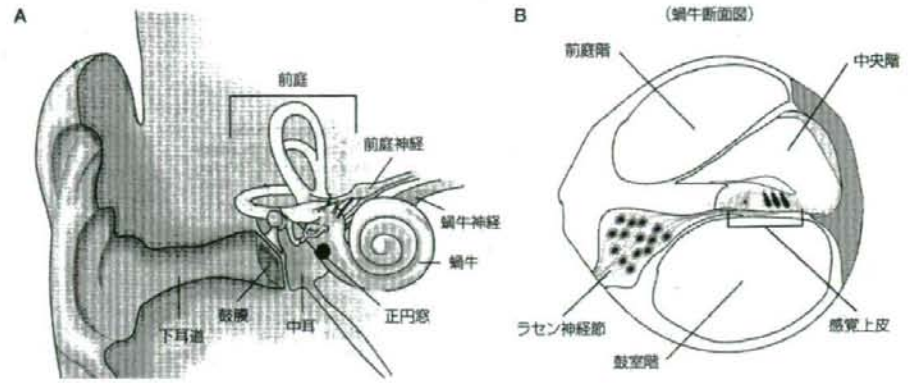

図 1. 内耳，蝸牛の解剖
A：内耳は，蝸牛と前庭からなり，中耳のさらに深部に存在し，そのほとんどは骨に囲まれている．正円窓のみ膜様構造物で内耳と中耳は接している．
B：蝸牛の断面図を示す．蝸牛は，前庭階，中央階，鼓室階という3つのコンパートメントからなる．中央階と鼓室階の境界に感覚上皮をのせる基底板が存在し，ラセン神経節は蝸牛軸に存在する．

う1つは，内耳に直接的に薬物を投与する薬物局所投与技術の開発である．この場合，より低侵襲な外科的操作で，安定した薬物の内耳への送達が可能な手法が望ましい．

本稿では，後者の内耳薬物局所投与という方法へのドラッグデリバリーシステム(DDS)の応用に関する最近の知見について紹介したい．

1．これまでに用いられてきた内耳局所投与方法

蝸牛を含めた内耳は，ほとんどが骨で囲まれており，外部から遮断された構造を有しているが，蝸牛の正円窓という部分のみ膜様の構造物で，中耳腔と接している(図1)．この正円窓膜を介して薬物を内耳に投与しようとする試みは，決して新しいアイデアではなく，経鼓膜的に中耳腔に薬物を注入する方法が古くからおこなわれてきた[3][4]．方法は，鼓膜を注射針で貫通し，中耳腔に薬液を注入するというシンプルなもので，耳鼻咽喉科医にとっては基本的な手技であり，容易な方法である．急性中耳炎の基本的治療法として，鼓膜切開，排膿する方法があるが，これに準ずるものである．過去には，この方法を用いてステロイドを局所投与する方法が，感音難聴治療としておこなわれてきた．いくつかの報告では，その有用性が報告されているが[5]，この方法では，治療効果を予見することが困難であることが指摘されている[6]．経鼓膜的な中耳への薬物注入は，簡単で安全な方法であるが，内耳に移行する薬物量のコントロールや持続的投与は不可能といえる．

より制御された持続的投与方法として，浸透圧ポンプを用いた投与方法が開発されている[7][8]．この方法は，カテーテルの先端部を正円窓膜付近に留置し，耳介後部に埋め込んだ浸透圧ポンプから持続的に薬液が正円窓膜上に投与されるというものであり，薬物の投与量や投与期間を厳密に制御できるという利点をもつ．しかし，この方法では，一般的な鼓室形成術と同等の手術侵襲がポンプとカテーテルの留置に求められ，さらに，治療後にこれらのデバイスを摘出する必要がある．一部の報告では，この方法によるステロイドの投与の急性高度難聴に対する有効性が報告されている

が[7][8]，広く普及するには至っていない．

2．DDS の内耳治療への応用

一方，最近のテクノロジーの進歩により，DDS は急速な進歩をみせている．現在開発されている DDS のいくつかは，薬物投与だけではなく，遺伝子導入にも応用できる可能性が高く，内耳の遺伝子治療への応用との観点からも注目すべきテクノロジーといえる．DDS において，薬物を徐放するための材料として，人工合成物と天然材料の 2 種類が用いられている．人工合成物の代表的なものとして，シリコンポリマーを挙げることができる．シリコンポリマーは，経皮吸収目的の徐放に使われる代表的な材料であり，気管支拡張薬，局所麻酔薬，さらには，禁煙目的のニコチン投与などに応用されている最もなじみのある DDS といえる．生分解性ポリマーとして最も広く用いられている素材としては，ポリ乳酸(PLA)，ポリグリコール乳酸(PLGA)が知られる．これらの材料は，手術で用いる吸収糸などとして臨床使用されており，組織工学の分野でも広く用いられている．天然素材では，ゼラチンやヒアルロン酸が用いられている．それぞれ，正円窓膜を介した内耳への薬物投与への応用が試みられているが，内耳への薬物の徐放動態には違いがある(図 2)．シリコンポリマーでは，単純な拡散により，含有されている薬物が徐放され，正円窓膜を通過して蝸牛内に入る．PLGA ナノパーティクルでは，ナノパーティクルとして蝸牛内に入り，蝸牛内での PLGA の加水分解に伴い，含有されている薬物が徐放される．ゼラチンハイドロゲルでは，ゼラチンポリマーが中耳腔で分解させるのに伴い，ポリマーに静電的に結合していた薬物が徐放され，蝸牛内に入っていく．それぞれ，ポリマーと薬物を結合させるメカニズム，方法が異なるため，使用できる薬物もこれに応じて異なる．以下の項で，シリコンポリマー，PLGA ナノパーティクル，ゼラチンハイドロゲルの内耳薬物投与への応用について，詳説する．

3．シリコンポリマー

シリコンは，安定した物質で生体内で分解されない．手術後のドレナージチューブに用いられている．薬物の徐放に関しては，マトリックス状のシリコンが汎用されている．シリコンゲルの中に薬物が含まれ，薬物は拡散により徐放される．この方法で徐放される薬物は，脂溶性で分子量が低いものが好ましい．内耳への応用としては，ステロイドを含有させたシリコンポリマーについての報告がなされている[9]．ステロイドを含有したシート状のシリコンの正円窓膜上への留置による，蝸牛外リンパ液へのステロイドの徐放が確認されている．また，このシリコンの留置が，蝸牛に組織および機能的障害を与えないことが示されている．しかしながら，薬物の徐放期間が終了しても，シリコンは中耳腔に残存したままとなる．このため，単回投与は可能だが，反復投与ではシリコンシートを除去する必要が生じる．また，正円窓膜に正確に接着させるという点でも安定性にかける．

4．PLGA ナノパーティクル

PLGA は，生体分解性素材として，医療に広く用いられている素材である．近年の PLGA ナノパーティクル精製技術の進歩により，比較的幅広い薬物を PLGA ナノパーティクル化することが可能になっている．われわれ[10]は，PLGA ナノパーティクルの内耳への薬物投与システムとしての有効性をモルモットを用いて検討した．蛍光色素であるローダミンを含有する PLGA ナノパーティクルを用い，正円窓膜の透過性を検討した．ローダミン含有 PLGA ナノパーティクルを正円窓膜上に留置し，24 時間後の蝸牛内の分布を組織学的に調べたところ，蝸牛の広い範囲にローダミン含有ナノパーティクルが認められた．これにより，PLGA ナノパーティクルは正円窓膜を通過することが可能であり，蝸牛外リンパ液中を拡散することがわかった．したがって，突発性難聴治療

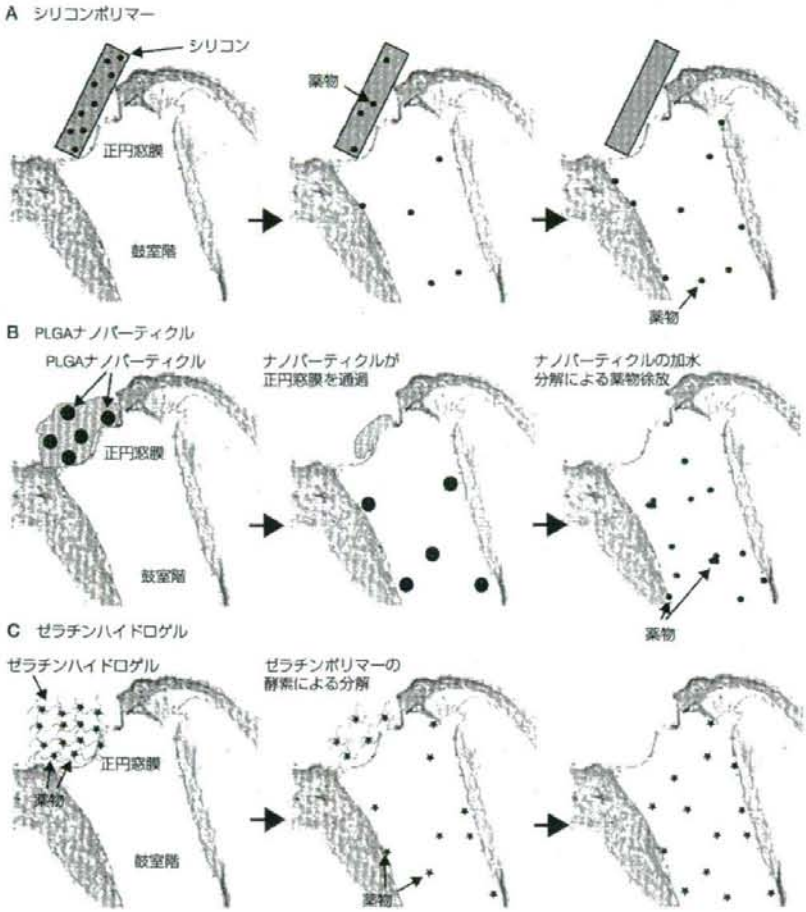

図 2. 内耳 DDS の薬物徐放機構（Nakagawa T *et al*[11]より改変引用）
A：シリコンポリマー．正円窓膜上に留置されたシリコンから，拡散により薬物が蝸牛鼓室階に徐放される．
B：PLGA ナノパーティクル．ナノパーティクルが正円窓膜を通過し，蝸牛鼓室階内で加水分解され，薬物が徐放される．
C：ゼラチンハイドロゲル．正円窓膜上でゼラチンが酵素分解され，薬物が徐放され，蝸牛鼓室階に移動する．

