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The Laryngoscope
Lippincott Williams & Wilkins, Inc.
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Drug Delivery to the Cochlea Using PLGA Nanoparticles

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Objectives: **This study aimed to investigate the efficacy of encapsulating therapeutic molecules in poly lactic/glycolic acid (PLGA) nanoparticles for drug delivery to the cochlea.** ***Study Design:*** **An experimental study.** ***Methods:*** **We examined the distribution of rhodamine, a fluorescent dye, in the cochlea, liver, and kidney of guinea pigs. Intravenous injection of rhodamine or rhodamine-encapsulated PLGA nanoparticles was used to target the fluorescent dye systemically to the liver, kidney, and cochlea, and these molecules were applied locally to the round window membrane (RWM) of the cochlea. The localization of rhodamine fluorescence in each region was quantitatively analyzed.** ***Results:*** **After systemic application of rhodamine nanoparticles, fluorescence was identified in the liver, kidney, and cochlea. The systemic application of nanoparticles had a significant effect on targeted and sustained delivery of rhodamine to the liver but not the kidney or cochlea. Rhodamine nanoparticles placed on the RWM were identified in the scala tympani as nanoparticles, indicating that the PLGA nanoparticles can permeate through the RWM. Furthermore, the local application of rhodamine nanoparticles to the RWM was more effective in targeted delivery to the cochlea than systemic application.** ***Conclusions:*** **These findings indicate that PLGA nanoparticles can be an useful drug carrier to the cochlea via local application.** ***Key Words:*** **Drug delivery, nanoparticle, cochlea, inner ear, rhodamine.**
Laryngoscope, **115:2000–2005, 2005**

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Editor's Note: This Manuscript was accepted for publication July 20, 2005.

This study was supported by a Grant-in-Aid for Regenerative Medicine Realization from the Ministry of Education, Science, Sports, Culture and Technology of Japan.

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DOI: 10.1097/01.mlg.0000180174.81036.5a

INTRODUCTION

The advancement of inner ear medicine will require the development of a means of nontraumatic and nontoxic delivery of therapeutic molecules to the cochlea. However, drug delivery to the cochlea presents a number of technical challenges, which have hindered the development of therapeutic strategies for the treatment of sensorineural hearing loss and related inner ear disorders. Reasons for the difficulty of drug delivery to the cochlea include the limited blood flow to the cochlea[1] and the existence of the blood-labyrinth barrier, which limits the transportation of molecules from blood to cochlear tissues.[2] The sustained delivery of therapeutic molecules is also critical for the efficient treatment of the cochlea, because bioactive molecules usually require a period of minutes or hours over which to produce their pharmacological actions. Consequently, a number of researchers are currently working to solve these problems and develop methods for the local application of molecules into the cochlea.[3,4]

Encapsulating bioactive molecules in nanoparticles consisting of biodegradable polymers such as poly-lactic/glycolic acid (PLGA) enables the sustained release of bioactive molecules in a controlled manner.[5] Recent advances in this field have made it possible to prepare PLGA nanoparticles using relatively simple techniques.[6] The present study aimed to examine the potential of PLGA nanoparticles for use as a vehicle for systemic and local drug delivery to the cochlea. We prepared PLGA nanoparticles encapsulating rhodamine, a red fluorescent dye, and administered these systemically or locally to adult guinea pigs. The profiles of rhodamine delivery to the cochlea were then analyzed using histologic techniques.

MATERIALS AND METHODS

Preparation of Rhodamine Nanoparticles

A PLGA formulation with a lactic/glycolic acid ratio of 50/50 was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). PLGA nanoparticles were prepared by an oil-in-water solvent diffusion method described elsewhere.[5] Briefly, a mixture of 20 μL of 0.5 mol/L zinc acetate aqueous solution and 0.7 mL of acetone dissolved in 20 mg of PLGA (Mw 8000) and 1 mg of rhodamine B (Sigma Chemical Co., St. Louis, MO) was added to 5 mL of a 0.5% (w/v) egg yolk lecithin (Sigma) aqueous

suspension. To chelate the zinc, 1 mL of 0.5 mol/L EDTA aqueous solution (pH 7.5) was added to the resulting suspension of nanoparticles. The nanoparticles were purified from unencapsulated rhodamine by ultrafiltration (YM-50, Millipore Co., Bilenica, MA) and subsequent gel filtration (PD-10 column, Amersham Biosciences, Tokyo, Japan). The diameter of the nanoparticles ranged from 140 to 180 nm.

Animals

Pigmented guinea pigs weighing 250 to 300 g were purchased from Japan SLC Inc. (Hamamatsu, Japan) for use in this study. The Animal Research Committee, Graduate School of Medicine, Kyoto University approved all experimental protocols, and animal care was supervised by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Systemic Application

Experimental animals were anesthetized with ketamine (20 mg/kg IM; Sankyo Co., Tokyo, Japan) and xylazine (5 mg/kg IM; Bayer, Tokyo, Japan). We exposed the right femoral vein and injected PLGA nanoparticles encapsulating rhodamine B (nanoRho; 0.25 mL, 20 μg/mL physiological saline) into 12 animals and normal, unencapsulated rhodamine B (Rho; 0.25 mL, 20 μg/mL physiological saline) into eight animals. At 10 or 120 minutes after injection of rhodamine, the animals were killed by cervical rotation under anesthesia with a lethal dose of ketamine and xylazine. The left temporal bones, kidney, and liver were immediately excised from the animals. The cochleae were dissected in cold 0.01-mol/L phosphate-buffered saline at pH 7.4 (PBS), and the kidney and liver were cut into approximately 1 cm^3 blocks. The specimens were immersed in 10% trichloroacetic acid in PBS at 4°C for 24 hours. After washing with PBS, specimens were embedded in OCT compound (Tissue-Tek, Sakura Finetechnical, Tokyo, Japan) and frozen at −80°C until use.

Local Application

Under general anesthesia with ketamine and xylazine, the bulla of the left temporal bone was exposed using a retroauricular approach. A small hole was made on the bulla to expose the round window membrane (RWM). A piece of gelfoam immersed with nanoRho (0.25 mL, 20 μg/mL physiological saline) was placed on the RWM of four animals. The same amounts of unencapsulated Rho were applied to another four animals. The cochleae were then collected 24 hours after application. Tissue preparation was performed according to the procedure described above for samples from systemically treated animals. To examine the dynamics of these molecules in the cochlea, we used a glass pipette to inject 10 μL of nanoRho through the RWM into the scala tympani of three animals, at the same concentration as that placed on the RWM. The temporal bones were collected 24 hours after the injection and used for histologic analysis.

Analysis of Rhodamine Distribution

Tissue specimens were cut into 10-μm thick sections. Four mid-modiolus sections from the cochleae of each animal were used for histological analysis. The specimens were covered with Vector Shield (Vector Laboratories Inc., Burlingame, CA), and viewed with a Nikon ECLIPSE E600 fluorescence microscope (Nikon, Tokyo, Japan). We counted the number of red fluorescent rhodamine particles within the cochlea in every section and calculated the mean number of particles from four sections, for each animal, for statistical analyses.

Four randomly selected sections from the liver and kidney of each animal were also used for histologic analysis. The numbers of rhodamine particles in five random fields of 0.4 mm^2 were counted in each section. The mean number of rhodamine particles from the four sections was determined for each animal.

