで安全に蝸牛に導入することができるなら，有毛細胞の再生の可能性がある．

別の観点では，内耳の画像化技術への応用が考えられる．難聴治療の問題点として，難聴の非侵襲的な部位診断が困難であることを挙げた．現在，マイクロCT[36,37]やマイクロMRI[38]を用いた蝸牛内構造の可視化が試みられているが，臨床的に用いる段階には至っていない．内耳DDSを用いて内耳形態の描出，あるいはカリウムイオンやpHの可視化ができれば，難聴の病態診断となり得る．

内耳DDSは，聴覚障害に対する治療開発の臨床応用というアウトプットへの鍵となる因子である．これから更なる広がりが期待される．

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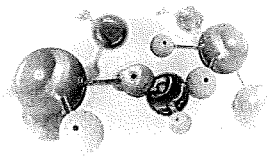

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The Laryngoscope

Sustained Delivery of Lidocaine Into the Cochlea Using Poly Lactic/Glycolic Acid Microparticles

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Objectives/Hypothesis: Lidocaine is a local anesthetic that is known to suppress tinnitus via systemic or local application; however, this effect has only limited duration. The current study aimed to establish a method for the sustained delivery of lidocaine into the cochlea using poly lactic/glycolic acid (PLGA) microparticles.

Study Design: Experimental study.

Methods: Lidocaine-loaded PLGA microparticles were produced and their in vitro-release profiles were examined. The lidocaine concentrations in the perilymph were measured at different time points following the application of the lidocaine-loaded PLGA microparticles to the round-window membranes of guinea pigs. The possible adverse effects of the local application of lidocaine-loaded PLGA microparticles were also examined.

Results: The in vitro analyses revealed that the microparticles were capable of the sustained delivery of lidocaine. The in vivo experiments demonstrated the sustained delivery of lidocaine into the cochlear fluid, and the maintenance of high lidocaine concentrations in the perilymph for up to 3 days after application. Nystagmus and inflammation in the middle ear mucosa were not detected after the local application of lidocaine-loaded PLGA microparticles, although temporary hearing loss was observed.

Conclusions: Lidocaine-loaded PLGA microparticles were shown to be capable of the sustained delivery of lidocaine into the cochlea, suggesting that they could be used for the attenuation of peripheral tinnitus.

Key Words: Biomaterial, drug-delivery system, inner ear, poly lactic/glycolic acid, tinnitus.

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INTRODUCTION

Tinnitus is the perception of sound within the human ear in the absence of corresponding external stimuli. Tinnitus is not a disease, but rather a symptom resulting from a range of underlying causes. It can be a serious problem for patients, because it can lead to severe depression with negative effects on the activities of daily life. Tinnitus is encountered by otolaryngologists worldwide; however, at present, only limited treatment options are available. Many different drugs have been used for the treatment of tinnitus, but with little success. One of the major obstacles to developing efficient treatments for tinnitus is the fact that tinnitus has various forms and underlying mechanisms. Theories about tinnitus pathophysiology emphasize abnormal peripheral or central neural activity in the auditory system.[1]

Several studies have reported on the efficacy of the local or systemic administration of lidocaine,[2–4] which is the most commonly used local anesthetic and is also employed as an antiarrhythmic agent. Various reports have suggested the cochlea, auditory nerve, and central auditory pathway as sites of lidocaine action on tinnitus.[5] Several studies have shown that the systemic application of lidocaine can alleviate tinnitus; however, this effect has only limited duration, and the treatment carries a risk of serious side effects, including cardiac arrhythmia and central nervous system excitation or

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depression.[2] The local application of lidocaine into the intratympanic space can avoid such systemic toxicity, but causes vertigo or dizziness.[4] Tinnitus suppression by local lidocaine application also has limited duration.[3]

Rapid recent technological progression has made possible the sustained and/or targeted delivery of drugs. We have developed a local system for the sustained delivery of growth factors for the treatment of inner ear disorders.[6] Local application can eliminate systemic side effects and deliver drugs to targeted organs at high concentrations. The use of drug-delivery systems in local treatment prolongs the therapeutic effects, and is sometimes necessary to achieve biological effects. We proposed that the use of drug-delivery systems for local lidocaine application could help to prolong the suppression of tinnitus caused by cochlear dysfunction. The current study thus aimed to develop a system for the sustained delivery of lidocaine to the cochlea, and to examine the possible adverse effects. Poly lactic/glycolic acid (PLGA), which is a material used for absorbable sutures, was investigated as a biomaterial for the sustained delivery of lidocaine. PLGA microparticles encapsulating lidocaine were produced, and their release profiles were examined both in vitro and in vivo. The effects on hearing, vestibular function, and histology of the middle ear mucosa were determined to evaluate the risk of adverse effects.

MATERIALS AND METHODS

Animals

Hartley guinea pigs (female; weight, 300–500 g; N = 74) were purchased from Japan SLC Inc. (Shizuoka, Japan). The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Kyoto, Japan, approved all experimental protocols. Animal care was supervised by the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with the National Institutes of Health, Guidelines for the Care and Use of Laboratory Animals.

Preparation of Lidocaine-Loaded PLGA Microparticles

PLGA with a lactic/glycolic acid ratio of 70:30 (molecular weight, 10,000 Daltons) was obtained from Polysciences Inc. (Warrington, PA). Polyvinyl alcohol (PVA), UP180 (degree of polymerization, 1,800), and UMR10H (degree of polymerization, 250) were purchased from Japan Vam & Plval Co., Ltd. (Osaka, Japan). Lidocaine powder and phosphate buffered saline (PBS) were purchased from Nakarai Tesque (Kyoto, Japan). Acetonitrile and Tween-80 were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Dichloromethane (DCM) was purchased from Fisher Scientific (Tokyo, Japan). All solvents were high-performance liquid chromatography (HPLC) grade.

Microparticles were prepared using emulsification by the homogenization-solvent evaporation method. Briefly, an organic phase was prepared consisting of the polymer (PLGA) and the drug (lidocaine), dissolved in an organic solvent (DCM). The organic phase was added to an aqueous phase containing a surfactant (PVA) to form an emulsion. This was broken down into microdroplets by applying external energy, and the droplets formed microparticles on solvent evaporation.

The effect of lidocaine loading on the release profile was examined in two kinds of PLGA microparticles. The microparticles were produced using 2 g PLGA or 2 g lidocaine dissolved in 5.5 mL DCM, or 500 mg PLGA or 500 mg lidocaine dissolved in 5 mL DCM, respectively. The organic phase was then mixed with 50 mL of 1% (wt/wt) PVA solution, and 50 mL of 1% (wt/wt) PVA solution was added as the aqueous phase. This was followed by stirring at 5,000 rpm for 5 minutes, and evaporation of the solvent for 3 days. The microparticles were collected by centrifugation at 5,000 rpm and 4°C, and then freeze dried. The lidocaine-free microparticles were prepared using a similar method, but in the absence of lidocaine. The surface morphology and average particle diameters of the microparticles were examined by scanning electron microscopy (SEM). The average size was estimated from the diameter of 100 randomly selected microparticles. Finally, lidocaine-loaded large (Lido-L) microparticles and lidocaine-loaded small (Lido-S) microparticles were prepared.