に用いられているステロイドやメニエール病に用いられているアミノ配糖体などをPLGAナノパーティクルに封入することにより，蝸牛への徐放が可能となると考えられる．今後，実際の薬物投与での有効性の検討が待たれる．また，このローダミン含有PLGAナノパーティクルを正円窓膜から蝸牛内に注入した場合，大量のパーティクルが蝸牛全体に認められ，一部は蝸牛組織内に移行しているものも認められた．PLGAナノパーティクルに遺伝子プラスミドを封入し，正円窓膜から注入することにより，蝸牛内の細胞に遺伝子導入をおこなえる可能性がある．すでに，経正円窓膜的にリポフェクションで遺伝子を蝸牛内の細胞に導入できることが示されている[12]．したがって，理論的には，PLGAナノパーティクルを用いて，蝸牛内細胞への遺伝子導入も可能と考えられる．

5．ゼラチンハイドロゲル

ゼラチンハイドロゲルは，ゼラチンをカチオン化あるいはアニオン化させ，逆の静電特性をもつ薬物と結合し，ポリマーを形成する．ゼラチンが生体内で加水分解されるのにしたがい，静電的に結合していた薬物を徐放するDDSである．ゼラチンハイドロゲルによるDDSの特徴は，薬物の担体となる物質と薬物のポリマー形成が容易な点にある．すなわち，薬物とその薬物の静電特性に対応したゼラチンハイドロゲルを投与30～60分前に室温で含浸させるのみであり，蛋白やペプチドの変性をきたすことはなく，細胞増殖因子や神経栄養因子などのポリペプチドの投与に用いることができる．この特徴に着目し，われわれ[13][14]は，脳由来神経栄養因子(BDNF)およびインスリン様細胞成長因子(IGF)1の経正円窓膜的投与にゼラチンハイドロゲルの応用を試みた．BDNF含浸させたハイドロゲルをモルモット正円窓膜上に留置し，経時的に蝸牛外リンパ液中のBDNF濃度を調べたところ，1週間以上の徐放効果が確認された．また，ゼラチンハイドロゲルによるBDNF投与で蝸牛ラセン神経節の保護効果を検証したところ，組織学的，機能的な保護効果が認められた．IGF 1については，音響外傷に対する蝸牛保護効果を検討した．その結果，ゼラチンハイドロゲルによるIGF 1投与により，音響外傷による感音難聴を防止することができ，組織学的に蝸牛有毛細胞の細胞死を防御できることが判明した．以上の結果から，ゼラチンハイドロゲルは蝸牛への細胞増殖因子や神経栄養因子などのポリペプチドの投与方法として優れた方法であることがわかった．現在，ゼラチンハイドロゲルを用いて，IGF 1投与による急性高度難聴治療の臨床応用の準備をおこなっている．

おわりに

近年の内耳障害，ことに感音難聴に関する基礎的研究成果には目をみはるものがあり，将来の遺伝子治療や細胞治療などの新しい治療法の開発を期待させる．しかしながら，このような新しい治療法の臨床応用に際しても，いかにして内耳に分子あるいは薬物を到達させるのかという問題がある．本稿で述べた内耳DDSは，このような新しい治療法の研究開発にも応用可能であり，内耳障害治療に対する応用実験の展開が期待される．また，感音難聴の薬物治療の分野における早期の臨床応用が望まれる．

文　献

1) Angelborg C *et al*：The microsphere method for studies of inner ear blood flow. *ORL J Otorhinolaryngol Relat Spec* **50**：355-362, 1998
2) Juhn SK *et al*：Blood-labyrinth barrier and fluid dynamics of the inner ear. *Int Tinnitus J* **7**：72-83, 2001
3) Ersner MS：Transtympanic injection of anesthetics for the treatment of Menière's Syndrome. *AMA Arch Otorhinolaryngol* **54**：43-52, 1951
4) Schuknecht HF：Ablation therapy for the relief of Meniere's disease. *Laryngoscope* **66**：

859-870, 1956
5) Guan-Min H *et al* : Effectiveness of intratympanic dexamethasone injection in sudden-deafness patients as salvage treatment. *Laryngoscope* **114** : 1184-1189, 2004
6) Salt AN *et al* : Local inner-ear drug delivery and pharmacokinetics. *Drug Discov Today* **10** : 1299-1306, 2005
7) Lefebvre PP *et al* : Steroid perfusion of the inner ear for sudden sensorineural hearing loss after failure of convensional therapy : a pilot study. *Acta Otolaryngol* **122** : 698-702, 2002
8) Plontke S *et al* : Outcomes research analysis of continuous intratympanic glucocorticoid delivery in patients with acute severe to profound hearing loss : basis for planning randomized controlled trials. *Acta Otolaryngol* **125** : 830-839, 2005
9) Arnold W *et al* : Novel slow- and fast-type drug release round-window microimplants for local drug application to the cochlea : An experimental study in guinea pigs. *Audiol Neurotol* **10** : 53-63, 2005
10) Tamura T *et al* : Drug delivery to the cochlea using PLGA nanoparticles. *Laryngoscope* **115** : 2000-2005, 2005
11) Nakagawa T *et al* : Drug delivery systems for the treatment of sensorineural hearing loss. *Acta Otolaryngol* in press
12) Maeda Y *et al* : *In vitro* and *in vivo* suppression of GJB 2 expression by RNA interference. *Hum Mol Genet* **14** : 1641-1650, 2005
13) Endo T *et al* : A novel strategy for treatment of inner ears using a biodegradable gel. *Laryngoscope* **115** : 2016-2020, 2005
14) Iwai K *et al* : Cochlear protection by local IGF-1 application using biodegradable hydrogel. *Laryngoscope* **116** : 526-533, 2006

Regenerative Medicine

I．再生医療を実現するDrug Delivery System技術

DDSを用いた感覚器領域における再生医療

Regenerative medicine using DDS in sensory organs

Keywords
ドラッグデリバリーシステム
感音難聴
視力障害

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Summary

Drug delivery to the cochlea is hampered by blood-inner ear barrier together with its dense bony capsule. The round window is a possible access to deliver drugs effectively into the cochlea. Intratympanic injections and osmotic mini-pump has been utilized to get access to the round window membrane (RWM). Gelatin hydrogel immersed with brain derived-neurotrophic factor (BDNF) or IGF1 is successfully applied to the RWM to protect spiral ganglion neurons or cochlear hair cells. PLGA nano-particles are shown to penetrate round window membrane, and are expected to provide sustained-release in the cochlea. Drug delivery to the retina is also a challenge due to the existence of blood-retina barrier. Photo dynamic therapy and intraocular implants are clinically utilized methods to overcome this difficulty. Iontophoresis using drug immersed hydrogel as a contact electrode effectively brings drugs into vitreous. PLGA nano-particles with pigment epithelium-delived factor protected the retina from ischemic injury. PLGA nano-particles may also be used as vehicles to transfect cells with plasmid DNA. Pegaptanib, an RNA aptamer which inhibit vascular endothelium-delived growth factor, is used for ocular vascular disease. PLGA nano-particles will also be used for better sustained-release of pegaptanib.

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はじめに

耳や眼は外界と接した臓器であり，アクセスは一見容易に思えるが，全身投与でも局所投与でも薬剤がターゲットとなる細胞に到達するまでにはさまざまな阻害要因があり，効率的な薬物投与は決して容易ではないため，これまでもDrug Delivery System (DDS) 的な工夫がなされてきた．本稿では，この領域で使われているDDSを紹介し，その再生医療としての応用について述べる．

感音難聴に対するDDS

感音難聴は非常に頻度の高い身体障害で，その原因としては，音響外傷，耳毒性薬剤，遺伝子異常，老化，メニエール病を含む内リンパ水腫関連の疾患などが挙げられるが，多くは蝸牛の障害が難聴を引き起こしている．空気

の疎密波として鼓膜に到達した音は，3つの耳小骨を介して蝸牛に伝えられ，蝸牛内では感覚上皮が振動する．感覚上皮に存在する感覚細胞（有毛細胞）では，振動が神経伝達物質の放出を引き起こし，一次感覚ニューロンであるらせん神経節細胞を興奮させ，中枢へと伝達される（図1）．多くの感音難聴ではこの有毛細胞やらせん神経が傷害されているため，治療の際には蝸牛がターゲットとなる．感音難聴に対して臨床的に実際に行われている治療はステロイドやビタミン剤，循環改善薬などの全身投与（経口，または経静脈）であるが，効果のある症例は限られている．感音難聴が治りにくい第一の理由は，いったん傷害された有毛細胞やらせん神経節細胞が再生しないことがまず挙げられるが，もう一つの理由が蝸牛への薬剤到達の難しさである．蝸牛を含めた内耳は非常に密な骨に囲まれており，血流も限られていること，さらに，「血液－脳関門」と同様の「血液－内耳関門」が存在することが蝸牛への薬物到達を難しくしている．ただ，蝸牛には正円窓という小さな開窓部があって，薄い正円窓膜が蝸牛を満たす外リンパ液をシールしているため，薬物投与のルートとしては可能性がある．ただし，膜を損傷して外リンパ瘻を起こすと難聴やめまいを引き起こし，またもう1枚の偽性膜が存在するために確実に正円窓膜上に投与できない例も多いといわれており，取り扱いに注意を要する．鼓膜を穿刺してあるいは鼓膜穿孔があればそこから薬液を鼓室内に充満させる方法（鼓室内投与法）は，簡便であるが，薬物は耳管を通じて鼻腔へ容易に排出されるため，蝸牛への到達の程度も決して高くはなく，その効果を持続させることも難しい[1]．マイクロカテーテルを正円窓膜近傍に留置する方法も報告されていて，より確実に正円窓に向けて薬物を持続投与できる．突発性難聴に対するステロイド投与法として臨床的に有効であることも示されているが[2]，マイクロカテーテル留置には中耳手術と同程度の侵襲が必要であり，普及するには至っていない．

