表 3　コクリン N 末端のアミノ酸配列解析結果
今回の実験で得られたウシ・コクリンの N 末端アミノ酸配列を，既知のヒト，マウス，チキンの cDNA から類推されるアミノ酸配列と比較した。左から，アイソフォームのナンバー，種，配列，ヒト COCH 遺伝子のアミノ酸に相当する部位，ウシアミノ酸配列とそれぞれの種のアミノ酸との相同性を表示してある。内部配列も合わせると全体でヒト COCH 遺伝子と 89.3%という非常に高い相同性が得られ，これらの蛋白スポットはヒト・コクリンホモローグであることが示された。

protein		sequence	position in hCOCH	sequence identity
p63-1 to 8	human	EGAAPIAITCFTRGLDIRKEKADVLCPGG	25-53	83%
	bovine	---V--P--*S--------------*---		—
	mouse	---V--PV---------------------		86%
	chiken	DSS-SN----------L---TE-----AN		48%
p44-1 to 5	human	ATGQAVSTAHPPTGKRLKKTPE	133-154	95%
	bovine	-----------A----------		—
	mouse	---R--------S---------		86%
	chiken	-V-RS-A--R-A----P---L-		68%
p40-1 to 3	human	TPEKKTGNKDCKADIAFLIDGSFNIGQRRF	152-181	97%
	bovine	----------*-------------------		—
	mouse	------------------------------		97%
	chiken	-L---A----------------Y-------		87%

(3) ゲル・イメージのコンピューター画像解析

ImageMaster 2D Elite software（Amersham Pharmacia Biotech）を用いて内耳二次元電気泳動の各蛋白泳動スポットのパターンを解析した。ソフトウェアーをまず 2D SDS-PAGE standards（Bio-Rad Industries）を用いてキャリブレーションし，各スポットの分子量，等電点，ゲル上の前蛋白スポットに対する比率を計算した。

(4) 蛋白の内部構造の解析―ペプチドマップ作成

これは蛋白全体像を比較する方法であり，In-gel digestion 法，またはクリーブランド法と呼ばれている。Lys-C をあらかじめゲル内に溶かし込んでおき，蛋白を stacking ゲル内で部分消化させる。それを電気泳動するとその蛋白独自のパターンが得られる。

2）結果と考察

本来プロテオーム解析とは，当該臓器の蛋白を二次元電気泳動マッピングした「基本地図」を作成し，健康な状態と病的状態，発生過程，刺激と非刺激状態などの蛋白発現を網羅的に比較することによって，蛋白発現レベルの変化や細胞間相互作用などを解明することを目的としている。今回われわれはウシ内耳蛋白の「二次元電気泳動マッピング」を行った結果，画像解析ソフトで認識された 135 個のうち，89 個のスポットを解析・同定した[4]。

マッピングの過程でコクリンに関して非常にユニークな知見を得た[14]。その結果を箇条書きにすると，以下のようになる。

①コクリンは 3 つの異なった N 末端をもち，分子量がそれぞれ 63 kDa，44 kDa，40 kDa の 3 種類のアイソフォーム p63s, p44s, p40s に分類される（表 3，図 3，4）。

②コクリン・アイソフォームはさらに等電点が異なる合計で 16 個の蛋白から構成されている（表 3，図 3）。

③コクリンは内耳の構成蛋白の 70%を占める（コラーゲンは本泳動条件では検出されないため，非コラーゲン構成蛋白という条件付きである）（表 4）。

④DFNA9 患者にみられる突然変異部位は全て p63s にのみ含まれており，p44s, p40s には含まれていなかった（図 2）。

⑤さらに，この突然変異部位は COCH 遺伝子の LCCL 領域のみにあり，この LCCL 領域が

図 3　ウシ内耳蛋白二次元電気泳動結果

a：ウシ内耳蛋白を二次元電気泳動し，クマシーブルーにより染色したゲルの全体像を示す。代表的な既知の蛋白，1：BSA，2：α tublin，3：β tublin，4：OCP-2 を示してある。この蛋白泳動パターンは内耳特有のもので，他の臓器，組織からはこのパターンは得られない。
b：二次元電気泳動上のコクリン・アイソフォーム。3a のボックス部分を拡大したもの。コクリンのアイソフォームをアルカリ側から酸性側にナンバーを付け，p63-1 から p63-8，p44-1 から p44-5，p40-1 から p40-3 と表示してある。p63-1 から p63-8 とは，分子量がほぼ同じであるが等電点の異なる 8 つのアイソフォームが存在していることを意味している。

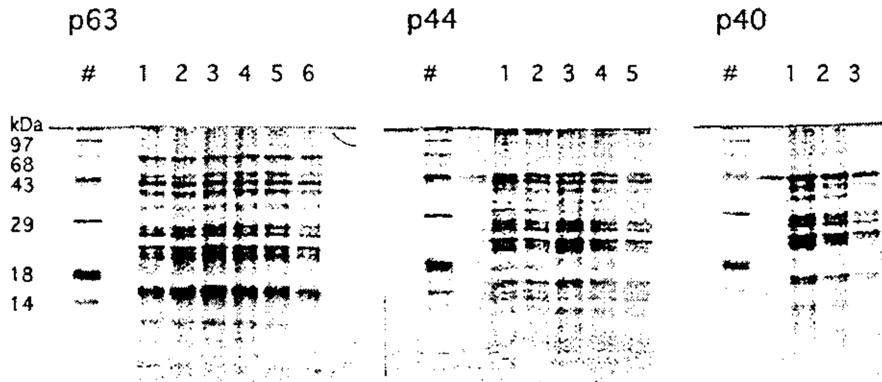

図 4　コクリン各アイソフォームのペプチドマップ

ウシ・コクリン各アイソフォームをクリーブランド法により部分消化して得られたペプチドマップ。消化された蛋白のバンドが染色されている。p63，p44，p40 の各アイソフォームの蛋白の内部構造（一次構造）がほぼ同一であることを示す結果が得られた。

COCH 遺伝子の機能に重要な部分であることが予測される（図 2）。

2. 研究その 2：コクリン・アイソフォーム特異的抗体によるヒト内耳蛋白の解析[15]

1）方法

(1) 抗コクリン・アイソフォーム特異的抗体の作成

①抗原のデザイン

二次元電気泳動解析で同定したコクリン・アイソフォームに特異的な抗体を 4 種類作成した。抗体を作成する際に，より免疫原性の高いエピトープをコンピューターで予測するソフトが開発されており，今回は Epitope Advisor Program（富士通九州システムエンジニアリング）を用いた。抗原ポリペプチドとして 14 から 19 アミノ酸より成るポリペプチドを選択した。通常のポリクローナル抗体作成法に準じて，KLH（キーホール・リンペット・ヘモシアニン）を担体蛋白とした。ウサ

表 4 ゲルイメージコンピューター解析
ウシ内耳蛋白二次元電気泳動パターンのゲルイメージコンピューター解析の結果。各アイソフォームの全蛋白に占める割合，等電点，分子量が表示してある。コクリンは，内耳全蛋白中 70%を占める非常に多量に存在する蛋白であることが示された。ただし，われわれの方法ではコラーゲンなどの線維性蛋白はゲルにのらず，これらの蛋白を除いた場合の比率である。

protein	norm. Vol.%	pI	MW (kDa)
p63-1	1.7	7.4	63.0
p63-2	6.9	7.2	63.4
p63-3	9.8	7.0	64.0
p63-4	6.6	6.8	64.2
p63-5	4.6	6.6	65.0
p63-6	2.2	6.4	65.7
p63-7	0.4	6.3	65.9
p63-8	0.1	6.1	65.9
	32.2 (p63s subtotal %)		
p44-1	3.9	7.1	43.8
p44-2	2.4	6.9	45.2
p44-3	4.6	6.6	45.5
p44-4	1.8	6.4	45.9
p44-5	0.2	6.2	46.6
	12.9 (p44s subtotal %)		
p40-1	20.2	6.1	40.1
p40-2	3.9	6.0	40.6
p40-3	1.2	5.8	41.5
	25.3 (p40s subtotal %)		
	70.4 (total %)		

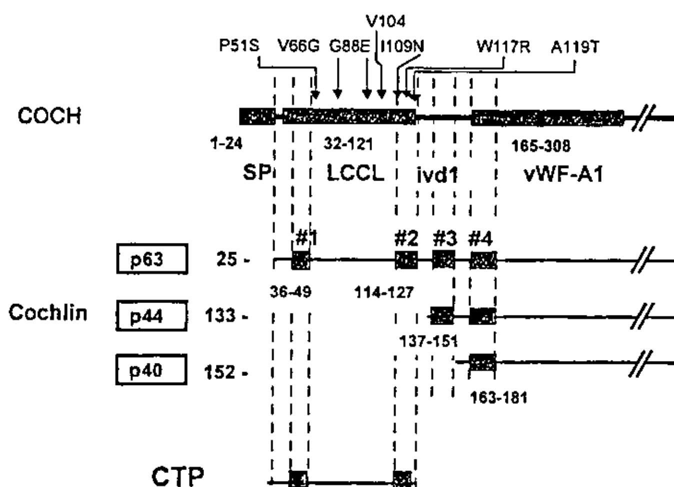

図 5 コクリン・アイソフォーム特異的抗体作成用の抗原ペプチド
1～4 は，アイソフォームを特異的に認識するようにデザインされた抗原ペプチドの位置を示す。

ギの皮下に 2～3 週間ごとに繰り返し免疫し，Protein A とアフィニティーカラムクロマトグラフィーを用いて抗体を精製した[15]。

下記に抗原として用いたペプチド配列を，図 5 にその部位を示した。

Peptide #1；"anti-LCCL-N", 18-mer (TRGLDIRKEKADVLCPGG：amino acid residue 36-49) 全長コクリン p63 の LCCL domain の N

