持細胞が再び分裂，増殖する可能性がある．p27Kip1の発現制御メカニズムとしては，p27Kip1遺伝子からの転写，翻訳レベルとp27Kip1タンパク質の細胞内での分解レベルの2つがある．後者のメカニズムにかかわるのが，Fボックスタンパク質の1つであるskp2である．skp2はp27Kip1のユビキチン化に関与し，skp2などの働きでユビキチンを付加されたp27Kip1はプロテアソームにて分解される．

発達段階に応じてマウス内耳感覚上皮でのskp2の発現変化を組織学的に解析することで，内耳感覚上皮で細胞増殖が活発な時期には，skp2によりp27Kip1の発現が抑制されており，内耳感覚上皮で有毛細胞，支持細胞への分化運命が決定されるタイミングにおいては，p27Kip1の発現は支持細胞のみに限定され，有毛細胞では消失することが明らかになった．この有毛細胞でのp27の発現の消失にskp2は関与せず[8]，遺伝子からの転写レベルよりも上流でp27Kip1の発現が制御されている．したがって，成熟した内耳感覚上皮支持細胞でのp27発現抑制には，skp2過剰発現によるタンパク質分解の促進もしくはRNA干渉による翻訳の抑制が有効な手段と推察される．蓑田らは，ウイルスベクターを用いたskp2の内耳感覚上皮での過剰発現が細胞増殖を誘導すると報告している[9]．しかし，細胞周期にリエントリーすることは，細胞死の誘導につながる可能性もあり[10]，今後の検討が注目される．

内耳再生への試み(2)：細胞移植

哺乳類内耳の再生能力が限られているのならば，再生能力のある細胞を内耳に送り込んでやればよいのではないか，という発想で内耳への細胞移植実験は開始された．最初の内耳への細胞移植実験は，内耳と同じ外胚葉系の幹細胞である神経幹細胞を用いたものである．有毛細胞障害を惹起した内耳に神経幹細胞を移植すると，内耳組織内に移植細胞の侵入を示唆する所見が認められた．また，ごく限られてはいるが，前庭感覚上皮内に侵入した移植細胞の一部が内耳有毛細胞のマーカーであるミオシン7aを発現している所見が認められた[11]．この結果は，神経幹細胞は障害を受けた内耳感覚上皮には侵入できるということを示しており，有毛細胞に分化する可能性があることを呈示するものとして内外の注目を集めた．内耳組織由来の細胞や胚性幹細胞由来の細胞移植でも，感覚上皮内に移植細胞が侵入し，有毛細胞様の細胞に分化することが報告されているが，機能再生に関連する報告はなされていない．いかに，有毛細胞や支持細胞といった感覚上皮特有の細胞に効率よく分化する細胞を開発するか，また，移植した細胞をいかにして感覚上皮内へと誘導するかが，解決すべき問題点として残されている．

細胞移植による内耳再生研究では，ラセン神経節細胞が主な研究対象とされている．現在，高度難聴に対する唯一の治療法は人工内耳であるが，人工内耳を埋め込んでもラセン神経節細胞に障害がある場合，良好な聞き取りは得られない．細胞移植によって，ラセン神経節細胞が再生すれば，人工内耳での聞き取りも向上すると期待される．このような臨床的背景に加え，神経細胞は種々の細胞から比較的分化誘導しやすいことにより，細胞移植によるラセン神経節細胞再生に関する研究が活発に行われている．種々の細胞が移植細胞のソースとして用いられているが，最も神経細胞の再生能力が高い細胞が胚性幹細胞（ES細胞）といえる．ES細胞では，高率に神経細胞へと分化誘導する方法が確立されており，*in vitro*, *in vivo*での移植実験がいくつか行われている．最も注目すべき点としては，*in vivo*の移植実験で機能回復を示唆する所見が認められているという点である．

神経細胞へ分化誘導したマウスES細胞を，ラセン神経節変性をあらかじめ誘導したモルモットに移植し，4週間後に，電気刺激聴性脳幹反応にて機能評価したところ，コントロールとしたシャムオペ（偽手術）群よりも有意に機能が回復していることが認められた[12]．組織学的にも，移植細胞由来神経細胞の蝸牛軸での局在が確認されている．今後，移植細胞由来神経細胞が直接的に機能回復に寄与しているのか，栄養因子の供給などの間接的な効果なのかを検討する必要がある．

臨床応用への展望

第一に内耳に内在する細胞を活性化し，内耳の再生を誘導し，内耳性難聴の治療に応用する試みについては，内耳再生を妨げている因子を抑制し，再生を誘導する方法が最も臨床に近い方法といえる．しかしながら，この方法による機能回復に関する知見は，報告されておらず，今後の課題といえる．

Overview

細胞移植による内耳再生では，人工内耳とも深いかかわりをもつラセン神経節細胞の再生が最も臨床応用に近い位置にある．すでに霊長類での実験が開始されている．最大の問題は，移植細胞ソースにかかわる倫理的な面といえる．ES細胞は他の幹細胞に比べて，均一な性質をもつ細胞を大量に準備することが可能であり，ラセン神経節細胞再生に関しても現在のところ最も能力の高い細胞といえる．しかし，わが国では，ヒトES細胞を使用した研究承認へのハードルはきわめて高い．このような背景から，ES細胞をソースとした研究で得られた成果を他の自己由来細胞で実現しようとする方向性が模索されている．最近，線維芽細胞からES細胞と同様の性質をもった細胞をつくることが可能であることも報告されており[13]，自己由来細胞からES細胞様の細胞をつくることが可能となれば，大きく展開は変わる可能性がある．

文献

1) Kelly, M. W.：Int. J. Dev. Biol., 51：571-583, 2007
2) Yamamoto, N. et al.：J. Mol. Med., 84：37-45, 2006
3) Lowenheim, H. et al.：Proc. Natl. Acad. Sci. USA, 96：4084-4088, 1999
4) Stone, J. S. & Cotanche, D. A.：Int. J. Dev. Biol., 51：633-647, 2007
5) Kawamoto, K. et al.：J. Neurosci., 23：4395-4400, 2003
6) Izumikawa, M. et al.：Nature Med., 11：271-276, 2005
7) Hori, R. et al.：Neuroreport, in press (2007)
8) Dong, Y. et al.：Neuroreport, 14：759-761, 2003
9) Minoda, R. et al.：Hear. Res., 232：44-51, 2007
10) Laine, H. et al.：J. Neurosci., 27：1434-1444, 2007
11) Tateya, I. et al.：Neuroreport, 14：1677-1681, 2003
12) Okano, T. et al.：Neuroreport, 16：1919-1922, 2005
13) Takahashi, K. et al.：Cell, 126：663-676, 2006

参考図書

『Development of the Inner Ear』Kelley, M. W. et al., Springer, 2005

『ここまで進んだ幹細胞研究と再生医療2006』（田賀哲也，中畑龍俊／編），実験医学増刊号，Vol.24 No.2，羊土社，2006

『中枢神経の機能再生─細胞治療と人工臓器─』，Medical Bio，オーム社，2007年7月号

筆頭著者プロフィール

中川隆之：1989年大阪市立大学医学部卒業，'95年同大学院医学研究科修了，内耳のアポトーシス研究を行い，医学博士取得．2001年より京都大学大学院医学研究科耳鼻咽喉科頭頸部外科助教．現在，内耳再生，特に新しい感音難聴治療方法の開発を目的として研究を行っている．

DDSを用いた内耳疾患の治療

中川隆之*

感音難聴は，最も頻度の高い身体障害であるが，治療法はきわめてかぎられている．解決すべき問題に，適切に内耳へ薬物を移行させる技術開発がある．この問題の解決手段として，中耳の正円窓膜を介した薬物の内耳局所投与に，ドラッグデリバリーシステムの応用が試みられている．ポリグリコール乳酸に薬物を封入したナノパーティクルは，正円窓膜を通過することができることから，ステロイドなどの内耳への徐放に応用が可能と考えられる．また，ゼラチンハイドロゲルは，内耳に神経栄養因子や細胞増殖因子を徐放できる簡便なシステムとして，臨床応用が期待されている．

はじめに

感音難聴は，最も頻度の高い身体障害の1つとされている．感音難聴の多くは，内耳の蝸牛障害に起因する．音響刺激は，空気の疎密波として，鼓膜を振動させる．鼓膜の振動は，3つの耳小骨を介して蝸牛に振動を伝える．耳小骨の振動は，蝸牛の感覚上皮を振動させ，感覚上皮の感覚細胞（有毛細胞）が振動エネルギーを神経信号に変換し，蝸牛軸に存在する蝸牛の一次神経節であるラセン神経節細胞を興奮させ，中枢に伝達される（**図1**）．感音難聴の原因としては，音響外傷，耳毒性薬物，遺伝子異常，老化がある．また，メニエール病などの内リンパ水腫に関連する難治性疾患も感音難聴を引き起こす．聾もしくは高度難聴は新生児の1000〜2000人に1人認められ，2000人に1人は成人するまでに高度難聴を発症する．70歳を超えた人口の6割には，高度難聴が存在する．このような背景があるにもかかわらず，感音難聴に対する治療オプションはごくかぎられているのが現状である．この現状を打破すべく，多くの基礎的研究がなされ，いくつかの治療薬としてのポテンシャルをもつ薬物が同定されている．しかしながら，臨床応用に至った基礎的研究成果は，ほとんどない．その大きな理由のひとつとして，内耳に適切に薬物を到達させる手段の欠如が挙げられてきた．