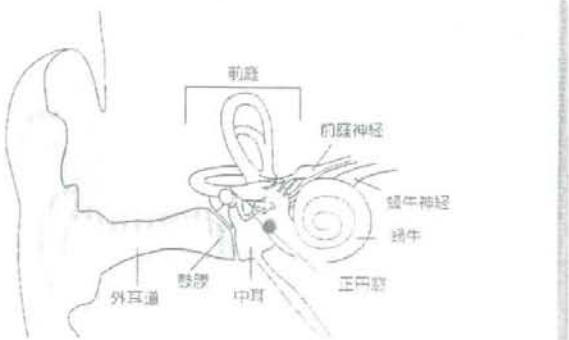

図1 外耳・中耳・内耳

ハイドロゲル

ハイドロゲルとは，ゼラチンやコラーゲン，ヒアルロン酸，アルギン酸などの親水性高分子化合物を架橋してできるゼリー状物質の総称である．特に，カチオン化，あるいはアニオン化したゼラチンを用いたハイドロゲル（ゼラチンハイドロゲル）は，静電気的に薬物と結合し，生体内で加水分解されるに従って薬物を徐放することが知られている．結合が静電気的であるため薬物を修飾する必要がなく，その架橋の程度を変えることで分解速度を制御できるので，DDS基材としては使いやすい．

Endoら[3]は，脳由来神経栄養因子（BDNF）を浸潤させたハイドロゲルをモルモットの正円窓膜上に留置して，蝸牛におけるその効果を検証した．外リンパ液中のBDNF濃度を調べると，1週間以上にわたってBDNFは徐放されており，また，らせん神経節細胞に対する保護効果が組織学的，機能的に確認された．また，Iwaiら[4]は

DDSを用いた感覚器領域における再生医療

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IGF1を含ませたゼラチンハイドロゲルを正円窓膜上に留置して，音響外傷による感音難聴に対する予防効果を検討した．その結果，機能的には難聴の防止効果が，組織学的には蝸牛有毛細胞の細胞死を防御する効果があることを確認した．現在我々はゼラチンハイドロゲルを用いたIGF1投与による急性高度難聴治療の臨床応用を準備中である．

生体適合性プラスチックによるナノパーティクル

PLGA (copoly lactic acid/glycolic acid) は，手術用吸収糸や創傷被覆膜として医療で広く用いられている生体適合性高分子である．材料である乳酸とグリコール酸の比率を変えることで分解を遅延することができ，生体内で加水分解されたあとの単量体は無害である．最近の微粒化技術の進歩によって，幅広い薬剤をPLGAナノパーティクル化できるようになり，新しいDDS基材として注目されている．

Tamuraら[10]は，PLGAナノパーティクルが正円窓膜を超えて蝸牛まで到達できるかどうかを調べた．ローダミン含有PLGAナノパーティクルを蝸牛の正円窓膜上に留置して24時間後の蝸牛内の分布を組織学的に調べると，ローダミン含有PLGAナノパーティクルは蝸牛の正円窓に近い基底回転から頂回転までの広い範囲にわたって存在しており，PLGAナノパーティクルは正円窓膜を通過して，蝸牛の外リンパ液中を拡散することがわかった．ハイドロゲルでは薬物はいったん徐放されてから拡散してターゲットへと輸送されていくが，ナノパーティクルの場合はパーティクルのままターゲットに輸送され，その場で徐放するので，薬物の安定性やターゲティング性能などの点で有利である．突発性難聴に用いられているステロイドやメニエール病の治療に用いられるアミノ配当体，さらに前述のBDNFやIGF1などをPLGAナノパーティクル化して用いれば，全身的な副作用などを回避しながら，蝸牛のみに高濃度かつ長期間，薬物を維持することができるので，今後の有効性の検証・臨床応用が待たれる．

視力障害に対するDDS

重篤な視力障害の原因として頻度の高いものは，糖尿病網膜症，緑内障，加齢黄斑変性症，網膜色素変性症などがあげられるが，その主な障害部位は網膜・視神経である（図2）．網膜において幹細胞の存在が明らかになっているが，それが傷害された網膜を再生させるという報告はいまだなく，細胞障害を受けると修復されることは困難であるということを意味する．眼球は強膜，結膜による強固なバリアーや涙によるクリアランスのほかに，「血液ー網膜関門」[11]が存在するため，点眼や全身投与を行っても，眼球後方への薬物投与の効率は低い．

滲出性加齢黄斑変性症は，脈絡膜に新生血管が生じ，出血，網膜剥離，浮腫などが生じ，病変が中心窩に及ぶと重大な視力低下をきたす疾患である．これに対して，光線力学的療法（photo dynamic therapy：PDT）が行われている．PDTは，新生血管集積性の光反応薬剤を全身投与した後，病

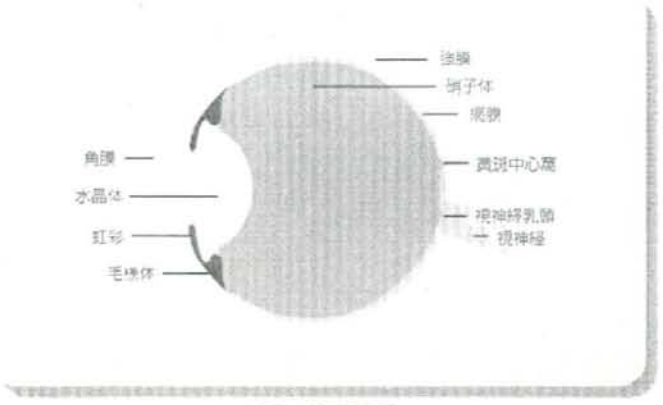

図2 眼球断面図

変部分である網膜の脈絡膜に弱いレーザーを照射することで局所の血管を閉塞させるという，ターゲティングDDSである。

また，日本国内では未承認であるが，ステロイド徐放インプラント（Retisert®：フルオシノロンをペレット状にしてポリビニルアルコールとシリコンで被覆して徐放化）[11]や抗ウイルス薬徐放インプラント（Viterasert®：ガンシクロビルペレットをポリビニルアルコールとエチレン酢酸ビニル共重合体で被覆して徐放化）[12]といった，眼球内に埋め込む形の徐放剤が実用化されている。

ハイドロゲル，PLGAナノパーティクルによる徐放

ポリヒドロキシエチルメタクリレート（HEMA）は，コンタクトレンズの材料として1960年代から用いられている高分子素材である。Eljarrat-Binstockら[13,14]はゲンタマイシンで膨潤させたHEMAハイドロゲルを角膜表面に，あるいはHEMAにエチレングリコールジメタクリレート（EGDMA）を組み合わせたハイドロゲルを強膜面にあて，1分程度通電することで房水，あるいは硝子体に効率的にゲンタマイシンを輸送できることを示した。このようなイオントフォレーシスと呼ばれる方法は，耳鼻咽喉科臨床的には局所麻酔薬を鼓膜に効率よく到達させるために古くから利用されているが，眼球後方の薬物到達が困難な領域へのDDSとしても，簡便で有効な方法である。

Liら[15]は，網膜血管新生を抑制する作用をもつ色素上皮由来因子（pigment epithelium-delived factor；PEDF）をPLGAナノパーティクルに封入し，網膜虚血モデルマウスの眼球内に投与した。PEDF封入PLGAナノパーティクルを使うことで，PEDF単体で投与するよりも長期にわたって網膜神経節細胞と内網状層の保護できることを示した。

核酸導入

*In vivo*で用いられている遺伝子導入法は，ウイルスベクターによるものがほとんどである。ウイルスベクターによるものは遺伝子導入効率は高いが，ウイルス蛋白に対する免疫反応，増殖能をもったウイルス混在の可能性が排除できないこと，ベクターがゲノムに挿入される場合には他の遺伝子に影響する可能性があることなどが問題になる。非ウイルスベクター法として，プラスミドDNAとカチオン性脂質との複合体（リポフレックス）を用いるリポフェクションと，プラスミドDNAとカチオン性ポリマーとの複合体（ポリフレックス）を用いるポリフェクションなどが用いられている。現状ではリポフェクションのほうが頻用されているが，ポリフェクションは1）均一で安定な複合体形成，2）遺伝子導入率が高い，3）血清の影響を受けやすいなどの利点がある[16]。前述のように，PLGAナノパーティクルが正円窓から蝸牛に導入できることから，同じ方法で蝸牛への遺伝子導入ができると期待される。

