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Ⅱ．分担研究報告書

小動物における ABR 検査の実際

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研究要旨
実験動物の聴力障害の程度を評価する方法の一つに聴性脳幹反応(ABR)を用いる方法がある。聴性脳幹反応を安定して正確に記録するためには、検査機器の整備と検査方法に習術することが必要である。今回はサイトメガロウイルスに感染させたモルモット、ラット、マウスなどの聴力障害を評価する方法として ABR を用いるため、まず感染していない小動物を用いて実際の ABR 記録の装置を整備し ABR の記録を行った。

A. 研究目的

先天性 CMV 感染による聴覚障害の発症貴女を解明するためには、CMV 感染小動物を用いた他覚的聴力検査が必須である。この研究の目的は、聴性脳幹反応（ABR）を安定して正確に記録するシステムを構築することであり、検査機器の整備、検査方法の習熟が必要不可欠である。本年度は最新の ABR 検査装置、電気シールド防音室を整備し、実際にコントロールとして感染していない小動物の ABR の記録を行った。

B. 研究方法

I. ＡＢＲを計測する際に必要な機器、環境の整備

ＡＢＲを計測するためには刺激音発生装置とＡＢＲ検査装置が必要である。刺激発生装置は powerlab（ADInstruments 社製、オーストラリア）を用い、刺激音は tone burst を用いた。ＡＢＲ検査装置は Tucker Davis 社製（オーストラリア）を用いた。できるかぎり電磁波などの影響の少ない環境とするために、金網で囲まれた聴力検査室において、動物、アイソレーター準備しアースをとり測定した。

II. ＡＢＲ記録法（麻酔、電極の取り付け）

動物（ラット）に対して、ジエチルエーテルで吸入麻酔後、フェントバルビタール（商品名：ネンブタール）を 30mg/kg で腹腔内投与し図 1 のように電極を刺入し、音刺激（刺激音は 12000Hz の tone burst）を与え、ABR を測定した。

（倫理面の配慮）

実験動物の取り扱いに関しては福島県立医科大学動物実験ガイドラインに沿って、愛護的に行った。

C. 研究結果

刺激音の周波数は 12000Hz で刺激音圧は 83.2dbSPL から 23.2dBSPL まで変化させ、ABR を測定した。そのうち、図 2 に 83.2dBSPL、53.2dBSPL、23.2dBSPL における ABR の波形を示す。

D. 考察

本装置を用いることにより、小動物（ラット）の ABR を安定して記録することが可能であった。本法はモルモットに対しても同一手技で ABR を測定することが可能であり、CMV 感染モデルにおける聴力の評価、聴力障害の程度を推察することが可能であると考えられた。今後は経胎盤感染するモルモット CMV の感染実験系を用い、ABR を計測し、聴覚障害をきたす動物実験系の確立をめざす。

E. まとめ

当科における小動物に対する ABR 測定の実際について報告した。小動物に対する ABR 評価のための聴力検査システムを整備した。

F. 健康危険情報

なし

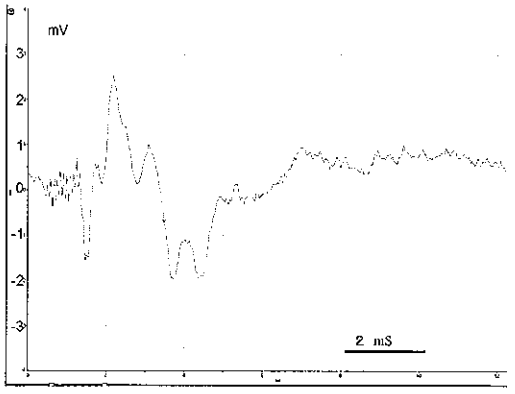
G. 研究発表

なし

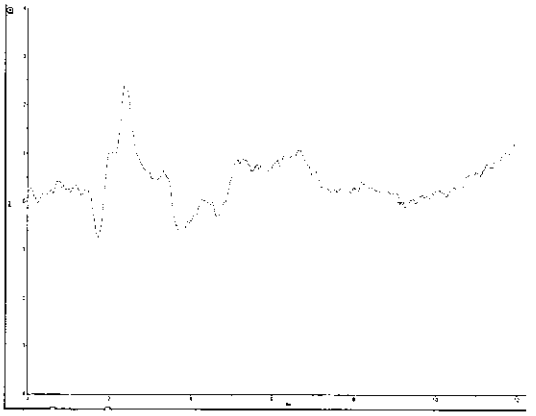
H. 知的財産の出願・登録状況

なし

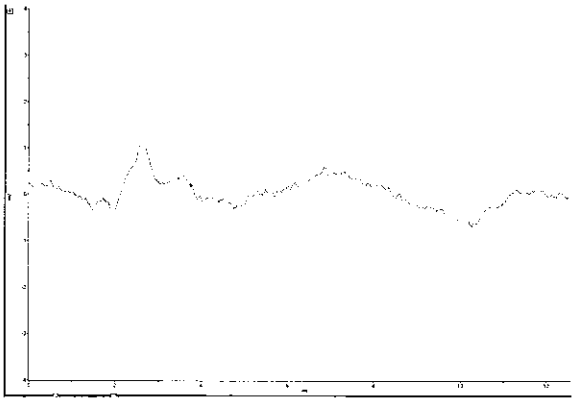
図１　ジエチルエーテルで吸入麻酔後フェントバルビタールを腹腔内投与し、左右乳突部に電極を挿入し左耳にスピーカをあて、音刺激を与えているところ。刺激音は 12000Hz の tone burst を用いた。



83. 2dBSPL



53. 2dBSPL



23. 2dBSPL

図２　刺激音圧が小さくなるとともに波形が小さくなる、

モルモットサイトメガロウイルスを用いた実験的ウイルス性内耳障害

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研究要旨

サイトメガロウイルスによる先天性難聴の発症機構を分子レベルでとらえることを目的に、動物実験モデルとして従来から用いられているモルモットの実験系を再考察、再興し、その病理組織学的解析を行なった。モルモットの内耳への感染実験では前庭階、鼓室階、らせん神経節のリンパ球浸潤、出血が見られ、ライスナー膜での巨細胞性変化、血管条の萎縮などの変化が認められた。内耳以外にも唾液腺、腎臓、膵臓、脈絡叢などに巨細胞封入体の出現が認められた。妊娠モルモットへの感染実験では親モルモットの胎盤内にウイルス感染細胞を認め、胎児の内耳にはライスナー膜、蝸牛神経節、鼓室階等における巨細胞の出現とともに、神経細胞の変性壊死、脱落、血管条の空胞変性など、ウイルス感染による変化が認められた。さらに胎児の中には蝸牛の奇形を持ったものも見られた。本実験系を用い、今後、サイトメガロウイルスによる先天性難聴におけるウイルス感染機構とその病態の関連を分子レベルで解析することが可能と考えられる。