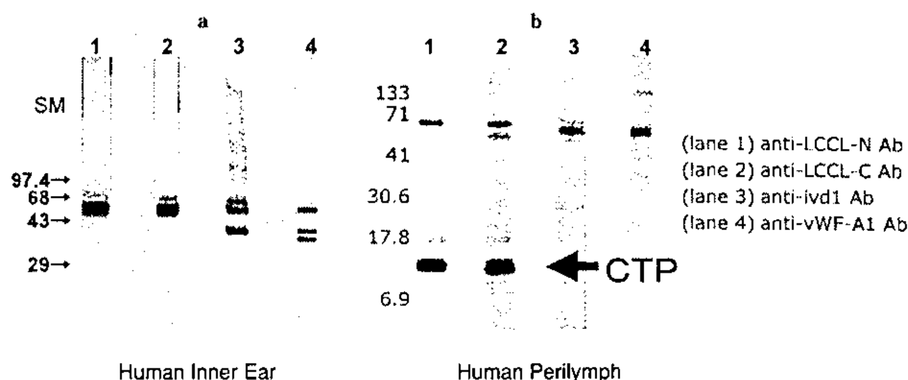

図 6 ヒト内耳 (a)，外リンパ (b) のコクリン・アイソフォーム
ヒト内耳 (a)，外リンパ (b) を泳動したブロットを短冊状に切り，4 種類の抗体で染色した結果を示す。(a) ヒト内耳では，ウシと全く同様に 3 種類のアイソフォーム p63，p44，p40 が発現していることがわかる。(b) ヒト外リンパでは，極少量の p63 と，16kDa の CTP が検出された。(a)，(b) の泳動結果は，図 2 で示したコクリン・アイソフォームの模式図をもとに予測した通りの結果であり，予測されないバンドは検出されなかった。つまり，われわれが提唱しているコクリン・アイソフォームの構造が正しいことを裏づけている。

末端に該当。

Peptide #2；“anti-LCCL-C”，14-mer (LSRWSASFTVTKGK：amino acid residue 114-127) 全長コクリン p63 の LCCL domain の C 末端に該当し，アイソフォーム p44s には含まれない部分。

Peptide #3；“anti-ivd1”，15-mer (AVSTAH-PATGKRLKK：amino acid residue 137-151) LCCL domain と vWF-A1 domain の間に該当し，アイソフォーム p44s には含まれるがアイソフォーム p40s には含まれない。

Peptide #4；“anti-vWF-A1”，19-mer (KADIAFLIDGSFNIGQRRF：amino acid residue 163-181) vWF-A1 domain の中にあり，p63s, p44s, p40s 全てに含まれる。

②抗体の特異性の確認

抗体の特異性は，ドットブロット並びにウシの内耳組織より調製した内耳蛋白質溶液（陽性コントロール）を抗原としたウエスタンブロッティングにより確認した。

③検討対象となる試料

聴神経腫瘍手術時に採取したヒトの内耳組織。

④一次元ウエスタンブロッティング，抗体反応，検出反応

通常の一次元ウエスタンブロッティングを行い PVDF membrane に蛋白をブロットした。次に，化学発光法による検出および解析を行った。転写後のニトロセルロース膜を，非特異的反応をブロックするために blocking buffer 中に 4℃で一晩浸した。これを washing buffer で 5 分間，3 回洗浄し，一次抗体と反応させた。一次抗体としては，抗コクリン・アイソフォーム特異的抗体 4 種類を antibody dilution buffer で 1,000 倍に希釈して，ニトロセルロース膜に添加した。反応は，振動させながら 2 時間行った。二次抗体としては，ヤギ由来 anti rabbit IgG antibody（HRP 標識）を，前記 antibody dilution buffer で 1,000 倍に希釈したものを用い，振動させながら 1 時間反応させた。これを前記 washing buffer で 15 分間，3 回洗浄した。その後，化学発光キット（ECL plus）を用いて化学発光させ，発生したシグナルをフィルム (Kodak Scientific Imaging Film) に感光させた。フィルムへの露光時間は約 1 分とした。

2) 結果と考察

既に報告したウシ内耳コクリン・アイソフォーム解析結果と全く同様にヒト内耳組織中にも p63s，p44s，p40s が認められた（図 6a)。ほかに，ウシ，ラット，モルモットでも検討したが，アイソフォーム発現パターンは全て同じであった。この結果は，ほ乳類ではコクリン・アイソフォームのプロセシングが保存されていることを示している。遺伝子によっては，動物種によって異なった

表 5 一次元電気泳動によるコクリン・アイソフォーム解析

ほ乳類培養細胞（HeLa cell, 293T cell）に COCH 遺伝子を導入した実験では，細胞内には 60，63kDa の蛋白が検出された。培養液中には 60，50 ならびに 69kDa の蛋白が検出された。一方，ヒト，ウシの内耳組織には，p63，p44，p40 アイソフォームが存在している。外リンパには，p63 と CTP（16kDa）が発現していた。この結果は，コクリン・アイソフォームの発現調節は，内耳細胞特異的であることを示している。

使用した細胞	cell lysates	culture media
HeLa cell（16）	60	60 50
293T cell（17）	63	69
使用した種	内耳組織	perilymph
ヒト（15）	63 44 40	63 16
ウシ（15）	63 44 40	63 16

＊単位は kDa
（ ）内は文献

発現パターンを示すものもあるが，コクリンの発現に関する限り，齧歯類内耳を用いた研究を行うことが可能であることを示す重要な知見である。

内耳以外の細胞を用いた研究では，コクリンの発現はどうであろうか。コクリン蛋白に関する世界の研究状況をみてみたい。分子生物学では，セルラインに遺伝子を導入・発現させてその解析をするという手法がよく用いられる。Grabski ら[16]（Cell Biology, University of Alabama）は子宮癌細胞セルライン HeLa cells（約 50 年前に子宮癌で亡くなったアメリカ人，Henrietta Lacks さん由来細胞）を用いて，また COCH 遺伝子発見者の Robertson ら[17]（Obstetrics, Gynecology and Reproductive Biology, Harvard Medical School）は，293T（human embryonic kidney cells）を用いて，COCH 遺伝子リコンビナント蛋白を作成した。その結果表 5 に示すように，それぞれ細胞内には 63 kDa，60 kDa の 1 種類の蛋白のみしか検出されなかった。この事実は何を示しているのだろうか。われわれは，さらに外リンパの解析を加えることで，内耳細胞特異的なコクリン代謝過程が存在していることを示した。

3. 研究その 3：ヒト外リンパの解析による新たなアイソフォームの同定[15]

1）方法

上記抗体を用いてヒト・ウシ外リンパを一次元，二次元ウエスタンブロッティングで解析した。

表 6 二次元電気泳動による CTP の解析

	pH	分子量
酸性側	CTP（pH7.7）	18.8 to 23.1 kDa
アルカリ性側	CTP（pH7.9）	17.7 to 22 kDa

2）結果と考察

ヒト外リンパでは，約 16 kDa に明瞭な細目のバンドが検出され，これはその分子量ならびに抗コクリン N 末端・C 末端抗体両方で全く同じパターンで認識されていることからコクリン-Tomoprotein（CTP）（コクリン-N 末端フラグメント蛋白）と考えられた（全長コクリン p63s の N 末端が切り取られているという意味を込めて，tomo-という接頭辞を付けた）[15]（図 2，6b）。

ウシ外リンパでは 16 kDa よりやや分子量の大きい幅の広いバンド（17〜23 kDa）が検出され，種により CTP の発現には微妙な差異があることを示している。ウシ外リンパの二次元電気泳動ウェスタンブロット解析では酸性側に CTP（pH7.7）分子量 18.8 to 23.1 kDa，アルカリ性側に CTP（pH7.9）分子量 17.7 to 22 kDa の CTP が検出された（表 6）。また，このパターンは抗コクリン N 末端・C 末端抗体両方で全く同じパターンで認識された。CTP にも等電点，分子量の異なるアイソフォームが認められたことは，CTP にもコクリンに認められているような発現の異質性（heterogeniety）があることを示している。