米国では，加齢性黄斑変性症などによる毛細血管新生を抑制して視力障害の進行を抑える効果をねらって，血管内皮増殖因子（VEGF）抑制性のRNAアプタマー（pegaptanib，Macugen®）が使用されており[17]，日本でも臨床試験が行われている。アプタマーとは標的蛋白と特異的に結合するようにデザインされた核酸分子であるが，pegaptanibの場合，核酸残基の置換，40kDaのポリエチレングリコール（PEG）との結合によって，硝子体液中での安定性と反応特異性を向上させた[18]。これをPLGAマイクロスフェア化することでさらなる徐放効果をねらう試みも行われている[19]。

おわりに

感覚器領域においても，昨今の再生医学研究の成果で，これまで機能回復が難しかった病態に対して治療に用いることのできる多数の因子が同定されつつあり，それとともに，それぞれに適したDDSの開発も進みつつあり，近い将来の臨床応用が期待される。さらに我々は，骨髄由来幹細胞を末梢血管から導入するとマクロファージ様の細胞が内耳に集積することに着目して，これを利用したDDSを模索している。

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●文　献

1) Angelborg C, Hillerdal M, Hulterantz E, et al : The microsphere method for studies of inner ear blood flow. ORL J Otorhinolaryngol Relat Spec **50** : 355-362, 1988
2) Juhn SK, Hunter BA, Odland RM : Blood-labyrinth barrier and fluid dynamics of the inner ear. Int Tinnitus J **7** : 72-83, 2001
3) Ho HG, Lin HC, Shu MT, et al : Effectiveness of intratympanic dexamethasone injection in sudden-deafness patients as salvage treatment. Laryngoscope **114** : 1184-1189, 2004
4) Salt AN, Plontke SK : Local inner-ear drug delivery and pharmacokinetics. Drug Discov Today **10** : 1299-1306, 2005
5) Lefebvre PP, Staecker H : Steroid perfusion of the inner ear for sudden sensorineural hearing loss after failure of conventional therapy : a pilot study. Acta Otolaryngol **122** : 698-702, 2002
6) Plontke S, Lowenheim H, Preyer S, et al : Outcomes research analysis of continuous intratympanic glucocorticoid delivery in patients with acute severe to profound hearing loss : basis for planning randomized controlled trials. Acta Otolaryngol **125** : 830-839, 2005
7) Endo T, Nakagwa T, Kita T, et al : Novel strategy for treatment of inner ears using a biodegradable gel. Laryngoscope **115** : 2016-2120, 2005
8) Iwai K, Nakagawa T, Endo T, et al : Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. Laryngoscope **116** : 529-533, 2006
9) Tamura T, Kita T, Nakagawa T, et al : Drug delivery to the cochlea using PLGA nanoparticles. Laryngoscope **115** : 2000-2005, 2005
10) Cunha-Vaz JG : The blood-retinal barriers system. Basic concepts and clinical evaluation. Exp Eye Res **78** : 715-721, 2004
11) Jaffe GJ, Yang CH, Guo H, et al : Safety and pharmacokinetics of an intraocular fluocinolone acetonide sustained delivery device. Invest Ophthalmol Vis Sci **41** : 3569-3575, 2000
12) Musch DC, Martin DF, Gordon JF, et al : Treatment of cytomegalovirus retinitis with a sustained-release ganciclovir implant. The Ganciclovir Implant Study Group. N Engl J Med **337** : 83-90, 1997
13) Eljarrat-Binstock E, Raiskup F, Frucht-Pery J, et al : Hydrogel probe for iontophoresis drug delivery to the eye. J Biomater Sci Polym Ed **15** : 397-413, 2004
14) Eljarrat-Binstock E, Raiskup F, Stepensky D, et al : Delivery of gentamicin to the rabbit eye by drug-loaded hydrogel iontophoresis. Invest Ophthalmol Vis Sci **45** : 2543-2548, 2004
15) Li H, Tran VV, Hu Y, et al : A PEDF N-terminal peptide protects the retina from ischemic injury when delivered in PLGA nanospheres. Exp Eye Res **83** : 821-833, 2006
16) 有馬英俊：遺伝子導入法としてのポリフェクション　α-シクロデキストリンを基本素材とする高機能性遺伝子導入用ベクターの構築を中心として　．薬学雑誌 **124** : 451-464, 2004
17) Rakic JM, Blaise P, Foidart JM : Pegaptanib and age-related macular degeneration. N Engl J Med **352** : 1720-1721, 2005
18) Ng EW, Shima DT, Calias P, et al : Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nat Rev Drug Discov **5** : 123-132, 2006
19) Carrasquillo KG, Ricker JA, Rigas IK, et al : Controlled delivery of the anti-VEGF aptamer EYE001 with poly (lactic-co-glycolic) acid microspheres. Invest Ophthalmol Vis Sci **44** : 290-299, 2003

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Drug delivery systems for the treatment of sensorineural hearing loss

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Abstract
Sensorineural hearing loss is one of the most common disabilities in our society. Experimentally, many candidates for therapeutic molecules have been discovered. However, the lack of safe and effective methods for drug delivery to the cochlea has been a considerable obstacle to clinical application. Local application of therapeutic molecules into the cochlea has been used in clinic and in animal experiments. Advances in pharmacological technology provide various drug delivery systems via biomaterials, which can be utilized for local drug delivery to the cochlea. Recent studies in the field of otology have demonstrated the potential of synthetic and natural biomaterials for local drug delivery to the cochlea. Although problems still remain to be resolved for clinical application, introduction into clinical practice of these controlled-release systems may be reasonable because of their certain advantages over previous methods.

Keywords: *Drug delivery, topical application, hearing loss, inner ear, biodegradable material*

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in industrial countries. Excessive noise, ototoxic drugs, genetic disorders and aging can all initiate SNHL. Endolymphatic hydrops-associated diseases including Meniere's disease also cause SNHL. Severe to profound SNHL affects 1 in 1000 newborns, and another 1 in 2000 children before they reach adulthood. About 60% of individuals older than 70 years will manifest SNHL. Despite the high prevalence of SNHL in our society, therapeutic strategies for the treatment of SNHL today are limited to hearing aids and cochlear implants. These therapeutic tools do not provide complete restoration of hearing ability, although they have significant clinical benefits. Based on such backgrounds, many attempts have been made to provide alternative means of biological therapy, which have identified a number of candidates for therapeutic molecules. Experimentally, protective effects of neurotrophins have been demonstrated [1,2], and inhibitors of apoptosis and glutamate antagonists have also shown the ability to promote hair cell survival [3–5]. Recently, local application of genes by virus vectors was shown to induce hair cell regeneration in the mammalian auditory epithelium [6,7], and silencing the mutant gene by RNA interference restored hearing loss in a genetic mouse model [8].

These therapeutic strategies are attractive and promising for restoring SNHL. However, clinical application is still limited. The problem of how to deliver therapeutic molecules to the inner ear has been a considerable obstacle to the development of treatments for SNHL. The systemic application of drugs carries the risk of unwanted side effects. In addition, the blood–inner ear barrier, which inhibits the transport of therapeutic molecules from the serum to the inner ear, represents a fundamental obstacle to systemic application [9]. The inner ear tissues are isolated from the surrounding organs by a bony construction, which allows the topical introduction of therapeutic molecules by local application. Therefore, development of strategies for local delivery into the inner ear is crucial for developing clinical therapies based on the experimental findings.

Previous methods for local application

Substances are applied intratympanically under the premise that they will enter the scala tympani (ST)

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through the round window membrane (RWM) and then be distributed throughout the inner ear fluids. The idea of a topical application of medicine to the inner ear is not new. Decades ago local anesthetics and aminoglycosides were applied through the tympanic membrane into the tympanic cavity to treat inner ear disorders [10–12]. Intratympanic injections have been used for local application of aminoglycosides or steroids in the therapy of Meniere's disease and sudden hearing loss. There are a number of clinical reports showing the efficacy of intratympanic injections of these drugs (reviewed by Salt et al. [13]). However, it is very difficult to predict the amounts of drugs that reach the inner ear fluid space. Some reports have indicated that this method led to varying results in the therapy of Meniere's disease [14–16]. An intratympanic injection is a simple and easy method; however, controlled and sustained release of drugs cannot be achieved by this method.

Recent animal studies have indicated the efficacy of growth factors, neurotrophins [1,2], antioxidants [5], and apoptosis inhibitors [3,4], which are locally applied to the inner ear, for otoprotection. Sustained treatment of inner ears by local viral gene transfer represents sufficient protection of inner ears from noise, drug toxicity, and reperfusion injury [17–21]. While basic studies have represented the benefits of local treatment with these substances, no cases have been approved for clinical application. Adenoviral vectors or adeno-associated viral vectors are being used most widely today for cochlear gene transfer. Despite their high efficiency for transfection, availability of high titers, or ease of production, they do not integrate into the genome, leading to transient expression, and their use potentially initiates an immune response resulting in destruction of recipient cochlear cells.

A controlled release system, in which the rate of release is determined by the design of the device, is required for certain biological effects of therapeutic molecules and elimination of unwanted side effects. For this purpose, implantable osmotic mini-pumps have been used for inner ear drug delivery in animal experiments [2,22]. This method, however, requires surgical treatment in the middle and inner ear, which may limit its clinical application. Previously, clinical efficacy of an implantable mini-pump, which delivers drugs via diffusion across the round window, has been described [23]. However, this technique has not been widely used in a clinical setting, because it requires surgical invasiveness almost equal to tympanoplasty. There remains intense interest in the development of safe and effective drug delivery systems for the inner ear, with a number of groups working on intracochlear catheter-based application systems. One approach has been to combine drug delivery with an existing device, such as by incorporating a drug delivery cannula into a cochlear implant electrode [24].

Candidates for therapeutic molecules for the treatment of SNHL are being discovered. It is therefore necessary to develop appropriate strategies for local delivery of therapeutic molecules. For clinical application, safe, effective, and direct methods for delivery of therapeutic molecules to the inner ear need to be developed.