A. 研究目的

サイトメガロウイルスは胎盤経由で胎児に垂直感染し、高度聴覚障害の原因となる。サイトメガロウイルスによる先天性難聴の動物実験モデルとしてモルモット(Guinea pig)やマウスが古くから用いられている。モルモットは妊娠期間が10週と比較的短期である上、胎盤の構造がヒトのそれに近似していること、解析しやすい大きさであることなどから垂直感染のモデルとして利用されてきた。1980年代にはこの実験系を用いた研究が盛んに行われ、その病理学的解析や聴覚機能を調査した実験結果の積み重ねにより、サイトメガロウイルス感染と内耳における組織障害の関連が実証され、サイトメガロウイルスの経胎盤感染が先天性聴覚障害の原因になるうることが明らかにされている[1,2,3]。近年の分子生物学の急激な進歩によりサイトメガロウイルス感染の分子生物学的な解析が進み、ウイルスの感染様式や感染機構、病態が分子レベルで明らかにされてきている。特に大きな進歩はウイルスの前初期遺伝子から始まる一連の遺伝子発現の仕組みが解明され、その病態との関連が分子レベルで明らかにされてきたことであろう。そこで、われわれはサイトメガロウイルスの垂直感染や内耳障害、聴覚障害の分子メカニズムを解明するため、従来から用いられているモルモットの実験系を再考察し、実験そのものを再興し、先天性難聴の発症機構を分子レベルでとらえることを目的とした。

B. 研究方法

1．内耳への感染実験

Hartley 系クリーンモルモット（体重 200g）８匹を用い、そのうち４匹を実験群として右中耳胞をメスで開放し正円窓より $5x10^5$ pfu /0.002ml の Guinea pig cytomegalovirus (GP-CMV) ウイルス液を歯科用 1/6 針を用いて注入した。対照群では同じ針で正円窓を穿刺した。ウイルス液は感染唾液腺乳剤を 10%DMSO/ハンクス液で調整した。実験群、対照群とも術後正円窓部をゼルフォームで覆った。

2．妊娠モルモットへの感染実験

妊娠３週の Hartley 系クリーンモルモットの背臀部皮下に $5x10^5$ pfu / 0.5 ml の GP-CMV ウイルス液を接種した。その３週後に麻酔下で胎児と母体の全臓器を摘出し 10%緩衝ホルマリンで固定した。

3．　病理標本の作製と免疫組織学的検索

耳を含めた全臓器の組織は 10%緩衝ホルマリンで固定し、蝸牛など硬組織を含むサンプルは 10% EDTA で 14 日間脱灰を行った。パラフィン包埋したブロックから 4 m 厚の切片を作製し、シランコートガラススライドに貼付した。脱パラフィン後、0.25%トリプシン処理にて抗原賦活化処理を行い、リン酸バッファー

で洗浄後、抗 GP-CMV マウスモノクローナル抗体（浜松医大病理　筒井祥博教授供与）を一次抗体として反応させた[4]。洗浄後、免疫組織化学染色ではビオチン標識抗マウスイムノグロブリン抗体を二次抗体として、三次抗体にはペルオキシダーゼ標識ストレプトアビジンを順次反応させ、ジアミノベンチジンで発色後、アルコール脱水、キシレン透徹、封入後、検鏡した。免疫蛍光染色では二次抗体に FITC 標識抗マウスイムノグロブリン抗体を用い、洗浄、封入後、蛍光顕微鏡にて観察した。また、免疫染色とは別に各標本はヘマトキシリン・エオジン（HE）染色で全体の形態変化を観察した。

C. 研究結果

1．内耳への感染実験

実験群の４匹の内耳には肉眼で出血性病変が確認された（図１，２）。病理組織学的変化として観察されたのは前庭階、鼓室階の著明な出血とリンパ球浸潤で、らせん神経節にもリンパ球浸潤、出血が確認された。また、蝸牛管ではライスナー膜に巨細胞性変化を来した細胞がみられ、蓋膜がライスナー膜への付着する像が見られた。また、血管条、らせん神経節において巨細胞封入体の出現がみられた。これらの巨細胞封入体には免疫組織化学、あるいは免疫蛍光染色で GP-CMV の抗原が認められた。内耳以外にも感染モルモットには様々な変化が観察され、唾液腺、腎臓、膵臓、脈絡叢に巨細胞封入体の出現が認められた。対照群のモルモットにはこれらの変化は認められなかった。