Statistics

We calculated the differences in the numbers of red fluorescent particles between nanoRho and Rho at 10 or 120 minutes after systemic application. The Mann-Whitney U test was used for all statistical calculations, and a probability (P) value of less than 0.05 was considered to be significant. Values are expressed as mean ± SE.

RESULTS

Liver and Kidney

In the liver, rhodamine fluorescence was found in every experimental group (Fig. 1). Rhodamine fluorescence was observed as moderate red fluorescent dots following application of Rho (Fig. 1C), and as intense red fluorescent dots following nanoRho application (Fig. 1A, B). A number of red fluorescent dots were observed at 10 minutes after Rho application (74.5 ± 4.5), but this figure had decreased significantly to 8.5 ± 0.6 at 120 minutes (P = 0.0008, Fig. 2A). Numerous red fluorescent dots were found in the liver after nanoRho application: 542.4 ± 61.2 at 10 minutes and 533.8 ± 24.8 at 120 minutes (Fig. 2A). There was no significant difference in numbers of red fluorescent dots between these two time points, indicating that the PLGA nanoparticles promoted sustained delivery of rhodamine to the liver. The application of nanoRho resulted in significantly higher numbers of red fluorescent dots in the liver than were seen after Rho application, at 10 (P = 0.0002) and 120 minutes (P = 0.0002) after application (Fig. 2A), showing that PLGA nanoparticles are significantly more effective at targeting delivery of Rho to the liver.

In the kidney, few or no red fluorescent dots were identified after application of Rho (10 min: 0.3 ± 0.3, 120 min: 0; Fig. 1D, 2B) or nanoRho (10 min: 4.4 ± 2.0, 120 min: 0.2 ± 0.2; Fig. 2B). There was no significant difference in the number of red fluorescent dots in the kidney between 10 and 120 minutes for either Rho or nanoRho application, or between these two preparations of Rho. These findings indicate that PLGA nanoparticles have no significant impact on the effectiveness of targeted delivery of rhodamine to the kidney.

Cochleae after Systemic Application

In the cochlea, no red fluorescent dots were observed after systemic application of Rho, whereas they were observed after systemic application of nanoRho (Fig. 1E-H). Rhodamine particles were localized in spiral prominence (Fig. 1E), stria vascularis (Fig. 1F), or the cochlear melodious (Fig. 1G, H). The regions in which rhodamine particles were localized corresponded to the location of blood vessels in the cochlea. Rhodamine fluorescence was found in the apical, middle, and basal portion of the cochlea. The number of red fluorescent dots after nanoRho application was 2.8 ± 0.3 at 10 minutes and 0.1 ± 0.1 at 120 minutes (Fig. 2C), and the difference between 10 and 120 minutes was significant at $P < 0.0001$. The numbers of rhodamine

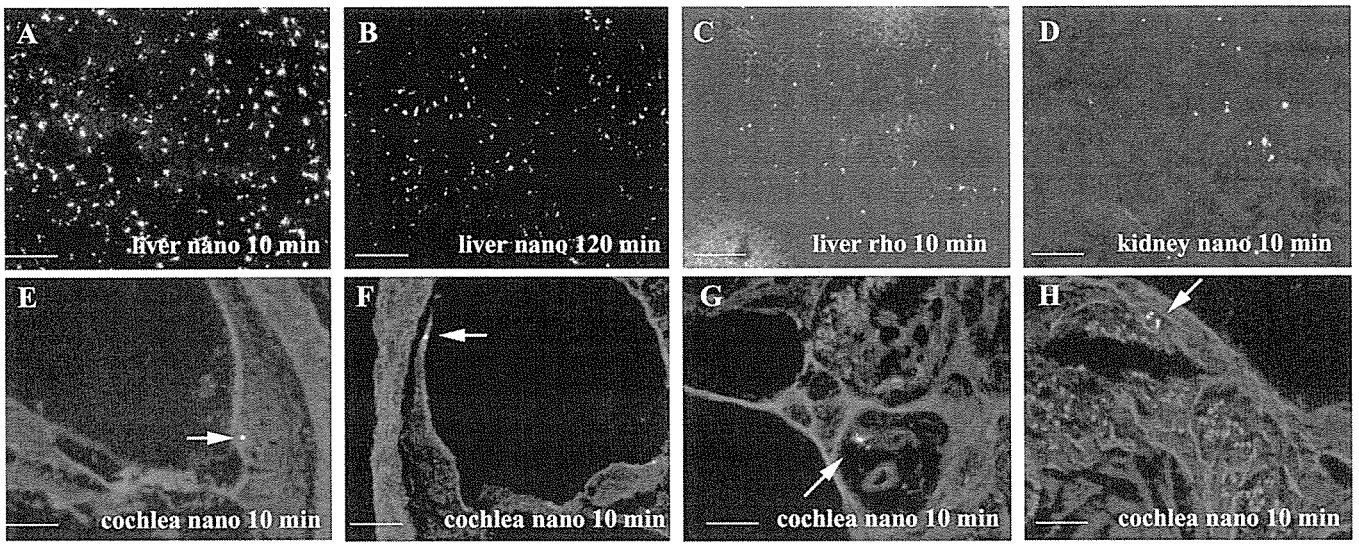

Fig. 1. Localization of rhodamine fluorescence in the liver, kidney, and cochlea after systemic application of rhodamine-nanoparticles or rhodamine Rhodamine fluorescence is frequently observed in the liver at 10 (**A**) and 120 minutes (**B**) after application of rhodamine-nanoparticles (nano). Rhodamine fluorescence is also identified in the liver at 10 minute after rhodamine (rho) application (**C**). A few dots with fluorescence are found in the kidney (**D**) and cochlea (**E-H**). In cochleae, rhodamine fluorescence is identified in the spiral prominence (arrow in E), stria vascularis (arrow in **F**), and modiolus (arrows in **G**, **H**). Scale bars represent 50 μm.

particles following systemic application of nanoRho were significantly higher than those following systemic application of Rho at 10 minutes ($P = 0.0001$), but not at 120 minutes ($P = 0.4142$; Fig. 2C).

Cochleae after Local Application

Following a local injection of nanoRho into the scala tympani, numerous rhodamine particles showing strong red fluorescence were found distributed from the base to the apex of the cochlea 24 hours after application (Fig. 3A). Rhodamine particles were located in the scala tympani and vestibule. After the application of nanoRho to the RWM, rhodamine fluorescence was found in the scala tympani of the basal and middle portion of the cochlea (Fig. 3C, D). The majority of rhodamine particles were located in the basal portion. The number of red fluorescent dots in the cochlea was 28.8 $\pm$ 4.5 after local nanoRho application on the RWM. There were residual rhodamine particles on the RWM (Fig. 3B), which showed intense fluorescence as well as those in the scala tympani. The number of rhodamine particles after local application of nanoRho was approximately 10-fold higher than that at 10 minutes after systemic application. Conversely, no rhodamine fluorescence was found in the cochlea after local application of unencapsulated Rho.