Controlled-release systems

In the past decade, pharmaceutical technologists have paid increasing attention to the controlled or sustained release technology via biomaterials for the delivery of drugs in order to avoid side effects and achieve sufficient drug levels in tissues. Such technology is utilized not only for drug delivery but also for gene delivery [25]. In an effort to develop controlled-release systems, a variety of methods using synthetic and natural materials have arisen. Recent publications have reported on the use of controlled-release systems for local drug delivery to the inner ear. Two synthetic materials, siloxane-based polymers [26] and poly lactic/glycolic acid (PLGA) polymers [27], and one natural material, gelatin-hydrogels [28,29], have been used for this purpose. Although these materials have been included in biomaterials for controlled-release systems, mechanisms for loading and releasing drugs apparently differ among these materials (Figure 1). In siloxane-based polymer systems, the drug dissolves in the polymer and then moves by diffusion [30]. For PLGA polymers, the drug is encapsulated in PLGA polymers and then released by hydrolysis of PLGA [31]. In gelatin-based release systems, the drug binds to gelatin carriers by polyion complexation and is released by enzymatic hydrolysis of gelatin polymers [32].

Siloxane-based polymers

Siloxane-based polymers have been used for years in medical applications in contact with the human body. Silicone-transdermal patches have been widely used in clinic. The drug release in this system is controlled by its diffusion through the silicone network [30]. The releasing rate in this system is determined by the composition of the polymer. This system is particularly suitable for application of lipophilic and low-molecular weight molecules. Arnold et al. [26] have utilized this system for local application of beclomethasone into cochlear fluids. A silicone-microimplant was placed onto the RWM

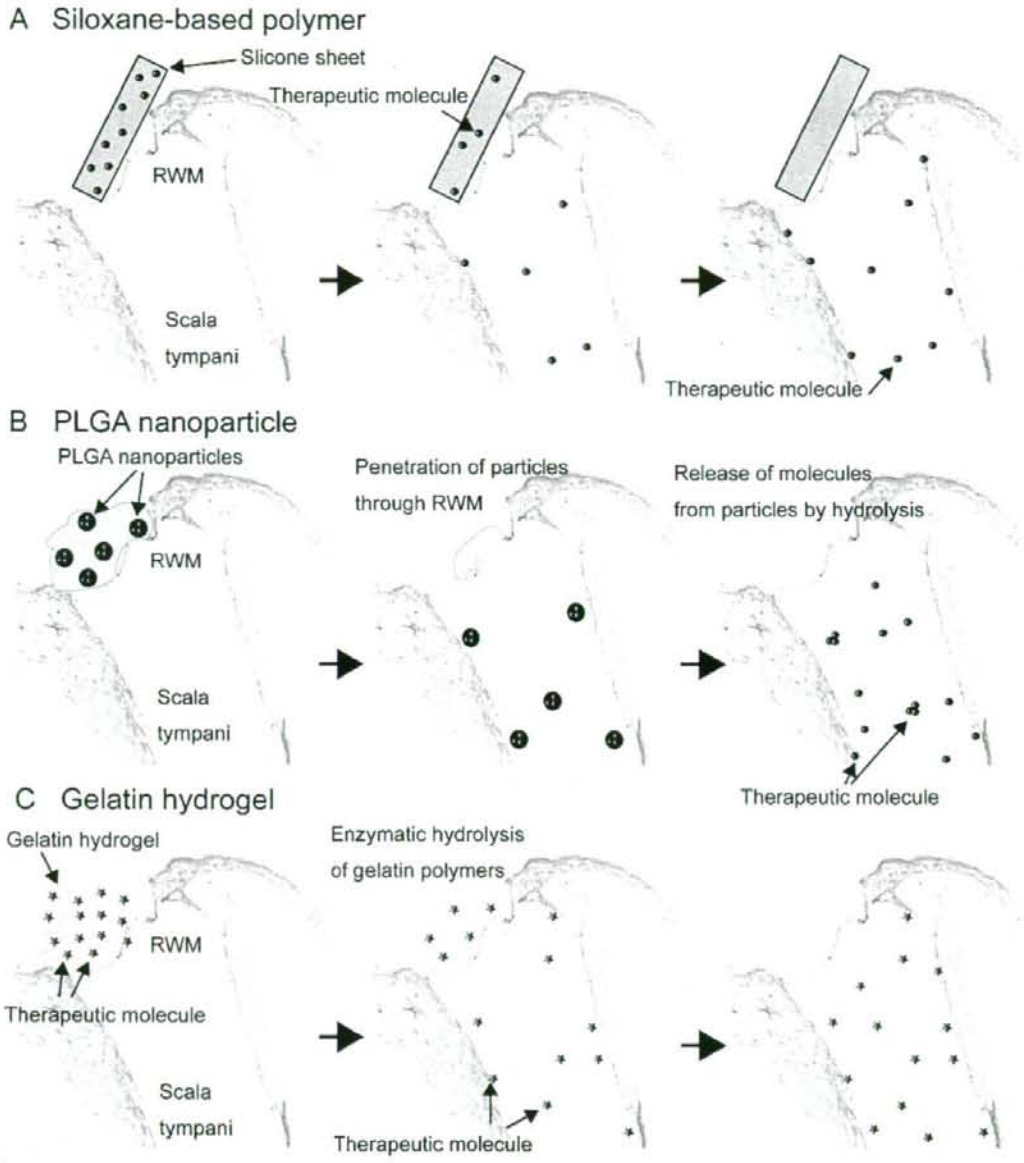

Figure 1. Mechanisms for controlled release of therapeutic molecules from biopolymers. (A) Therapeutic molecules dissolved in siloxane-based polymers move into the scala tympani by diffusion. A silicone sheet remains on the round window membrane (RWM). (B) Poly lactic/glycolic acid (PLGA) nanoparticles containing therapeutic molecules penetrate through the RWM. Therapeutic molecules are released from nanoparticles by their hydrolysis. (C) Therapeutic molecules bind to gelatin carriers by polyion complexation and are released by enzymatic hydrolysis of gelatin polymers.

of guinea pigs. Liquid chromatography demonstrated release of beclomethasone from the silicone-microimplant into cochlear fluids. In this system, a silicone-microimplant remains on the RWM (Figure 1A), although it does not induce functional and histological damage in the cochlea. Therefore, repeated treatment requires extirpation of the material that had been used previously. In addition, only a limited number of molecules can be used in this system.

PLGA nanoparticles

Encapsulating bioactive molecules in PLGA or polylactic acid (PLA) particles has been used as a method for controlled-release application. Water-insoluble, low-molecular weight agents were encapsulated in PLGA or PLA microparticles and nanoparticles, and provided for clinical use [33,34]. However, recent advances in this field enable encapsulation of water-soluble, low-molecular weight agents in PLGA nanoparticles [31]. Tamura et al. [27] have examined the potential of PLGA nanoparticles for drug delivery to the cochlea using guinea pigs. The distribution of PLGA nanoparticles encapsulating rhodamine (140–180 nm in diameter) in the cochlea following local application onto the RWM was evaluated. PLGA nanoparticles containing rhodamine were observed in the cochlea, indicating that PLGA nanoparticles can penetrate through the RWM. Rhodamine will be released from PLGA nanoparticles after penetration of PLGA nanoparticles through the RWM (Figure 1B). On the other hand, systemic application of PLGA nanoparticles has no significant effects on sustained, targeted delivery of rhodamine into the cochlea. These findings indicate that encapsulating therapeutic molecules in PLGA nanoparticles is suitable for local drug delivery to the cochlea.

In comparison with a silicone-microimplant, PLGA nanoparticles have advances including the ability of repeated application, because PLGA is dissolved by hydrolysis. Various therapeutic molecules for inner ear diseases can be encapsulated in PLGA nanoparticles, and applied as intratympanic drugs. The efficacy of encapsulating betamethasone phosphate in PLGA nanoparticles has already been confirmed using animal models for rheumatoid arthritis and autoimmune uveoretinitis [35,36]. Local gentamicin application has been used for the control of intractable vertigo in Meniere's disease [14–16]. PLGA nanoparticles can be utilized for controlled release of gentamicin. However, PLGA nanoparticles are not suitable for delivery of proteins or peptides. Hence, this system cannot use for controlled delivery of neurotrophins or growth factors.

Gelatin hydrogel

Gelatin is a commonly used natural polymer that is derived from collagen. Gelfoam, which is prepared from porcine-skin gelatin, has been used for drug delivery to the cochlea [37]. Recently, gelatin-based controlled-release systems have been developed [32]. The isoelectric point of gelatin can be modified during the fabrication process to yield either a negatively charged acidic gelatin or a positively charged basic gelatin, which allows electrostatic interactions to take place between charged therapeutic molecules and gelatin of the opposite charge, forming polyion complexes. The significance of this system is the ability for application of proteins and plasmid DNA. Previous reports have demonstrated its efficacy for controlled release of various growth factors or plasmid DNA in other fields [25,38,39]. In this system, therapeutic molecules are released by enzymatic degradation of gelatin (Figure 1C), the rates of which are determined by the crosslinking density of gelatin hydrogels.

Endo et al. [28] have demonstrated sustained release of brain-derived neurotrophic factors (BDNFs) into cochlear fluids by a gelatin hydrogel. BDNF concentrations in the cochlear fluid after placing a hydrogel containing this agent onto the RWM of guinea pigs were measured by enzyme-liked immunosorbent assay (ELISA), which reveals sustained delivery of BDNF into the cochlear fluid via the hydrogel. In addition, local BDNF delivery using a gelatin hydrogel sufficiently protects spiral ganglion neurons in functionality and histology. More recently, Iwai et al. (29) have described significant protection of auditory hair cells from noise trauma in rats using local application of insulin-like growth factor I via gelatin hydrogels. These findings demonstrate that the gelatin-based controlled-release system is a useful method for sustained delivery of neurotrophins and growth factors into the cochlea. Repeated applications using this system are possible. This system has several advances in comparison with the other two controlled-release systems: (1) easy loading of therapeutic molecules into biopolymers, (2) it is applicable for delivery of proteins, peptides, or plasmid DNA. These advances are favorable for the treatment of SNHL, because the efficacy of neurotrophins or growth factors and the potential of gene therapy for treatment of SNHL have been demonstrated.