〔キーワード〕
感音難聴
正円窓
ゼラチン
ポリグリコール乳酸
ナノパーティクル

* NAKAGAWA Takayuki/京都大学大学院医学研究科耳鼻咽喉科頭頸部外科

全身投与された薬物が蝸牛の存在する内耳に到達しにくい原因は，おおまかに2つある．1つは，内耳に到達する血流量がきわめて少ないという点である[1]．もう1つは，血液と内耳のあいだに「血液―内耳関門」が存在することである[2]．血液―脳脊髄液関門と同様に，多くの薬物は血液―内耳関門を通過することができない．以上の問題を解決するためには，2つのストラテジーが考えられる．1つは，薬物に化学的な修飾を加え，内耳に到達しやすくする工夫である．薬物の内耳へのターゲティングともいえる．この方法では，血液―内耳関門を通る性質をもたせ，なおかつ内耳に特異的に集積する正確をもたせることが求められる．も

Presented by Medical*Online

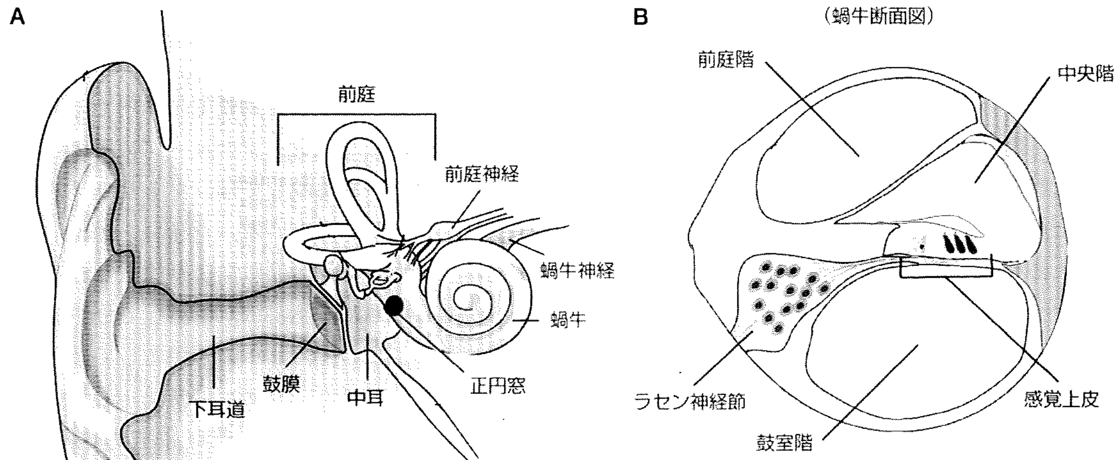

図 1. 内耳，蝸牛の解剖
A：内耳は，蝸牛と前庭からなり，中耳のさらに深部に存在し，そのほとんどは骨に囲まれている．正円窓のみ膜様構造物で内耳と中耳は接している．
B：蝸牛の断面図を示す．蝸牛は，前庭階，中央階，鼓室階という3つのコンパートメントからなる．中央階と鼓室階の境界に感覚上皮をのせる基底板が存在し，ラセン神経節は蝸牛軸に存在する．

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

Presented by Medical*Online

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

Presented by Medical*Online

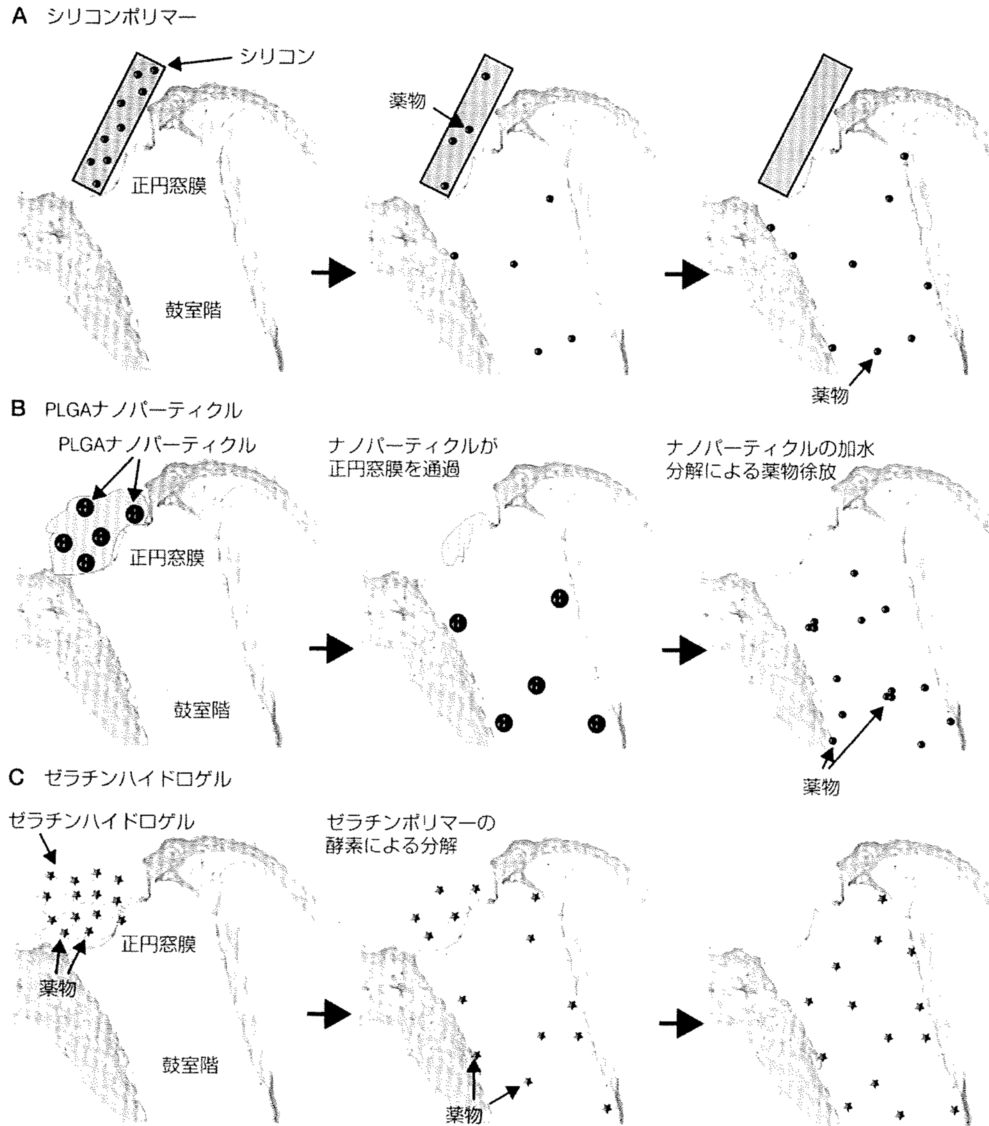

図 2. 内耳DDSの薬物徐放機構（Nakagawa T *et al*[11]より改変引用）

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．

Presented by Medical*Online

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**：

Presented by Medical*Online

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

Presented by Medical*Online

Otology & Neurotology
28:976–981 © 2007, Otology & Neurotology, Inc.

Novel Therapy for Hearing Loss: Delivery of Insulin-Like Growth Factor 1 to the Cochlea Using Gelatin Hydrogel

*‡Kyu Yup Lee, *Takayuki Nakagawa, *Takayuki Okano, *Ryusuke Hori, *Kazuya Ono, †Yasuhiko Tabata, ‡Sang Heun Lee, and *Juichi Ito

**Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, and †Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Science, Kyoto University, Kyoto, Japan; and ‡Department of Otolaryngology, Kyungpook National University Hospital, Daegu, Korea*

Hypothesis: Local application of recombinant human insulin-like growth factor 1 (rhIGF-1) via a biodegradable hydrogel after onset of noise-induced hearing loss (NIHL) can attenuate functional and histologic damage.
Background: The biodegradable gelatin hydrogel makes a complex with drugs by static electric charges and releases drugs by degradation of gelatin polymers. We previously demonstrated the efficacy of local rhIGF-1 application via hydrogels before noise exposure for prevention of NIHL.
Methods: First, we used an enzyme-linked immunosorbent assay to measure human IGF-1 concentrations in the cochlear fluid after placing a hydrogel containing rhIGF-1 onto the round window membrane of guinea pigs. Second, the functionality and the histology of guinea pig cochleae treated with local rhIGF-1 application at different concentrations after noise exposure were examined. Control animals were treated with a hydrogel immersed in physiologic saline alone.
Results: The results revealed sustained delivery of rhIGF-1 into the cochlear fluid via the hydrogel. The measurement of auditory brainstem responses demonstrated that local rhIGF-1 treatment significantly reduced the threshold elevation from noise. Histologic analysis exhibited increased survival of outer hair cells by local rhIGF-1 application through the hydrogel.
Conclusion: These findings indicate that local rhIGF-1 treatment via gelatin hydrogels is effective for treatment of NIHL.
Key Words: Biomaterial—Drug delivery—Growth factor—Noise trauma—Round window.
Otol Neurotol **28**:976–981, 2007.

Sensorineural hearing loss (SNHL) is one of the most common disabilities, but therapeutic options are limited to hearing aids and cochlear implants. Many investigations have therefore examined novel therapeutic molecules for SNHL and have discovered several agents exerting therapeutic activity against SNHL. Despite such progress in basic research, translation of these basic findings into clinical use is very rare. The lack of safe and effective methods for drug delivery to the cochlea has formed a considerable obstacle to clinical applications. Systemically applied drugs have great difficulty reaching cochlear cells because of 1) the blood-labyrinth barrier (1) acting as an obstacle to the transfer of drugs from serum to cochlear cells and 2) the limited blood flow to the cochlea (2). The development of local drug delivery systems to the cochlea thus remains crucial for the clinical application of basic findings in this field.