The lidocaine loading contents of the Lido-L and Lido-S microparticles were analyzed by the method reported previously.[7] The concentration of lidocaine in the supernatant was measured by an ultraviolet (UV) detector (wavelength, 263 nm), and the total amount of lidocaine in the particles was determined. The lidocaine contents (%) were calculated as follows: (weight of lidocaine in microparticles)/(total weight of microparticles)×100. Three independent measurements were performed for each condition.

In Vitro-Release Profile

The lidocaine-release profiles of the Lido-L and Lido-S microparticles were determined in vitro. A 2.5-mg sample of the microparticles was incubated in a tube containing 1.5 mL PBS (pH 7.4) with 0.2% (wt/wt) Tween-80 buffer. The samples were then placed in a shaken water bath regulated at 30 rpm and 37°C. Sampling of 1 mL of the buffer was performed at 1, 2, 4, 6, and 12 hours, and 4, 7, 14, 21, 28, and 32 days after incubation, followed by replacement with 1 mL of fresh buffer. The concentration of lidocaine in the samples was determined using a UV detector (wavelength, 263 nm). The cumulative amount of lidocaine released was calculated for each time point. The lidocaine-release profile of each particle (%) was calculated as follows: (cumulative amount of lidocaine released)/(total lidocaine content)×100. The amounts of released lidocaine per hour were also calculated for the following time points: 0 to 1 hour, 1 to 2 hours, 2 to 4 hours, 4 to 6 hours, 6 to 12 hours, 12 hours to 4 days, 4 to 7 days, 7 to 14 days, and 14 to 21 days. Three independent measurements were performed for each condition.

In Vivo-Release Profile

The lidocaine concentration in the perilymph was measured on days 1, 3, 7, and 14 after the application of 2.5 mg Lido-L microparticles to the round-window membrane (RWM) of the guinea pigs (n = 16). The animals were anesthetized with an intramuscular injection of midazolam (5 mg/kg; Astellas Co., Tokyo, Japan), medetomidine (18.5 μg/kg; Zenoac, Fukushima, Japan), and butorphanol tartrate (0.25 mg/kg; Bristol-Myers, K.K., Tokyo, Japan). A small hole was made in the bulla to expose the cochlea, and Lido-L microparticles were placed on the RWM. To measure the lidocaine concentrations in the perilymph, the cochleae were excised from the temporal bones under general anesthesia on day 1 (n = 7), day 3 (n = 4), day 7 (n = 6), and day 14 (n = 4). Each cochlea was punctured at the apical portion, and 3 μL perilymph was aspirated using a 30-gauge needle (BD and Company, Fukuoka, Japan). The perilymph samples collected from the animals on day 1 after the sham-operation (n = 4) were used as a negative control.

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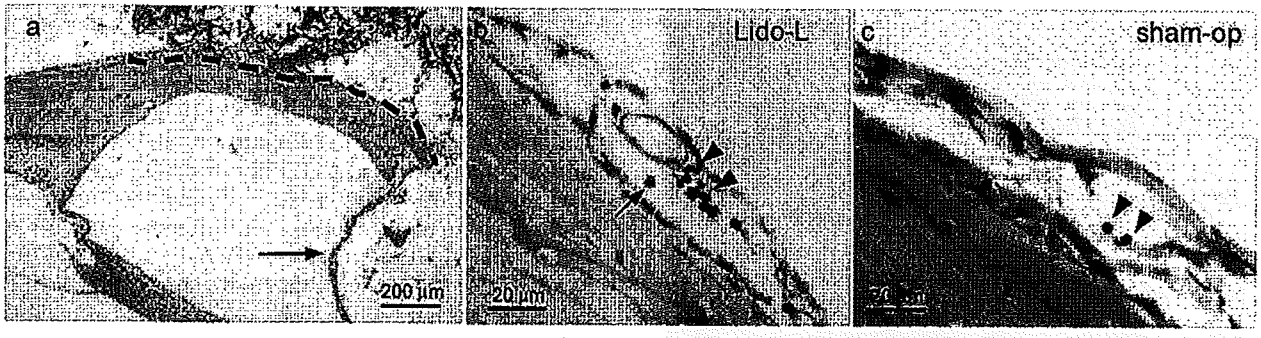

AQ5 Fig. 1. Histopathology of the middle ear mucosa 7 days after local application of Lido-L microparticles (Lido-L) or sham-operation (sham-op). (a) The dotted line indicates the evaluated area of the middle ear mucosa. The arrow shows the RWM. (b) The middle ear mucosa treated with Lido-L microparticles showed a few lymphocytes (arrowheads) and a neutrophil (an arrow). (c) A few lymphocytes (arrowheads) were found in a sham-operated specimen.

The concentration of lidocaine in the perilymph was measured by reverse-phase (RP)-HPLC using the Shiseido Nanospace SI-2 system (Shiseido, Tokyo, Japan). The lidocaine was separated on a Cosmosil C_{18}-AR-II reverse-phase column AQ2 (5 μm, 4.6 mm I.D. × 150 mm; Nacalai Tesque, Kyoto, Japan) at 35°C. The mobile phase consisted of water (0.1 M NaH_2PO_4/Na_2HPO_4, pH adjusted to 4.5) and methanol at a volume ratio of 40:60. The flow rate was 0.3 mL/minute. The detector potential was maintained at +1.0 V.

Effects on Auditory Function

To assess the effects of the local application of Lido-L microparticles on the auditory function, the auditory brainstem response (ABR) was recorded. Lido-L microparticles (2.5 mg) were placed on the left RWM of guinea pigs (n = 4) under general anesthesia. Lidocaine-free PLGA particles were applied to the controls (n = 4). ABR measurements were performed preoperatively (day 0), and on days 1, 3, 7, and 14 after application, according to previous studies.[6]

Effects on Vestibular Function

Vestibular dysfunction was assessed by the occurrence of nystagmus after the local application of either Lido-L microparticles (n = 16) or a piece of gelatin sponge immersed with 20 μL of 0.5%, 1%, 2%, or 4% lidocaine hydrochloride (Astra Zeneca K.K., Osaka, Japan; n = 2 for each concentration of lidocaine). The Lido-L microparticles (2.5 mg) or pieces of gelatin sponge immersed with lidocaine hydrochloride were placed on the RWM of the right ear under general anesthesia with sevoflurane (Abbott Japan Co., Ltd., Tokyo, Japan). The direction and the duration of nystagmus were recorded using an infrared video system in a dark room for 2 hours. In addition, for the Lido-L microparticle-treated animals, the occurrence of nystagmus was evaluated on days 1, 3, and 7 after application. The concentrations of lidocaine in the perilymph were also measured 5 minutes after the local application of 1% or 2% lidocaine hydrochloride (n = 4 for each concentration of lidocaine). The concentration of lidocaine was analyzed using the method described for the in vivo-release profile.