Conclusions for clinical application

The results in experimental studies using controlled-release systems are preferable; however, the delivery protocol in humans is likely to differ from that in animal experiments. The distribution of drugs applied in the cochlear fluid space depends on dispersal diffusion, which is influenced by the length and volume of the cochlear fluid space [13]. In addition, the round window niche membrane covers the round window niche in 57% of human subjects [40]. Therefore, it is necessary to remove tissues overlaying the RWM for drug penetration through the RWM in some cases. However, introduction into

clinical practice of these controlled-release systems may be reasonable since they have certain advantages over previous methods and implantable devices.

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References

[1] Miller JM, Chi DH, O'Keeffe LJ, Kruszka P, Raphael Y, Altschuler RA. Neurotrophins can enhance spiral ganglion cell survival after inner hair cell loss. Int J Dev Neurosci 1997;15:631–43.

[2] Shinohara T, Bredberg G, Ulfendahl M, Pyykko I, Olivius NP, Kaksonen R, et al. Neurotrophic factor intervention restores auditory function in deafened animals. Proc Natl Acad Sci U S A 2002;99:1657–60.

[3] Nakagawa T, Kim TS, Murai N, Endo T, Iguchi F, Tateya I, et al. A novel technique for inducing local inner ear damage. Hear Res 2003;176:122–7.

[4] Cunningham LL, Cheng AG, Rubel EW. Caspase activation in hair cells of the mouse utricle exposed to neomycin. J Neurosci 2002;22:8532–40.

[5] Duan ML, Ulfendahl M, Laurell G, Counter AS, Pyykko I, Borg E, et al. Protection and treatment of sensorineural hearing disorders caused by exogenous factors: experimental findings and potential clinical application. Hear Res 2002; 169:169–78.

[6] Kawamoto K, Ishimoto S, Minoda R, Brough DE, Raphael Y. Math1 gene transfer generates new cochlear hair cells in mature guinea pigs in vivo. J Neurosci 2003;23:4395–400.

[7] Izumikawa M, Minoda R, Kawamoto K, Abrashkin KA, Swiderski DL, Dolan DF, et al. Auditory hair cell replacement and hearing improvement by *Atoh1* gene therapy in deaf mammals. Nat Med 2005;11:271–6.

[8] Maeda Y, Fukushima K, Nishizaki K, Smith RJ. *In vitro* and *in vivo* suppression of *GJB2* expression by RNA interference. Hum Mol Genet 2005;14:1641–50.

[9] Juhn SK, Rybak LP. Labyrinthine barriers and cochlear homeostasis. Acta Otolaryngol (Stockh) 1981;91:529–34.

[10] Ersner MS. Transtympanic injection of anesthetics for the treatment of Menière's Syndrome. Arch Otorhinolaryngol 1951;43–52.

[11] Völger G. Beseitigung von Labyrinthliquordruckstörungen bei dem Menièrschen Symptomkomplex durch das Hyaluronidasepräparat Kinetin. HNO 1952;3:142–7.

[12] Schuknecht HF. Ablation therapy for the relief of Meniere's disease. Laryngoscope 1956;66:859–70.

[13] Salt AN, Plontke KR. Local inner-ear drug delivery and pharmacokinetics. Drug Discov Today 2005;10:1299–306.

[14] Lange G, Maurer J, Mann W. Long-term results after interval therapy with intratympanic gentamicin for Meniere's disease. Laryngoscope 2004;114:102–5.

[15] Thomsen J, Charabi S, Tos M. Preliminary results of a new delivery system for gentamicin to the inner ear in patients with Meniere's disease. Eur Arch Otorhinolaryngol 2000; 257:362–5.

[16] Schoendorf J, Neugebauer P, Michel O. Continuous intratympanic infusion of gentamicin via a microcatheter in Meniere's disease. Otolaryngol Head Neck Surg 2001;124: 203–7.

[17] Yagi M, Magal E, Sheng Z, Ang KA, Raphael Y. Hair cell protection from aminoglycoside ototoxicity by adenovirus-mediated overexpression of glial cell line-derived neurotrophic factor. Hum Gene Ther 1999;10:813–23.

[18] Staecker H, Li D, O'Malley BW Jr, Van De Water TR. Gene expression in the mammalian cochlea: a study of multiple vector systems. Acta Otolaryngol (Stockh) 2001;121: 157–63.

[19] Luebke AE, Foster PK, Muller CD, Peel AL. Cochlear function and transgene expression in the guinea pig cochlea, using adenovirus- and adeno-associated virus-directed gene transfer. Hum Gene Ther 2001;12:773–81.

[20] Nakaizumi T, Kawamoto K, Minoda R, Raphael Y. Adenovirus-mediated expression of brain-derived neurotrophic factor protects SGNs from ototoxic damage. Audiol Neurootol 2004;9:135–43.

[21] Hakuba N, Watabe K, Hyodo J, Ohashi T, Eto Y, Taniguchi M, et al. Adenovirus-mediated overexpression of a gene prevents hearing loss and progressive inner hair cell loss after transient cochlear ischemia in gerbils. Gene Ther 2003;10: 426–33.

[22] Ylikoski J, Pirvola U, Virkkala J, Suvanto P, Liang XQ, Magal E, et al. Guinea pig auditory neurons are protected by glial cell line-derived neurotrophic factor from degeneration after noise trauma. Hear Res 1998;124:17–26.

[23] Lefebvre PP, Staecker H. Steroid perfusion of the inner ear for sudden sensorineural hearing loss after failure of conventional therapy: a pilot study. Acta Otolaryngol (Stockh) 2002;122:698–702.

[24] Paasche G, Gibson P, Averbeck T, Becker H, Lenarz T, Stover T. Technical report: modification of a cochlear implant electrode for drug delivery to the inner ear. Otol Neurotol 2003;24:222–7.

[25] Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin. Gene Ther 2004;11: 1205–14.

[26] Arnold W, Senn P, Hennig M, Michaelis C, Deingruber K, Scheler R, et al. Novel slow- and fast-type drug release round-window microimplants for local drug application to the cochlea: an experimental study in guinea pigs. Audiol Neurotol 2005;10:53–63.

[27] Tamura T, Kita T, Nakagawa T, Endo T, Kim TS, Ishihara T, et al. Drug delivery to the cochlea using PLGA nanoparticles. Laryngoscope 2005;115:2000–5.

[28] Endo T, Nakagawa T, Kita T, Iguchi F, Kim TS, Tamura T, et al. A novel strategy for treatment of inner ears using a biodegradable gel. Laryngoscope 2005;115:2016–20.

[29] Iwai K, Nakagawa T, Endo T, Matsuoka Y, Kita T, Kim TS, et al. Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. Laryngoscope 2006;116:529–33.

[30] Colas A. Silicones in pharmaceutical applications. Dow-Corning Healthcare Industries. http://www.dowcorning.com/content/publishedlit/51-993a-01.pdf

[31] Ishihara T, Izumo N, Higaki M, Shimada E, Hagi T, Mine L, et al. Role of zinc in formulation of PLGA/PLA nanoparticles encapsulating betamethasone phosphate and its release profile. J Control Release 2005;105:68–76.

[32] Young S, Wong M, Tabata Y, Mikos AG. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. J Control Release 2005;109:256–74.
[33] Okada H, Yamamoto M, Heya Y, Inoue Y, Kamei S, Ogawa Y, et al. Drug delivery using biodegradable microspheres. J Control Release 1994;28:121–9.
[34] Niwa T, Takeuchi H, Hino T, Kunou N, Kawashima Y. Preparation of biodegradable nanospheres of water-soluble and insoluble drugs with D,L-lactide/glycolide copolymer by a novel spontaneous emulsification solvent diffusion method, and the drug release behavior. J Control Release 1993;25: 89–98.
[35] Higaki M, Ishihara T, Izumo N, Takatsu M, Mizushima Y. Treatment of experimental arthritis with poly(D, L-lactic/ glycolic acid) nanoparticles encapsulating betamethasone sodium phosphate. Ann Rheum Dis 2005;64:1132–6.
[36] Sakai T, Kohno H, Ishihara T, Higaki M, Saito S, Matsushima M, et al. Treatment of experimental autoimmune uveoretinitis with poly(lactic acid) nanoparticles encapsulating betamethasone phosphate. Exp Eye Res 2006; 82:657–63.
[37] Husmann KR, Morgan AS, Girod DA, Durham D. Round window administration of gentamicin: a new method for the study of ototoxicity of cochlear hair cells. Hear Res 1998; 125:109–19.
[38] Hatano T, Miyamoto S, Kawakami O, Yamada K, Hashimoto N, Tabata Y. Acceleration of aneurysm healing by controlled release of basic fibroblast growth factor with the use of polyethylene terephthalate fiber coils coated with gelatin hydrogel. Neurosurgery 2003;53:393–400.
[39] Park H, Temenoff JS, Holland TA, Tabata Y, Mikos AG. Delivery of TGF-beta1 and chondrocytes via injectable, biodegradable hydrogels for cartilage tissue engineering applications. Biomaterials 2005;26:7095–103.
[40] Alzamil KS, Linthicum FH Jr. Extraneous round window membranes and plugs: possible effect on intratympanic therapy. Ann Otol Rhinol Laryngol 2000;109:30–2.