Several methods of local drug delivery to the cochlea have been reported (3). Implantable minipumps has frequently been used for local drug delivery to the cochlea in animal experiments (4). In addition, several clinical reports have described the efficacy of local steroid application using a semi-implantable minipump (5,6). However, the use of implantable minipumps has not been widely adopted because of the need for surgical procedures similar to tympanoplasty. Local drug delivery using biodegradable polymers has thus gained attention as an alternative to implantable minipumps. In general, biodegradable polymers containing therapeutic molecules are

Address correspondence and reprint requests to Takayuki Nakagawa, M.D., Ph.D., Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, 606-8507 Kyoto, Japan; E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp
This study was supported by a grant-in-aid for Regenerative Medicine Realization Project from the Ministry of Education, Science, Sports, Culture, and Technology of Japan and by a grant-in-aid for Researches on Sensory and Communicative Disorders from the Japanese Ministry of Health, Labor, and Welfare.

Copyright © 2007 Otology & Neurotology, Inc. Unauthorized reproduction of this article is prohibited.

placed on the round window membrane (RWM), and therapeutic molecules released from the polymers transfer into the perilymph of cochleae through the RWM (3,7). We have reported the efficacy of gelatin hydrogel for sustained delivery of brain-derived neurotrophic factor (BDNF) to the cochlear perilymph in guinea pigs (8). Brain-derived neurotrophic factor delivered by gelatin hydrogels efficiently protect spiral ganglion neurons from secondary degeneration caused by hair cell loss. However, BDNF is not currently clinically applicable. Insulin-like growth factor 1 (IGF-1) is a mitogenic peptide that plays essential roles in the regulation of growth and development in the inner ear. The gelatin hydrogel system was examined as a vehicle to deliver recombinant human IGF-1 (rhIGF-1) to the cochlea because this drug has already been approved for clinical use. Local rhIGF-1 application through the gelatin hydrogel before noise exposure significantly protects cochleae from functional and histologic losses induced by noise trauma (9).

Our goal is the clinical use of local rhIGF-1 application via the gelatin hydrogel as a therapeutic option for the treatment of SNHL. The current study examined whether posttraumatic application of rhIGF-1 to the cochlea through gelatin hydrogel attenuates noise-induced hearing loss (NIHL). In addition, we examined IGF-1 concentrations in cochlear perilymph after placing rhIGF-1–containing hydrogel onto the RWM of guinea pigs to determine the efficiency of the gelatin hydrogel system for cochlear application of rhIGF-1.

MATERIALS AND METHODS

Experimental Animals

Twenty-six adult female Hartley guinea pigs weighing 250 to 300 g (Japan SLC, Hamamatsu, Japan) served as experimental animals. Animal care was conducted under the supervision of the Institute of Laboratory Animals at 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.

Biodegradable Gelatin Hydrogel

A biodegradable hydrogel has been developed for sustained delivery of peptides, including growth and trophic factors (10). In this approach, a positively charged protein is electrostatically complexed with negatively charged polymer chains, which form the components of the biodegradable hydrogel. The biodegradation of the polymer chains leads to the release of peptide. Biodegradable hydrogels are generated by glutaraldehyde cross-linking of gelatin. The rates of degradation are determined according to the concentration of glutaraldehyde. A previous analysis of in vitro IGF-1 release profiles from hydrogels has demonstrated that a hydrogel made with 10-mmol/L glutaraldehyde allows for optimal IGF-1 delivery (11). We therefore used this type of hydrogel in the present study.

In Vivo IGF-1 Release Profile

Guinea pigs were anesthetized using ketamine (dose, 80 mg/kg intramuscularly; Sankyo, Tokyo, Japan) and xylazine (dose, 9 mg/kg intramuscularly; Bayer, Tokyo, Japan). A sheet of hydrogels in dried condition was cut to a size of 1.5 to 2 mm^3 under microscopy. A piece of hydrogel immersed in rhIGF-1 (amount, 400 μg dissolved in 40-μL physiologic saline; Astellas, Tokyo, Japan) was positioned on the left RWM of each animal in the treated group. Perilymph was collected on Days 1, 3, or 7 (n = 4 each) after drug application in treated groups and from nontreated animals (n = 4). For each animal, a small hole was made in the basal turn of the cochlea 2 mm from the RWM, under general anesthesia, and 3 μL of perilymph was collected through the hole using a micropipette. The same amounts of cerebrospinal fluid (CSF) and serum were obtained from each animal. Insulin-like growth factor 1 proteins were quantified using enzyme-linked immunosorbent assay (ELISA), performed using a Quantikine human IGF-1 immunoassay kit according to standard protocols (R&D Systems, Minneapolis, MN, USA). Triplicate measurements were averaged.

Noise Exposure and Drug Application

Baseline auditory brainstem response (ABR) thresholds were measured within 7 days before initial noise exposure. Animals were then exposed to 4-kHz octave band noise at 120-dB sound pressure level for 5 hours in a ventilated sound exposure chamber. Sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure stimulus uniformity. Under general anesthesia with ketamine and xylazine, a piece of hydrogel immersed in rhIGF-1 at a concentration of 1 or 10 μg/μL in 40 μL physiologic saline (n = 5 for each concentration) was placed on the RWM in the left ear of animals 5 hours after noise exposure; then, the hydrogel immersed in physiologic saline was placed on the RWM of the right ear.

Functional Analysis

Auditory function was assessed by using ABR recordings. Measurements of ABR thresholds were performed at frequencies of 4, 8, and 16 kHz before noise exposure and on Days 3, 7, 14, and 21 after drug application. The animals were anesthetized using ketamine and xylazine and were kept warm with a heating pad. Generation of acoustic stimuli and subsequent recording of evoked potentials were performed using a PowerLab/4SP data acquisition system (ADInstruments, Castle Hill, Australia). Acoustic stimuli, consisting of tone-burst stimuli (0.1-ms cos 2 rise/fall and 1-ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) connected

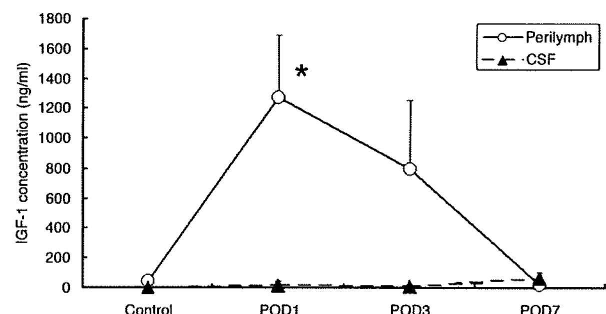

FIG. 1. Graph showing concentrations of human IGF-1 in cochlear perilymph and CSF. A significant increase in concentration of IGF-1 is found on postoperative Day 1 for perilymph (*$p <$ 0.05), whereas no significant changes are observed in concentrations of IGF-1 in CSF. Bars represent SEM. POD indicates postoperative day.

Copyright © 2007 Otology & Neurotology, Inc. Unauthorized reproduction of this article is prohibited.

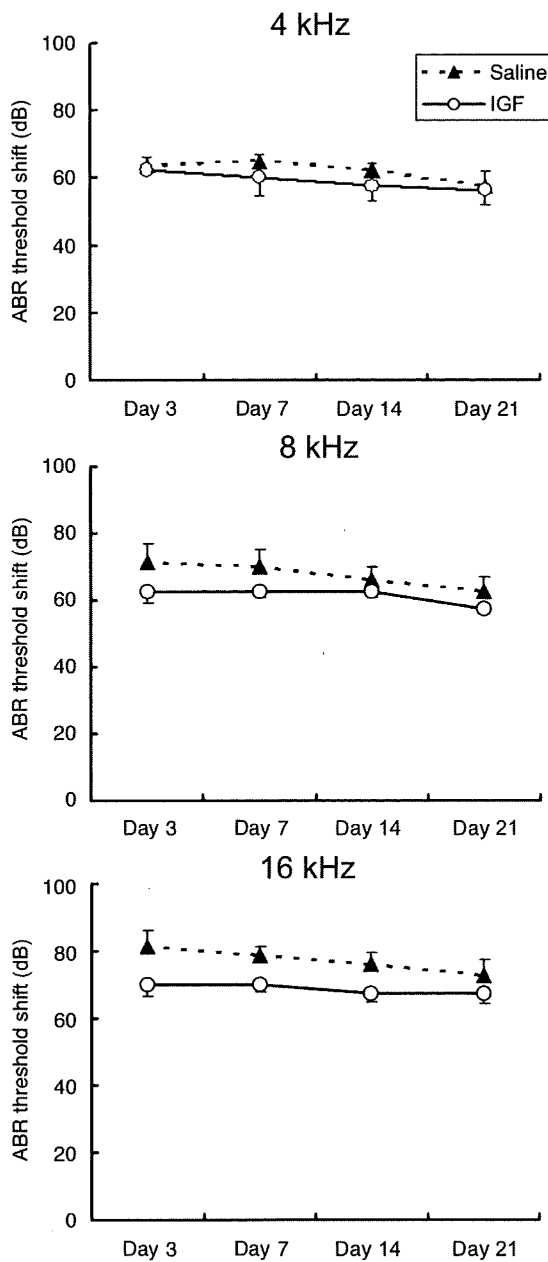

FIG. 2. Graphs showing time courses of alterations in threshold shifts of ABRs in cochleae treated with 1-µg/µl rhIGF-1 and cochleae treated with saline at frequencies of 4, 8, and 16 kHz. No significant differences are present in ABR threshold shifts between rhIGF-1– and saline-treated cochleae at any frequencies. Bars represent SEM.

to a funnel fitted into the external auditory meatus. To record bioelectric potentials, subdermal stainless steel needle electrodes were inserted at the vertex (ground), ventrolateral to the measured ear (active), and contralateral to the measured ear (reference). Stimuli were calibrated against a ¼-inch free-field microphone (ACO-7016; ACO Pacific, Belmont, CA, USA) connected to an oscilloscope (DS-8812 DS-538; Iwatsu Electric, Tokyo, Japan) or a sound level meter (LA-5111; Ono Sokki, Yokohama, Japan). The responses between the vertex and the mastoid subcutaneous electrodes were amplified using a digital amplifier (MA2; Tucker-Davis Technologies, Alachua, FL, USA). Thresholds were determined from a set of responses at varying intensities with 5-dB sound pressure level intervals; then, electric signals were averaged for 1,024 repetitions. The thresholds at each frequency were verified at least twice.