DISCUSSION

Most of the drugs for the treatment of inner ear diseases have been administered systemically. We then evaluated the effects of PLGA nanoparticles on drug delivery to the cochlea via systemic application. We used the liver as a control organ to analyze the distribution of rhodamine nanoparticles after systemic application, because the liver has a good blood supply, with abundant phagocytes, in which nanoparticles have a characteristic tendency to accumulate.[7] The presence of rhodamine fluorescence in the liver following systemic application of either nanoRho or Rho confirms the accuracy of systemic application of these molecules. The numbers of rhodamine fluorescence dots in the liver after application of nanoRho were significantly higher than those after application of Rho. Furthermore, the levels of fluorescence after the administration of nanoRho showed no significant decrease at 120 minutes after application, indicating targeted and sustained delivery of rhodamine to the liver by encapsulating PLGA nanoparticles. In contrast to the liver, no significant effects of PLGA nanoparticles on the delivery of rhodamine to the kidney were found in the present study. The differences in the distribution of rhodamine particles to the liver and kidney might be caused by the organ-specific characteristics, including the distribution of phagocytes.

The blood flow to the liver or kidney is much higher than that for the cochlea.[8] Rhodamine particles were found in the cochlea after systemic application of nanoRho, despite the presence of a small blood supply to this organ, suggesting the efficacy of PLGA nanoparticles for drug delivery to the cochlea. However, few rhodamine particles in the cochlea were identified 120 minutes after systemic application. The present findings also indicate that rhodamine particles observed in the cochlea are located in cochlear vessels. The PLGA nanoparticles used in the present study can take a couple of days to release 50% of the molecules they contain.[5] Consequently, rhodamine particles located in the cochlear vessels at 10 minutes after application might be removed via cochlear blood flow within 120 minutes. These findings indicate that systemic application of PLGA nanoparticles might not have significant effects on cochlear drug delivery under physiologic conditions.

Rhodamine particles were identified in the cochlea 24 hours after local application of nanoRho. PLGA is a bio-

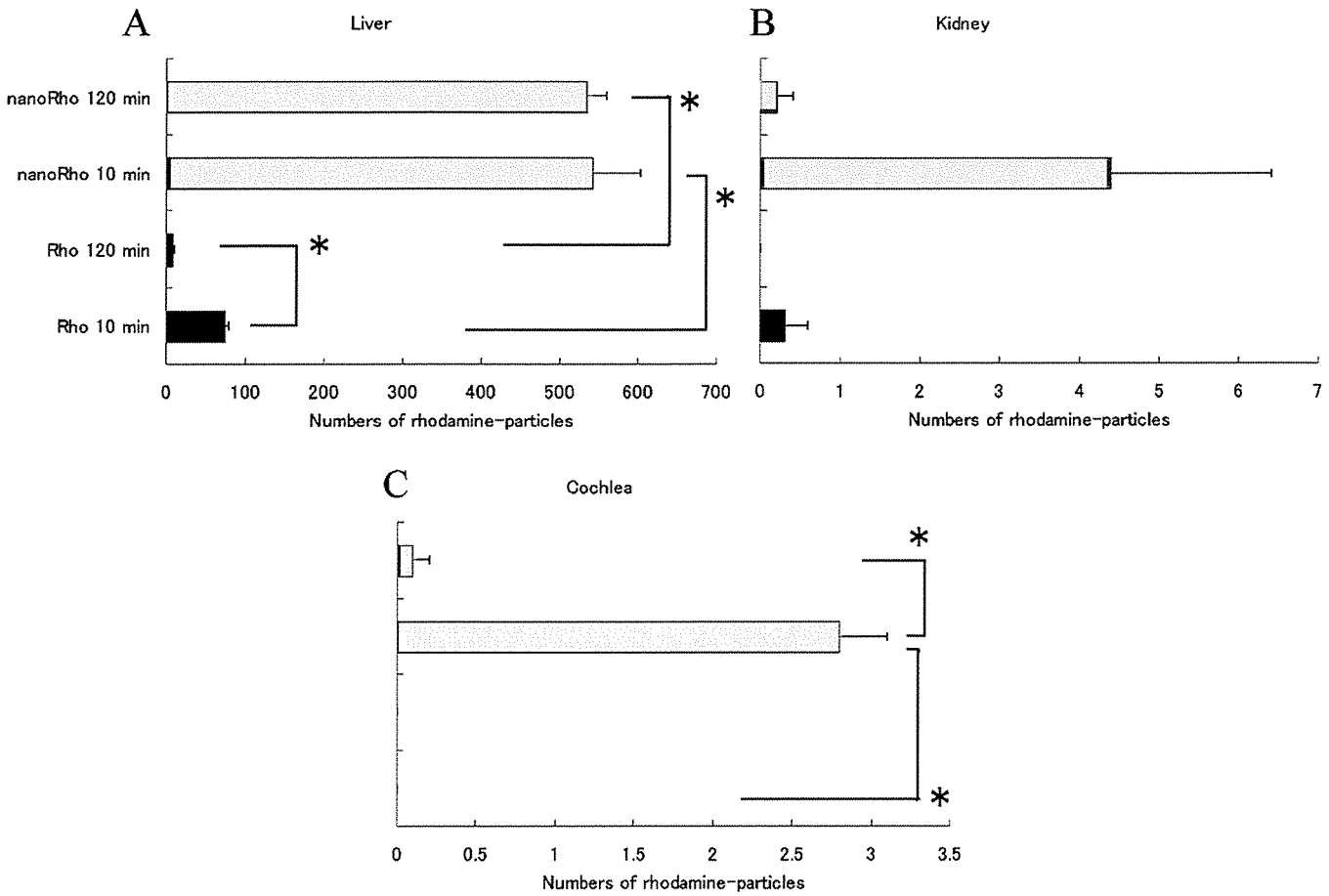

Fig. 2. The mean numbers of rhodamine particles in the liver, kidney, and cochlea at 10 or 120 minutes after systemic application of rhodamine nanoparticles (nanoRho) or normal rhodamine (Rho). The x-axis indicates the numbers of rhodamine-particles in 0.4 mm^2 for the liver and kidney and those in one section for the cochleae. Bars represent standard errors. Asterisks indicate statistical significance ($P < 0.005$, Mann-Whitney test).

degradable polymer, so rhodamine can be released from nanoparticles in both the middle and the inner ear. Rhodamine particles observed in the cochlea after local nanoRho application exhibited strong fluorescence, similar to that seen after systemic nanoRho application. Residual rhodamine particles on the RWM also exhibited strong red fluorescence. In addition, PLGA nanoparticles are small enough to pass through the RWM.[9] Therefore, the rhodamine fluorescence observed in the cochlea after local application of nanoRho application may result from rhodamine nanoparticles that have passed through the RWM and not from rhodamine released from PLGA nanoparticles in the middle ear. Previous studies have demonstrated that cochlear fluids have an extremely slow flow rate.[10] Rhodamine particles observed in the cochlea after local nanoRho application were located in the perilymphatic space, which indicates that the clearance of rhodamine particles depends on the flow of perilymph. If the flow of perilymph is slow, it would follow that the clearance of rhodamine particles from the cochlea might also be slow, thereby resulting in sustained release of rhodamine from rhodamine particles in the perilymph. In addition, the numbers of rhodamine particles in the cochlea after local nanoRho application are apparently higher than those after systemic application. Therefore, the local application of PLGA nanoparticles to the RWM may be an effective strategy for targeted and sustained drug delivery to the cochlea.