また，外リンパ中には全長コクリン p63s が少量検出されたが，p44s，p40s は認められなかった（図 6b）。このことは，「外リンパ中で全長コクリン p63s から CTP が切断されて生成される」というコクリン代謝パターンは否定的であることを示しているのではないかと考えている。

先に紹介した Grabski らや Robertson らの報告では，遺伝子導入細胞の lysate のみならず，その培養液の免疫沈降反応を行いかなり詳細に検討されている。なぜであろうか。COCH 遺伝子には signal peptide モチーフがあり，コクリンが分泌性蛋白であることを示唆している。それに加えて，コクリン・アイソフォーム解析の結果，p44s，

p40s では LCCL モチーフが欠損していることから，CTP の存在が理論的に予想されていたからである。しかし，彼らの報告をみると培養液を解析した結果得られたコクリン様蛋白は，それぞれ 60，50 kDa と 69 kDa であった（表 5)。このことはコクリンの転写後調節，蛋白レベルでのプロセシングは内耳細胞特異的であることを示唆している。

以下に，想定されるコクリンのプロセシングを示す。

(1) Transcriptional modifications

①Usage of a different (more 5') polyadenylation site

②A product of alternative splicing

③Exon skipping

④Differential use of promoter cap sites

(2) Translational modifications

①Proteolysis at peptide bond removing of other intervening or C-terminal sequences as for intracellular processing

②Action of proteolytic cascades in the metabolism of secreted molecules

③Chemical modification such as glycosylation phosphorylation and/or deamination

われわれは，おそらく未知のコクリン特異的代謝酵素が存在するのであろうと予測している。そのような「内耳特異的代謝酵素」が発見されれば，新たな遺伝性難聴の原因遺伝子候補となり得る。またコクリン代謝の複雑性は，COCH 遺伝子自体に mutation がなくてもコクリン代謝異常が内耳疾患の原因になり得ることを示唆している。同様な病態はアルツハイマー病におけるタウ蛋白でも報告されている。

CTP は，おそらく LCCL module そのものであることが推察される。LCCL module そのものが独立した蛋白として存在する例はほかには知られておらず，生物学的にも非常に興味深い分子である。今後，CTP の機能が判明すればコクリンの機能を推察するうえでも非常に役立つであろうと考えられる。また，CTP は非症候性優性遺伝性難聴 DFNA9 の突然変異部位全てを含んでおり，DFNA9 発症メカニズムを解明するうえでも重要な蛋白である。

IV. コクリン・アイソフォーム解析の臨床応用—外リンパ瘻診断—

基礎医学研究の目標は，研究成果を実地臨床現場に応用し患者の利益となるべく役立てることにある。コクリンは遺伝性難聴の原因遺伝子であり，その研究は将来的に遺伝性難聴の診断・治療に結びつく。われわれはコクリンの内耳発現特異性を利用した遺伝子治療法の開発も視野に入れているが[18]，実際に治療に用いるためにはまだまだ越えなければならないハードルがある。人工内耳術後の成績予測に難聴遺伝子変異解析が用いられつつある[19]。高度難聴となった DFNA9 患者の治療には人工内耳が用いられ，その治療成績は良好であることが知られている。

現在われわれは，CTP の外リンパ発現特異性を利用して，「外リンパ瘻診断」に応用する研究を行い，臨床検査センターとの共同研究が進んでいる。

1. 現在の外リンパ瘻診断法

確定診断には，試験的鼓室開放か内視鏡によって外リンパの漏出を確認する。しかし，鼓室開放術は侵襲的検査であることからその施行がためらわれる場合もあり，また実際に手術を行っても外リンパの漏出が確認できず確定診断がなされないケースも存在している。外リンパ瘻漏出の有無の判断は，術者（もしくは看護師を含めた診療チーム）によってなされているが，その客観性に問題があることは以前より指摘されている[20,21]。そこで，術者の主観的判断に左右されず，客観的に診断できる生化学的確定診断マーカーを模索して，様々な物質が報告されてきた。髄液漏の診断に用いられる β_2 transferrin は，髄液中に存在するが血清中には含まれていない。そこで外リンパ瘻の診断にも応用できるのではないかと期待され多くの論文が発表された。しかし最近，精度の高い検査法でヒト外リンパ 20 検体を検査したところ，β_2 transferrin 陽性は 1 例もなかったことが報告された[22]。今まで最も報告の多かった $\beta 2$ Transferrin であるが，その有無で外リンパ瘻診断を行うことはできないことが判明し，現在のところ臨床的に確立したマーカーは存在しない。

2. CTP は外リンパ瘻の診断マーカーになり得るか

アイソフォーム解析の結果，蛋白レベルでは全長コクリン p63 の発現は，内耳特異的であることが判明した[23]。そこで，さらに CTP の発現を調べてみた。人工内耳，アブミ骨手術，半規管瘻孔の術中に漏出したヒト外リンパを検査したところ，全検体が明らかに CTP 陽性だった。一方，中耳に存在し得る他の体液，つまり髄液，血液，中耳洗浄液，慢性中耳炎の耳漏，滲出性中耳炎の滲出液を検査したところ，これらは全例陰性であった。この結果は，CTP が外リンパ瘻診断の生化学的マーカーとして十分な発現特異性を兼ね備えていることを示している。

この種の臨床検査法を確立する場合，最も大切なことはその診断特異性である。特異性が高ければ偽陽性が少ない。逆に感受性が高いと偽陰性が少なくなる。外リンパ瘻疑い例に対して内耳窓閉鎖術を行うかどうか，CTP 検出法の結果を術前診断に用いるためには，先ず偽陽性が低いことが求められる。不必要な手術的（侵襲的）治療を行わないためである。さらに，内耳窓閉鎖術によって治療し得る疾患をもれなく正確に診断するため（偽陰性を低くする）には，その感受性を向上させる必要がある。このためには，CTP 検出抗体の特異感度の改善，ならびに検査システムの改善が求められ，数種類の抗体を作成し基礎研究を行っている。

現在われわれは，外リンパ瘻症例から得られた中耳洗浄液（手術中に，鼓室を 0.3 ml 生理食塩水で 3～4 回洗浄し回収）の検討を行っている[24]。実際の臨床症例の検討の結果，側頭骨骨折で蝸牛にも骨折が及んでいる症例，アブミ骨底陥入症例などの外傷性外リンパ瘻症例では中耳洗浄液は明らかに CTP 陽性であった。一方，いわゆる「特発性外リンパ瘻」（外傷，手術の既往のない，鼻かみ後などの発症）はどうであろうか。特に北米の報告ではその存在自体を疑問視する報告もある[20,21]。われわれの検討の結果，鼻かみ後発症の特発性外リンパ瘻で明らかに CTP 陽性の症例があり，特発性外リンパ瘻が明らかに疾患として存在していることを証明する結果となった。

V. おわりに

分子生物学の技術的進歩に伴い数多くの病因遺伝子が同定され，未知の遺伝子も新たに発見され，遺伝性難聴の研究は爆発的に進歩している。腫瘍などの特別な場合を除き，通常の内耳疾患ではバイオプシーは禁忌である。生体病理所見が得にくい内耳疾患の病態に，分子生物学的アプローチが新たな展望を開くことが期待される。

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外リンパ瘻共同研究のお願い

CTP 検出法が臨床検査法として有用かどうか判断するためには，ある程度の症例数の臨床データーを比較検討することが必要です。外リンパ瘻はそれほど頻度の高い疾患ではないため，是非諸先生方のご協力をお願いしたいと思います。下記の要領で採取したサンプルをお送り頂ければ，「レトロスペクティブな検討」になりますが後日結果をお知らせします（現在，検査所用時間の関係で術前診断は行っておりません）。

外リンパ瘻症例のサンプル採取方法

中耳洗浄液の回収量は症例により様々で，おそらく中耳腔の大きさの個人差によるものと思われます。われわれが行っている方法は下記の通りです。

(1) 外リンパ瘻診断基準に基づいて，もしくは明らかな直達外傷，手術などの病歴がある場合に，試験的鼓室開放術を行った症例を対象にしています。鼓室に血液がなるべく混入しないように注意して，生理食塩水を入れて中耳を 3〜4 回洗浄します。

(2) 生理食塩水は 0.3 ml 入れて 0.1 ml 回収するようにしています。0.1 ml 回収できないときはさらに 0.1 ml を入れて洗浄回収します。検査感度を向上させるため，極力洗浄液が希釈されないようにしています。

(3) 洗浄液をエッペンドルフチューブに入れ，エッペンドルフ用遠心器で 6,000 回転 15 秒遠心沈殿します。遠心器が近くにない場合は 30 分間静置し上澄みを採取し，赤血球を除去して上清を −20°〜−70° で凍結保存します。

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Survival of Fetal Rat Otocyst Cells Grafted into the Damaged Inner Ear

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Kojima K, Murata M, Nishio T, Kawaguchi S, Ito J. *Survival of fetal rat otocyst cells grafted into the damaged inner ear.* Acta Otolaryngol 2004; Suppl. 551: 53–55.