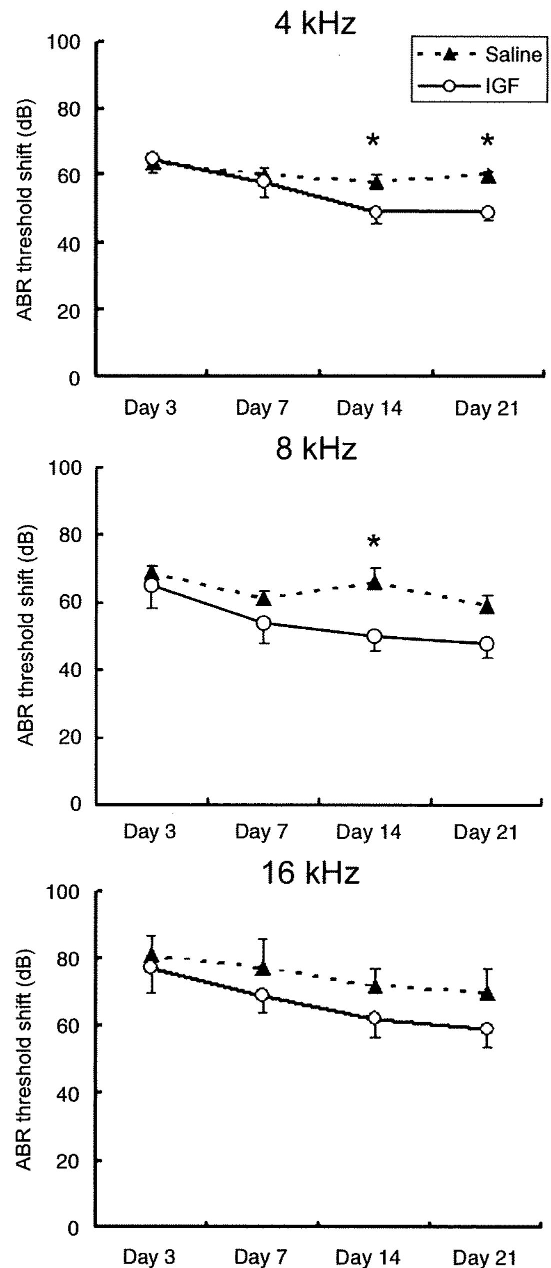

FIG. 3. Graph showing time courses of alterations in threshold shifts of ABRs in cochleae treated with 10-µg/µL rhIGF-1 and cochleae treated with saline at frequencies of 4, 8, and 16 kHz. The differences in ABR threshold shifts at 4 kHz between rhIGF-1– and saline-treated cochleae on Days 14 and 21 are significant, and the difference in ABR threshold shifts at 8 kHz on Day 14 is significant (*$p < 0.05$). Bars represent SEM.

Copyright © 2007 Otology & Neurotology, Inc. Unauthorized reproduction of this article is prohibited.

Histologic Analysis

On Day 21 after drug application, the animals were anesthetized with ketamine and xylazine, and the cochleae were exposed. After removal of the stapes, 4% paraformaldehyde in 0.01-mol/L phosphate-buffered saline (PBS) at pH 7.4 was gently introduced into the perilymphatic space of the cochleae. Temporal bones were then excised and immersed in the same fixative at a temperature of 4°C for 4 hours. After rinsing with PBS, cochleae were dissected from temporal bones and were subjected to histologic analysis in whole mounts. Three regions of cochlear sensory epithelia were used at a distance of 30 to 50% (apical), 50 to 70% (middle), or 70 to 90% (basal) from the apex for quantitative assessments of hair cell loss.

Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin were used to label the surviving inner hair cells (IHCs) and the outer hair cells (OHCs). Anti–myosin VIIa rabbit polyclonal antibody (dilution, 1:500; Proteus BioSciences, Ramona, CA, USA) was used as the primary antibody, and Alexa 546–conjugated goat anti–rabbit immunoglobulin G (dilution, 1:400; Molecular Probe, Eugene, OR, USA) was the secondary antibody. After immunostaining for myosin VIIa, the specimens were stained with fluorescein isothiocyanate–conjugated phalloidin (dilution, 1:300; Molecular Probe). Specimens were viewed under confocal microscopy (TCS SP2; Leica Microsystems, Wetzlar, Germany). Nonspecific labeling was tested by omitting the primary antibody from the staining procedures. The numbers of IHCs and OHCs in 0.2-mm-long regions of the apical, middle, or basal portion of cochleae were counted by the 3 authors (K.Y.L., T.N., and T.O.). The average of the values was used as the data for the animal.

Adverse Effects

We examined the incidence of loss of body weight at times of sample collection compared with weight at the beginning of the experiments. As a local adverse effect, incidence of otitis media was examined at times of sample collection. Otitis media was considered present if effusion was identified in the middle ear cavity.

Statistics

The overall effects of rhIGF-1 application on ABR threshold shifts were examined using two-way factorial analysis of variance. When interactions were significant, multiple comparisons using the Fisher protected least significant difference test were performed for pairwise comparisons. Differences in IHC and OHC numbers in each region of the cochlea between rhIGF-1– and saline-treated cochleae were examined using Student *t* test. Values of *p* less than 0.05 were considered statistically significant. Values are expressed as mean ± standard error of the mean (SEM).

RESULTS

In Vivo IGF-1 Release Profile

Enzyme-linked immunosorbent assay analysis of human IGF-1 proteins of the perilymph was performed to examine in vivo IGF-1 release profiles of hydrogels (Fig. 1). Very limited levels (mean ± SEM, 45.4 ± 31.0 ng/mL) of IGF-1 proteins were detected in samples obtained from nontreated cochleae. A marked increase in IGF-1 protein levels was observed on postoperative Days 1 (mean ± SEM, 1278 ± 413 ng/mL) and 3 (mean ± SEM, 801.6 ± 456 ng/mL). The IGF-1 protein levels then decreased to the control level by postoperative Day 7 (mean ± SEM, 23.7 ± 17 ng/mL). Differences in IGF-1 protein level between control and postoperative Day 1 and between postoperative Days 1 and 7 were significant (control versus postoperative Day 1, $p = 0.019$; postoperative Days 1 versus 7, $p = 0.017$). Level of IGF-1 protein in the CSF of each experimental group was almost the same as that in the perilymph of control animals (Fig. 1), and no significant differences in IGF-1 protein level were observed among experimental groups. The IGF-1 protein levels in the serum were undetectable in each experimental group.

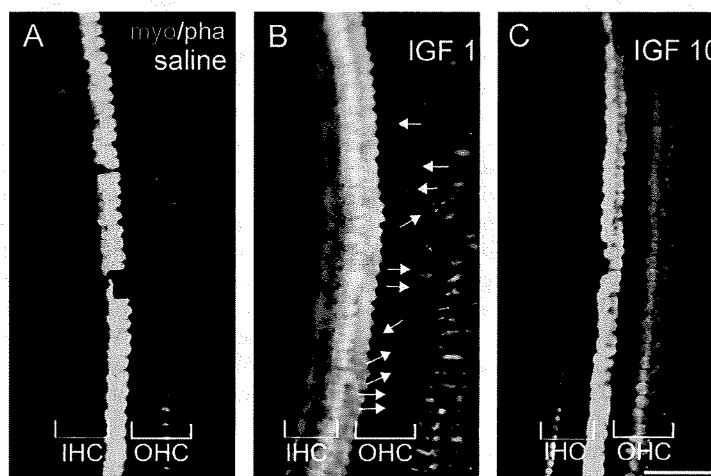

FIG. 4. Image showing immunostaining for myosin VIIa (*myo* [*red*]) and F-actin labeling with phalloidin (*pha* [*green*]) of cochlear sensory epithelia in the basal portion of cochleae. Severe loss of OHCs is observed in the saline-treated cochlea (*A*). Surviving OHCs in the specimen treated with 1-μg/μl recombinant human insulin-like growth factor 1 (rhIGF-1) exhibit scattered distribution (*B*; *arrows*). In cochleae treated with 10-μg/μl rhIGF-1, OHCs are comparatively preserved (*C*). Bar represents 50 μm.

Copyright © 2007 Otology & Neurotology, Inc. Unauthorized reproduction of this article is prohibited.