Rhodamine fluorescence was found from the basal to apical portion of the cochlea after a local injection of nanoRho into the scala tympani, whereas it was observed only to a limited degree in the basal portion of the cochlea after direct application of nanoRho to the RWM. The distribution of molecules within the cochlear fluid spaces is dominated by passive diffusion,[11] the rate of which depends on the physiologic characteristics of the molecules, particularly molecular weight.[12] Therefore, rhodamine released from PLGA nanoparticles in the perilymphatic space possibly spreads more toward the apical portions of the cochlea than PLGA nanoparticles applied to the RWM. Further studies are required to optimize the profile of nanoparticles in accordance with the desired distribution and release of drugs they contain.

Various therapeutic molecules for inner ear diseases can be encapsulated in PLGA nanoparticles and applied as intratympanic drugs. The efficacy of encapsulating betamethasone phosphate in PLGA nanoparticles has already been confirmed using animal models for rheumatoid

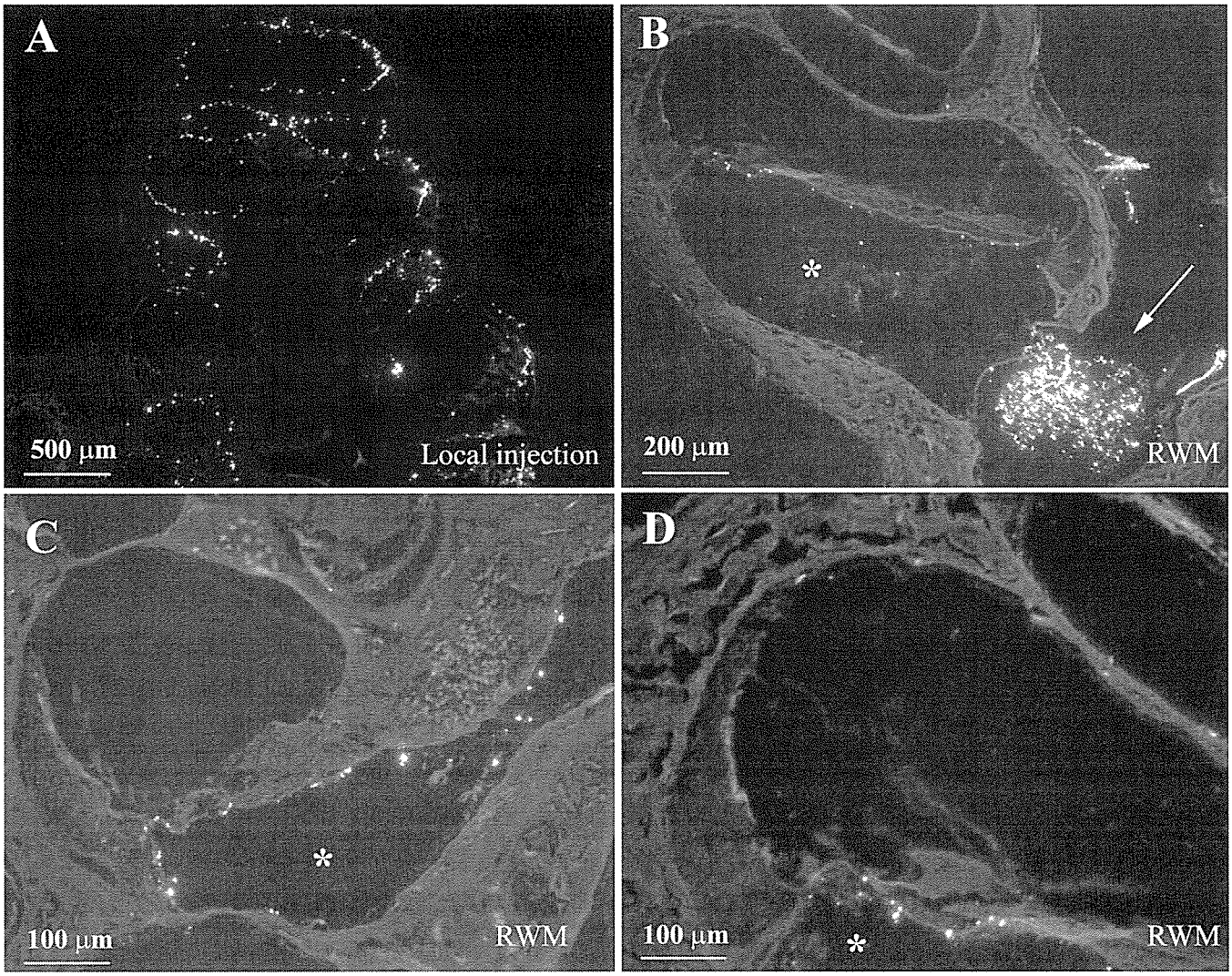

Fig. 3. Localization of rhodamine-particles in cochleae after local application of rhodamine nanoparticles After a local injection of rhodamine-nanoparticles into the scala tympani, numerous rhodamine-particles are found distributed from the basal to the apical portion of the cochlea (**A**). Rhodamine particles are identified in the scala tympani of the basal portion of cochleae after application of rhodamine-nanoparticles on the round window membrane (asterisks in **B–D**). Residual rhodamine particles on the round window membrane are indicated by an arrow (**B**). Scale bars represent 500 μm in **A**, 200 μm in **B**, and 100 μm in **C** and **D**.

arthritis.[13] Local gentamicin application has been used for the control of intractable vertigo in Ménière disease.[14] PLGA nanoparticles can be utilized for controlled release of gentamicin. Given these findings, we intend to examine the effects of PLGA nanoparticles encapsulating therapeutic molecule on models of inner ear diseases.

CONCLUSIONS

In the present study, rhodamine nanoparticles were identified in the cochlea after systemic or local application, suggesting that PLGA nanoparticles have a potential use in drug delivery to the cochlea. The transfer of PLGA nanoparticles through the RWM to the perilymph was also demonstrated, indicating the efficacy of encapsulating drugs in PLGA nanoparticles as a strategy for sustained and targeted drug delivery to the cochlea.

Acknowledgments

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.

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Serofendic acid promotes survival of auditory hair cells and neurons of mice

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Received 1 March 2005; accepted 2 March 2005

Serofendic acid is a newly discovered neuroprotective substance derived from fetal calf serum. It has previously been shown to protect cortical neurons from the cytotoxicity of nitric oxide, glutamate and oxygen species. In the present study, we examined the protective effects of serofendic acid on auditory hair cells exposed to aminoglycoside toxicity using explant cultures of mouse auditory epithelia. We also determined the effect of serofendic acid on auditory neurons experiencing neurotrophin deprivation using primary cultures of mouse spiral ganglion neurons. Supplementation with serofendic acid significantly promoted the survival of auditory hair cells and neurons, and its protective effects were stronger than those of the caspase inhibitor z-VAD-fmk. These findings demonstrate the great potential of serofendic acid for protection of the auditory system. *NeuroReport* 16:689–692 © 2005 Lippincott Williams & Wilkins.