Inflammatory Responses

The inflammatory responses in the middle ear following the local application of Lido-L microparticles were estimated histologically. On day 7 after the local application of Lido-L microparticles (2.5 mg) to the RWM of guinea pigs, the temporal bones were collected and fixed with 4% paraformaldehyde in PBS at pH 7.4 for 3 hours at room temperature (n = 4). Specimens obtained from sham-operated animals (n = 4), which underwent surgical procedures without substances being applied to the RWM, served as controls. The paraffin-embedded tissues were processed into 3 μm sections, and stained with hematoxylin and eosin. Two midmodiolar sections from each cochlea were subjected to quantitative analyses. The numbers of lymphocytes, neutrophils, and plasmacytes in the middle ear mucosa were counted in five randomly selected fields located within 1 mm of the edge of the RWM under a ×40 objective lens for each section (Fig. 1a). The distance from the edge of the AQ3 RWM was measured using Image J software (http://www.nist.gov/lispix/imlab/prelim/dnld.html). Cell counting was performed in a blind manner. The average cell number in five fields was used for each animal in the analysis.

Statistical Analyses

The differences in lidocaine concentrations among time points after Lido-L microparticle application were examined by analysis of variance (ANOVA) with the Tukey-Kramer test. The overall effect of the local application of Lido-L or lidocaine-free PLGA microparticles on the ABR thresholds was examined by 2-way factorial ANOVA. When the interactions were significant, multiple comparisons using the Tukey-Kramer test were performed for pair-wise comparisons. The numbers of infiltrated cells in the middle ear mucosa of the Lido-L microparticle-treated animals were compared with those in the sham-operated animals using the unpaired t test. A P value <0.05 was considered statistically significant. All data are represented as the mean ± standard error.

RESULTS

In Vitro-Release Profile

SEM demonstrated that the Lido-L and Lido-S microparticles had a smooth and round surface morphology (Fig. 2). The average outer diameter of the Lido-L F2 microparticles was 100.0 ± 3.0 μm, and that of the Lido-S microparticles was 5.0 ± 0.5 μm, indicating their stable production. The loading contents of lidocaine were 41.8% AQ4 ± 1.1% for the Lido-L microparticles and 5.0% ± 0.1% for the Lido-S microparticles. In general, the drug-release profiles of PLGA particles are partitioned into four phases: an initial burst period (phase 1), an induction period (phase 2), a slow-release period (phase 3), and a final release period (phase 4). The cumulative release

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Fig. 2. Surface morphology of lidocaine-loaded microparticles. The scale bar represents 20 μm.

profiles of lidocaine from the Lido-L and Lido-S microparticles are shown in Figure 3a. A burst period (phase 1) was observed for both types of microparticle at 24 hours, which was followed by phase 2. The Lido-L microparticles released 77.3% ± 2.3% of the lidocaine contents during phases 1 and 2 (days 0–7). By the end of phase 3 (days 7–56), 82.7% ± 3.3% of the lidocaine had been released. A final release period was not observed during the experimental period. The Lido-S microparticles released 76.6% ± 1.0% of the lidocaine during phases 1 and 2 (days 0–7). By the end of phase 3 (days 7–21), 94.7% ± 0.9% of the lidocaine had been released, and by the end of phase 4 (days 21–56) the amounts reached up to 98.9% ± 0.9%. The amounts of lidocaine released per hour from the Lido-L and Lido-S microparticles are shown in Figure 3b. The Lido-L microparticles exhibited the stable release of lidocaine during the initial 12 hours. In the first hour, lidocaine was released from these microparticles at a rate of 669.8 μg/hour. Between 1 and 6 hours, over 200 μg/hour lidocaine was released, whereas the rate fell to 79.7 μg/hour between 6 and 12 hours. Lidocaine was released at a rate of 10.5 μg/hour between 12 hours and 4 days, and this decreased to 2.4 μg/hour between 4 and 7 days. By contrast, the Lido-S microparticles released lidocaine at a rate below 30 μg/hour, even between 1 and 2 hours. These findings indicated that the Lido-S microparticles released the majority of the loaded lidocaine in an initial burst, which was not advantageous for sustained release. By contrast, the Lido-L microparticles showed the continuous release of comparatively large amounts of lidocaine during the initial 12 hours, indicating that local application might maintain high concentrations of lidocaine in the cochlear fluid for a few days. We therefore used Lido-L microparticles in the subsequent experiments.

In Vivo-Release Profile

RP-HPLC analyses demonstrated measurable concentrations of lidocaine in the perilymph samples collected from the cochleae 1 to 14 days after Lido-L microparticle application to the RWM (Fig. 4). The lidocaine concentrations were 744.0 ± 176.9 ng/mL on day 1, and 863.3 ± 366.3 ng/mL on day 3. The values decreased to 87.2 ± 27.2 ng/mL on day 7, and were maintained at 30.3 ± 13.6 ng/mL on day 14. The difference in lidocaine concentrations between days 3 and 7 was statistically significant (Tukey-Kramer test). These findings demonstrated that the lidocaine released from Lido-L microparticles was transferred into the perilymph through the RWM, and that high concentrations of lidocaine in the perilymph were maintained for at least 3 days, which was consistent with the in vitro-release profiles of the Lido-L microparticles.

Effects on Auditory Function

The ABR thresholds after the application of Lido-L or lidocaine-free microparticles are shown in Figure 5.