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Ginsenoside Rb1 protects against damage to the spiral ganglion cells after cochlear ischemia

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Abstract

The effects of transient cochlear ischemia on spiral ganglion cells (SGCs) were studied in Mongolian gerbils. Ischemic insult was induced by occluding the bilateral vertebral arteries of gerbils for 15 min. Seven days after ischemia, the percentage of SGCs decreased to 67.5% from the preischemic baseline in the basal turn. Evaluation with immunohistochemical staining showed TUNEL-positive reactions in the SGCs with fragmented nuclei. In addition, we investigated the protective effects of ginsenoside Rb1 (gRb1) against ischemic injury to SGCs. Seven days after ischemia, the auditory brainstem response threshold shift was significantly reduced and the percentage of SGCs decreased to 90.2% from the preischemic baseline in the basal turn in the gRb1-treated group. These findings suggest that gRb1 prevented hearing loss caused by ischemic injury to SGCs in Mongolian gerbils.

Keywords: Transient cochlear ischemia; Spiral ganglion cells; Ginsenoside Rb1; TUNEL staining; Bcl-xL protein

Ischemic injury is a major cause of acute sensorineural hearing loss [1,2,8]. We thus evaluated the pathology of transient cochlear ischemia in Mongolian gerbils. In these animals, the posterior cerebral communicating arteries are lacking and the labyrinthine arteries are nourished solely by the vertebral arteries. We previously showed that inner hair cells are more vulnerable to damage via the apoptotic process than outer hair cells and that transient ischemia for 5 min caused delayed cell death in the spiral ganglion [3,5,9,15,16]. However, the mechanism of neuronal damage to spiral ganglion cells (SGCs) has not been clarified.

Ginseng root is widely prescribed in Asian countries for a variety of ailments. Ginsenoside Rb1 (gRb1) is the main ingredient of ginseng root. In the brain, gRb1 has been shown to prevent apoptotic cell death via regulation of the Bcl-xL protein, which inhibits apoptosis by preventing the release of cytochrome *c* in the cell death pathway [17]. In the present study, we investigated the effects of transient cochlear ischemia on SGCs and evaluated whether postischemic intravenous infusion of gRb1 in this setting could prevent SGC degeneration.

The following study was conducted in accordance with the Guidelines for Animal Experimentation of Ehime University School of Medicine, Japan. Adult male Mongolian gerbils weighing 60–80 g were used as subjects. Transient cochlear ischemia was induced in the animals by temporarily occluding the vertebral arteries bilaterally in the neck, as described by Hata et al. [4].

The hearing ability of animals treated with gRb1 was assessed by sequential recording of ABR. Measurements were made before and 4 and 7 days ($n=6$ each) after the ischemia insult. The ABR in response to an 8000-Hz tone burst (0.5-ms rise/fall time and 10-ms duration) was measured using a signal processor (NEC Synax 1200; NEC Medical Systems, Tokyo, Japan). This frequency was selected because we previously found that the higher-frequency region of the cochlea was more vulnerable to ischemic injury than the middle or lower-frequency regions [9]. The average ABR thresholds in control and gRb1-treated group were analyzed using a two-tailed Mann–Whitney *U*-test.

Ginsenoside Rb1 was isolated and purified from the crude saponin fraction in the rhizome of Korean red ginseng (Panax

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ginseng, C.A. Meyer) by repeated-column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (65:35:10) and on octadecylsilyl (ODS) silica with MeOH–H_2O (1:1–7:3) [12]. GRb1 was then dissolved in isotonic saline. A total of 200 μL of gRb1 solution (50 μg/200 μL) was injected into the left femoral veins of the gRb1 group ($n = 3$) and 200 μL of saline was injected into the left femoral veins of the control group ($n = 3$) 1 h after ischemia.

To determine SGC survival, the six gerbils were anesthetized deeply, killed 7 days after ischemia, and fixed by cardiac perfusion (4% paraformaldehyde in phosphate buffer). After decalcification in EDTA and embedding in paraffin, 6-μm sections were cut from the gerbils in a paramodiolar plane. Every fourth section was mounted on a glass slide and stained with hematoxylin–eosin. Six sections were randomly selected from the 10 most mid-modiolar sections from each animal and used in the quantitative analysis of the SGCs. Counting was performed in a double-blind fashion. All neurons meeting the size and shape criteria to be considered type I SGCs within each profile of Rosenthal's canal in the basal turn were counted. The outline of the Rosenthal's canal profile was then traced to generate a SGC density, expressed as the density of SGC in an area of 10,000 μm^2. The baseline average number of SGCs was counted in six animals without ischemia. This baseline average was used to calculate the percentage of lost SGCs 7 days after ischemia. Statistical assessment of the differences in the SGC densities between the study groups was performed with analysis of variance and Student's *t*-test.

Double-stranded DNA breaks were detected by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) using specimens from six gerbils 1 day after ischemia. An apoptosis in situ detection kit (Roche, Mannheim, Germany) was used for the TUNEL staining. Deparaffinized sections were washed with distilled water and treated with proteinase K for 5 min at 37 °C. After washing three times with 0.01 M phosphate-buffered saline (PBS), the sections were treated with TdT solution for 10 min at 37 °C. To block endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide for 5 min at room temperature. Peroxidase-conjugated antibody was applied for 10 min at 37 °C. After washing with 0.01 M PBS, nick end-labeling was visualized with 3,3′-diaminobenzidine (DAB) solution.

For the Bcl-xL immunostaining, six gerbils were used 1 day after ischemia. The sections from both the gRb1 and control groups were deparaffinized and exposed to 5% normal horse serum (NHS) in PBS for 1 h. The sections were subsequently incubated for 12 h with rabbit anti-Bcl-xL antibody (1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), flooded for 1 h with biotin-conjugated goat anti-rabbit immunoglobulin (1:400 dilution; DAKO Cytomation Co., Kyoto, Japan) in 1% NHS–PBS, and incubated in biotinylated tyramine solution containing 0.01% H_2O_2. The sites of bound primary antibodies were visualized after incubating the sections with ABC reagent (Vector Laboratories, Burlingame, CA, USA). Sections from each animal were immunostained in the same run for between-group comparisons.

The fine structure of the SGCs was evaluated with transmission electron microscopy (TEM). Four animals were killed 1 day after ischemia. Immediately following removal of both otic bullae during deep anesthesia, the cochleae were perfused with 2.5% glutaraldehyde. The cochleae were then removed, immersed for 8 h in the same fixative at 4 °C, and dissected in PBS (pH 7.4). Pieces of the organ of Corti were used for whole-mount staining and TEM analysis. The specimens were postfixed with 2% osmium tetroxide for 1 h at room temperature. Following dehydration, the specimens were embedded in epoxy resin. Sections were cut with an ultramicrotome and stained with uranyl acetate and lead citrate. The cochleae were then observed with a TEM (H300; Hitachi, Tokyo, Japan).

The average increase in the ABR threshold was 22.5 ± 2.9 dB on the seventh day after inducing ischemia with saline treatment. In contrast, the ABR threshold increased to 14.2 ± 3.8 dB in ischemic animals treated with gRb1. The average increase in the ABR threshold 7 days after inducing ischemia was significantly lower in animals treated with gRb1 than in saline-treated animals ($P < 0.05$) (Fig. 1).

Fig. 2 shows the representative sections of Rosenthal's canal in the base of the cochlea from a control subject (Fig. 2a) and a gRb1-treated subject (Fig. 2b). Seven days after ischemia, the mean percentage decrease in SGCs was 30.6 ± 2.1, 16.0 ± 7.8, and 7.5 ± 1.8% at the basal, second, and apical turns, respectively. These results show that SGCs at the basal turn were more vulnerable than those at the second and apical turns. Therefore, we investigated the effect of gRb1 on ischemic damage to SGCs in the basal turn.

Before ischemia, the average number of SGCs in the basal turn was 28.1/10,000 μm^2. Seven days after ischemia, the average number of SGCs in the basal turn was 25.8/10,000 μm^2 in the gRb1 group and 19.3/10,000 μm^2 in the control group. The mean percentage loss of SGCs in the gRB1 group was 8.0 ± 4.7%, which was significantly smaller than the 30.6 ± 2.1% loss observed in the control group ($P < 0.05$; Fig. 3). Injection of gRb1 resulted in enhanced survival of SGCs 7 days after ischemia.

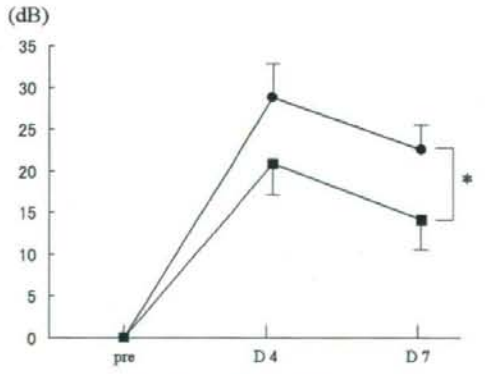

Fig. 1. Changes in the auditory brainstem response (ABR) threshold following transient cochlear ischemia. The ABR was measured in response to an 8000-Hz tone burst. The ABR threshold before ischemia was defined as 0 dB. *$P < 0.05$ vs. the control.