Key words: Cochlea; Hair cell; Ototoxicity; Protection; Spiral ganglion neuron

INTRODUCTION

Sensorineural hearing loss (SNHL) is one of the most prevalent disabilities affecting the aging populations of industrialized countries. At present, therapeutic strategies are limited to hearing aids and cochlear implants. Excessive noise, ototoxic drugs, genetic disorders and aging all contribute to the causes of SNHL. Previous studies on human temporal bones have indicated that the loss of auditory hair cells and/or neurons, spiral ganglion neurons (SGNs), is a major cause of SNHL [1,2]. The protection of auditory hair cells and neurons is therefore a crucial issue for the treatment of SNHL.

Aminoglycoside toxicity is a key cause of drug-induced hearing loss. Previous studies have indicated the activation of apoptotic pathways [3,4] and the involvement of nitric oxide (NO) [5], excitotoxicity [6] or oxidative stress [7,8] in the processes of aminoglycoside-induced hair-cell death. Deprivation of the neurotrophic support from hair cells is an important cause of SGN degeneration [9,10]. Deprivation of neurotrophins leads to the generation of reactive radical species and the activation of apoptotic pathways [11,12].

Serofendic acid (SFA), which is a recently identified lipophilic substance isolated from the ether extract of fetal calf serum, displays potent neuroprotective activity [13]. SFA is a 15-hydroxy-17-methlysulfinylatisan-19-onic-acid sulfur-containing atisane-type diterpenoid. Synthetic SFA can exert protective effects on neurons undergoing apoptosis induced by NO toxicity, excitotoxicity and oxidative stress [13–17]. SFA is therefore a good candidate for the protection of auditory hair cells from aminoglycoside toxicity and the protection of SGNs from neurotrophin deprivation.

In the present study, we examined the protective effects of SFA on auditory hair cells exposed to aminoglycoside toxicity using mouse auditory sensory epithelia explant cultures, and on SGNs exposed to neurotrophin deprivation using SGN primary cultures.

MATERIALS AND METHODS

Materials: Synthetic SFA was a gift from Eisai Co. Ltd (Tokyo, Japan). z-VAD-fmk, which is a general caspase inhibitor, was purchased from R&D Systems Inc. (Minneapolis, Minnesota, USA). SFA and z-VAD-fmk were dissolved in 0.1% dimethylsulfoxide (DMSO; Wako Pure Chemical Industries Ltd, Osaka, Japan) in double-distilled water before supplementation into the culture medium. The final concentrations of the agents supplemented in the culture medium are described below.

Experimental animals: Postpartum day 3 (P3) ICR (Institute for Cancer Research) mice were purchased from SLC Inc. (Hamamatsu, Japan). The experimental protocols and animal care procedures were approved by the Institute of Laboratory Animals Animal Research Committee, Graduate School of Medicine, Kyoto University, Japan. All experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Explant culture of auditory epithelia: P3 mice were killed by cervical dislocation under deep anesthesia with ether. The cochleae were immediately dissected out from the temporal bones. After removal of the bony walls, cochlear

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lateral walls and SGNs in phosphate-buffered saline (PBS; pH 7.4), the sensory epithelia were placed on a sterile filter membrane (Millicell 12 mm; Millipore, Billerica, Massachusetts, USA) in a standard medium composed of minimum essential medium (Invitrogen Corp., Carlsbad, California, USA) supplemented with 3 g/l glucose and 0.3 g/l penicillin G potassium salt (Nacalai Tesque Inc., Kyoto, Japan), and subsequently placed in a 24-well culture plate (Asahi Techno Glass Corp., Tokyo, Japan). The auditory epithelia were then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h.

The cultured auditory epithelia were divided into five experimental groups: the control group, the neomycin (NM) group, the NM + SFA group, the NM + z-VAD-fmk group and the NM + SFA + z-VAD-fmk group. In the control group (n=7 cultures), the specimens were cultured in the standard medium. In the NM group (n=8 cultures), the specimens were cultured in the control medium containing 0.6 mM NM (Wako Pure Chemical Industries Ltd) and 0.1% DMSO. The NM + SFA group was divided into three subgroups according to the concentration of SFA in the medium: auditory epithelia were incubated with the control medium supplemented with 0.6 mM NM and 1, 10 or 100 μM SFA (n=6 cultures in each subgroup). The specimens in the NM + z-VAD-fmk group (n=5 cultures) were incubated with the control medium containing 0.6 mM NM and 100 μM z-VAD-fmk. In the NM + SFA + z-VAD-fmk group, the specimens were cultured in the control medium supplemented with 0.6 mM NM, 100 μM SFA and 100 μM z-VAD-fmk. All the specimens were cultured for 72 h.

Primary culture of spiral ganglion neurons: The SGNs of P3 mice were harvested according to the methods described by Lallemend and colleagues [11]. Dissociated SGNs were seeded in a four-well plate (Nalge Nunc International, Rochester, New York, USA) on 12-mm round coverslips coated with poly-D-lysine-laminin (BIOCOAT; Becton Dickinson Labware, Bedford, Massachusetts, USA) and maintained in Dulbecco's modified Eagle's medium (Invitrogen Corp.) supplemented with N1 (Sigma-Aldrich Corp., St Louis, Missouri, USA), 6 g/L glucose, 50 ng/ml human recombinant neurotrophin-3 (hrNT-3; R&D Systems Inc.) and 50 ng/ml brain-derived growth factor (hrBDNF; R&D Systems Inc.). After the initial 24 h incubation, the neurotrophins were withdrawn and replaced with the culture medium supplemented with 100 μM SFA or 100 μM z-VAD-fmk instead of neurotrophins. Control cultures were incubated with the culture medium supplemented with hrNT-3 and hrBDNF. SGN cultures with no supplements were included in order to determine the effect of neurotrophin withdrawal on SGN survival. All SGN cultures were incubated for an additional 48 h.

We monitored pH of each culture medium at the beginning and endpoints of cultures. The pH of the culture medium of each experimental group was maintained between 7.2 and 7.4.

Histological analysis: At the end of the culture period, specimens were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and processed as whole mounts for histological analysis. Specimens were permeabilized in 0.5% Triton X-100 in PBS for 30 min at room temperature and then incubated with the BlockAce blocking solution (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan) for 30 min at room temperature. Immunocytochemistry for myosin VIIa was employed for analysis of the surviving hair cells. The number of surviving SGNs was evaluated by combining the expression of βIII tubulin with the nuclear morphology determined through the use of DAPI (Molecular Probes, Eugene, Oregon, USA). SGNs with nuclear pyknosis were excluded from the quantitative analysis. Anti-myosinVIIa rabbit polyclonal antibody (1:500; a gift from Tama Hasson, University of California, San Diego, California, USA) and anti-βIII tubulin rabbit polyclonal antibody (1:500; clone TUJ1; Covance Inc., Princeton, New Jersey, USA) were used as primary antibodies. Alexa-488-conjugated anti-rabbit goat IgG (Molecular Probes) was used as a secondary antibody. Samples were mounted onto glass slides, coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, California, USA) and viewed using a Leica TCS SP2 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany).