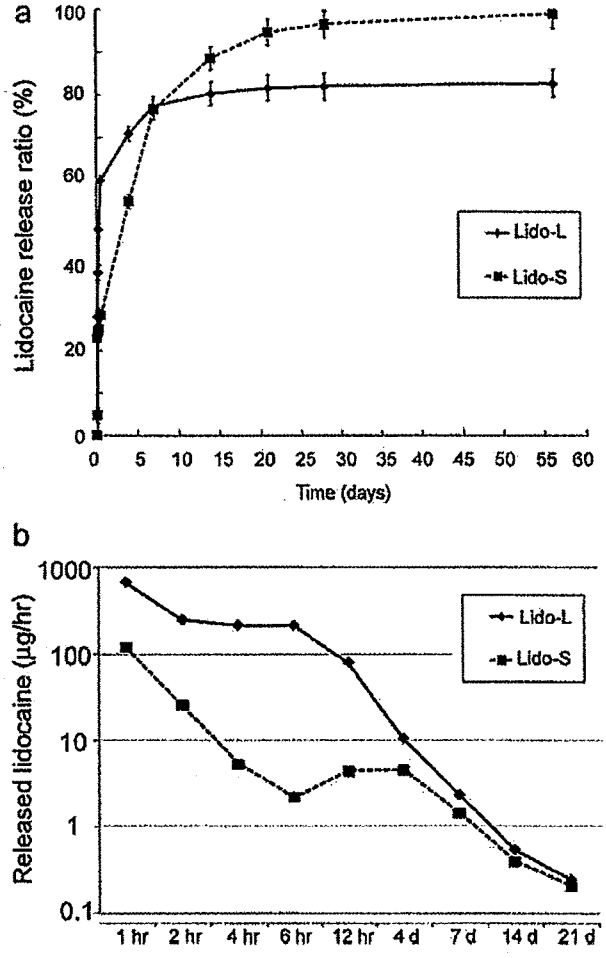

Fig. 3. In vitro-release profiles of lidocaine from Lido-L and Lido-S microparticles. The closed squares show the data for Lido-S microparticles, and the closed diamonds show the data for Lido-L microparticles. (a) Cumulative amounts of lidocaine released from Lido-L or Lido-S microparticles at each time point. The value (%) was calculated as follows: (cumulative amount of lidocaine released)/(total lidocaine content)×100. (b) The amount of lidocaine released per hour at the following time points: 0 to 1 hour, 1 to 2 hours, 2 to 4 hours, 4 to 6 hours, 6 to 12 hours, 12 hours to 4 days, 4 to 7 days, 7 to 14 days, and 14 to 21 days.

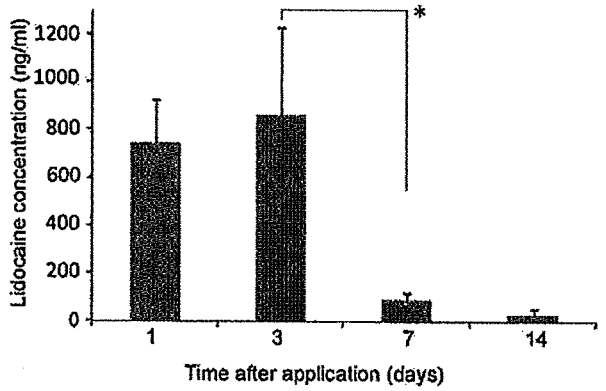

Fig. 4. Lidocaine concentrations in the perilymph following local application of Lido-L microparticles. The bars represent the standard errors. *Indicates a significant difference by analysis of variance with the Tukey-Kramer test.

Interestingly, the animals treated with Lido-L microparticles showed a temporal elevation of the ABR thresholds on day 7 at each frequency, although the lidocaine concentration in the perilymph decreased significantly at this time point. The overall effects of Lido-L microparticle application on the ABR thresholds were statistically significant in comparison with those in animals treated with lidocaine-free particles (4 kHz, $P = .0295$; 8 kHz, $P = .0016$; and 16 kHz, $P = .001$). In the Lido-L microparticle-treated animals, pair-wise comparisons demonstrated significant differences in the ABR thresholds between day 7 and before or day 1 or 14 at 4 and 8 kHz, and between day 7 and before or day 1 at 16 kHz (Fig. 5). By contrast, the animals administered lidocaine-free particles showed no significant differences among the time points. The differences in the ABR thresholds between the Lido-L microparticle-treated and lidocaine-free particle-treated animals were significant on day 7 at all tested frequencies (Fig. 5).

Effects on Vestibular Function

Before testing the Lido-L microparticles, we measured the duration of nystagmus following the local application of lidocaine hydrochloride at various concentrations, and the lidocaine concentration in the perilymph 15 minutes after the local application of 1% or 2% lidocaine hydrochloride, to examine the efficacy of our system for the estimation of nystagmus. The local application of 0.5% lidocaine hydrochloride did not cause nystagmus. However, nystagmus was induced in the animals treated with 1%, 2%, or 4% lidocaine hydrochloride. The latency time was 2 minutes with 1%, 2%, or 4% lidocaine hydrochloride treatment, and nystagmus with horizontal components developed toward the treated side for approximately 10 minutes. The nystagmus then gradually disappeared in the animals treated with 1% lidocaine hydrochloride. In those treated with 2% or 4% lidocaine hydrochloride, paralytic nystagmus with horizontal components developed toward the opposite side and persisted for over 2 hours. RT-HPLC analyses showed that the lidocaine concentrations in the perilymph were 10,708 ± 4,606 ng/mL after the application of 1% lidocaine hydrochloride and 17,384 ± 4,027 ng/mL after the application of 2% lidocaine hydrochloride. These findings confirmed that our system detected the nystagmus induced by lidocaine delivered into the cochlear fluid. We then examined the occurrence of nystagmus following local Lido-L microparticle application in 16 animals. Nystagmus and abnormal behaviors indicating vestibular dysfunction were not observed in any of these guinea pigs.

Inflammatory Responses

No severe inflammatory responses, including effusion or swelling of the mucosa, were identified in either Lido-L microparticle-treated or sham-operated ears. However, inflammatory cells were present in the middle

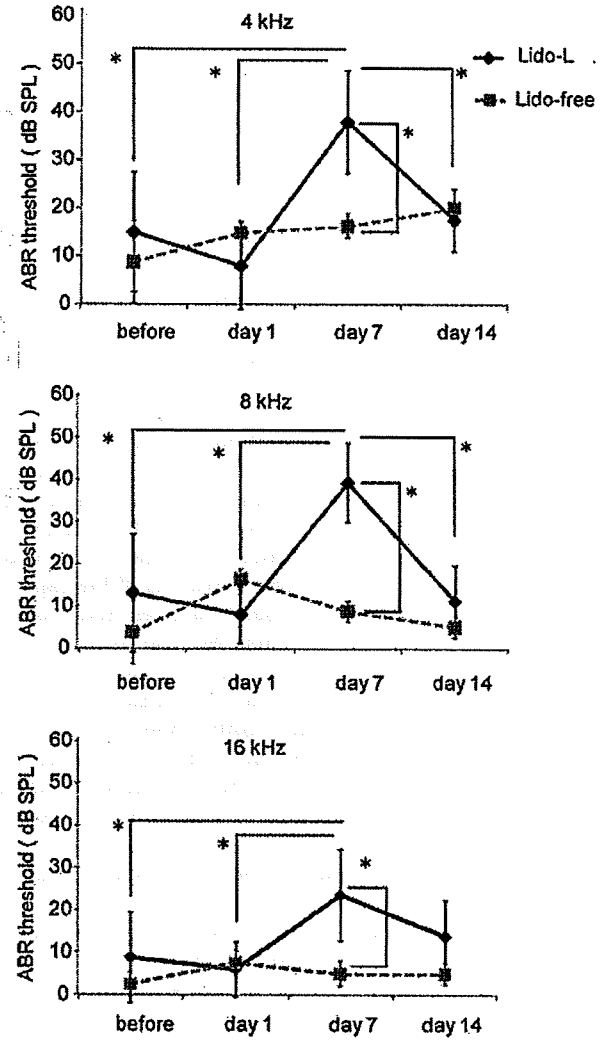

Fig. 5. Auditory brainstem response (ABR) thresholds 4, 8, and 16 kHz before and after local application of Lido-L microparticles or lidocaine-free poly lactic/glycolic acid microparticles. The closed diamonds show the data for Lido-L microparticles (Lido-L), and the closed squares show the data for lidocaine-free particles (Lido-free). *Indicates significant differences according to the Tukey-Kramer test.