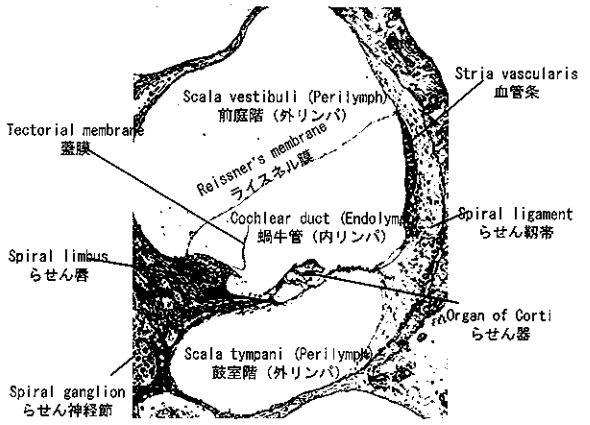

図１　蝸牛の構造

蝸牛の断面を示す。ヘマトキシリンエオジン染色。

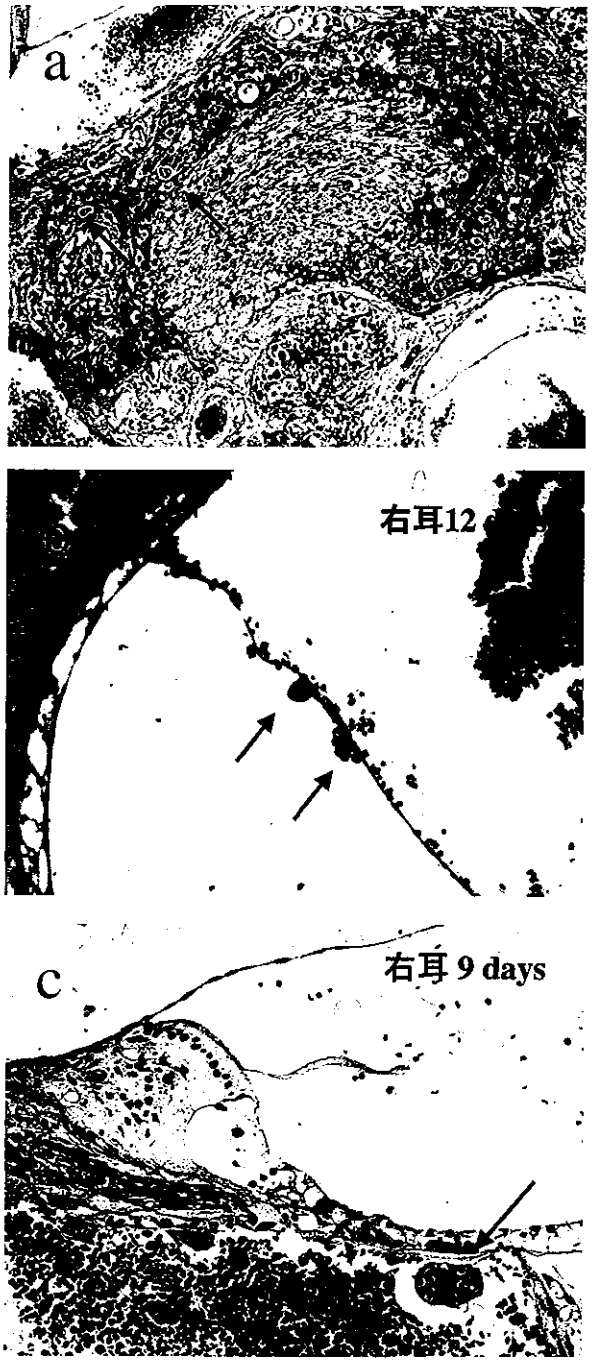

図２　内耳への接種後の蝸牛の変化

(a) らせん神経節に見られた巨細胞封入体（矢印）。出血が著明でリンパ球浸潤も認められる。周辺の前庭階、鼓室階にも出血が著明である。(b) ライスナー膜に見られた巨細胞封入体（矢印）。蛍光免疫染色で GP-CMV 抗原が確認されている。前庭階には出血も見られる。(c) らせん唇、コルチ体周辺に見られた巨細胞封入体（矢印）。鼓室階に存在し、鼓室階には出血が著明である。らせん唇の変化は著明でないがコルチ体周辺の細胞は萎縮しており、空胞変性しているものも見られる。

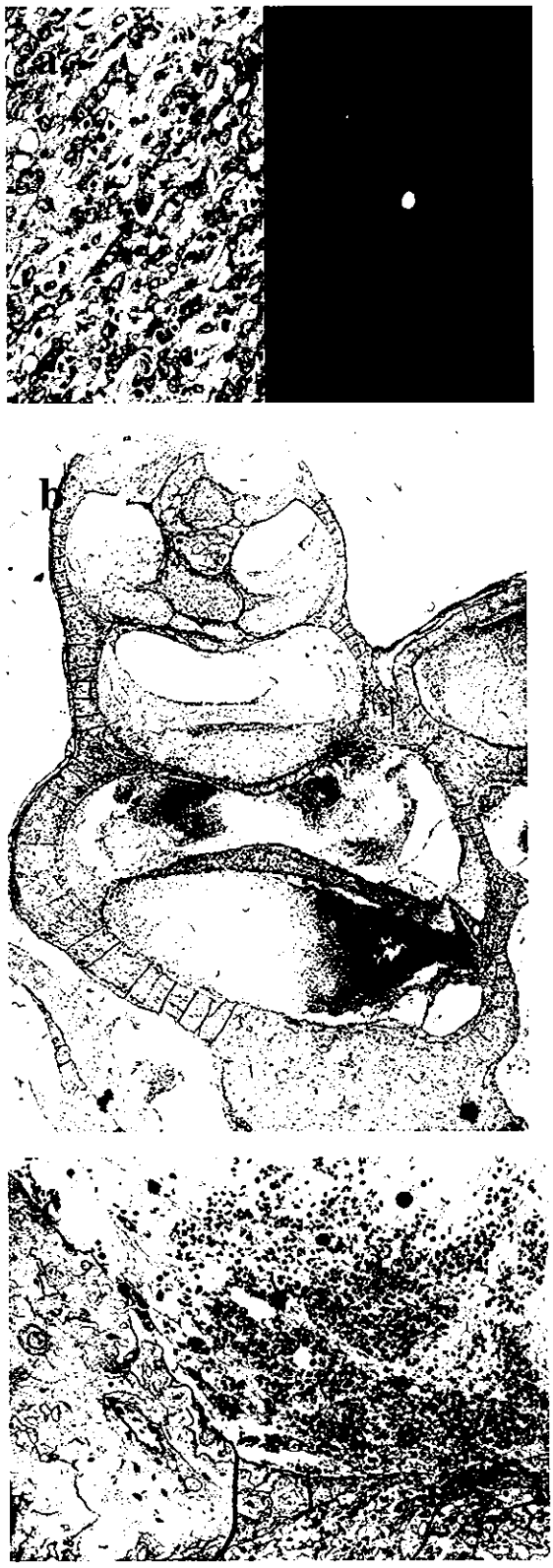

図３　妊娠モルモットへの感染実験

(a) 親モルモットの胎盤。核内封入体を持った細胞が認められ(矢印)、免疫蛍光染色でGP-CMVの抗原が検出された。

(b) 胎児の蝸牛全景。鼓室階、前庭階に出血が著明である。

(c) 鼓室階に見られた巨細胞封入体（矢印）。出血が著明である。

２．妊娠モルモットへの感染実験

親モルモット１０匹に接種実験を行ったところそのうちの１匹に全身CMV感染症が認められた。このモルモットの蝸牛神経節には多くのCMV感染細胞と広範な神経細胞の変性壊死、脱落が認められている。１０匹のモルモットからは３２匹の胎児が得られ、５匹（３腹）は死亡胎児であった。また、明らかな発育遅滞が３匹（２腹）に認められた。免疫組織化学では胎盤には明らかなCMV感染細胞が認められた（図３a)。胎児の内耳にはライスナー膜、蝸牛神経節における巨細胞の出現、神経細胞の変性壊死、脱落、血管条の空胞変性などが認められ、免疫組織化学でもCMV感染が確認された（図３)。さらに蝸牛の奇形を持った胎児も発見され、蝸牛管とコルチ体の過形成や、蝸牛先端が対称性になっているなどの変化が認められた。