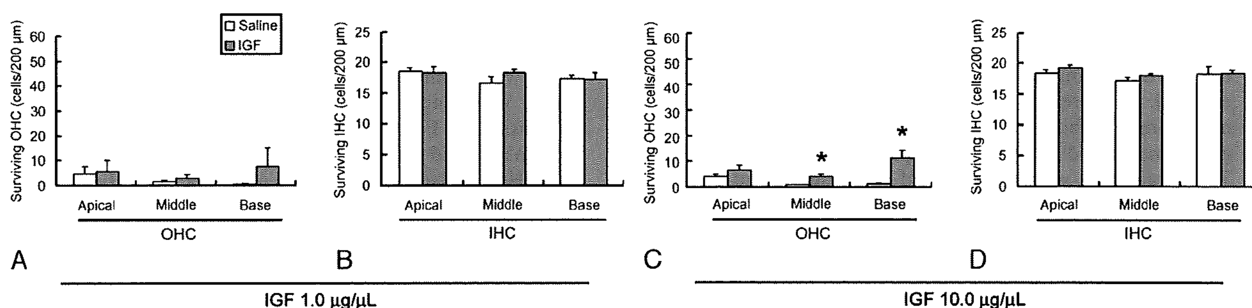

FIG. 5. Graphs showing numbers of surviving inner and outer hair cells in apical, middle, and basal portions of cochleae. No significant differences in numbers of OHCs (*A*) or IHCs (*B*) are found between saline- and 1-µg/µl rhIGF-1–treated cochleae. Differences in numbers of surviving OHCs between saline- and 10-µg/µl rhIGF-1–treated cochleae are significant in basal and middle portions of cochleae (*C*; $^*p < 0.05$), whereas significant differences in numbers of IHCs are not found between the 2 groups (*D*). Bars represent SEM.

Auditory Function

Time courses of alterations in ABR threshold shifts after drug application at 4, 8 and 16 kHz are shown in Figures 2 and 3. Local rhIGF-1 treatment at the concentration of 1 µg/µL demonstrated no significant effects on ABR threshold shifts at each frequency, although a trend toward lower threshold shifts was observed in comparison with controls (Fig. 2). Conversely, the overall effect of rhIGF-1 application on ABR threshold shifts at a concentration of 10 µg/µL was significant at 4 and 8 kHz, but not at 16 kHz (Fig. 3; $p = 0.014$ for 4 kHz, 0.005 for 8 kHz, and 0.074 for 16 kHz). Differences in threshold shifts at 4 kHz between 10-µg/µL rhIGF-1– and saline-treated cochleae on Days 14 and 21 were significant at multiple comparisons using the Fisher protected least significant difference test (Day 14, $p = 0.01$; Day 28, $p = 0.01$), and the difference in threshold shifts at 8 kHz on Day 14 was significant ($p = 0.016$).

Histologic Damage

Immunostaining for myosin VIIa and phalloidin staining demonstrated severe degeneration of OHCs in the apical, middle, and basal portions of saline-treated cochleae. Surviving OHCs were rarely found in middle and basal portions of saline-treated specimens (Fig. 4A). Outer hair cells degeneration in 1-µg/µL rhIGF-1–treated specimens was also severe. Surviving OHCs exhibited scattered distribution (Fig. 4B). In specimens treated with 10-µg/µL rhIGF-1, loss of OHCs was still observed; however, the degree of OHC degeneration was moderate (Fig. 4C). Inner hair cells were well maintained in all the experimental groups (Fig. 4, A–C). Quantitative assessments revealed no significant differences in numbers of surviving OHCs in any cochlear portion between saline- and 1-µg/µL rhIGF-1–treated cochleae (Fig. 5A). Differences in numbers of surviving OHCs between saline- and 10-µg/µL rhIGF-1–treated cochleae were significant in basal and middle portions of cochleae but not in the apical portion (Fig. 5C; basal and middle turns, $p = 0.009$; apical turn, $p = 0.387$). No significant differences were observed in numbers of surviving IHCs between saline- and 1- or 10-µg/µL rhIGF-1–treated cochleae (Fig. 5, B and D).

Incidence of Adverse Effects

No experimental animals exhibited loss of body weight. Scar formation was identified at the surgical site of the bulla, but no cochleae exhibited collection of effusion in the middle ear cavity indicative of otitis media.

DISCUSSION

These findings demonstrate that local rhIGF-1 treatment using gelatin hydrogel is effective for the treatment of NIHL in guinea pigs. Enzyme-linked immunosorbent assay in the present study revealed sustained delivery of rhIGF-1 to the cochlear fluid for 3 days, which is reasonable given the previous findings regarding the in vitro release profile of the hydrogel (11). The hydrogel used in the present study releases approximately 80% of IGF-1 into PBS for 3 days in the presence of collagenase, which is also present in the middle ear (12,13). The present findings from ELISA measurements also demonstrated no influences of cochlea rhIGF-1 application on levels of IGF-1 in CSF or serum. In addition, no systemic or local adverse effects were found in experimental animals. These findings indicate that rhIGF-1 application using the hydrogel offers safe and efficient delivery to the cochlea.

In the present study, ABR measurements exhibited significant effects of local rhIGF-1 treatment through gelatin hydrogels on attenuation of threshold shifts due to noise exposure. Although a tendency toward reduced threshold shifts was observed, local rhIGF-1 application at 1-µg/µL concentration showed no significant reduction in threshold shifts, whereas local rhIGF-1 application at 10-µg/µL concentration (the concentration recommended by the supplier for clinical use) exhibited significant attenuation of threshold shifts at frequencies of 4 and 8 kHz. Local rhIGF-1 application via hydrogels also exhibited significant histologic protection. Similar to functional protection, local rhIGF-1 treatment at a

Copyright © 2007 Otology & Neurotology, Inc. Unauthorized reproduction of this article is prohibited.

concentration of 10 μg/μL significantly increased the numbers of surviving OHCs, whereas treatment at a concentration of 1 μg/μL had no significant effect on the numbers of surviving OHCs. These findings indicate dose dependency for the effects of local rhIGF-1 treatment on the attenuation of NIHL.

Previous studies have demonstrated that several agents ameliorate NIHL when they are applied before noise exposure; however, only limited agents show protective effects by postexposure administration. Local application of D-JNK-1 peptide, an inhibitor of c-Jun N-terminal kinase, 12 hours after noise exposure attenuates NIHL (14). The efficacy of D-JNK-1 peptide has been demonstrated by application via an osmotic minipump or a hyaluronic acid gel. Postexposure administration of edaravone, a free-radical scavenger, also rescues cochleae from NIHL (15). Locally applied edaravone via an osmotic minipump can rescue OHCs even when it is applied 21 hours after noise exposure. In addition, these agents offer stronger protection of cochleae than does rhIGF-1. The drug delivery system via a gelatin hydrogel may be used for cochlear delivery of D-JNK-1 peptide because the gelatin hydrogel is suitable for sustained delivery of peptides (7–11). However, D-JNK-1 peptide is not clinically applicable. On the other hand, edaravone is clinically available; however, how to deliver edaravone into the cochlea continuously is an obstacle for clinical use. Gelatin hydrogels are not suitable for sustained delivery of edaravone because edaravone is not soluble in water (7). Therefore, other drug delivery systems that are fit for delivery of water-insoluble agents, including encapsulating in polylactic/glycolic acid particles (16), may be required for sustained delivery of edaravone.

The present findings indicate the effectiveness and safety of local rhIGF-1 treatment using gelatin hydrogels for NIHL. Clinical use of gelatin hydrogel as a drug delivery system has already started for angiogenesis of the inferior limb in Japan (17). In addition, rhIGF-1 is clinically applicable. The present findings may help advance the clinical application of local rhIGF-1 treatment using gelatin hydrogel for the treatment of SNHL.

REFERENCES

1. Juhn SK, Rybak LP. Labyrinthine barriers and cochlear homeostasis. *Acta Otolaryngol* 1981;91:529–34.
2. Angelborg C, Hillerdal M, Hultcrantz E, Larsen HC. The microsphere method for studies of inner ear blood flow. *ORL J Otorhinolaryngol Relat Spec* 1998;50:355–62.
3. Salt AN, Plontke S. Local inner-ear drug delivery and pharmacokinetics. *Drug Discov Today* 2005;10:1299–306.
4. Takemura K, Komeda M, Yagi M, et al. Direct inner ear infusion of dexamethasone attenuates noise-induced trauma in guinea pig. *Hear Res* 2004;196:58–68.
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* 2002;122:698–702.
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* 2005; 125:830–9.
7. Nakagawa T, Ito J. Drug delivery systems for the treatment of sensorineural hearing loss. *Acta Otolaryngol* 2007;557:30–5.
8. Endo T, Nakagawa T, Kita T, et al. A novel strategy for treatment of inner ears using a biodegradable gel. *Laryngoscope* 2005;115: 2000–5.
9. Iwai K, Nakagawa T, Endo T, et al. Cochlear protection by local IGF-1 application using biodegradable hydrogel. *Laryngoscope* 2006;116:526–33.
10. Young S, Wong M, Tabata Y, et al. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *J Control Release* 2005;109:256–74.
11. Holland TA, Tabata Y, Mikos AG. Dual growth factor delivery from degradable oligo(poly[ethylene glycol] fumarate) hydrogel scaffolds for cartilage engineering. *J Control Release* 2005;101: 111–25.
12. Antonelli PJ, Schultz GS, Kim KM, et al. Alpha 1-antitrypsin and ilomastat inhibit inflammatory proteases present in human middle ear effusions. *Laryngoscope* 2003;113:1347–51.
13. Jang CH, Shin SH, Cho HH, et al. Expression of matrix metalloproteinase-9 and -2 in pediatric chronic otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 2006;70:1155–8.
14. Wang J, Ruel J, Ladrech S, et al. Inhibition of the c-Jun N-terminal kinase-mediated mitochondrial cell death pathway restores auditory function in sound-exposed animals. *Mol Pharmacol* 2007;71:654–66.
15. Tanaka K, Takemoto T, Sugahara K, et al. Post-exposure administration of edaravone attenuates noise-induced hearing loss. *Eur J Pharmacol* 2005;522:116–21.
16. Tamura T, Kita T, Nakagawa T, et al. Drug delivery to the cochlea using PLGA nanoparticles. *Laryngoscope* 2005;115:2000–5.
17. Arai Y, Marui A, Komeda M. Regenerative medicine with the sustained release system of basic fibroblast growth factor. *Nippon Rinsho* 2006;64:2142–7.