Hair cell loss induced by aging, ototoxic drugs and noise leads to irreversible hearing loss and balance disorders in mammals due to the failure of hair cells to regenerate. To investigate the possibility of transplantation therapy to repair damaged inner ear, we have examined whether grafted fetal otocyst cells could survive and migrate into injured sensory organs. We obtained otocyst cells from green fluorescein protein (GFP)-transgenic rats on embryonic day 12.5, then transplanted these cells into the inner ears of young rats previously exposed to intense sound. One month after transplantation, the grafted inner ear sensory organs were examined immunohistochemically. Grafted otocyst cells had survived and demonstrated special morphological features in the host organs; cells that migrated into the organ of Corti were similar to supporting cells. These results indicate that injured sensory organs express some kind of scaffolding that plays important roles in the survival and differentiation of the grafted otocyst cells. *Key words: acoustic overstimulation, fetal otocyst cell, hair cell progenitor, immunostaining, inner ear, neural repair, sensory epithelium, transplantation.*

INTRODUCTION

Aging and severe damage to the inner ear (e.g. acoustic overstimulation or ototoxins) induce hair cell loss that results in irreversible hearing and balance disorders because hair cell regeneration scarcely occurs in mammalian inner ears. Similarly to inner ear sensory epithelia, it has also been thought that axonal or neuronal regeneration never occurs in the mammalian central nervous system (CNS) (1). However, several studies reported that transplantation of immature neuronal progenitor cells (e.g. neural stem cells) induced differentiation of the progenitor cells to mature neuronal or glial cell types to support survival and axonal elongation of neurons (2–4). It is anticipated that immature neural progenitor cells may be developed into materials for cell transplantation therapy for neural repair of the CNS after damage (5–7). These findings indicate that immature progenitor cells survive and express their potential to differentiate mature principal cell types in the host organs. In this study, we assessed the possibility of cell transplantation therapy to repair damaged inner ear by investigating whether grafted fetal otic epithelial cells can survive in host inner ear that was damaged by acoustic overstimulation.

MATERIALS AND METHODS

Fifteen postnatal day 21 (P21) Sprague-Dawley (SD) rats and 30 embryos from three pregnant dams of the same strain were used in this study. All experiments were performed with the approval of the Animal Care and Use Committee of the Graduate School of Medicine, Kyoto University.

Donor cells were obtained from green fluorescein protein (GFP)-transgenic SD rats (8). The expression of GFP in the somatic cells of the transgenic animals was driven by an actin promoter. Most of the somatic cells of the GFP-transgenic animals expressed GFP, except for nail or hair. When the cells of GFP-transgenic rats as donors were transplanted into the inner ear of wild type rats as recipients, GFP in the grafted cells allowed the fate of the grafted cells to be traced by immunohistochemistry. Thirty embryonic day 12 (E12) GFP-transgenic SD rat embryos were removed from three dated pregnant rats. Sixty otocysts were dissected from the E12 embryos in phosphate buffered saline without calcium or magnesium (PBS−), then mechanically dissociated in PBS−. After centrifugation of the cell suspension, PBS− was added to a 15 ml tube containing cell pellet to establish a donor cell suspension with a concentration of 1×10^8 cells/ml.

To damage the inner ear of recipient animals, 10 host animals were exposed to white noise for 1 h at a 120 dB sound pressure level with a range from 1 kHz to 20 kHz. After acoustic overstimulation, the lateral bone of the cochlea at the level of the middle turn was bored using a hand drill, and 0.1 μl of cell suspension containing otic epithelial cells derived from GFP-transgenic rats was injected through the hole in the lateral walls of the middle turn of the cochlea. Five animals received transplantation of the fetal otocyst cells without acoustic overstimulation.

One month after the transplantation, recipient animals were fixed transcardially with 4% paraformaldehyde, and then the inner ears were removed and immersed in PBS−, followed by decalcification in 5% of EDTA solution at 4°C for 3 days. The decalcified

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inner ears were immersed in 20% sucrose/PBS solution for cryoprotection at 4°C overnight. These inner ears were mounted in OCT compound and transected at 20 μm thick on a cryostat.

The semi-thin sections of inner ear were immersed in PBS containing 0.2% Triton for 30 min, then incubated in PBS containing 1% bovine serum albumin (BSA) and a primary antibody, i.e. polyclonal anti-GFP IgG (1:250; Molecular Probes, Eugene, OR, USA) at 4°C overnight. After washing the specimens twice in PBS containing 0.2% Triton, they were incubated in 1% BSA/PBS with a secondary antibody, i.e. Alexa Fluor 594-labeled anti-rabbit IgG (1:250; Molecular Probes) at 4°C for 3 h. Cell nuclei of the specimens were stained by incubation with DAPI (4′,6-diamidino-2-phenylindole; 0.1 μg/ml; Molecular Probes) solution at room temperature for 15 min. After washing twice, the specimens were mounted in antifade reagents (Molecular Probes), and covered with thin coverglasses. Fluorescence images of the specimens were observed under fluorescence microscope Nikon Eclipse E600 equipped with a digital image capture system (Nikon, Tokyo, Japan).

RESULTS

Eight host animals with acoustic overstimulation and four host animals without acoustic overstimulation survived after transplantation. In all of the surviving experimental animals, grafted cells were observed in the host inner ear. Only three host animals with acoustic overstimulation and one host animal without acoustic overstimulation demonstrated grafted cells in the cochlea ducts with injection tracts through the lateral walls. In the other animals, grafted fetal otocyst cells were observed in the lateral walls, modiolus and space of the scala tympani or scala vestibuli. Most grafted otocyst cells were observed along the tracts of the needle injection for transplantation, and these had survived and integrated into the host mesenchymal tissues (Fig. 1A and B). When the cells were introduced into the perilymphatic space of scala tympani or vestibuli, these cells formed agglomerations or lined the inner lumen of the scala tympani and vestibuli. Only a small number of grafted cells had migrated to and survived on the base membrane where sensory cells of the organ of Corti had existed before being broken by acoustic overstimulation (Fig. 1C). A few grafted fetal otic epithelia cells were integrated in the supporting cell layers of the damaged cochlea, and expressed shapes similar to the supporting cells (Fig. 2). In this study, we could not observe integration of the grafted cells into the hair cell layers in the organ of Corti.

DISCUSSION

Several reports have indicated that immature cells transplanted into the organs can survive and be induced to differentiate into organ-specific cell phenotypes (e.g. neural stem cells differentiate into blood cells), suggesting that the microenvironment of organs provide some kind of cue to the grafted cells and induce the cells to differentiate into the mature cell phenotypes that the recipient organs are composed of (5). Our study showed that grafted otocyst cells from E12 rat embryos survived in the inner ear sensory organs of recipient animals. Moreover, when grafted immature otocyst cells integrated in the supporting cell layers, the integrated cells expressed morphological similarities to normal supporting cells. These findings support a hypothesis that inner ear sensory organs provide cues that play important roles in the

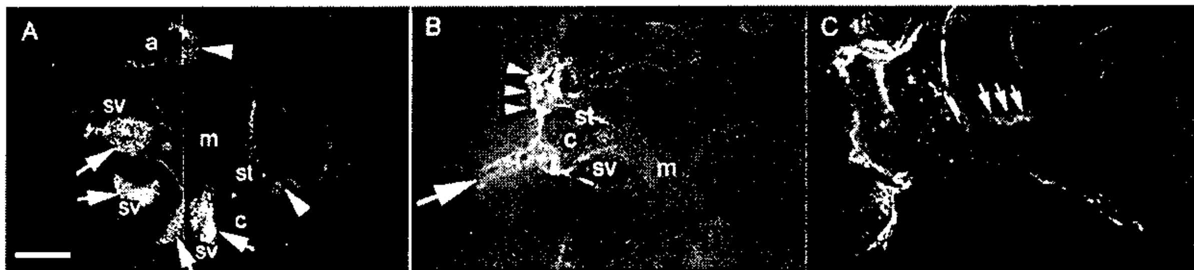

Fig. 1. Grafted GFP-positive cells survived in the inner ear. (*A*) A confocal laser image for GFP. GFP-positive grafted cells survived to form aggregations (*arrows*) and attached to the inside of the perilymphatic spaces (*arrowheads*). (*B*) A fluorescent microscope image for GFP showing that most GFP-positive cells existed along the tract of the needle injection (*arrow*). Some GFP-positive cells migrated to the lateral walls of the apical turn of the cochlea. (*C*) A fluorescent microscope image for GFP showing that a small number of grafted cells (*arrows*) migrated and survived on the damaged epithelial layers where Claudius cells had existed before damage. (a = apical turn; c = cochlea duct; m = modiolus; st = scala tympani; sv = scala vestibuli.) Scale bar represents 200 μm (*A*); 100 μm (*B*).