D. 考察

モルモットの内耳はヒトと近似した構造を持ち、聴覚機能において最も重要な役割を果たす蝸牛にCMV 感染による変化が認められることが高度聴覚障害の原因となる。蝸牛管では感覚細胞である外毛細胞とそれにつながるらせん神経節に出血、壊死などの変化を認めたことはCMV 感染が感覚器細胞を直接、あるいは間接的に高度な傷害を与えることを意味する。らせん唇や血管条、らせん靭帯はこれら感覚器細胞の構造を支えるいわば支持器としての役割を果たすと考えられるが、これらの構造が壊されても聴覚障害の原因になることが予想される。ヘルペスウイルスの内耳への感染経路は正円窓あるいは卵円窓から感染するリンパ液を使ったルートと血行性ルート、さらに顔面神経を通った神経系のルートの３つが考えられている。単純ヘルペスウイルス及びGP-CMVをモルモットの内耳に注入した倉田・野村らの報告[3]では内耳に現れる最初の変化は外リンパ液中に見られるマクロファージの滲出で、その後、外リンパ液を伝わり、ウイルスが周辺組織に破壊的に浸潤していく過程が報告されている。出血、炎症部位は鼓室階、前庭階に激しく見られる点も今回の結果と一致するが、これは外リンパ液の流れに沿ってウイルスが伝幡していることを示している。血管条も豊富な血流を反映してか、ウイルスの標的になりやすく、萎縮や空胞変性などの変化がしばしば認められた。一方で感覚器細胞であるコルチ器の細胞は蝸牛管の中に存在するため比較的健全で、コルチ器のみが選択的に傷害される像は見られないことから、周辺の支持組織、あるいは神経組織が破壊され、聴覚障害が起こるというのがこの動物モデルにおけ

る聴覚障害の発症メカニズムと考えられる。

先天性ウイルス性聴覚障害では経胎盤感染であることは分かっているがその後、ウイルスが内耳までどのように到達し、どこから病変が形成されるか、明らかでない。本研究で観察された変化は感染がかなり進み、比較的激しい状態のものであり、内耳内での最初の感染の標的がどこであるかは明らかでない。血管条が標的の一つとなっているので血行性感染が考えられるが、神経細胞の変化や内外リンパ液中に感染細胞が見つかっていることから、神経系のルートやリンパ行性のルートも考えられる。蝸牛の奇形が発症するメカニズムも今後の研究課題である。奇形が発症するためにはウイルスがかなり早い時期に胎盤から胎児側に感染している必要があり、感染時期なども問題になろう。

CMV の垂直感染モデルとしてのモルモットを用いた動物実験モデルは様々な利点がある。モルモットの胎盤はヒトと同じ単層の栄養芽細胞を持つ。このことはウイルスが胎盤を経由して胎児に感染しやすい条件といえる。また、妊娠期間 10 週はウイルス感染後の変化や奇形を観察するのに十分な長さがある。今回の観察から、現在でもこの実験系は十分に機能し、分子機序を調べる上できわめて有用な実験系と考える。

E. 結論

サイトメガロウイルスによる先天性難聴の動物実験モデルとしてモルモットの実験系を再考察、再興した。内耳への感染実験では内耳のおける巨細胞封入体の出現など、多くの変化が認められた。妊娠モルモットへの感染実験では親モルモットの胎盤内にウイルス感染細胞を認め、胎児の内耳にも巨細胞の出現や神経細胞の変性壊死、脱落、血管条の空胞変性など、ウイルス感染による変化が認められた。本実験系を用い、今後、サイトメガロウイルスによる先天性難聴におけるウイルス感染機構とその病態の関連が分子レベルで解析が可能と考えられる。

（倫理面への配慮）

動物実験は当該施設（国立感染症研究所）の承認の後、国立感染症研究所動物実験ガイドラインに沿って行われた。

F. 健康危険情報

本研究で得られた成果に関して健康危険情報として報告しなければならない情報はない。

G. 参考文献

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I. 知的財産権の出願・登録状況

なし

Ⅲ. 研究成果の刊行に関する一覧表

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報道

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Ⅳ．研究刊行物・別刷

■ 子どもと親の健康と幸福を願って ■

臍の緒を利用した診断技術

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突然ですがサイトメガロウイルスというウイルスをご存じでしょうか？インフルエンザウイルスはとても有名ですが、それは冬にインフルエンザが流行して身近な話題となるからでしょう。それとは対照的にサイトメガロウイルスは多くの方が子どものうちに感染し、症状を起こすことがほとんどありません。話題になることもなく、あまり一般には知られてないウイルスでしょう。しかし完全におとなしいウイルスかというとそうでもありません。抵抗力が落ちている人には病気を起こします。また妊娠中の女性がこのウイルスに感染するとお腹の赤ちゃんにうつってしまい、赤ちゃんが先天性サイトメガロウイルス感染症という病気になってしまいます。

お母さんのお腹の中で感染したとしても、その赤ちゃんのほとんどは無症状です。しかし約一％の赤ちゃんには成長・発達の遅れや耳の聞こえが悪くなる（難聴）などの症状が出てきます。生まれてすぐに症状がある赤ちゃんの診断は比較的簡単なのですが、生まれてしばらく経ってから難聴などの症状が出てくることもあります。生まれてから三週間以上経ってしまうと、生まれた後に感染したものかお母さんのお腹の中で感染したものかを区別をすることが出来なくなってしまいますので、そのような場合はこの病気を疑ってもはっきり診断することは出来ませんでした。

そこで私たちは、日本人なら誰でも持っている臍の緒を利用して診断する方法を考えました。臍の緒は生まれたばかりの赤ちゃんの情報がそのまま残っているので、そこからサイトメガロウイルスが検出されれば、たとえその赤ちゃんが現在二十歳になっていても、過去に戻って生まれる前の感染なのかを診断することが可能になるのです。実際、この方法でサイトメガロウイルスが検出され診断がついたお子さんがいらっしゃいます。今後この方法を用いて原因がわからない発達の遅れや難聴を持つお子さんにこの感染症がどれほど関係しているかを調べていく予定です。

残念ながら、今のところこの病気に対するきちんとした予防法や治療法はまだありません。しかし近い将来、この研究を足がかりに効果的な予防法、治療法を確立していきたいと考えております。

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Expression of β-Catenin in Developing Auditory Epithelia of Mice

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Takebayashi S, Nakagawa T, Kojima K, Kim T-S, Kita T, Dong Y, Endo T, Iguchi F, Naito Y, Omori K, Ito J. *Expression of β-catenin in developing auditory epithelia of mice*. Acta Otolaryngol 2004; Suppl. 551: 18–21.