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ear mucosa of both groups (Fig. 1b, 1c). The numbers of lymphocytes, neutrophils, and plasmacytes in the Lido-L microparticle-treated specimens were 15.3 ± 3.6, 2.8 ± 1.2, and 6.8 ± 1.3, and those in the sham-operated specimens were 16.0 ± 2.8, 4.8 ± 1.1, and 7.3 ± 2.5, respectively. There were no significant differences in the numbers of lymphocytes, neutrophils, or plasmacytes between the Lido-L microparticle-treated and sham-operated specimens.

DISCUSSION

The analyses of the in vitro-release profiles demonstrated that the Lido-L microparticles released lidocaine in a typical sustained-release fashion, whereas the Lido-S microparticles released the majority of the loaded lidocaine during an initial burst. The lidocaine concentrations in the perilymph after the local application of Lido-L microparticles to the guinea pig cochleae demonstrated sustained delivery into the cochlear fluid. High amounts of lidocaine in the perilymph were found a couple of days after the local application, and measurable concentrations were still present after 14 days. These findings demonstrated that Lido-L microparticles were capable of sustained lidocaine delivery into the perilymph after application to the guinea pig RWM.

Local lidocaine application has the advantage of the elimination of systemic side effects compared with systemic lidocaine application. However, local application has side effects including vestibular dysfunction. We examined the occurrence of nystagmus to evaluate the risk of vestibular dysfunction following the local application of Lido-L microparticles. Nystagmus was not observed after the local application of Lido-L microparticles to guinea pigs, although the local application of 2% or 4% lidocaine hydrochloride caused severe nystagmus, as previously reported in rats.[8] The deficit of nystagmus after local application of Lido-L microparticles may be due to a lower concentration of lidocaine in the perilymph than that necessary for anesthetic actions on vestibular peripheral systems. We also examined the inflammatory responses in the middle ear mucosa of guinea pigs following the local application of Lido-L microparticles. No significant infiltration of inflammatory cells was observed in the Lido-L microparticle-treated specimens compared with the sham-operated specimens. Local application using lidocaine-loaded PLGA microparticles, therefore, appeared to be a safe strategy for the sustained delivery of lidocaine into the cochlear fluid.

The present study demonstrated interesting effects of the local application of Lido-L microparticles on hearing. ABR recordings showed a temporal elevation of thresholds on day 7 after application, but no permanent threshold shifts. Histological analyses revealed no significant damage to the middle ear mucosa on day 7 after the local application of Lido-L microparticles, indicating that the observed ABR threshold shifts were not conductive hearing loss. In the ABR recording experiments, lidocaine-free PLGA particles were applied locally to control animals, which showed no significant alterations in the ABR thresholds. The temporal elevation of ABR thresholds observed on day 7 might have been caused by the effects of sustained lidocaine delivery into the cochlear fluid on the auditory pathway. These findings demonstrate that the local application of Lido-L microparticles have certain effects on the auditory system, which can be associated with a beneficial (silencing tinnitus), or an adverse effect (progression of hearing impairment). We should precisely examine the risk for progression of hearing impairment by local application of Lido-L microparticles before clinical application.

The therapeutic effects of lidocaine on the Purkinje fibers in the heart are generally associated with plasma levels of 6 to 25 μM (1.5–6 μg freebase per mL).[9] In general, the suppression of tinnitus by systemic lidocaine application has occurred at equal or lower plasma levels of lidocaine. The present findings demonstrated that the lidocaine concentrations in the perilymph during the initial 3 days after application were maintained at approximately 0.8 μg freebase per mL. We therefore propose that the concentrations of lidocaine in the perilymph after the local application of Lido-L microparticles might be sufficient for tinnitus suppression if the targets are located in the cochlea. Experimental studies have indicated that the spiral ganglion neurons might be one origin of tinnitus in the cochlea.[10] Lidocaine is known to cause the failure of depolarization in neurons by blocking sodium channels, resulting in anesthetic effects. The anesthetic effects of lidocaine on the spiral ganglion neurons require local lidocaine application at a concentration of over 40 mM (1% lidocaine hydrochloride).[11] In the present study, 15 minutes after the local application of 1% lidocaine hydrochloride, the concentration of lidocaine in the perilymph reached 10 μg/mL, which was higher than the maximum concentration of lidocaine following local Lido-L microparticle application. However, a study using ^{14}C-lidocaine demonstrated the accumulation of lidocaine in the cochlear modiolus, where the spiral ganglion neurons are located, after the systemic application of lidocaine.[12] It is therefore possible that locally applied lidocaine accumulates in the spiral ganglion neurons, which could explain the temporal threshold shifts observed on day 7 in the present study. However, lidocaine also affects various types of channel and receptor, including potassium channels and N-methyl-D-aspartic acid (NMDA) receptors.[5] The blocking of potassium currents by lidocaine occurs in the outer hair cells, leading to the reduction of cochlear microphonics.[13] The involvement of NMDA receptors in the generation of peripheral tinnitus has also been indicated in an animal model.[14] Hence, further studies are needed to elucidate the mechanisms of lidocaine action in the cochlea.

CONCLUSION

We produced lidocaine-loaded PLGA microparticles that were capable of the sustained delivery of lidocaine into the cochlea after local application to the RWM. The local application of lidocaine-loaded PLGA microparticles had no significant adverse effects, including vertigo and

otitis media, although the ABR thresholds were temporally elevated. These findings suggest a potential use of lidocaine-loaded PLGA microparticles for the attenuation of peripheral tinnitus. The established animal models of tinnitus[15] could be used to test the efficacy of lidocaine-loaded PLGA microparticles for the attenuation of peripheral tinnitus. In addition, it is also crucial to develop methods for diagnosis of peripheral tinnitus before clinical application.

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The Laryngoscope

Transplantation of Bone Marrow-Derived Neurospheres Into Guinea Pig Cochlea

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Objectives/Hypothesis. To investigate the potential of neurally induced bone marrow stromal cells (BMSCs) as transplants for replacement of spiral ganglion neurons.

Methods. BMSCs were harvested from the femurs and tibias of adult guinea pigs. BMSCs were cultured with neural induction media and formed spheres. The capacity of BMSC-derived spheres for neural differentiation was examined by immunocytochemistry in vitro. BMSC-derived spheres were injected into the modiolus of the intact cochleae or those locally damaged by ouabain, followed by histological and functional analyses.

Results. In vitro analysis revealed a high capacity of BMSC-derived spheres for neural differentiation. After transplantation into the cochlear modiolus, the survival and neural differentiation of BMSC-derived spheres was observed in both the intact and damaged cochleae. In intact cochleae, transplants settled in various portions of the cochlea, including the cochlear modiolus, whereas in damaged cochleae, transplants were predominantly observed in the internal auditory meatus. Transplantation of BMSC-derived spheres resulted in no functional recovery of the cochlea or protection of host spiral ganglion neurons.