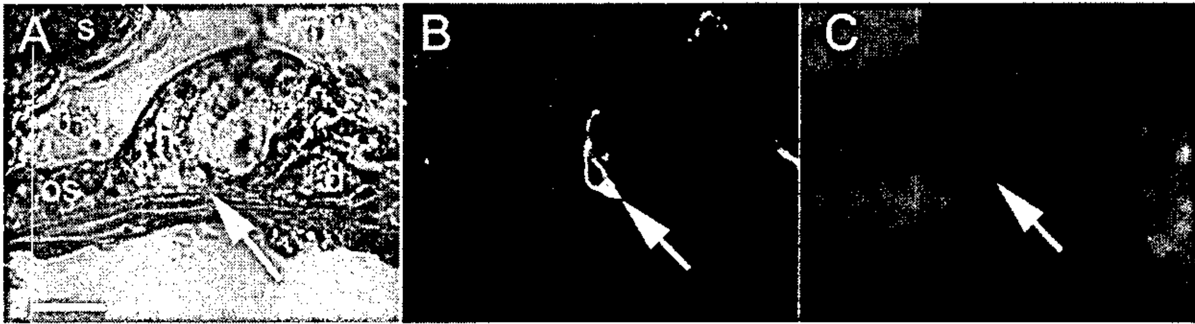

Fig. 2. Integration of a grafted cell into the supporting cell layer. (*A*) Hematoxylin-eosin staining. One cell transplanted into an inner ear damaged by acoustic overstimulation (*arrow*) was integrated as a supporting cell, Hensen's cell layer. (*B*) Fluorescent microscope image. The shape of the GFP-positive cell (*arrow*) was similar to that of Hensen's cells. (*C*) Nuclear staining by DAPI. The GFP-positive cell (*arrow*) possessed a nucleus, indicating that the grafted GFP-positive cell survived in the Hensen's cell layer. (*A–C*) Same field images. (d = Deiters cell layer; h = Hensen cell layer; os = outer sulcus; s = stria vascularis.) Scale bar represents 30 μm.

survival and differentiation of grafted immature cells as described in our previous report (9).

The findings in this study indicate that grafted embryonic otocyst cells differentiate into mature supporting cell types morphologically when integrated into the inner ear epithelia. However, when introduced to the perilymphatic space, the grafted cells aggregated or attached to the wall of the scala tympani and vestibuli. It was difficult technically to induce the cells to integrate into the epithelia. Moreover, in this study, the lateral walls, especially the stria vascularis, was broken when cell suspensions were injected into the endolymphatic space of the cochlea duct. For this reason, we could not clearly evaluate whether grafted otocyst cells could survive in normal cochlear duct environments, such as endolymph with a high potassium concentration. However, we continue to anticipate neural repair of the damaged inner ear by cell transplantation therapy, when the difficulties and technical problems of cell transplantation are resolved (10).

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Transplantation of Neural Stem Cells into the Modiolus of Mouse Cochleae Injured by Cisplatin

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Tamura T, Nakagawa T, Iguchi F, Tateya I, Endo T, Kim T-S, Dong Y, Kita T, Kojima K, Naito Y, Omori K, Ito J. *Transplantation of neural stem cells into the modiolus of mouse cochleae injured by cisplatin.* Acta Otolaryngol 2004; Suppl. 551: 65–68.

This study aimed to examine the possibility of restoration of spiral ganglion neurons, which transmit sound stimulation to the brain, by transplantation of fetal neural stem cells (NSCs) into the modiolus of cochleae. Fetal mouse NSCs expressing green fluorescence were injected into the modiolus of cisplatin-treated cochleae of mice. The temporal bones were collected 14 days after transplantation, and provided histological examination. The cell fate of transplants was determined by immunohistochemistry for a neural or glial cell-marker. Histological analysis 2 weeks after transplantation revealed robust survival of transplant-derived cells in the modiolus of the cochlea. NSCs injected in the basal portion of cochleae migrated as far as the apical end of the modiolus. Grafted NSCs expressing a neural cell marker were identified, but the majority of grafted NSCs differentiated into glial cells. These findings suggest the possible use of NSCs in cell therapy for restoration of spiral ganglion neurons. However, further treatments are required to increase the number of NSC-derived neurons in the modiolus to realize functional recovery. *Key words: cell therapy, neurosphere, regeneration, spiral ganglion neuron, stem cell.*

INTRODUCTION

Hearing impairment is the most frequent disability of people in industrialized countries. The loss of spiral ganglion neurons (SGNs) is a major cause for profound hearing loss (1). SGNs are auditory primary neurons in Rosenthal's canal in the modiolus of the cochlea. Sound stimulation is adapted by auditory hair cells, converted into electronic stimulation in hair cells, and transmitted to SGNs. The survival of SGNs is therefore a critical issue for maintenance of hearing function, and for obtaining the clinical benefits of cochlear implants, which are implantable devices designed to stimulate SGNs electronically. Regeneration of SGNs has thus been an important issue for restoration of hearing because their regenerative activity is very limited (2). New experimental approaches including cell transplantation are, thus, being pursued to restore hearing (3–5).

The aim of this study was to examine the potential of cell transplantation for restoration of SGNs. Fetal neural stem cells (NSCs) that have the potential for differentiation into neurons (4–7) were used as donor cells, and adult mice affected by cisplatin, in which severe degeneration of SGNs is induced (8), were the recipient animals.

MATERIALS AND METHODS

The Animal Research Committee, Graduate School of Medicine, Kyoto University approved all experimental protocols. Animal care was under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

Fetal mouse NSCs expressing green fluorescence were used as transplants (4, 5). NSC spheres were obtained from the neuroepithelium of the dorsal telencephalon of embryos at embryonic day 11.5 of C57BL/6JTg14 (Act-EGFP) Obs-Y01 transgenic mice (9) using the neurosphere culture medium (10, 11). Secondary neurospheres, which exhibited expression of nestin, but no expression of neuron-specific class III beta-tubulin (TuJ1) or glial fibrillary acidic protein (GFAP), were collected, dissociated and suspended for transplantation at a density of 1×10^5 cells/μl in the neurosphere culture medium. C57BL/6 mice at 6 weeks of age ($n = 4$) were used as recipient animals. A cisplatin solution (2.5 mg/ml in physiological saline; Sigma, St Louis, MO, USA) was injected from the left posterior semicircular canal. The procedures used are described in detail elsewhere (12). In this model, about 60% of SGNs disappear at 14 days after cisplatin treatment (8). The left cochlea of recipient animals was exposed under anesthesia with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (9 mg/kg) 14 days after treatment of local application of cisplatin. The medium containing NSC aggregates (2 μl) was then injected into the cochlea through the round window toward the direction of the cochlear modiolus using a Hamilton syringe and infusion pump. The round window membrane was then covered with connective tissue and adherent agents.

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On day 14 after transplantation, the left cochlea of recipient animals was re-exposed after administration of lethal doses of ketamine and xylazine, and 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS) at pH 7.4 was gently perfused into the perilymph from the round window. The animals were then sacrificed by cervical dislocation. The temporal bones were collected and immersed in the same fixative for 4 h at 4°C. Cryostat sections 10 μm thick were made, and the mid-modiolus sections were used for histological analysis.

The cell fates of grafted NSCs were determined by immunohistochemistry for TuJ1, a marker for neurons, or GFAP, a marker of glial cells. Anti-TuJ1 rabbit polyclonal antibody (1:300; Covance Research Product, Berkeley, CA, USA) or anti-GFAP rabbit polyclonal antibody (1:200; Sigma) was used as the primary antibody, and Alexa Fluor 594-conjugated anti-rabbit or goat IgG (1:200; Molecular Probes, Eugene, OR, USA) was as the secondary antibody. Counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was performed for demonstration of nuclear chromatin. The specimens were viewed with a Nikon ECLIPSE E600 fluorescence microscope (Nikon, Tokyo, Japan).

The numbers of transplant-derived cells in the modiolus in one section and the ratios of positivity for each marker in transplant-derived cells were examined. Both EGFP- and DAPI-positive cells were defined as transplant-derived cells. The average of two sections for each animal was defined as the number for that animal. The ratio of positivity for each marker in transplant-derived cells in the modiolus was then calculated.