This study investigated the role of β-catenin in the development of mouse auditory epithelia. Inner ears obtained from embryonic and newborn mice were used. Expression of β-catenin was examined together with the expression of Ki-67, a marker for proliferating cells, or myosin VIIa, a marker for differentiated hair cells. In the early phase of development, intense expression of β-catenin was found in auditory epithelia in which a number of Ki-67-positive cells were identified. Together with a decrease in proliferating cells, the intensity and area of β-catenin expression were reduced. In addition, during differentiation and maturation of hair cells, the area of β-catenin expression was further limited. These findings suggest that patterns of expression of β-catenin are closely linked with the status of auditory epithelia development. *Key words: β-catenin, cell proliferation, cochlea, development, differentiation, hair cells.*

INTRODUCTION

Beta-catenin is firstly identified as an intracellular domain of cadherin-mediated adherens junction (1, 2). Beta-catenin is also a component of adherens junctions in sensory epithelia of the inner ear, and plays a role in restoration of epithelial architecture (3, 4). However, β-catenin plays a role as a mediator in the WNT signaling pathway (5–7). Translocation of β-catenin from the cytoplasmic membrane to the nucleus initiates cell proliferation. Hence, β-catenin could play a role in the regulation of cell proliferation of sensory epithelia of the inner ear. In fact, the contribution of β-catenin has been indicated in the early phase of morphogenesis of the inner ear (8–10). However, the role of β-catenin in development of the inner ear has not been elucidated in detail. This study therefore investigated the role of β-catenin in cell proliferation and differentiation in the auditory epithelia of mice during development.

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.

On embryonic day 12 (E12; $n = 3$), E13 ($n = 3$), E15 ($n = 3$) or E17 ($n = 3$), ICR mice pups were harvested from pregnant females that had been deeply anesthetized with xylazine. The pregnant females were then sacrificed by cervical dislocation. The pups themselves were anesthetized via anesthesia of the mother. ICR mice on postnatal day 7 (P7; $n = 3$) were deeply anesthetized with diethylether and sacrificed by cervical dislocation. Whole embryos (at E12, E13, E15 and E17) and dissected temporal bones (at P7) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) at 4°C for 12 h. The samples were then placed into 30% sucrose in PBS and incubated at 4°C overnight, prior to embedding in OCT compound (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) and frozen at −80°C until use. P7 inner ears were dissected and fixed as described above, prior to incubation in 5% EDTA for 24 h at 4°C for decalcification. Then 10 μm-thick cryostat transverse or coronal sections were mounted on γ-aminopropyl triethoxysilane-coated glass slides.

Immunohistochemistry for β-catenin was performed to demonstrate the localization of β-catenin in developing auditory epithelia. Immunohistochemistry for Ki-67 was employed to evaluate cell proliferation activity in auditory epithelia, and that for myosin VIIa was used to evaluate hair cell differentiation. Cryostat sections were permeablized in 0.2% Triton X-100 in PBS for 30 min at room temperature. Anti-Ki-67 rabbit monoclonal antibody (1:100; Lab Vision, Fremont, CA, USA) or anti-myosin VIIa rabbit polyclonal antibody (1:100; Affinity Bioreagents, Golden, CO, USA) was used as the primary antibody. The samples were incubated with primary antibodies at 4°C overnight. After two washes in PBS, the sections were incubated with Alexa Fluor 594-conjugated anti-mouse or -rabbit goat IgG (1:200; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Then, after two washes in PBS, the specimens were incubated with FITC-conjugated anti-β-catenin mouse monoclonal antibody (1:50; BD Biosciences, San Jose, CA, USA) for 3 h at 4°C.

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At the end of staining procedures, specimens were stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) for demonstration of nuclear chromatin.

RESULTS

Ki-67, which labels cells in G1, S, G2 or M phase, was used as a marker of proliferating cells in this study. On E12, Ki-67-positive cells demonstrated a diffused pattern in the ventromedial wall of the cochlear duct (Fig. 1A), which comprises the primordial auditory sensory epithelium (11). Intense expression of β-catenin was found in the apical surface of the auditory epithelia. Beta-catenin was predominantly distributed to the area corresponding to the cytoplasmic membrane (Fig. 1A). Beta-catenin expression was identified from the base to apex of epithelial cells. In addition, there were no apparent differences in expression patterns of β-catenin observed among epithelial regions. Some epithelial cells exhibited cytoplasmic accumulation of β-catenin.

On E13, Ki-67 was expressed in the inner and outer pharyngeal region, but not in the immature organ of Corti (OC; Fig. 1B). Beta-catenin expression was found in the apical surface of all epithelial cells. However, the areas in which Ki-67-positive cells were localized exhibited expression of β-catenin not only in the apical portion but also the basal portion, while β-catenin was expressed only in the apical portion in the premature OC (Fig. 1B). On E15, the number of Ki-67-positive cells in auditory epithelia significantly decreased (Fig. 1C). Expression of Ki-67 was found in the inner pharyngeal region, named the greater epithelial ridge (GER). At this stage, β-catenin expression was limited to the apical surface of epithelial cells (Fig. 1C). Expression of β-catenin at the basal portion of auditory epithelia became weak. In addition, an area exhibiting weak β-catenin expression appeared in the OC.

On E17, hair cells in the OC were distinguished from supporting cells by expression of myosin VIIa (Fig. 2A). Strong β-catenin expression was limited to the apical portion of the outer hair cells (OHCs) and GER (Fig. 2A). Weak β-catenin expression was identified in the apical portion between the OHCs and GER. On P7, the OC was morphologically matured. Immunohistochemistry for myosin VIIa clearly demonstrated single inner hair cells and three OHCs (Fig. 2B). Strong expression of β-catenin was observed in the apical portion of the OHCs and GER similar to the findings obtained at E17. However, the distribution of β-catenin in OHCs was further limited in the apex of OHCs corresponding to the location of adherens junctions. At this stage, pillar cells exhibited intense expression of β-catenin. In addition, the apical portion of inner hair cells (IHCs) lacked β-catenin expression.