Statistical analyses: Statistical analyses were performed using one-way factorial analysis of variance (ANOVA). Each experiment was performed in triplicate and repeated on at least three different occasions. Individual differences were examined using Scheffe's post-hoc test for significance ($p<0.05$). The number of inner and outer hair cells (IHCs and OHCs, respectively) was counted in a 0.2-mm long region of the middle portion (approximately 1.2 mm from the apical edge) of the auditory epithelia. The number of surviving SGNs was counted in each well, and neuronal survival was shown as a percentage of the mean of the control cultures with neurotrophins. Data were expressed as the mean ± standard deviation (SD).

RESULTS

Explant culture of auditory epithelia: Myosin VIIa immunohistochemistry in the control group specimens revealed a single row of IHCs and three rows of OHCs (Fig. 1a and d). In contrast, a massive loss of hair cells was seen in the auditory epithelia of the NM group (Fig. 1b and e): a 94% loss of OHCs and a 40% loss of IHCs was observed in the middle portion of the auditory epithelia (Fig. 1g and h). These findings demonstrate that NM caused significant damage to the auditory hair cells. Specimens supplemented with SFA tended to show improved survival of the hair cells (Fig. 1c). In specimens treated with 100 μM SFA, a single row of IHCs was identified in the middle portion of the auditory epithelia (Fig. 1f). The characteristic three rows of OHCs were not observed in the same portion of the auditory epithelia, although surviving OHCs were identified (Fig. 1f). SFA supplementation exerted protective effects on OHCs in a dose-dependent manner (Fig. 1h). The differences in the number of OHCs between the NM and NM + 10 or 100 μM SFA groups were statistically significant (p=0.0463 for 10 μM and p=0.0023 for 100 μM). In contrast, no significant differences were observed in the number of IHCs between the NM and NM + SFA groups (Fig. 1g), although increasing numbers of IHCs were observed in the SFA-supplemented groups.

z-VAD-fmk, which is a general caspase inhibitor, also tended to increase the number of surviving OHCs, although no significant difference was observed in the number of OHCs between the NM and NM + z-VAD-fmk groups (Fig. 1h). However, the specimens treated with both SFA

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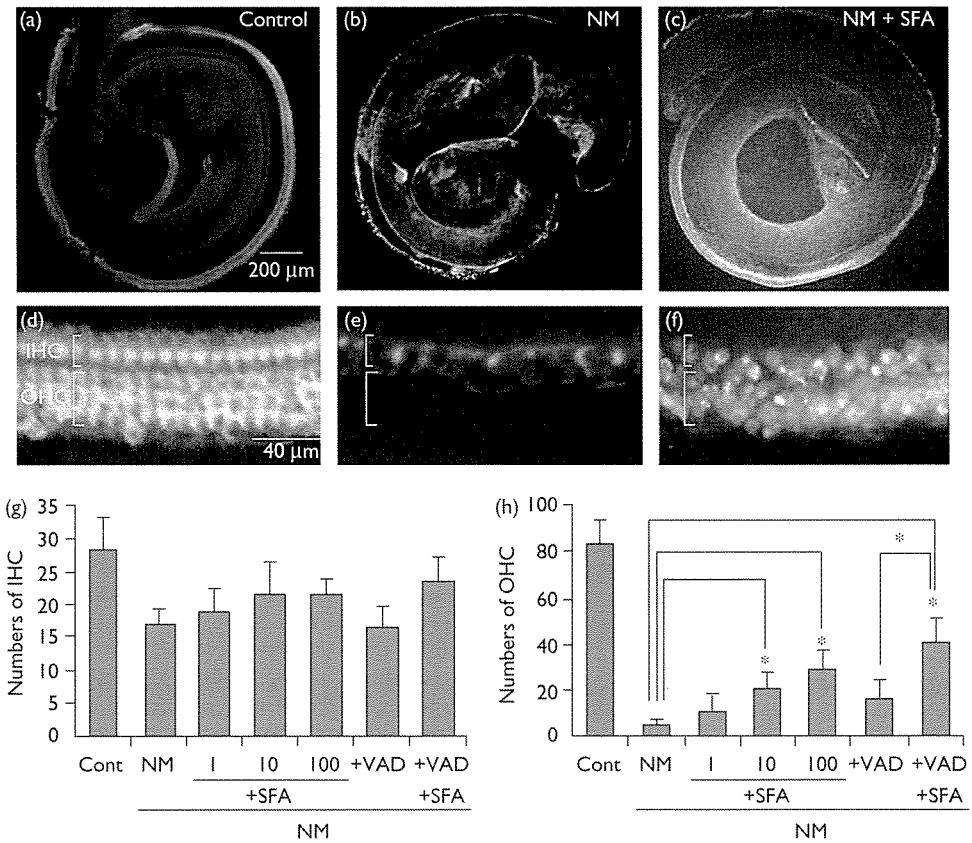

Fig. 1. Serofendic acid (SFA) effects on hair cell survival against neomycin (NM) toxicity. Immunostaining for myosin VIIa demonstrates surviving hair cells in cultured auditory epithelia (**a–f**). The upper panels (a–c) show gross views of cultured auditory epithelia and the lower panels (d–f) show high magnification views of their middle portions. The auditory epithelia cultured with the control media (a,d) demonstrate a single row of inner hair cells (IHCs) (small bracket) and three rows of outer hair cells (OHCs) (large bracket), while those cultured with the medium containing NM (b,e) demonstrate partial loss of IHCs and an almost total loss of OHCs. Supplements of 100 μM SFA (c,f) rescue hair cells from NM toxicity. The scale bars represent 200 μm in a–c and 40 μm in d–f. The numbers of surviving IHCs and OHCs in a 0.2-mm long region in the middle portion of the auditory epithelia are shown (**g**, **h**). Significant differences are observed in OHC numbers between the NM and NM + SFA (10 or 100 μM) groups, between the NM and SFA + z-VAD-fmk groups, and between the NM + z-VAD-fmk and NM + SFA + z-VAD-fmk groups (*$p < 0.05$, ANOVA with Scheffe's post-hoc test). The error bars represent the SDs.

and z-VAD-fmk exhibited the highest number of surviving OHCs (Fig. 1h). The difference in the number of OHCs between the SFA (100 μM) and SFA + z-VAD-fmk groups was not significant; however, that the difference between the z-VAD-fmk and SFA + z-VAD-fmk groups was significant (p=0.0023), indicating that the application of SFA had additional effects on the promotion of OHC survival by z-VAD-fmk. No protective effect of z-VAD-fmk was found with respect to the number of IHCs (Fig. 1g).

Primary culture of spiral ganglion neurons: The neuronal survival of SGNs cultured without neurotrophins was reduced to 37.8±8.6% of that of the controls (Fig. 2), indicating that the withdrawal of neurotrophins caused significant SGN death. Supplementation with z-VAD-fmk caused no significant promotion of SGN survival, whereas supplementation with SFA significantly increased neuronal survival to 107.9±10.1% (p=0.0021) (Fig. 2).

DISCUSSION

The present findings demonstrate that SFA efficiently protects auditory hair cells against aminoglycoside toxicity and protects SGNs against neurotrophin deprivation *in vitro*. Previous studies have indicated that apoptotic pathways play an important role in the degeneration of hair cells due to aminoglycosides [3,4] and the degeneration of SGNs due to neurotrophin deprivation [11,12]. The apoptotic pathways involved activation of a number of caspases [18]. General caspase inhibitors can therefore protect these cells from dying. In fact, previous studies have indicated the efficacy of general caspase inhibitors for the protection of hair cells [4] and SGNs [13]. On the basis of these findings, we used a general caspase inhibitor as a benchmark to measure the protective effects of SFA for hair cells and SGNs. Interestingly, SFA exhibited stronger protective effects on OHCs and SGNs than those of the general caspase inhibitor z-VAD-fmk. These findings indicate that SFA has the potential to act as a protective mechanism for auditory hair cell and SGN death.