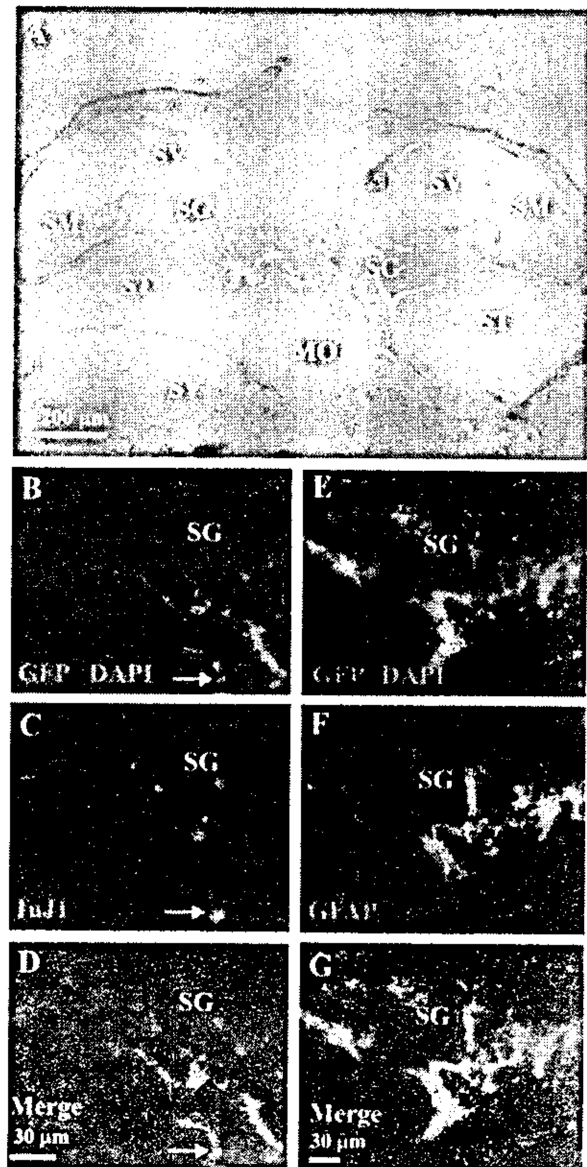

Fig. 1. Survival and differentiation of neural stem cells (NSCs) grafted into the modiolus of injured cochleae. (*A*) NSC-derived cells expressing green fluorescence protein (GFP) settled in the apical portion of the modiolus. (*B*–*D*) Immunohistochemistry for neuron-specific class III beta-tubulin (TuJ1: *red*) demonstrates that a few of the NSC-derived cells differentiated into neurons (*arrows*). (*E*–*G*) Expression of glial fibrillary acidic protein (GFAP; *red*) is frequently observed in NSC-derived cells. (SG = spiral ganglion; MO = modiolus; ST = scala tympani; SV = scala vestibuli; SM = scala media.) Scale bar represents 200 μm (*A*) or 30 μm (*B*–*G*).

RESULTS

We confirmed robust survival of the injected cells in cochleae of all experimental animals. NSC-derived cells were located in the modiolus of cochleae or scala tympani (Fig. 1A). NSC-derived cells injected into the basal part of the modiolus were also found in the apical end of the modiolus, indicating the migration activity of NSCs in the modiolus of cochleae. Some NSC-derived cells settled in spiral ganglions. The mean and standard deviation of numbers of NSC-derived cells in the modiolus was 190.5 and 60.8.

NSC-derived cells in the modiolus exhibited expression of TuJ1 (Fig. 1B–D) or GFAP (Fig. 1E–G). TuJ1-positive grafted cells were predominantly located in the osseous spiral lamina or spiral ganglion. Expression of TuJ1 was observed in about 10% of NSC-derived cells. Immunohistochemistry for TuJ1 also demonstrated an apparent decrease of SGNs. The majority of NSC-derived cells were positive for GFAP (Fig. 1E–G). Expression of GFAP was observed in about 80% of NSC-derived cells. GFAP-positive grafted cells were found in the modiolus and spiral ganglion. Interestingly, TuJ1-positive grafted cells

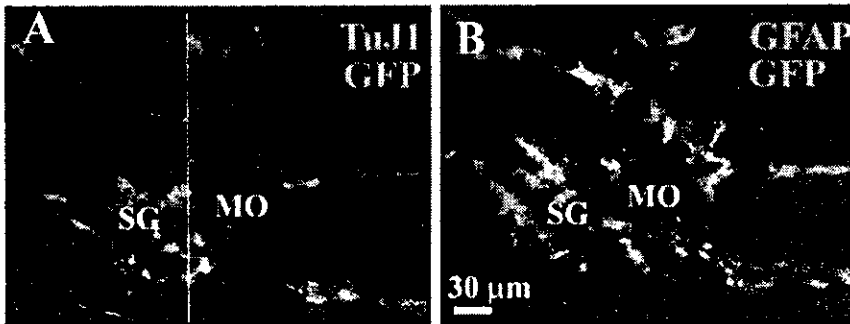

Fig. 2. Distribution of neuron-specific class III beta-tubulin (TuJ1)- or glial fibrillary acidic protein (GFAP)-positive grafted cells in the modiolus. TuJ1-positive grafted cells are positioned on the periphery of the mass of grafted cells (*A*), while GFAP-positive ones are central (*B*). (GFP = green fluorescence protein.) Scale bar represents 30 μm.

were positioned on the periphery of grafted cells (Fig. 2A), while GFAP-positive grafted cells were mainly located in the center of grafted cells (Fig. 2B).

DISCUSSION

Previous studies have demonstrated that NSCs grafted into the inner ear survive in the cochlear cavity of newborn rats (3) and adult mice (4). In addition, it has been demonstrated that NSCs grafted into the cochlear cavity migrate into the modiolus and sensory epithelia of cochleae damaged by aminoglycosides (5). These findings suggest that NSCs can be a source of transplants for cell therapy of degenerative inner ear diseases. It has also been revealed that grafted NSCs surviving in the cochlear cavity differentiate into neural cells in normal inner ears (4) and in injured inner ears (5), suggesting that NSCs can be transplants for restoration of SGNs. We thus used NSCs as transplants for restoration of SGNs in this study. In previous studies on NSC transplantation into the inner ear, injection of NSCs into the cochlear duct (3, 5) or semicircular canal (4) was used as procedures for transplantation. However, no or few NSC-derived cells in the modiolus have been identified in these studies. We thus used a direct injection into the modiolus as a method for NSC transplantation to introduce NSCs into the modiolus.

The present findings demonstrate the robust survival of NSC-derived cells in the modiolus. In addition, NSCs injected in the basal portion of the modiolus settled in the apical end of the modiolus and osseous spiral lamina, indicating the high potential of NSCs for migration. The migration capacity of NSCs has also been reported in the spinal cord (13). Therefore, an injection of NSCs in the basal portion of the modiolus is an effective method for introduction of NSCs into the modiolus from the base to the apex.

Immunohistochemistry for TuJ1 and GFAP revealed the cell fate of grafted NSCs surviving in the modiolus. NSC-derived cells differentiated into neurons in the modiolus, indicating that NSC transplantation into the modiolus may be utilized for restoration of SGNs. However, only 10% of NSC-derived cells differentiated into neurons, similarly to previous findings when NSCs are grafted into the fluid space of the inner ear (4, 5). On the other hand, it is noteworthy that TuJ1-positive grafted cells distributed on the periphery of the mass of grafted cells. SGNs are located between the modiolus and organ of Corti. Therefore, NCS-derived cells tend to differentiate into neurons in the near area corresponding to the known area of auditory nerves.

Functional recovery of SGNs requires more abundant neurons derived from transplants. Therefore, promotion of the activity of neural differentiation or increase of numbers of surviving NSC-derived cells was crucial for functional recovery of SGNs. Neurotrophins reportedly stimulate neural differentiation of NSCs (14). In addition, several neurotrophins have effects for promotion of neurite outgrowth from NSCs (15). Therefore, the supplement of neurotrophins may be a cue for the promotion of the survival of NSCs grafted in the inner ear and functional recovery. On the other hand, the microenvironments where NSCs settled influence the fate of grafted NSCs. Cytokines associated with inflammation influence the survival and differentiation of NSCs transplanted in the nervous system (16). The microenvironments of the cochlea also alter depending on the time after traumatic treatment (8). Optimization of the timing of transplantation of NSCs is hence required for increased numbers of NSC-derived neurons in the modiolus.

In conclusion, an injection of NSCs into the modiolus of injured cochleae results in robust survival of grafted NSCs in the modiolus. Grafted NSCs differentiate into neurons in the modiolus, although their number is limited. NSC is a possible candidate of cell therapy for restoration of SGNs.

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Trophic support of mouse inner ear by neural stem cell transplantation

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In the auditory system, efforts to reduce degeneration of spiral ganglion neurons have the immediate objective of improving clinical benefits of cochlear implants, which are small devices designed to stimulate spiral ganglion neurons electronically. Recent studies have indicated several neurotrophins can enhance survival of spiral ganglion neurons. However, the strategy for application of neurotrophins in inner ear is still a matter of debate. In this study, we examined the potential of cell therapy as a strategy for application of neurotrophins in the inner ear. Neural stem cells obtained from green fluorescent protein-transgenic mice were used as donor cells. Medium containing neural stem cells was injected into mouse inner ear. Histological analysis 4 weeks later revealed that transplant-derived cells survived in inner ear and that most transplant-derived cells in the cochlea had differentiated into glial cells. Moreover, expression of glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor was observed in transplant-derived cells. These findings indicate that transplantation of neural stem cells can be a useful strategy for application of neurotrophins in inner ear. *NeuroReport* 14:77–80 © 2003 Lippincott Williams & Wilkins.