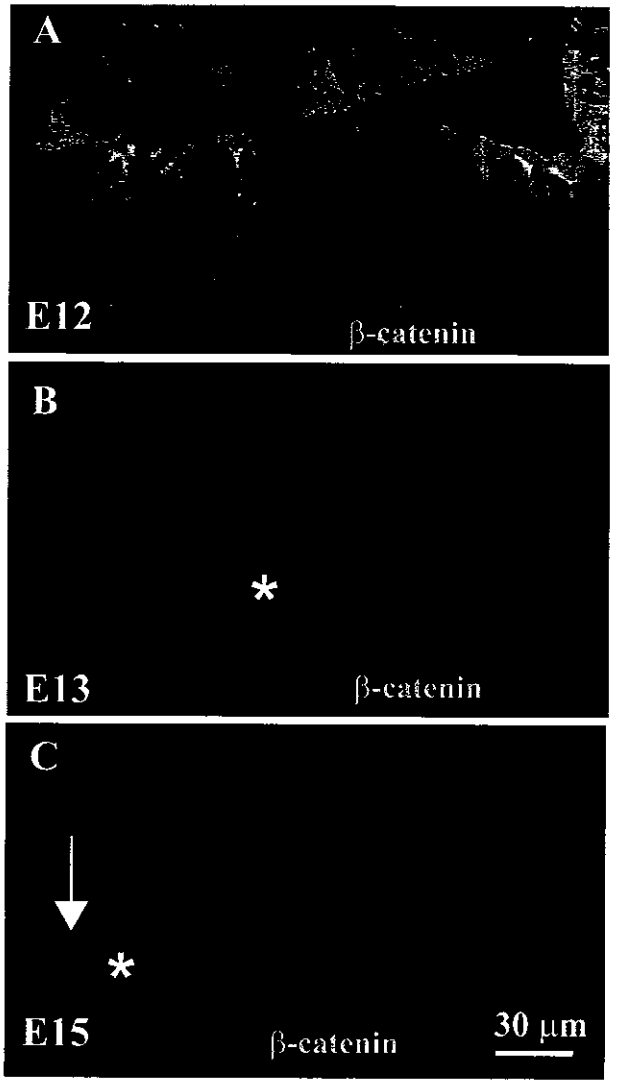

Fig. 1. Correlation of cell proliferation and β-catenin expression in developing auditory epithelia of mice. (*A*) On embryonic day 12 (E12), Ki-67-positive cells (*red*) were diffusely localized in the auditory epithelium. Beta-catenin (*green*) was expressed from the base to apex of epithelial cells. (*B*) On E13, in the immature organ of Corti (OC; *asterisk*), there were no Ki-67-positive cells observed, and β-catenin expression was limited in the apical portion of the OC. (*C*) On E15, the number of Ki-67-positive cells was apparently decreased. Beta-catenin expression was limited in the apical portion of epithelial cells. An area showing faint β-catenin expression appeared in the immature OC (*arrow*). Scale bar represents 30 μm.

DISCUSSION

The present findings demonstrate that patterns of β-catenin expression in auditory epithelia alter with the

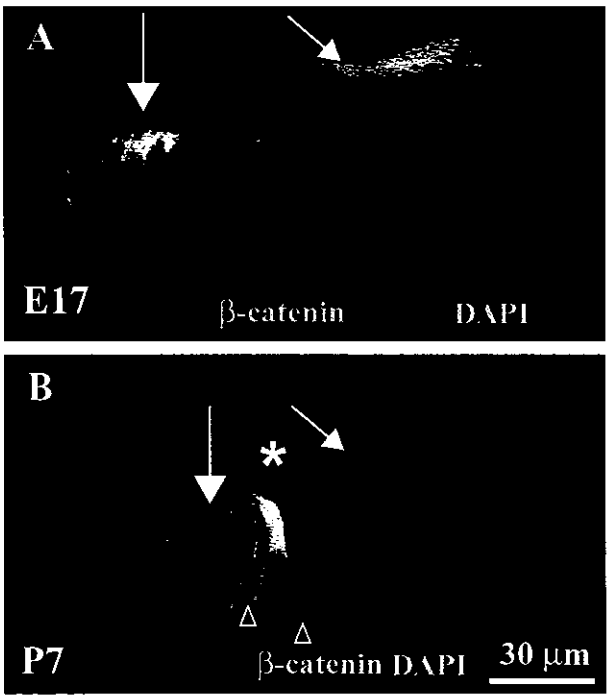

Fig. 2. Correlation of structural maturation of the organ of Corti (OC) and β-catenin expression in the developing auditory epithelia of mice. (*A*) On embryonic day 17 (E17), hair cells were identified by expression of myosin VIIa (*red*). Strong expression of β-catenin (*green*) was limited to the apical portion of outer hair cells (OHCs; *large arrow*) and the greater epithelial ridge (GER; *small arrow*). DAPI (*blue*) demonstrated the location of nuclei. (*B*) On postnatal day 7 (P7), the structure of the OC had matured. Immunohistochemistry for myosin VIIa clearly demonstrated cochlear hair cells (*red*). Strong β-catenin expression was also observed in the apical portion of OHCs (*large arrow*) and the GER (*small arrow*). However, β-catenin expression in OHCs was further limited to the apical surface. At this stage, strong β-catenin expression appeared in pillar cells (*triangles*). The apical portion of inner hair cells (*asterisk*) lacked expression of β-catenin. Scale bar represents 30 μm.

development of auditory epithelia. In the mouse cochlea, precursor cells enter terminal mitoses between E12 and E15 (12). During this period, the extended expression of β-catenin was observed in auditory epithelial cells, especially in the area in which active cell proliferation was occurring in this study. In the area with proliferating cells, β-catenin expression was identified from the base to apex of epithelial cells, while in quiescent areas expression was limited to the apical portion of epithelial cells. During the early stage, cytoplasmic accumulation of β-catenin was occasionally observed. These findings suggest that the cellular distribution of β-catenin is associated with cell proliferation in developing auditory epithelia. However, accumulation of β-catenin in the nucleus, which mediates up-regulation of the WNT signaling pathway (5–7), was not clearly observed in auditory epithelia during the observation period in this study. The present findings are therefore insufficient to determine the involvement of WNT signaling pathways in cell proliferation during the development of auditory epithelia.