In the present study, the application of SFA enhanced the protective effects of z-VAD-fmk on OHC survival, indicating that SFA might also block cell-death pathways that are not mediated by caspases. Recent studies have indicated that the death process of hair cells following ototoxic treatments involves necrotic [19] or other protease-mediated, but not caspase-mediated, pathways [20]. However, the generation of reactive oxygen species in hair cells is a key factor in the induction of various cell-death pathways [21]. Previous studies on cortical neurons have indicated

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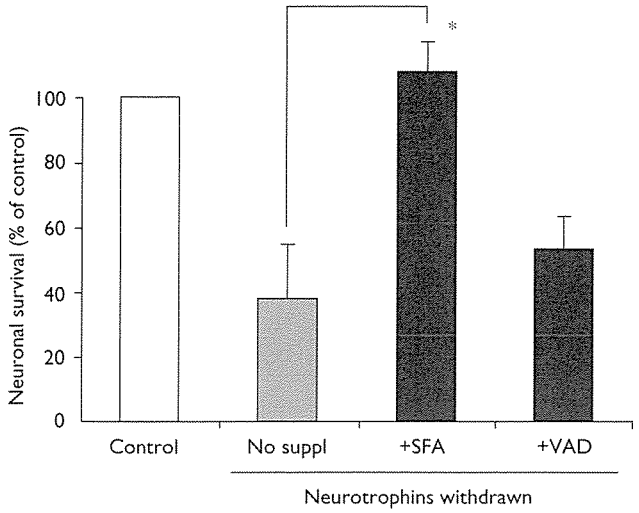

Fig. 2. Quantitative analysis of the survival of cultured spiral ganglion neurons (SGNs) after neurotrophins were withdrawn. The *y*-axis depicts the mean percentage numbers of surviving SGNs in the control specimens cultured with neurotrophins. Withdrawal of neurotrophins caused a remarkable decrease in the number of surviving SGNs. Supplements of z-VAD-fmk showed no significant promotion of SGN survival, while supplements of serofendic acid (SFA) significantly increased neuronal survival (*$p < 0.05$, ANOVA with Scheffe's post-hoc test). The error bars represent the SDs.

that SFA rescues neurons by scavenging reactive oxygen species [17]. Similar mechanisms might occur in the protection of hair cells and SGNs by SFA.

SFA can rescue cortical neurons from glutamate and NO toxicity without influencing their physiological functions [13]. These effects of SFA are suitable for the treatment of auditory systems. Glutamate and NO also play roles in physiological auditory function [22,23]. Therefore, the use of *N*-methyl-D-aspartate antagonists and nitric oxide synthase inhibitors involves the risk of affecting physiological auditory function, although their protective effects on hair cells and SGNs have been reported [24,25]. Considering the influences on glutamate-mediated neurotransmission, SFA appears to be more suitable than *N*-methyl-D-aspartate antagonists and nitric oxide synthase inhibitors.

CONCLUSION

The present findings demonstrate the great potential of SFA for the promotion of survival of auditory hair cells and neurons of P3 mice *in vitro*, suggesting that this newly discovered neuroprotectant could be utilized for the protection of auditory systems. It will therefore be important to ascertain the mechanisms of otoprotection by SFA and to estimate SFA actions on auditory functions in future studies.

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Acknowledgements: This study was supported by a Grant-in-Aid for Scientific Research, a Grant-in-Aid for Exploratory Research and a Grant-in-Aid for the Regenerative Medicine Realization Project from the Ministry of Education, Science, Sports, Culture and Technology of Japan. We would like to thank Eisai Co. Ltd (Tokyo, Japan) for providing the SFA, Professor Tama Hasson (Department of Biology, University of California, San Diego, USA) for providing the anti-myosinVIIa antibody and Toru Higashi and Yoko Nishiyama for technical assistance.

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耳鼻臨床　98：1；1～4，2005

論　　説

内耳への新しい薬物投与方法

—徐放性ドラッグデリバリーシステム—

伊　藤　壽　一

New Method for Drug Application into the Inner Ear

—Biodegradable Drug Delivery System—

Juichi Ito

(Kyoto University)

It has been believed that inner ear sensory cells are very weak and recovery of function after damage is very difficult. That is why sensory neural hearing loss is very difficult to treat. Recently, several drugs and chemicals have been reported to protect, or to recover, inner ear function. However, application of those drugs to the inner ear is difficult because of its specific anatomical and physiological system.

A new drug delivery system (DDS) is introduced.

In particular, the application of nerve growth factor via biodegradable hydrogel is thought to be effective for the protection and recovery of inner ear function.

Key words : DDS, regeneration, inner ear hair cell, biodegradation

再生医学の内耳障害回復への可能性

内耳の感覚細胞（有毛細胞）は一度障害を受けると回復することは難しく，そのために生じる感音難聴，高度の平衡障害は治療が困難となる．

一方，最近特に注目を浴びているのが再生医学（医療）である．再生医学は，生体には元々，障害を受けると再生しようとする能力が備わっており，その再生現象を誘導し，治療に役立てようとする考え方である．

再生医療の目的は，障害された生体組織の再生あるいは代替を行うことである．再生医療が成り立つには，①障害を受けた組織（細胞）に代わりうる「細胞」を供給すること．②その細胞が生着するための適切な環境（「足場」）を提供すること．③「細胞」，「足場」が効率よく機能するための「環境因子」を供給すること．この３つが重要な要素となる．図１に再生医学の３要素を示す．細胞に関しては，近年特に発展の著しい「幹細胞」技術が応用されている．しかし，いかに幹細胞医学が進歩しても細胞が活動する周囲環境が適切に機能しないと組織の再生は誘導されない．この適切な周囲環境，場を構築するための医工学技術，方法論を「生体組織工学 tissue engineering」と呼ぶ．

組織（臓器）によっては適切な足場を供給するだけで再生が誘導される場合もある．耳鼻咽喉科領域で，単純な鼓膜穿孔の場合，コラーゲン膜，ベスキチン膜などを足場として置くだけで鼓膜が再生されるのも，この考え方を応用したものである．しかし，組織の再生能力が低い場合には，細胞を提供し，足場を作るだけでは組織再生は期待できない場合もある．そこで必要になるのが「環境因子」である．この「環境因子」の１種と考えられ再生医療で対象となるものに，細胞の増殖・分化促進作用

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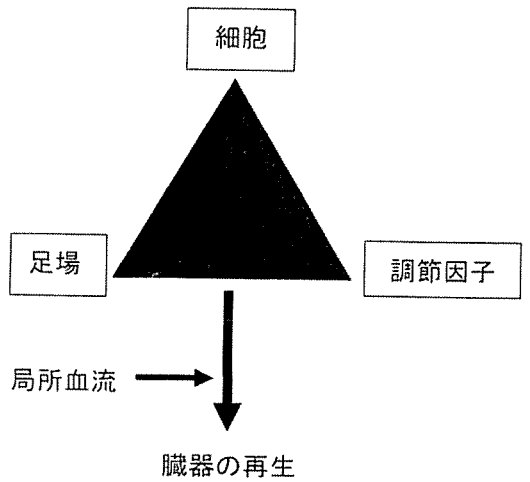