Key words: Cochlear implants; Neurotrophin; Spiral ganglion; Stem cell; Transplantation

INTRODUCTION

Hearing impairment is the most frequent disability of people in industrialized countries, affecting more than one in seven individuals. One child in every 2000 is born deaf from genetic causes. However, options for improvement of hearing are limited to hearing aids and cochlear implants (CIs). CIs are small devices that are surgically implanted in the inner ear to stimulate the spiral ganglion neurons. CIs have significantly contributed to restoration of hearing in deafened individuals for decades. However, the CI depends on remaining spiral ganglion neurons, and their loss severely compromises its efficacy [1,2]. Therefore, survival of spiral ganglion neurons is a critical issue for maintaining the hearing benefits provided by CIs. Recent studies have revealed the efficacy of application of several neurotrophins in increasing the survival of spiral ganglion neurons [3,4]. An osmotic infusion pump or gene transfection has been used as a strategy for administration of neurotrophins in experimental models. However, these methods can provide inner ear with neurotrophins for only a limited duration of time. On the other hand, cell therapy has the potential to enable long-term application of neurotrophins. Cell transplantation has been reported to be a strategy for application of peptides or proteins in the CNS [5,6]. For cell therapy to promote the viability of spiral ganglion neurons, the potential for survival of donor cells and their production of neurotrophins in the inner ear are required. Neural stem (NS) cells have been considered donor cells for cell therapy in a variety of systems [7,8]. After transplantation, most NS cells differentiate into glial cells in brain or retina. Glial cells can be a source for several neurotrophins. Therefore, transplantation of NS cells into the inner ear has the potential for application of neurotrophins in the inner ear. In this study, we examined the ability of NS cells to survival in the inner ear and the potential for production of neurotrophins after transplantation into mouse inner ear.

MATERIALS AND METHODS

Preparation of donor cells: As a source of neural stem cells, we used enhanced green fluorescent protein (EGFP)-transgenic mice (kindly given by Dr Yamada of the Institute for Virus Research, Kyoto University). EGFP-transgenic mouse embryos were removed from staged pregnant females that were painlessly euthanised early on the 12th day of gestation. The neuroepithelium of the dorsal telencephalon of embryonic mice at embryonic day 11.5 was transferred into neurosphere culture medium (DMEM/F-12 (1:1), Gibco, Grand Island, NY) supplemented with 100 μg/ml transferrin (Sigma, St. Louis, MO), 25 μg/ml

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insulin (Sigma), 20 nM progesterone (Sigma), 30 nM sodium selenite (Sigma), 60 μM putrescine (Sigma), 20 ng/ml epidermal growth factor (Gibco), and 20 ng/ml basic fibroblast growth factor (Gibco), washed with 0.01 M phosphate-buffered saline (PBS) at pH 7.4 once, and dissociated completely by pipetting. The cell suspension (100 μl, at a density of 1×10^6 cells/ml) was plated in each well of 96-well ultra-low attachment plates (Corning, Corning, NY). At day 3, primary spheres were collected, spun down at 1000 r.p.m. for 5 min, and digested with 0.25% trypsin–EDTA and DNase I (50 μg/ml) for 10 min at 37°C. After supplementation of 0.25% trypsin inhibitor and PBS, the cells were spun down, resuspended in the neurosphere culture medium, and dissociated completely by pipetting. The obtained cell suspension (100 μl, at a density of 1×10^6 cells/ml) was plated in each well of 96-well ultra-low attachment plates. At day 6, secondary spheres were collected for transplantation, dissociated in the same way and suspended at a density of 1×10^5 cells/μl in the neurosphere culture medium.

Surgical procedures: We injected 10 μl of the medium including NS cells into the inner ear of five adult female C57/BL6 mice at 6 weeks of age. Animals were anaesthetized with ketamine (100 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.). We used a retroauricular incision for approach to the temporal bone. A small hole was made in each of the lateral and posterior semicircular canals of the left ear. A glass needle was inserted into the lateral semicircular canal, and the medium containing NS cells was injected using an infusion pump. Then, the holes in the semicircular canals plugged with connective tissue and covered with an adhesive agent. The surgical procedures were completed by suturing of the skin incision.

Tissue preparation: At day 28 after transplantation, the mice were deeply anesthetized with a lethal dose of ketamine and xylazine and were perfused intracardially with normal saline, followed by 4% paraformaldehyde in PBS. The left temporal bones were removed and immersion-fixed in the same fixative at 4°C for 4 h. After decalcification with 0.1 M EDTA for 24 h at 4°C, 10 μm cryostat sections of the temporal bones were prepared. The sections were then mounted on α-aminopropyl triethoxysilane (APS)-coated slide glasses. Mid-modiolus sections from each animal were provided for histological analysis.

Histological analysis: The fate of transplant-derived cells was determined by immunohistochemistry for microtubule-associated protein 2 (MAP2), a cell marker of neural cells, and glial fibrillary acidic protein (GFAP), a cell marker for glial cells. The ability for production of neurotrophins was examined by immunohistochemistry for glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). Anti-MAP2 mouse monoclonal antibody (1:500 in PBS containing 1% bovine serum albumin (BSA-PBS), Sigma), anti-GFAP rabbit monoclonal antibody (1:200 in BSA-PBS, DAKO, Carpinteria, CA), anti-GDNF rabbit polyclonal antibody (1:200 in BSA-PBS, Santa Cruz, Santa Cruz, CA) and anti-BDNF rabbit polyclonal antibody (1:200 in BSA-PBS, Santa Cruz) were used as primary antibodies. The sections were permeabilized in 0.5% Triton X-100 in PBS for 20 min. After blocking procedures for non-specific reaction, the samples were incubated overnight at 4°C with a primary antibody. Samples were washed and incubated for 1 h at room temperature with a rhodamine-conjugated anti-rabbit goat IgG (1:200 in BSA-PBS; Chemicon,Temecula, CA) or an Alexa Fluor 594-conjugated anti-mouse goat IgG (1:200 in PBS; Molecular Probes, Eugene, OR). At the end of staining procedures, specimens were incubated with PBS containing 2 μg/ml 4′,6-diamino-2-phenylindole dihydrochloride (DAPI, Molecular Probes) for demonstration of nuclear chromatin. Non-specific labeling was tested by omitting the primary antibody from the staining procedures. The specimens were viewed with a Nikon Eclipse E600 fluorescence microscope.

Quantification: The numbers of transplant-derived cells in the cochlear second turn and the rates of positivity for each marker in transplant-derived cells were examined. Both EGFP- and DAPI-positive cells were defined as transplant-derived cells. The numbers of transplant-derived cells in the scala vestibuli, in the scala tympani, in the scala media and the total number were counted. The average of 4 sections for each animal was defined as the number for that animal. Then, the percentages of the numbers of transplant-derived cells in the scala vestibuli, scala media and scala tympani were caluculated. We then calculated the rate of positivity for each marker in transplant-derived cells in the second turn of the cochlea. The rate of positivity was determined as the number of rhodamine- or Alexa Fluor 594-positive cells with EGFP and DAPI signals divided by the number of transplant-derived cells. The average of the rates in 2 sections was used as the rate for the animal and the mean and s.d. of each parameter in four experimental animals was determined.

All experimental protocols were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. Animal care was under the supervision of the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University.

RESULTS

Transplant-derived cells were found in every turn of the cochlea in all the experimental animals. Transplant-derived cells were predominantly located in the perilymphatic space of the cochlea (scala vestibuli: 62.0%, 322 in 519 transplant-derived cells, scala tympani: 37.6%, 195 in 519). Most transplant-derived cells were attached to the cochlear architecture, and only a few of them were as floating aggregated cells. These findings demonstrate that NS cell-derived cells can survive in the perilymphatic space in the cochlea for at least 28 days.

Expression of GFAP and MAP2 was found in transplant-derived cells (Fig. 1). GFAP-positive transplant-derived cells were frequently observed in cochleae. The mean (± s.d.) rate of positivity for GFAP was 0.96 ± 0.10, and for MAP2, 0.16 ± 0.09. This indicated that most NS cells transplanted into the cochlea had differentiated into glial cells. On the other hand, transplant-derived cells in cochleae exhibited immunoreactivity for neurotrophins (Fig. 1). Most transplant-derived cells were positive for GDNF, and 50% were