With differentiation of the OC, the area expressing β-catenin became limited. Strong β-catenin expression was found only in the areas of OHCs and the GER, and IHCs exhibited no or faint expression of β-catenin. On P7, the distribution of β-catenin in OHCs was limited in the apical portion, and strong expression in pillar cells appeared. Alteration of the distribution of β-catenin in the OC observed in this study is associated with functional maturation of the OC. Morphological criteria correlated with the onset of cochlear function, including opening of the tunnel of Corti, was completed on P9–10 in mice (13). It is noteworthy that the onset of intense β-catenin expression in pillar cells was almost identical to the formation of the tunnel of Corti. Mouse OHC stereocilia appear on E15, and complete structural maturation on P10–14 (11). Therefore, alteration in the distribution of β-catenin in OHCs may be correlated with structural maturation of the apical surface of OHCs. These findings suggest that β-catenin plays a role in the structural maturation of the OC.

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Transplantation of Neural Stem Cells into Explants of Rat Inner Ear

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Damage and loss of hair cells in the inner ear is the most frequent cause of hearing loss and balance disorders. Mammalian hair cells do not regenerate in the conventional ways. To regenerate the hair cell in the mammalian inner ear we transplanted neural stem cells into explants of rat inner ear. The stem cells integrated successfully into the sensory epithelium of the vestibular organs, but not into the organ of Corti. This method is useful to investigate efficient ways to transplant stem cells into the inner ear. *Key words: balance disorders, hair cell, hearing loss, organ culture.*

INTRODUCTION

Ototoxic drugs and excessive noise exposure can cause cochlear and vestibular hair cell damage that result in hearing loss and balance disorders. Spontaneous recovery from damage in mammalian hair cells is limited (1, 2), while that in birds is evident (3, 4). Many attempts have been made to regenerate damaged hair cells, including application of neurotrophic factors (5) and gene manipulation (6). Another possible method of recovering auditory function involves transplantation of multipotential stem cells into the inner ear. The neural stem cell (NSC) is a multipotential cell that can differentiate into neurons or glial cells isolated from the cerebral cortex and hippocampus (7–9). In other parts of the brain and sensory organs, grafted NSCs survived and differentiated into neurons with region-specific functions (10, 11). We have previously transplanted NSCs into the rat cochlea *in vivo* (12). These NSCs successfully integrated into the sensory epithelia. However, the number of integrated cells was not sufficient and differentiation into the hair cell was not certain. To determine the optimal method of increasing the rate of integrated neurons and promoting stem cell differentiation into hair cells, it is useful to use *in vitro* preparation. In the present study, we transplanted rat NSCs into explants of rat inner ear. Cells successfully integrated into the sensory epithelia of otolith organs.

MATERIALS AND METHODS

Three-day-old Wistar rats (Japan SLC Inc., Hamamatsu, Japan) were deeply anesthetized with ether and decapitated. The organ of Corti, utricle and saccule were dissected from the temporal bone by the method described previously (13). Individual isolated organs were placed on mesh (Millicell-CM 12 mm; Millipore, Bedford, MA, USA) in the 24-well culture dish with the sensory epithelium facing upwards. Organs were cultured in the medium (MEM; Invitrogen Corp., Carlsbad, CA, USA) with 0.3% glucose and 0.03% Penicillin G) at 37°C in the CO_2 incubator. After 1 day of incubation, 0.5–3 mM gentamicin (Nacalai Tesque, Kyoto, Japan) was added to the medium for 12–36 h to damage the hair cells.

The primary adult rat NSCs (PZ5) were generously provided by Dr Fred Gage (9). NSCs were genetically marked with the β-galactosidase gene. Cells were maintained in medium (D-MEM F12 (Invitrogen Corp.), 1% N2-supplement, 0.002% basic FGF), and passages were carried out every 3 days. After gentamicin was washed from explants of inner ear, medium that included 2000–4000 NSCs was dropped onto the recipient organs. After transplantation, the organs were cultured in the same medium used for NSCs.

At 4–10 days after transplantation, the organs were fixed with 0.5% glutaraldehyde. The specimens were frozen and sectioned to 10 μm slices. They were stained with a solution containing X-gal (2% X-gal (5-bromo-4-chloro-1H-indol-3-yl β-D-galactopyranoside; Wako Pure Chemical Industries, Osaka, Japan), 5 mM K-ferricyanide, 5 mM K-ferrocyanide, 2 mM $MgCl_2$ in PBS) for 6–12 h at 37°C. β-Galactosidase-positive NSCs were specifically stained in blue with X-gal. For the negative control, the organ without transplantation was processed in the same way. For the positive control, NSCs in which lacZ was expressed were stained with X-gal using the same procedure.

RESULTS

NSCs were transplanted into eight utricles, four saccules and four organs of Corti. Integration of NSCs into the organs was observed in three utricles and one saccule, but not in any organ of Corti. All specimens in which NSCs integrated were exposed to low concentration (≤ 1 mM) of gentamicin. The interval between transplantation and fixation was between 7 and 10 days in the organs where integration

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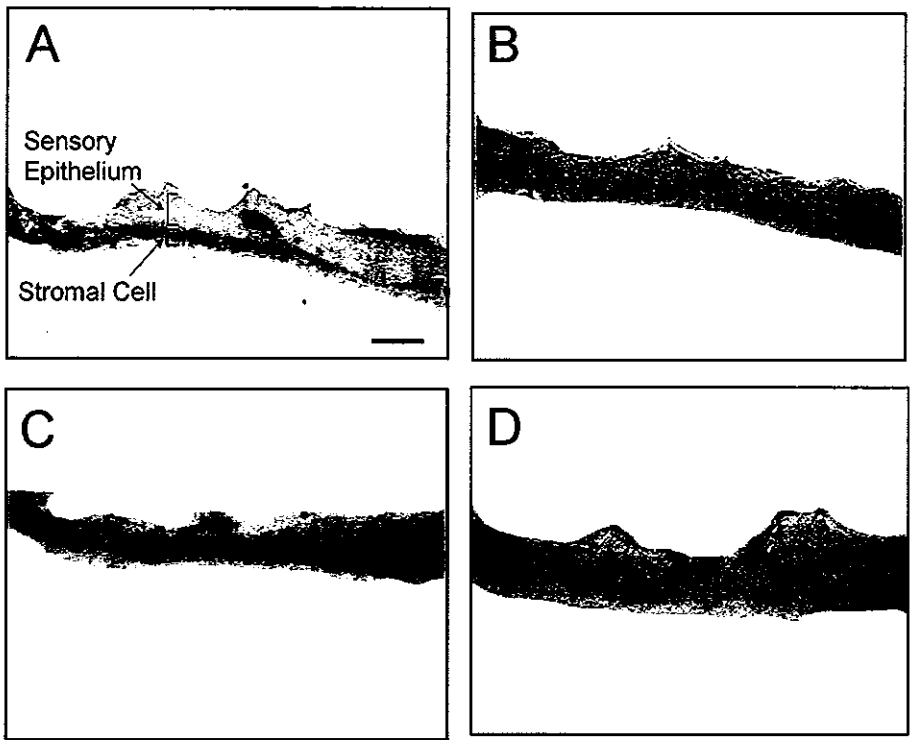