Copyright © 2007 Otology & Neurotology, Inc. Unauthorized reproduction of this article is prohibited.

Annals of Otology, Rhinology & Laryngology 116(10):762-769.
© 2007 Annals Publishing Company. All rights reserved.

Drug Delivery System of Hepatocyte Growth Factor for the Treatment of Vocal Fold Scarring in a Canine Model

Tsunehisa Ohno, MD; Shigeru Hirano, MD; Shin-ichi Kanemaru, MD;
Masaru Yamashita, MD; Hiroo Umeda, MD; Atsushi Suehiro, MD;
Yoshihiro Tamura, MD; Tatsuo Nakamura, MD; Juichi Ito, MD; Yasuhiko Tabata, PhD

Objectives: Vocal fold scarring remains a therapeutic challenge. Previous studies have indicated that hepatocyte growth factor (HGF), a strong antifibrotic element, has therapeutic potential for restoring scarred vocal folds. To enhance the effect of HGF in vivo, we developed a novel drug delivery system (DDS) in which HGF is embedded in gelatin hydrogel and continuously released over a period of 2 weeks. In the present study we investigated the therapeutic efficacy of the HGF DDS on vocal fold scarring by using a canine model.

Methods: The vocal folds of 8 beagles were unilaterally scarred by stripping the entire layer of the lamina propria. The contralateral vocal folds were kept intact as normal controls. One month after the procedure, hydrogels (0.5 mL) containing 1 μg of HGF were injected into the scarred vocal folds of 4 dogs (HGF-treated group), whereas hydrogels containing saline solution were injected in the other 4 dogs (sham group). Histologic and vibratory examinations were completed for each group 6 months after the initial surgery.

Results: The excised larynx experiments showed significantly better vibration in terms of mucosal wave amplitude and glottal closure in the HGF-treated group compared to the sham group. Histologic evaluation of the vocal folds indicated remarkable reduction in collagen deposition and tissue contraction, with favorable restoration of hyaluronic acid and elastin in the HGF-treated group.

Conclusions: The present findings suggest that the novel HGF DDS may provide favorable effects in restoring the vibratory properties of scarred vocal folds.

Key Words: drug delivery system, gelatin hydrogel, hepatocyte growth factor, vocal fold scarring.

INTRODUCTION

Vocal fold scarring may occur after injury or inflammation. Scarring disrupts the layered structure of the lamina propria, thus changing the viscoelasticity of the vocal fold mucosa and often resulting in severe and intractable dysphonia. Although various injectable materials such as bovine autologous or homologous collagen and autologous fat have been used in an attempt to soften scarred vocal folds,[1-4] it is difficult to restore vocal fold scars to a normal state.

Previous histologic studies have revealed dense and/or disorganized collagen deposition, decreased elastin, and occasional decreases of hyaluronic acid (HA) in the lamina propria of scarred vocal folds.[5] Hyaluronic acid is thought to play a major role in maintaining the viscoelasticity of the lamina propria in the vocal fold, and is believed to be closely associated with scarless wound healing.[6,7] Thus, a material that increases HA production and reduces collagen synthesis from fibroblasts in the vocal fold lamina propria might be useful in treating vocal fold scarring.

Hepatocyte growth factor (HGF) has strong antifibrotic activity, and has been proven to contribute to the prevention or complete resolution of fibrosis in the liver, kidney, and lung in animal models.[8] It has also been found to have some therapeutic potential in restoring scarred vocal folds in animal models.[9-11] However, the biological activity of HGF may be limited in vivo because of rapid disposal by diffusion.[12] Hirano et al[11] attempted to treat scarred vocal folds in a canine model by local administration of HGF solution. Although encouraging results were

From the Department of Otolaryngology–Head and Neck Surgery, Kyoto University Graduate School of Medicine (Ohno, Hirano, Kanemaru, Yamashita, Umeda, Suehiro, Tamura, Ito), and the Department of Bioartificial Organs (Nakamura) and the Department of Biomaterials, Field of Tissue Engineering (Tabata), Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan. This study was supported by the Naito Foundation.

Presented at the meeting of the American Broncho-Esophagological Association, San Diego, California, April 26-27, 2007.

Correspondence: Tsunehisa Ohno, MD, Dept of Otolaryngology–Head and Neck Surgery, Kyoto University Graduate School of Medicine, 54, Shogoin-Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan.

noted with regard to vibratory function and some histologic aspects, the effects were limited by the extensive individual variation in mucosal wave patterns. Histologic examinations indicated a resolution of excessive collagen deposition and tissue contraction, but there was no improvement in the loss of elastin. These results warranted examination of this strategy for better regeneration of vocal folds.

A biodegradable hydrogel has been developed to enhance the in vivo regenerative effects of growth factors such as HGF, basic fibroblast growth factor, platelet-derived growth factor, and epidermal growth factor, and has been successful in the controlled release of biologically active growth factors in other parts of the body.[12-18] In the present study, we employed a hydrogel drug delivery system (DDS) with HGF to enhance the regenerative effects of HGF for treatment of vocal fold scarring. In this system, HGF was embedded in gelatin hydrogel and gradually released in a continuous fashion over a 2-week period in vivo. In the current study we aimed to investigate the therapeutic efficacy of the HGF DDS on vocal fold scarring using a canine model.

MATERIALS AND METHODS

Animals. Eight beagles weighing 10 to 12 kg were used in this study. All experimental protocols were approved by the Animal Research Committee of the Graduate School of Medicine, Kyoto University. Animal care was provided under the supervision of the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University.

Preparation of HGF Hydrogel. Human recombinant HGF (lot 0203S201-A) was obtained from PeproTech EC, Ltd, London, England. Biodegradable hydrogels were developed by the Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University.[12,13] Briefly, the hydrogel was constituted by chemical cross-linking of acidic gelatin with glutaraldehyde. Fifty milliliters of acidic gelatin aqueous solution (5% wt/wt) was mixed with 50 µL of glutaraldehyde aqueous solution (25% wt/wt) to give a final concentration of 6.25 mmol/L. The water content of the hydrogel was 94.8%. Next, 1 µg of HGF solution in 20 µL of phosphate-buffered saline solution (PBS) was dripped onto the gelatin hydrogels and left overnight at 4°C to create HGF hydrogel. It has been confirmed that once the HGF hydrogel is administered in vivo, HGF is gradually and continuously released at the site over a 2-week period.[12]

Surgical Procedure. All animals were sedated under general anesthesia with intramuscular injections of ketamine hydrochloride (15 mg/kg) and xylazine hydrochloride (6 mg/kg). A direct laryngoscope was inserted into the larynx to visualize the glottis. The vocal folds were unilaterally scarred by stripping the entire layer of the lamina propria down to the muscle, and the contralateral vocal folds were kept intact to use as normal controls. The sides used for scarring were randomly selected.

At 1 month after the procedure, 0.5 mL of hydrogel solution containing 1 µg of HGF was injected into the scarred vocal folds of 4 dogs (HGF-treated group) with a transoral intracordal injector, and 0.5 mL of hydrogel solution containing 1 µg of PBS was injected into the scarred folds of the other 4 dogs (sham group). All animals were euthanized 6 months after surgery by intracardiac injection of pentobarbital sodium. Whole larynges were harvested and used for excised larynx experiments to examine vibratory function, and then were subjected to histologic examination after the excised larynx experiments.

Vibratory Examination With Excised Larynx Setup. Vocal fold vibration was examined with an excised larynx setup. To better visualize the vocal folds, we removed supraglottic structures such as the epiglottis, false vocal folds, and aryepiglottic folds after resection of the superior portion of the thyroid cartilage. The arytenoid adduction procedure was bilaterally performed with 3-0 nylon suture used to close the glottis. The larynx was mounted on a table, and an endotracheal tube was inserted into the trachea and tightly clamped. Airflow was directed through the tube to generate vocal fold vibrations. During the vibratory examination, saline solution was dripped onto the vocal folds to prevent dehydration. A high-speed digital imaging system (Memrecam, NAC Image Technology, Osaka, Japan) was used to record vocal fold vibrations from the superior view. The camera was mounted 50 cm above the larynx, and the image was viewed on a monitor. The images were recorded at a frame rate of 1,000 frames per second, and the amplitude of the mucosal wave and the glottal gap were compared for the HGF-treated and the sham groups. The amplitude of the mucosal wave and the glottal gap were measured with image analysis software (Scion Image beta3b, Frederick, Maryland). The distance (d1) from the midline of the glottis to the free edge of the vocal fold was measured at the anteroposterior middle portion of the vocal fold during the closed phase, and then the same distance (d2) was measured at the maximum open phase. The mucosal wave amplitude was defined by subtracting d1 from d2, and was normalized by dividing by the length (L) from the anterior commissure to the vocal process [normalized mucosal wave amplitude = $(d2 - d1)/L \times 100(u)$]. The glottal gap was examined on the images during