Conclusions. The present findings indicate that BMSC-derived spheres can be a source for replacement of spiral ganglion neurons, although further manipulations are required for functional recovery.

Key Words: Allograft, cell therapy, cochlea, neurosphere, spiral ganglion neuron, regeneration.

Laryngoscope, 000:000–000, 2009

INTRODUCTION

Treatment options for sensorineural hearing loss are currently limited to cochlear implants and hearing aids. Hence, there is a requirement for alternative means of biological therapy, including cell-based therapy. Indeed, recent studies have indicated that cell-based therapy could be utilized as a therapeutic option for inner ear disorders.[1–3] Spiral ganglion neurons (SGNs), primary auditory neurons, are located in the modiolus of the cochlea and transmit sound stimulation to the central auditory system. The loss of SGNs, therefore, compromises auditory function. In addition, SGN loss also reduces the effectiveness of cochlear implants, which can improve impaired hearing by stimulating SGNs. SGNs are, therefore, a primary target for cell transplantation in the auditory system.

Bone marrow stromal cells (BMSCs) are a heterogeneous population of stem/progenitor cells with pluripotent capacity to differentiate toward a neuronal phenotype,[4,5] and consequently the possible use of BMSCs for the treatment of neurological diseases has acquired enormous importance. BMSCs have great potential as therapeutic agents, because they are easy to isolate and expand. Previously, the potential of BMSC transplantation for the treatment of inner ear disorders has been investigated.[6–9] These previous studies have demonstrated that undifferentiated BMSCs are able to settle in the cochlea and have a high capacity for migration. However, limited numbers of transplants differentiated into neurons after transplantation into the intact or damaged cochlea,[6–8] which indicates that neural induction of BMSCs before transplantation is required for SGN replacement by BMSC transplantation.

The aim of this study was to elucidate the neural expression profile of neurally induced BMSCs of guinea pigs and their ability to retain neural differentiation potential when transplanted into the intact or damaged cochleae of guinea pigs. In addition, we examined the capacity of neurally induced BMSCs for functional and histological replacement of SGNs.

MATERIALS AND METHODS

Experimental Animals

A total of 18 Hartley-strain guinea pigs were purchased from Japan SLC Inc. (Hamamatsu, Japan). The Animal

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Research Committee of the Graduate School of Medicine, Kyoto University, Kyoto, 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.

BMSCs

Bone marrow was isolated from the femurs and tibias of 6- to 8-week-old guinea pigs (n = 4, Japan SLC Inc.). Under general anesthesia with midazolam (8 mg/kg, intramuscular injection) and xylazine (8 mg/kg, intramuscular injection), the epiphyses of the femurs and tibias were removed, and the marrow was flushed out into a 100-mm culture dish. The isolated bone marrow, composed of hematopoietic and stromal cells, was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Thermo Trace Ltd., Noble Park, Victoria, Australia) and 1% antibiotic-antimycotic (Invitrogen) at 37°C with 5% CO_2. The medium was changed twice weekly until the cells were 80% confluent. Nonadherent cells were removed during the medium-change procedure. The BMSCs were passaged three to five times before use. BMSCs at this stage were defined as undifferentiated.

Neural Induction of BMSCs

For neural induction, cultured BMSCs were enzymatically detached from culture dishes. The BMSCs were plated into 100-mm culture dishes at a density of 2×10^6 cells/well, and cultured in serum-free DMEM/F-12 medium (Invitrogen) supplemented with B27 (Invitrogen), 20 ng/mL of basic fibroblast growth factor (bFGF, Invitrogen), and 20 ng/mL of epidermal growth factor (EGF, Invitrogen). We added the same amounts of bFGF and EGF every 3 days. After 7 days of culture, BMSC-derived spheres were collected and fixed with 4% paraformaldehyde (PFA) for 15 minutes. The characteristics of BMSC-derived spheres were examined by immunohistochemistry for nestin and Musashi-1. Anti-nestin rabbit polyclonal antibody (1:500; BD Biosciences, San Jose, CA) or anti-Musashi rabbit polyclonal antibody (1:500; Chemicon, Billerica, MA) was used as the primary antibody. Alexa Fluor 488-conjugated anti-mouse donkey IgG (1:1000; Molecular Probes, Eugene, OR) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (1:1000; Molecular Probes) were used as the secondary antibodies. We counted the numbers of spheres and the number of marker-positive spheres in five randomly selected fields (3.4 mm^2 in area), and then calculated the ratio of nestin or Musashi-1 expressing spheres to the total number of spheres. Four independent cultures were performed.

In Vitro Neural Differentiation

To investigate the ability of BMSC-derived spheres to neurally differentiate, BMSC-derived spheres were plated onto 8-well chamber slides at a density of 100 spheres/well in serum-free DMEM/F-12 medium supplemented with B27, retinoic acid (1 mM, Sigma, St. Louis, MO), and dibutyryl cyclic adenosine monophosphate (AMP) (1 mM, Sigma). After 7 days of culture, the cells were fixed with 4% PFA for 15 minutes and immunostained for beta-III tubulin. Anti-beta-III tubulin rabbit polyclonal antibody (1:500; Sigma) was used as the primary antibody, and Alexa Fluor 488-conjugated anti-mouse donkey IgG (1:1000; Molecular Probes) was used as the secondary antibody. We counted the total numbers of the cells and the number of beta-III tubulin-positive cells in five randomly selected fields (3.4 mm^2 in area), and then calculated the ratio of beta-III tubulin expressing cells to the total number of cells. Four independent cultures were performed.

Transplantation Procedure

After labeling with DiI (Invitrogen, 5 μg/mL), the cell suspension of BMSC-derived spheres (10^5 cells in 10 μL DMEM) was injected into the cochlear modiolus of guinea pigs weighing 300 to 330 g as described previously.[10] Briefly, under general anesthesia with midazolam and xylazine, a small hole was made on the left otic bulla to expose the round window niche and the basal turn of the cochlea. After cochleostomy in the basal turn of the cochlea, a glass pipette, which was connected to a microsyringe (Hamilton, Reno, NV), was inserted into the cochlear modiolus of the basal portion of the cochlea. The glass pipette was removed 1 minute after completion of the infusion. Finally, the cochleostomy site was closed with a fat graft and then covered with fibrin glue.

BMSC-Derived Sphere Transplantation

BMSC-derived spheres were transplanted into intact or damaged cochleae of guinea pigs weighing 300 to 330 g. Four weeks after transplantation, four intact guinea pigs were transcardially perfused with phosphate buffered saline (PBS) at pH 7.4, followed by 4% PFA, and sacrificed under general anesthesia with an overdose of pentobarbital. The temporal bones were immediately dissected out and immersed in the same fixative for 4 hours at 4°C.