Fig. 1. Integrated neural stem cells (black or dark gray spots) stained by X-gal in the utricles (*A–C*) and saccule (*D*). Scale bar represents 20 μm for A, B, C and 15 μm for D.

was observed. Fig. 1 shows integrated NSCs in the utricle or saccule. In all specimens, the integrated cells were found in the sensory epithelia, and sometimes in the supporting cell layers. NSCs were found in two to four slices per specimen. Fig. 2 shows the X-gal-stained NSCs as positive controls.

DISCUSSION

The present study showed that the grafted NSCs migrated and integrated into the sensory epithelium of the vestibular organs. We have previously transplanted NSCs *in vivo*, and obtained successful integration into the cochlea (12). However, the number of integrated cells was not sufficient. There has not yet been any report describing integration of NSCs into the vestibular organs. In the cerebral cortex, grafted NSC was integrated into the host cortical circuitry and formed functional synapses (11). In the sensory organs, grafted NSCs integrated well into damaged retina, but not into normal retina (10, 14). It is possible that some types of trophic factors that promote migration and survival of grafted cells were secreted from the damaged tissue. Another possibility is that damage to intercellular adhesion facilitates the migration of grafted cells. In this study, we damaged the hair cells using aminoglycoside antibiotics. We found that NSCs did not integrate into organs that were severely damaged with a high concentration (> 1 mM) of gentamicin. A high concentration of aminoglycoside may damage not only hair cells but also supporting cells that are essential for integration of NSCs. The goal of the next study is to promote differentiation of the integrated cells into hair cells as occurs in other organs (15).

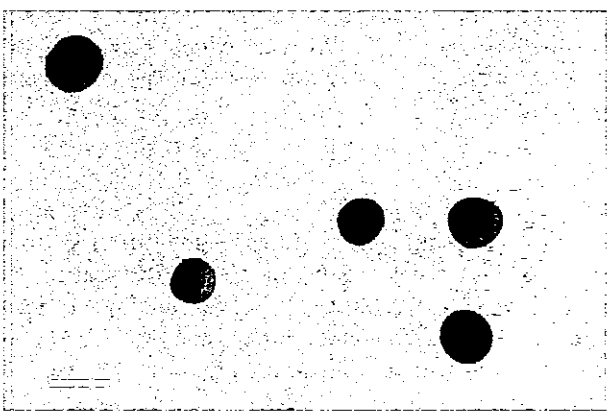

Fig. 2. X-gal-stained neural stem cells as positive controls. Scale bar represents 10 μm.

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Surgical Techniques for Cell Transplantation into the Mouse Cochlea

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Iguchi F, Nakagawa T, Tateya I, Endo T, Kim T, Dong Y, Kita T, Kojima K, Naito Y, Omori K, Ito J. *Surgical techniques for cell transplantation into the mouse cochlea.* Acta Otolaryngol 2004; Suppl. 551: 43–47.

This study investigated surgical procedures for cell transplantation into the mouse inner ear. Female C57BL/6 mice were used as recipient animals. Fetal mouse neural stem cells expressing green fluorescence were used as donor cells. Two methods, an injection of transplants from the lateral semicircular canal (LSCC) and from the cochlear lateral wall (CLW), were examined. Two weeks after transplantation, the distribution of transplant-derived cells in the cochlea was examined. Effects on auditory function were assessed by measurement of auditory brain stem responses (ABRs). Cochleae receiving cell transplantation from the LSCC exhibited robust survival of transplant-derived cells mainly in the scala vestibuli and scala tympani. Transplantation from the LSCC caused elevation of ABR thresholds by less than 10 dB SPL. However, transplantation from the CLW resulted in considerable hearing loss, even though transplant-derived cells settled in the scala media. These findings demonstrate that an approach from the LSCC can be utilized for cell transplantation into the perilymph without causing apparent auditory disorder, while an approach from the CLW delivers cells to the endolymph but appears to cause auditory dysfunction. *Key words: hearing loss, lateral semicircular canal, neural stem cell.*

INTRODUCTION

Recently, a number of studies have been performed on the use of stem cells for regenerating various organs including the inner ear. To utilize cell therapy for inner ear diseases, adequate donor cells and the technique for transplantation into the inner ear are required.

Many attempts have been made to introduce drugs or genes into the inner ear (1–3). The strategies developed for this purpose are not suitable for cell transplantation because of size differences in the administered materials. Therefore, surgical techniques for cell transplantation into the inner ear need to be established in order to realize regeneration of inner ear tissues by stem cell transplantation. This study investigated surgical procedures that can achieve transplantation at desirable sites of the inner ear with minimal invasiveness. Here we report our surgical procedures for cell transplantation into the mouse inner ear and discuss the efficacy of each procedure and surgical damage to auditory function.

MATERIALS AND METHODS

Preparation of donor cells

As a source of neural stem cells, we used enhanced green fluorescent protein (EGFP)-transgenic mice (generously donated 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 twelfth day of gestation (4). The neuroepithelium of the dorsal telencephalon of embryonic mice at embryonic day 11.5 was transferred to neurosphere culture medium (DMEM/F-12 (1:1) (Gibco, Grand Island, NY, USA) supplemented with 100 μg/ml transferrin (Sigma, St Louis, MO, USA), 25 μg/ml 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 completely dissociated by pipetting. 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, USA). At day 3, primary spheres were collected, spun down at 1000 rpm 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 neurosphere culture medium, and completely dissociated 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. On 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 neurosphere culture medium.

Surgical procedures

Female C57BL/6 mice at 10 weeks of age ($n = 10$) were used as recipient animals. We used two different surgical approaches to the mouse inner ear. One is an approach from the lateral semicircular canal (LSCC; $n = 5$) and another is that from the cochlear

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