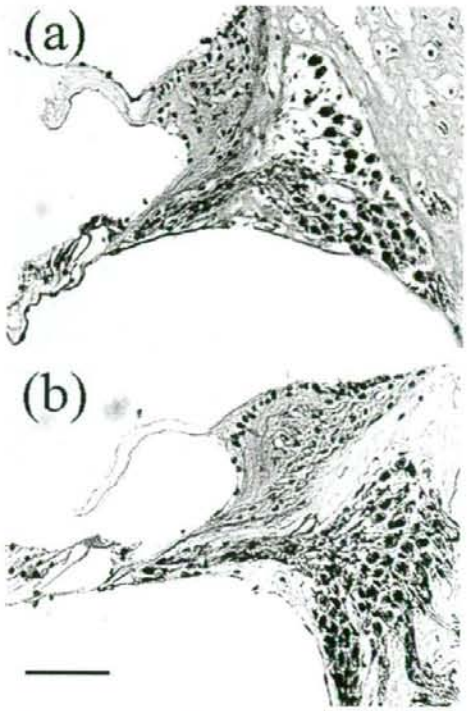

Fig. 2. Representative sections of Rosenthal's canal in the base of the cochlea from a control subject (a) and a gRb1-treated subject (b). A clear difference was observed in the survival of these subjects. Scale bar = 60 μm.

Ischemia induced TUNEL-positive reactions in SGCs with fragmented nuclei (Fig. 4a). Few TUNEL-positive cells were observed in the basal turn of the cochleae in animals administered gRb1 (Fig. 4b). The mean percentage of TUNEL-positive cells in the specimens 1 day after ischemia was 24.4 ± 7.8% for animals treated with saline and 4.1 ± 2.9% for animals treated with gRb1.

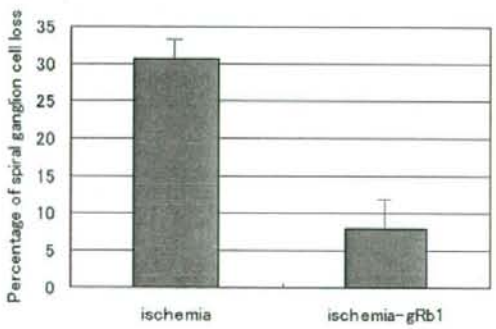

Fig. 3. The percentage decrease of SGCs in the basal turn 7 days after ischemic injury. SGC degeneration was significantly lower in the gRb1-treated group compared to the control group ($P < 0.01$).

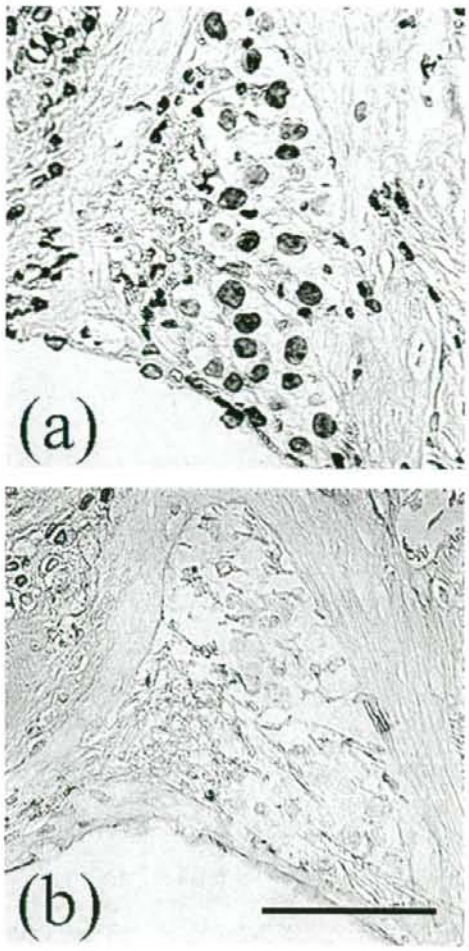

Fig. 4. Light microscopic photograph of TUNEL staining in the spiral ganglion. TUNEL-positive cells were observed in the spiral ganglion of control animals (a), whereas no TUNEL-positive cells were observed in gRb1-treated animals (b). Scale bar = 60 μm.

After Bcl-xL immunostaining, strongly stained cells were present throughout the basal turn in the gRb1 group (Fig. 5c). Weak immunopositive cells were detected in the control group (Fig. 5b) or in specimens not exposed to the antibodies (data not shown). The results of the immunostaining for Bcl-xL were classified by two evaluators, who were blind to the treatment the animals had received, into four categories: no, mild, moderate, and strong staining. Table 1 summarizes the results of the immunostaining for Bcl-xL into four categories.

The ultrastructure of the SGCs was analyzed in the cochlear sections. Under electron microscopy, we observed the appearance of nuclear fragmentation and irregularly shaped nuclei in the control group (Fig. 6a), whereas most of the cells in the gRb1-treated group had regularly shaped nuclei (Fig. 6b). We further

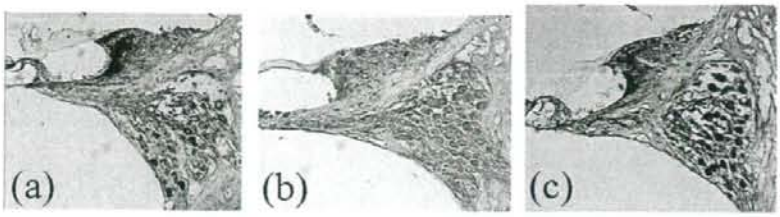

Fig. 5. Immunostaining for Bcl-xL before ischemia (a), 1 day after ischemia in the spiral ganglion of controls (b) and gRb1-treated animals (c). Ginsenoside Rb1 increased the expression of Bcl-xL 1 day after ischemia in the spiral ganglion. Scale bar = 60 μm.

Table 1
Summary of immunostaining for Bcl-xL

Control	Day 1	Day 1 + gRbl
++	+	++ to +++

+: mild staining; ++: moderate staining; +++: strong staining; +gRb1: animals given ginsenoside Rb1; control: before the induction of ischemia; Day 1: 1 day after the induction of ischemia.

distinguished SGCs as types I and II under electron microscopy. Dysmorphologic nuclei were observed in 36.9% of type I SGCs and 18.5% of type II SGCs. We postulate that type I SGCs were more vulnerable than type II SGCs.

As primary auditory neurons, SGCs play an important role in hearing. In patients with severe sensorineural hearing loss, the only therapeutic intervention is a cochlear implant, which electrically stimulates residual primary auditory neurons to provide the patients with auditory cues required for speech perception. Most studies in humans and animals have shown that spiral ganglion neurons are lost following the loss of hair cells, particularly as a result of ototoxic drugs, noise exposure, and aging [6,7,11]. Results from these studies suggest that degeneration of the auditory nerve is a slow process that occurs over months or years. In contrast, the application of ouabain, which binds to Na^+, K^+-ATPase and blocks its activity in the cochlea, has been reported to result in a rapid loss of SGCs via apoptotic cell death [10,13]. However, the mechanism of ischemic injury has not been elucidated. We induced ischemic injury in gerbils for 15 min and observed an agglomeration of chromatin and aberrant nuclei via electron microscopy 1 day after ischemia. Additionally, apoptotic cells were detected by the TUNEL method. These findings indicate that ischemic injury is caused by apoptotic cell death in SGCs. The percentage of SGCs decreased to 68.5% from the preischemic baseline in the basal turn 7 days after ischemic injury. This is the first study to report how ischemic injury induces apoptotic cell death in SGCs and the degree of degeneration that ensues.

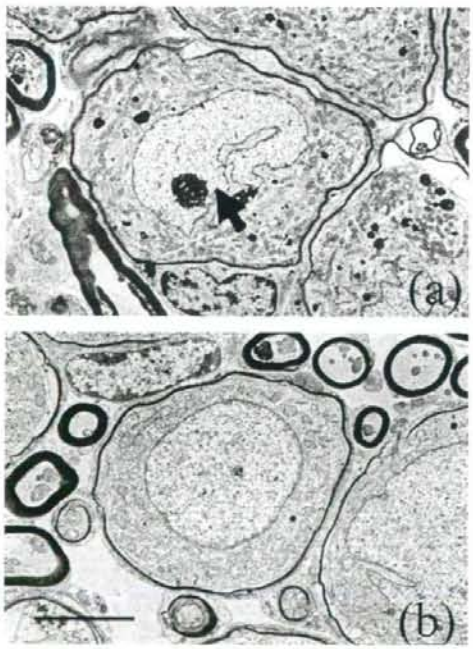

Fig. 6. Electron micrographs of the spiral ganglion 1 day after ischemia in controls (a) and gRb1-treated animals (b). (a) The SGC of the control animal has an indented nucleus with condensed chromatin (arrow). (b) The SGC of the gRb1-treated animal appears normal. Scale bar = 5 μm.

Ginseng root is an important component of treatment in many Asian countries and consists of two major ingredients: crude ginseng saponin and nonsaponin fractions. To date, more than 40 saponins have been isolated from ginseng root and identified chemically. The saponins can be classified into three major groups according to their chemical structures: protopanaxadiol, protopanaxatriol, and oleanolic acid. Ginsenoside Rb1, ginsenoside Rg1, and ginsenoside Ro are representative substances [14]. Zhang et al. reported that gRb1 rescued cortical neurons in the ischemic penumbra and reduced the cortical infarct volume by approximately 50%. Their report demonstrated that gRb1 deregulated the expression of Bcl-xL, which is known to suppress activation of procaspase-9 by forming a complex with Apaf-1. This, in turn, prevents the release of cytochrome *c* from mitochondria, thereby maintaining cell viability and cell survival [17]. In the present study, postischemic administration of gRb1 preserved the configuration of the spiral ganglion and decreased the number of TUNEL-positive cells with obvious expression of Bcl-xL 1 day after ischemia. We postulated that gRb1 may suppress apoptotic cell death in SGCs by activating the Bcl-xL signaling pathway. We observed decreased degeneration of SGCs in the basal turn of gRb1-treated animals 7 days after ischemia compared to untreated controls. Our results suggest