Ten guinea pigs received local application of ouabain (5 μL at a concentration of 5 mM in saline; Sigma), which causes SGN degeneration,[9] through the round window membrane under general anesthesia with midazolam and xylazine. One week after application, the electrically evoked auditory brainstem response (eABR), which has been used for functional evaluation of SGNs, was measured before cell transplantation as previously described.[10] Eight animals that showed no eABRs were used in the following experiments. Immediately after the eABR measurements, four animals received transplantation of BMSC-derived spheres similar to intact guinea pigs, and the other four animals received an injection of the culture media and were used as controls. Four weeks later, the cochlear specimens were collected after eABR recording.

Specimens (10-μm thick) were prepared using a cryostat after decalcification with 0.1 M ethylenediaminetetraacetic acid in PBS for 3 weeks at 4°C. Then, immunostaining for beta-III tubulin was performed, followed by nuclear staining with 4',6-diamino-2-phenylindole dihydrochloride (DAPI; 2 μg/mL PBS, Molecular Probes). Specimens were viewed with a Leica TCS-SPE confocal laser-scanning microscope (Leica Microsystems Inc., Wetzlar, Germany). Five midmodiolar sections (each separated by 30 μm) were provided for quantitative analyses from each tissue sample. We defined the cells that were positive for DiI with a distinct nucleus identified by DAPI as surviving transplants. The numbers of transplants were counted in the internal auditory meatus and in five cochlear compartments (the modiolus, the scala vestibuli, the scala media, the scala tympani, and the lateral wall). We also counted the numbers of beta-III tubulin-positive transplants, and calculated the ratio of beta-III tubulin expressing transplants to total surviving transplants. In addition, the densities of SGNs in the Rosenthal canals were quantified in ouabain-treated specimens as described previously.[11] All data are presented as the mean ± 1 standard deviation.

Statistical Analyses

We statistically compared the total numbers of surviving transplants and the ratios for beta-III tubulin expression

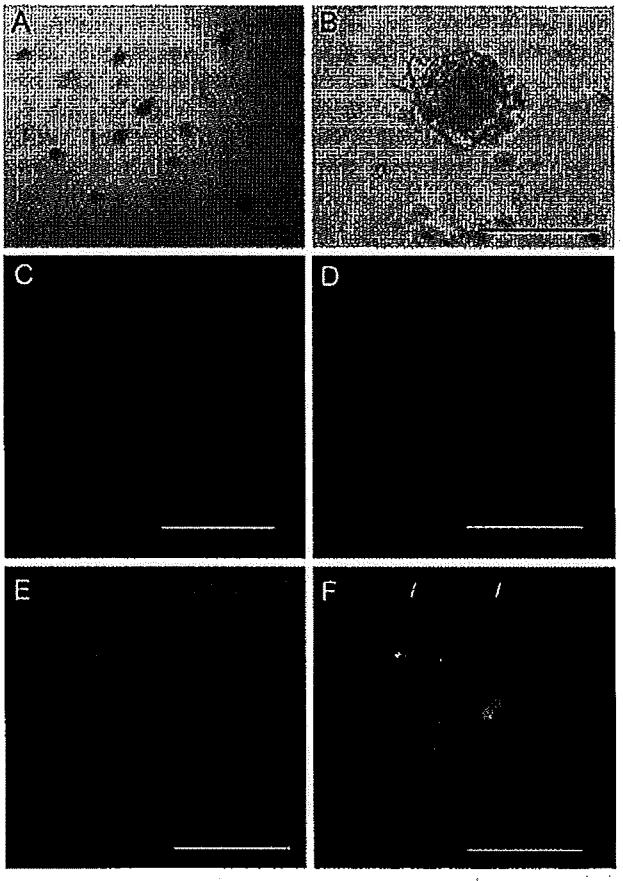

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Fig. 1. BMSC-derived spheres. (A, B) Phase contrast images. (C) 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) staining. (D) Immunostaining for nestin. (E) Immunostaining for Musashi-1. (E) Merged image. Scale bars = 500 μm.

between transplants in damaged cochleae and those in intact cochleae, using unpaired t tests. The difference in the locations of surviving transplants between damaged and intact cochleae was examined by two-way analysis of variance. In damaged models, the difference in the density of remaining SGNs between control and transplanted cochleae were compared using unpaired t tests. P values of $< .05$ were considered to be statistically significant.

RESULTS

Neural Induction of BMSCs

After 2 to 3 days in vitro, BMSCs began to form spheres. On day 7, $1.28 \pm 0.71 \times 10^4$ spheres were identified in each culture dish (Fig. 1A, 1B). Immunohistochemistry revealed the expression of nestin and Musashi-1 in the BMSC-derived spheres (Fig. 1C–1E). The expression of nestin was found in 91.9 ± 4.7% of total BMSC-derived spheres, and that of Musashi-1 was found in 93.6 ± 2.9%, suggesting that neurospheres were generated from guinea pig BMSCs.

F1

In Vitro Neural Differentiation

We transferred the BMSC-derived spheres into differentiation medium containing retinoic acid and dibutyryl cyclic AMP to examine their capacity for neural differentiation. Sphere-forming cells attached to culture dishes and the cells migrated from the sphere (Fig. 2A). Then, some of the cells extended processes (Fig. 2B). On day 7, 89.2 ± 2.8% of the cells expressed beta-III tubulin (Fig. 2C, 2D), indicating that BMSC-derived spheres have the capacity to differentiate into neurons.

F2

Transplantation Into Intact Cochleae

DiI-positive transplants were found in all intact cochleae following transplantation of BMSC-derived spheres. In each midmodiolar section, 74.1 ± 44.4 transplants were found. Transplants were located in multiple regions of the cochlea, predominantly in the scala tympani and the modiolus (Fig. 3A, 3C). Transplants were rarely found in the internal auditory meatus. The expression of beta-III tubulin was observed in 18.6% ± 6.4% of transplants (Fig. 3B).

F3

Transplantation Into Damaged Cochleae

DiI-positive transplants were also identified in all transplanted cochleae that had been damaged by ouabain. The number of surviving transplants in each midmodiolar section was 72.1 ± 53.1. There was no significant difference in the number of surviving transplants between intact and damaged cochleae, whereas the locations of surviving transplants in the damaged cochleae significantly differed from those in the intact cochleae ($P = .007$). In the damaged cochleae, the settlement of transplants was observed in the modiolus, similar to observations in the intact cochleae; however, the most prominent region for settlement of transplants

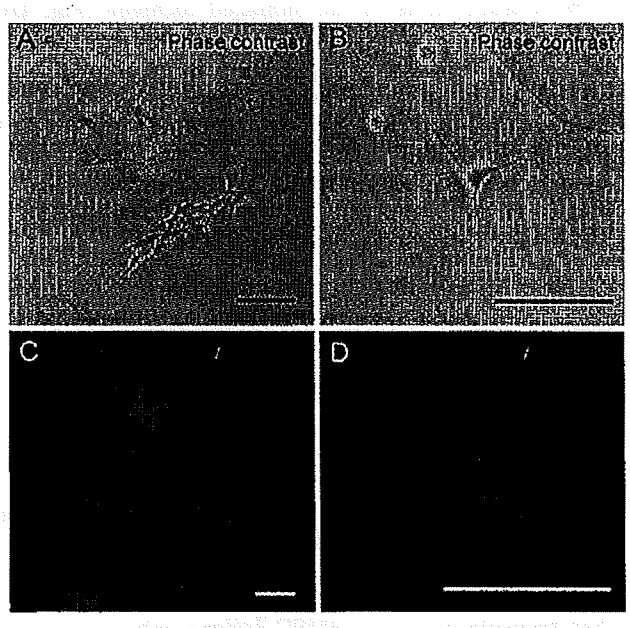

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Fig. 2. Neural induction of BMSC-derived spheres in vitro. (A) Phase contrast image on day 3 in vitro. (B) Phase contrast image on day 7 in vitro. (C, D) Immunostaining for beta-III tubulin and nuclear staining with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI). Scale bars = 20 μm.