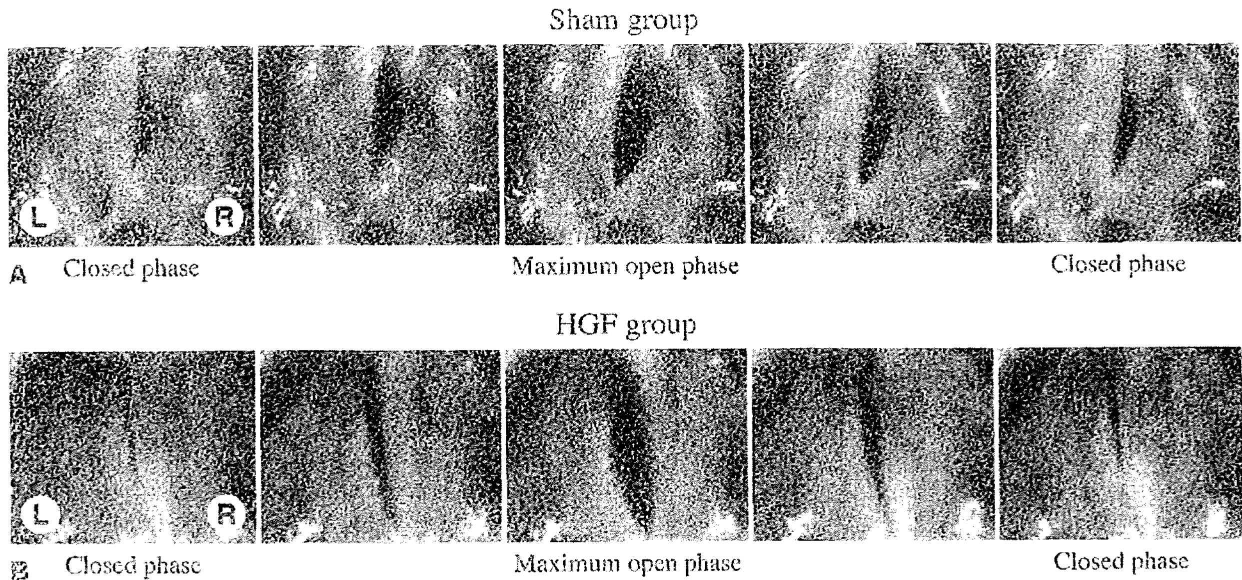

Fig 1. Vibratory patterns of treated vocal folds on digital high-speed imaging in excised larynx experiments. **A)** Sham group. Right vocal fold was scarred. **B)** Hepatocyte growth factor (HGF)–treated group. Left vocal fold was treated with HGF drug delivery system.

the closed phase, and the glottal area (a) was measured and then normalized by dividing by L^2 [normalized glottal gap = $a/L^2 \times 100$(u)].

Histologic Examination. The collagen, elastin, and HA in the lamina propria of each vocal fold were examined under light microscopy. The thickness of the lamina propria was also assessed to determine the degree of scar contraction. After the excised larynx experiments, the vocal fold tissues were fixed with 10% formaldehyde and then embedded in paraffin. Four-micrometer-thick serial sections were prepared in the coronal plane. The sections were made in the same systematic manner, beginning from the anteroposterior middle portion and proceeding through the anterior and posterior portions of the vocal fold. Elastica–van Gieson staining was performed to identify collagen and elastin separately. The hyaluronidase digestion technique was used to detect HA. For the hyaluronidase digestion procedure, 50 mg of bovine testes hyaluronidase (Sigma, St Louis, Missouri) was diluted in 100 mL PBS, and each section was incubated in this solution for 1 hour at 37°C. Next, the sections were stained with Alcian blue (pH 2.5). Hyaluronic acid was detected by comparing the sections without digestion to those with digestion. Images were captured with an Olympus BX50 microscope (Olympus Corporation, Tokyo, Japan). Collagen and HA were examined at 2× microscopic power, and elastin was examined at 20× microscopic power in the lamina propria. The thickness of the lamina propria was determined by measuring the distance from the free edge of the vocal fold down to the muscle layer at 2× microscopic power with Adobe Photoshop Image Analysis Software (Adobe Systems Inc, San Jose, California). The thickness of the treated lamina propria was represented by the ratio of the pixel size of the treated side to that of the normal side.

Statistical Analysis. Statistical analyses were performed for comparisons of the normalized mucosal wave amplitude, the normalized glottic gap, and the thickness of the lamina propria between the HGF-treated and the sham groups by use of the Mann-Whitney U-test. A p value of less than or equal to .05 was considered statistically significant.

RESULTS

Vibratory Data. Vibratory examinations showed better mucosal vibration in the HGF-treated group than in the sham group. Incomplete glottal closure and volume deficiency of the vocal fold were significant in the sham group (Fig 1A), whereas a smaller glottal gap and an improved vibratory pattern were observed in the HGF-treated group (Fig 1B).

The normalized mucosal wave amplitude was significantly greater in the HGF-treated group than in the sham group ($p < .05$; Fig 2A). Since the normal range of the normalized mucosal wave amplitude in dogs has been reported to be 6 to 10 units,[11] all normalized mucosal wave amplitude values for the current HGF-treated group were within the normal range, whereas the values in the sham group were shown to be beyond the normal range. Although there was still glottal incompetence in the HGF-treated group, the normalized glottal gap was

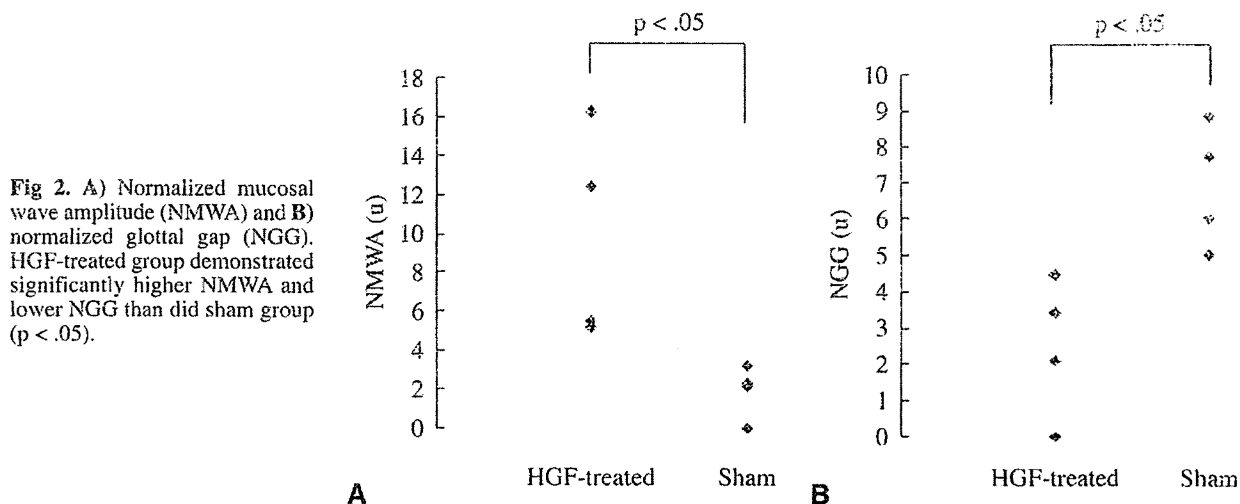

Fig 2. A) Normalized mucosal wave amplitude (NMWA) and **B**) normalized glottal gap (NGG). HGF-treated group demonstrated significantly higher NMWA and lower NGG than did sham group ($p < .05$).

significantly smaller ($p < .05$) in the HGF-treated group than in the sham group (Fig 2B).

Histologic Data. Collagen and elastin were located mainly in the superficial layer of the lamina propria, whereas HA was distributed throughout the entire layer in the normal control vocal folds. In the sham group, remarkable tissue contraction and disorganized collagen deposition were noted throughout the scarred lamina propria compared with the normal side (Fig 3A,B), and elastin and HA were remarkably decreased on the sham side compared to the normal side (Fig 3C-F).

In the HGF-treated group, tissue contraction and collagen deposition were found to be minimal (Fig 4A,B), and the restoration of HA was well identified on the HGF-treated side (Fig 4C,D). Elastin was also restored in the superficial lamina propria (Fig 4E,F).

The thickness of the lamina propria was nearly normal in the HGF-treated vocal folds, whereas it was significantly ($p < .05$) thinner in the sham group than in the HGF-treated group (Fig 5).

DISCUSSION

Vocal fold scarring remains a therapeutic challenge to date. In the Chevalier Jackson Lecture at the annual meeting of the American Broncho-Esophagological Association in 1995, Hirano[19] stated that problems in vocal fold scar treatment awaited improvements, and that the most promising future direction would be to develop techniques to replace the scarred tissues with normal pliable mucosa, or to innovate techniques to soften scarred tissues with certain therapeutics and/or physical treatments. Many experimental studies have since revealed the histologic and rheological changes in scarred vocal folds.[5-7,20-22] There have been some discrepancies among the findings on the use of extracellular matrix (ECM) in vocal fold scarring, possibly due to differences in the animal species used, the type of injury, and/or the duration of injury. However, these studies suggested that disorganization of ECM components may affect the tissue properties of the vocal fold mucosa. Excessive or disorganized collagen, reduced elastin, increased fibronectin, and decreased decorin have all been frequently noted. The density of HA did not change in most cases, although the total amount of HA decreased. Although the effects of these changes in ECM molecules on the tissue properties of the vocal fold remain unclear, it appears to be useful to restore these histologic changes to the normal state in the treatment of vocal fold scarring. Based on these findings, various therapeutic strategies for the treatment of scarred vocal folds have attempted to use tissue engineering and regenerative medicine.[10,11,23,24]

Therapeutic strategies in regenerative medicine include cell therapy, growth factor therapy, and implantation of regenerative materials. Growth factors are one of the most potent regulatory elements that affect cells and tissue remodeling. Recently, growth factor therapy has been thought to be a notable treatment in various fields of regenerative medicine. Hepatocyte growth factor has strong antifibrotic activity that stimulates HA production in mesenchymal cells and dissolves the collagen matrix. Previous studies using animal models have revealed that injection of HGF solution into scarred vocal folds has therapeutic potential.[9-11] However, incomplete regeneration was also noted, probably due to the insufficient retention time of HGF in vivo.[12] In order to enhance the effects of HGF in vivo in the present study, we employed a DDS developed at Kyoto University that allowed a gradual release of HGF from