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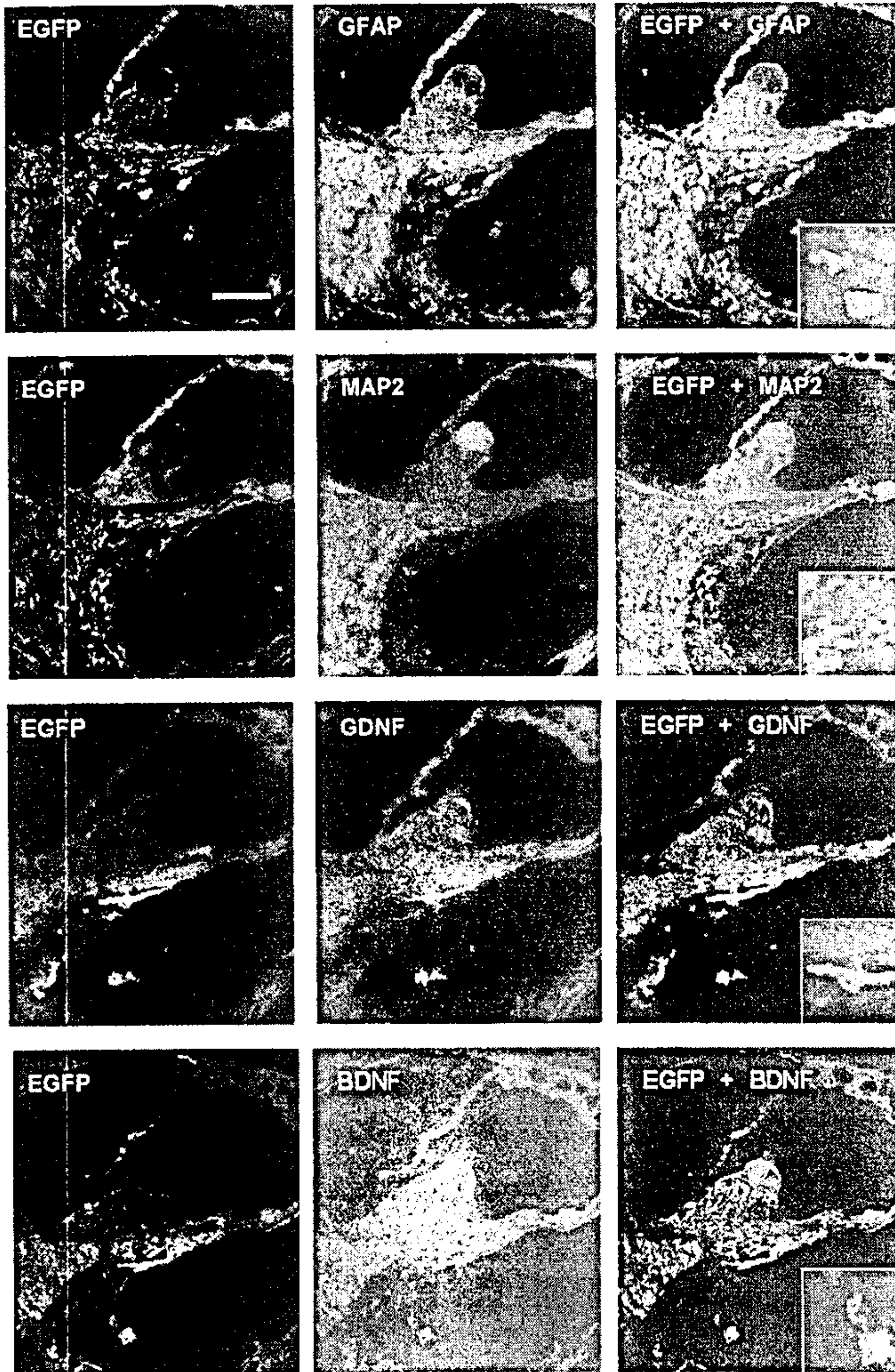

Fig. 1. Transplanted neural stem cells differentiate into glial and neuronal cells and exhibit immunopositivity for neurotrophins. Left line, enhanced green fluorescent protein (EGFP) shows localization of transplant-derived cells. Middle line, immunostaining for glial fibrillary acidic protein (GFAP), microtubule-associated protein 2 (MAP2), glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). Right line, merged images of EGFP and immunostaining. Double positive cells are also demonstrated at a high magnification in small squares. Bar = 100 μm.

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positive for BDNF. The rates of positivity for GDNF and BDNF were 0.90 ± 0.07 and 0.49 ± 0.29, respectively. This indicated that NS cell-derived cells can spontaneously produce GDNF and/or BDNF in the cochlea.

DISCUSSION

The present findings demonstrate that NS cell-derived cells can survive for 4 weeks after transplantation into the inner ear. This suggests that multi-potential NS cells can be utilized for treatment of inner ear diseases. In this study, most transplant-derived cells differentiated into glial cells, identical to previous findings obtained when NS cells were transplanted into brain or retina [7,9]. We focused on the ability of transplant-derived cells to produce neurotrophins in the cochlea in this study. Immunohistochemical analysis revealed that transplant-derived cells had the potential to produce neurotrophins. Therefore, transplantation of NS cells can be a strategy for long-term application of neurotrophins in the inner ear.

In clinical cell transplantation into the inner ear, transplantation can be performed at the time of CI surgery, which requires opening of the scala tympani. The present findings demonstrated that transplant-derived cells had the potential to produce GDNF and BDNF, which have been reported to have efficacy for protection of spiral ganglion neurons from various toxic insults [3,4,10,11]. Therefore, neurotrophins derived from NS cell-derived cells will support the survival of spiral ganglion neurons after CI surgery, which can contribute to the maintaining hearing benefits provided by CIs. In addition, recent studies have indicated that neurotrophins enhance the sensitivity of neurons [4,12]. Therefore, supplementation of neurotrophins by transplantation of NS cells may enhance the sensitivity of surviving spiral ganglion neurons to electronic stimuli from CIs. Consequently, we believe that transplantation of NS cells in combination with CIs can enhance the clinical efficacy of CIs.

In conclusion, transplanted NS cells survived in the cochlea for 28 days, and differentiated into glial and neuronal cells. In addition, transplant-derived cells had the potential to produce neurotrophins. This suggests that cell therapy is a promising strategy for maintenance of spiral ganglion neurons, especially in combination with CI.

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SURGICAL MANAGEMENT OF SPECIAL CASES OF INTRACTABLE MENIERE'S DISEASE: UNILATERAL CASES WITH INTACT CANALS AND BILATERAL CASES

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If a clinician seeks to allow patients with vertigo to return to work as soon as possible, it is very important to determine the appearance of vestibular symptoms during convalescence just after treatment, as well as the long-term results. Apprehensive patients with vertigo may undergo severe psychological torment if treatment requires long-term rest in bed before they can return to daily life. In this paper, we observed postoperative vestibular symptoms (subjective sensation and objective nystagmus) in 50 patients with intractable Meniere's disease, including cases from our previous preliminary report, during the period of convalescence just after endolymphatic sac drainage and steroid instillation surgery (EDSS). All symptoms were eliminated within 8 days after EDSS. There was no significant difference in the duration of any vestibular symptoms between bilateral (n = 8) and unilateral cases (n = 42). This result indicates that EDSS could be as safe a treatment for bilateral Meniere's disease as for unilateral disease. In unilateral cases with intact semicircular canal function (n = 17), postoperative evoked vestibular sensation, positional, and positioning (Dix-Hallpike) nystagmus disappeared significantly earlier than in those with canal paresis (n = 25). This result indicates that EDSS could keep the vestibular peripheral function of patients with unilateral Meniere's disease with intact canals quite stable after surgery. Therefore, EDSS could be recommended as an initial, less-invasive surgical treatment for intractable Meniere's disease, especially in unilateral cases with intact canals and in bilateral cases.

KEY WORDS — bilateral Meniere's disease, dizziness, elderly patient, endolymphatic sac drainage, intact semicircular canal, nystagmus, steroid.

INTRODUCTION

Most patients with Meniere's disease are relieved of severe vestibulocochlear symptoms and achieve a stable condition by means of oral medication.[1] Some patients, however, do not have good results with pharmacotherapy and experience frequent repeated vertigo, progressive sensorineural hearing loss, persistent tinnitus, and headache. In bilaterally affected cases, their daily social life is severely handicapped.[2,3] Therefore, various types of surgical therapies should be taken into consideration for such intractable cases; the prevalent types for Meniere's disease consist of endolymphatic sac surgery,[4-6] vestibular neurectomy,[7,8] and middle ear administration of steroids[9-11] or gentamicin.[12-15]

When surgical therapies are selected for intractable cases, long-term positive results regarding vertigo, tinnitus, and hearing improvement are often overemphasized.[16] However, it is also absolutely important for apprehensive patients with vertigo to have relief from therapy-induced dysequilibrium during convalescence just after therapy as quickly as possible, because they would undergo severe psychological torment if a long-term rest in bed were required before returning to daily life. Patients with bilateral Meniere's disease should be especially carefully treated in terms of possible vestibular peripheral damage induced by surgery, because peripheral functions contralateral to the operated side may be initially as weak as the ipsilateral ones.[17,18] In addition, even unilateral cases with intact semicircular canal function should also be carefully treated, because severe vestibular peripheral imbalance induced by surgical invasion would impair the patient's daily life.[13,19,20]

We have focused on the endolymphatic sac as a therapeutic window according to some basic experimental reports,[21,22] and we treated 50 patients with intractable Meniere's disease with endolymphatic sac drainage and steroid instillation surgery (EDSS) as described previously.[23,24] In this article, putting long-term results of our surgery aside to assess them in later communications, we first observed not only subjective vestibular sensations but also objective vestibular findings in all 50 patients using electronystagmography (ENG) during the period of convalescence after EDSS. We then compared the severity of these postoperative symptoms between bilateral and unilateral cases and between unilateral cases with canal paresis (CP) and those with no CP. It was our hope that this study would give evidence that EDSS

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