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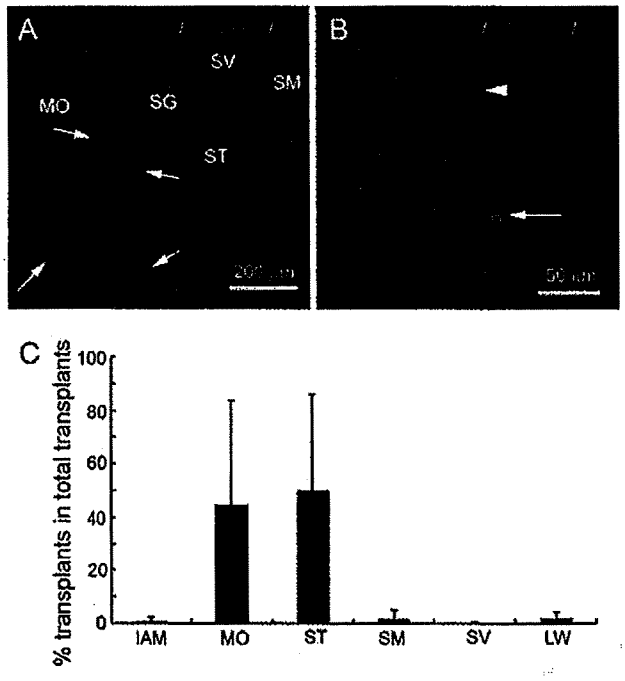

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Fig. 3. Transplantation into the intact cochleae. (A) Transplants labeled by DiI (red) are located in the modiolus (MO) of the cochlear basal portion (arrows). (B) Transplant labeled by DiI (arrow) is positive for beta-III tubulin and another (arrowhead) is negative. (C) The locations of transplants in the cochlea and in the internal auditory meatus (IAM). SV = scala vestibule; SG = spiral ganglion; SM = scala media; ST = scala tympani; LW = lateral wall. Bars represent a standard deviation.

was the internal auditory meatus (Fig. 4A, 4C). The expression of beta-III tubulin was observed in 24.1 ± 5.3% of transplants in the damaged cochleae (Fig. 4B). There was no significant difference in the ratio of beta-III tubulin expressing transplants to total number of transplants between intact and damaged cochleae. These findings indicated that SGN degeneration prior to transplantation caused the migration of BMSC-derived spheres into the internal auditory meatus, and had no effects on the survival and neural differentiation of BMSC-derived spheres after transplantation.

Effects of Transplantation on Cochlear Function

We used eABR recording to monitor SGN function. All the animals receiving eABR evaluation showed no responses before an injection of a cell suspension or a culture medium. Four weeks postoperation, positive eABRs were identified in two of four animals in each group. Thresholds of eABRs in the two animals that showed positive responses in the transplanted group were 300 and 400 μA, and those in the sham-operated group were 250 and 650 μA. These findings indicated that transplantation of BMSC-derived spheres into the cochlear modiolus induced no significantly functional recovery of the cochlea.

We quantified SGN densities after cell transplantation or sham operation to evaluate the effects of BMSC-derived spheres on enhancement of the survival of remaining host SGNs. Local ouabain application caused severe degeneration of SGNs, especially in the basal turn of cochleae. No significant differences in the SGN density of the basal, second, or third turn of cochleae were found between transplanted and sham-operated specimens (Fig. 5), indicating that transplantation of BMSC-derived spheres did not promote the survival of remaining host SGNs, which is consistent with eABR results.

DISCUSSION

The present findings demonstrate that guinea pig BMSCs are able to form spheres that have the capacity to differentiate into neurons in vitro. We aimed to replace SGNs, which are located in the cochlear modiolus, with BMSC-derived neurons. We thus directly injected BMSC-derived spheres into the modiolus of intact or damaged cochleae of guinea pigs. For accurate introduction of the cells into the cochlear modiolus, the size of the cochlea is a critical issue. Previously, we tried to introduce transplants into the cochlear modiolus of mice,[12] in which the success rate for the settlement of the transplants was not satisfactory. In addition, functional evaluation following cell transplantation is virtually impossible. On the other hand, guinea pig[10,13] or chinchilla[6] model systems exhibited better settlement

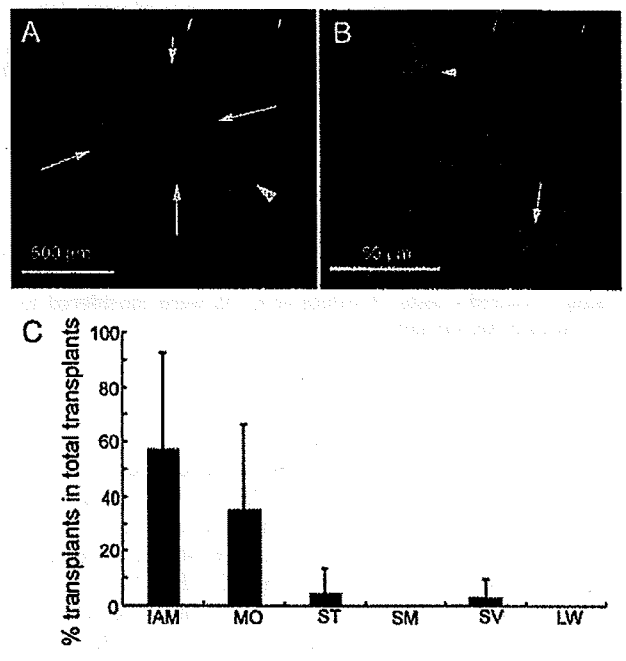

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Fig. 4. Transplantation into the damaged cochleae. (A) In the internal auditory meatus (IAM), transplants labeled by DiI (red) are observed (arrows). An arrowhead indicates the location of the glial-schwann junction. (B) A transplant labeled by DiI (arrow) is positive for beta-III tubulin and another (arrowhead) is negative. (C) The locations of transplants in the cochlea and in the internal auditory meatus (IAM). MO = modiolus; ST = scala tympani; SM = scala media; SG = spiral ganglion; SV = scala vestibule; LW = lateral wall. Bars represent a standard deviation.

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