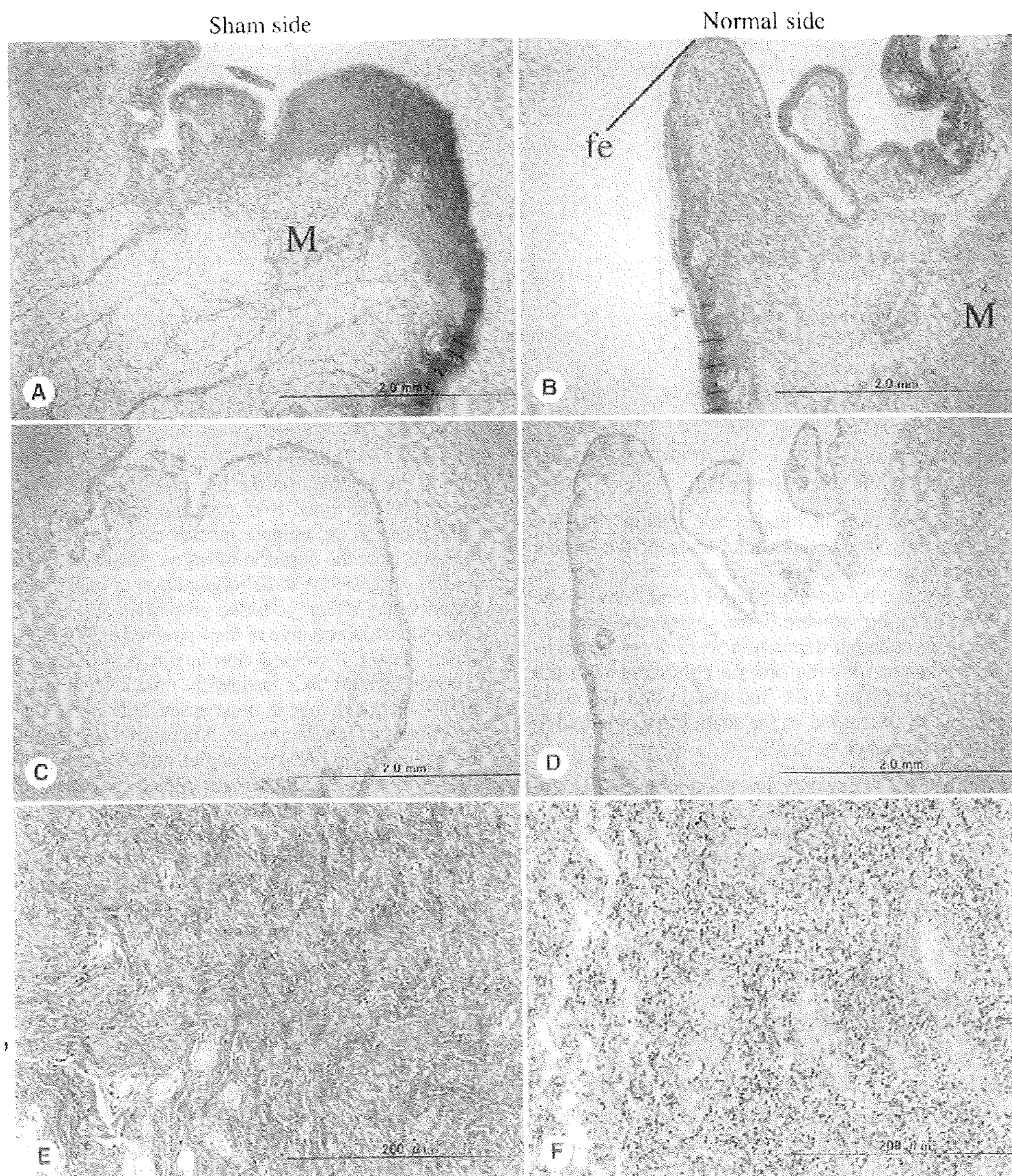

Fig 3. Histologic findings in sham group. **A,B)** Elastica–van Gieson stain, original ×2. Compared to normal side, remarkable tissue contractions and increased disorganized collagen depositions (red stain) were observed throughout scarred lamina propria of sham side. M — thyroarytenoid muscle; fe — free edge of vocal fold. **C,D)** Alcian blue stain, original ×2. Hyaluronic acid (blue stain) was markedly decreased compared to normal side. **E,F)** Elastica van Gieson stain, original ×20. Elastin density (black stain) was markedly reduced on sham side.

gelatin hydrogels in a continuous manner. Indeed, this DDS of HGF has been proven to be useful in preventing the progression of heart failure in stroke-prone spontaneously hypertensive rats.[12,14]

In this study, excised larynx experiments showed a significant improvement of mucosal vibration in terms of mucosal wave amplitude and glottal closure in the HGF-treated group. The effects on muco-

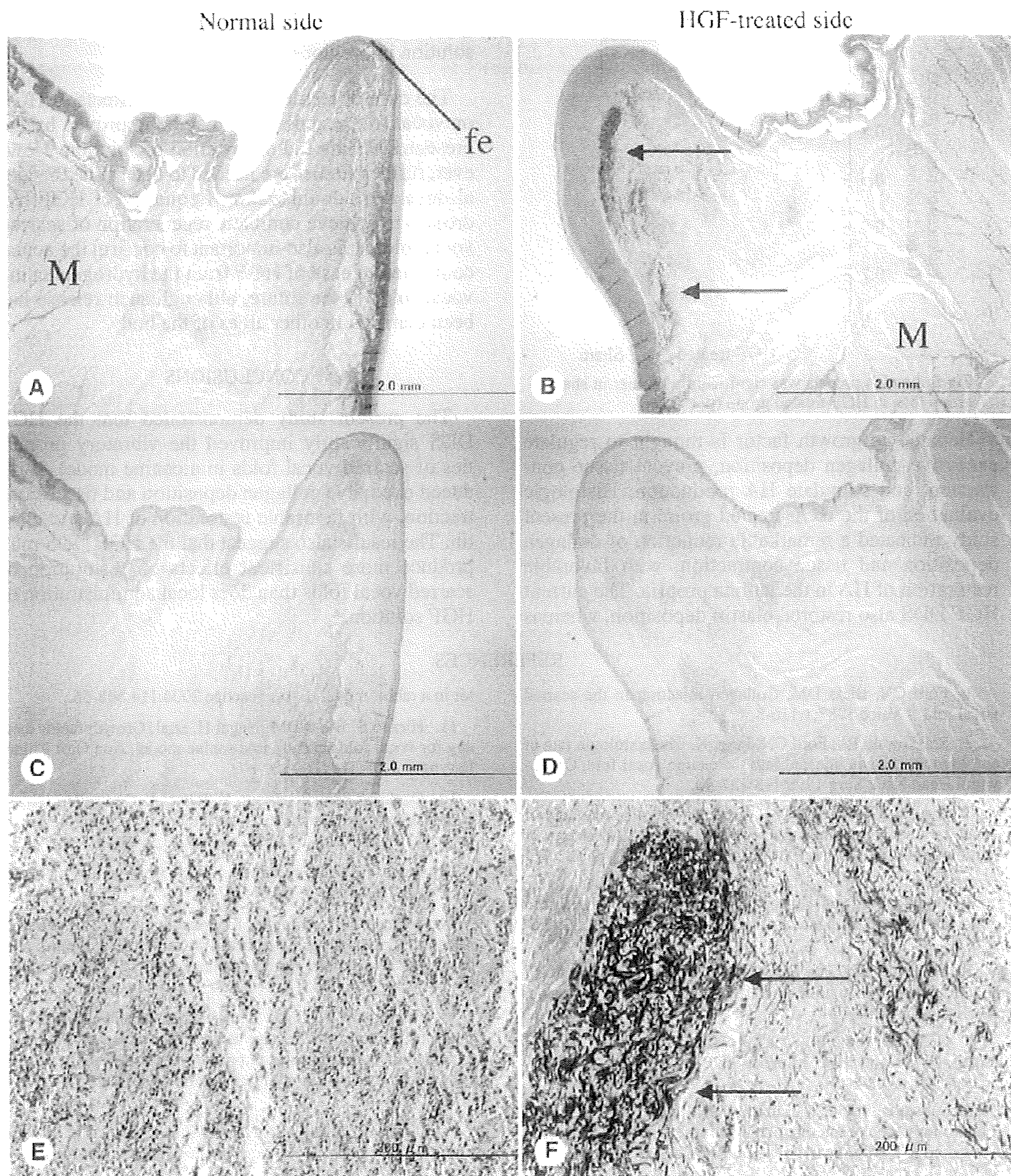

Fig 4. Histologic findings in HGF-treated group. **A,B)** Elastica–van Gieson stain, original ×2. Tissue contraction and collagen deposition (red-stained region as indicated by arrows) were found to be minimal on HGF-treated side. **C,D)** Alcian blue stain, original ×2. Restoration of hyaluronic acid (blue stain) was well identified on HGF-treated side. **E,F)** Elastica–van Gieson stain, original ×20. Elastin (black stain as indicated by arrows) was present in superficial portion of HGF-treated fold.

sal wave amplitude were consistent across the animals, whereas previous studies using HGF solution demonstrated considerable individual variation.[10,11] Our results suggest that the current HGF DDS may facilitate continuous and retentive release of the active factor to elicit consistent effects in decreasing stiffness of the scarred vocal folds, thus resulting in improvement of the vibratory properties.