図1　組織（臓器）再生の3要素

をもつ細胞成長因子（growth factor）（「細胞増殖因子」とも呼ぶ）と呼ばれる物質が挙げられる．細胞成長因子は細胞の増殖あるいは分化を制御しているタンパク質（糖タンパク質）であり，発生の段階，再生の途上で重要な役割を果たすこともある．細胞成長因子は分子生物学や細胞生物学などの進歩に伴って，その作用メカニズムが明らかになるとともに，遺伝子操作により大量生産が可能となっている．細胞の増殖や分化，形態形成などに働くこれらの因子を利用すれば組織の再生促進が期待できる．

再生を促進する物質とその投与方法

内耳に関してもこれまでいくつかの物質が感覚細胞保護，または再生に有効であると報告されている．しかし，細胞成長因子は一般にはタンパクであり，生体内では非常に不安定で，生体に投与してもすぐ代謝され，期待する組織再生効果は得られないことが多い．またこれらの物質の投与方法として，例えば全身投与する場合は内耳に作用させるためには大量頻回投与しか方法がなく，薬物が正常細胞にも高濃度で作用することが考えられ，副作用を引き起こす可能性がある．理想的には必要な物質を必要な部位に，必要な量だけ，適切な期間投与するのが理想である．物質の濃度を，必要な場所で必要な期間にわたって有効値に保つ技術をドラッグデリバリーシステム（DDS）と呼ぶ[1]．本システムは，投与する物質をあるキャリヤーと呼ばれる物質（少しずつ放出することが望ましいので，「徐放キャリヤー」とも呼ぶ）にくっつけ，また封入することにより，プログラムされた期間で，期待する量を目標とする組織に投与する方法である．表1は投与する物質，ここでは細胞成長因子と徐放キャリヤーとの組合せによる組織再生の報告例である．細胞成長因子を徐放キャリヤーと一緒に用いることによって組織再生が報告されるようになり，特にこの徐放キャリヤーの開発の必要性が重要視されるようになった．

表1　細胞成長因子の徐放化の研究例

細胞成長因子	キャリヤー材料	再生・新生組織
BMP	ポリ乳酸	長管骨
	コラーゲンスポンジ	顎骨
EGF	アガロース	血管
bFGF	アガロース	血管
	ゼラチン	神経，血管，皮膚
NGF	ゼラチン	神経
TGF-β	アガロース	血管
VEGF	ポリ乳酸	血管
bFGF/rhBMP-2	多孔質HA	頭蓋骨

薬物徐放化技術と再生医療

内耳など全身的な薬物投与効果の少ない部位では，局所にしかも薬物を少しずつ徐放できる技術が必要である．特に内耳感覚細胞の発達・再生に役立つと考えられている細胞成長因子は現在ではまだ入手が困難な状況にあり，できる限り少量で最大限の効果が期待できる投与法の開発が望まれる．これらの薬物の投与法の可能性を以下に示す．

1）生体吸収性ゼラチンハイドロゲルを用いた薬物の徐放

薬物の徐放に，生体吸収性ハイドロゲル（biodegradable hydrogel）を徐放キャリヤーとして用いる方法が試みられている．このハイドロゲルは徐放したい薬物（主にタンパク質である）に対して刺激が少なく，安全性が確立されているため，徐放キャリヤーとして有用である．薬物を効果的に局所に投与するためには少なくとも数日間から数週間の期間で徐放するよう操作できることが必要である．また徐放キャリヤーが生体内に残存することは好ましくなく，最終的には生体内で吸収されることが要求される．徐放期間の調節に関しては，徐放キャリヤーであるハイドロゲルと薬物の間に生じる分子間相互作用力が利用されている．つまり生体内で薬物がハイドロゲルとの分子間相互作用により一定の期間局所に留まり，

ハイドロゲルに分解酵素が作用することによりそれと同時に薬物が徐々に放出されるしくみである[2]．このハイドロゲルには生体内での安全性と生体内での吸収性，さらに徐放したい薬物との分子間親和性が要求される．これらの要望を満たす材料として，コラーゲン，ゼラチン，ヒアルロン酸，アルギン酸などがあり，それら単独またはいくつかの物質の組み合わせで架橋を作製し徐放キャリヤーとして用いる．

2）薬物を他の物質に封じ込めて投与する方法

投与する薬物が水溶性のタンパク質の場合，油溶性物質に封じ込める，または混合して徐放する方法がある．この物質で一般的に用いられるのは油溶性の乳酸グリコール酸共重合体である．この際問題となるのがこれらの油溶性物質と投与する薬物の間に相互作用が生じ，薬物の活性が落ちることである．このため，ポリ乳酸にポリエチレングリコールを共重合させてこれらの油溶性物質の親水性を高め，投与する薬物との親和性を高めて薬物の活性の低下を最小限にしてから徐放する方法が試みられている．

3）薬物を放出する細胞の導入

細胞に期待される薬物を放出するような遺伝子を導入し，その細胞を投与することにより薬物（細胞成長因子など）を投与する方法[3]．遺伝子プラスミドを生体内で徐放化することによって細胞内への導入効率や細胞成長因子の分泌量を高める方法[4]．薬物放出が期待される細胞を直接投与する方法[5]，などが考えられる．

徐放性ゼラチンハイドロゲルを用いた内耳への細胞成長因子の投与

細胞成長因子（ここでは BDNF：brain-derived neurotrophic factor）の水溶液を凍結乾燥ゼラチンハイドロゲルへ滴下し，一定時間放置しておくと細胞成長因子はゼラチンハイドロゲル内へ固定化される．このハイドロゲルは時間経過とともに分解し，その分解速度（徐放期間）はハイドロゲルの架橋の作製の仕方により調節できることが確かめられている[6]．ハイドロゲルの生体内での吸収性と薬物の徐放にはよい相関があることも確かめられている．例えばハイドロゲルの架橋の作り方により，薬物を1週間で分解するようプログラムを組むことができる．

内耳へ薬物を徐放する基礎実験として，BDNF を組み込んだハイドロゲルをモルモットの正円窓に留置した．留置後3日後に蝸牛より外リンパ液を採取し，BDNF が内耳リンパ液中に放出されているかどうかを確かめた．コントロールとして，BDNF を含まないハイドロゲルのみの群，BDNF を直接正円窓から微小針で投与した動物と比較検討した．その結果，ハイドロゲルに BDNF を組み込んだ場合，一定の濃度の BDNF が外リンパ液から検出された．一方 BDNF を含まないハイドロゲルの場合は当然のことであるが，外リンパ液中には BDNF は検出されず，また急速投与した場合も3日後にはほとんど BDNF は検出されず，代謝されたか脳脊髄液に流失したなどの可能性が考えられた．以上の研究結果により徐放性のハイドロゲルに組み込まれた BDNF は徐々に内耳に放出され，一定の期間内耳である濃度を保ちながら留まることが確認された．この徐放性ゼラチンハイドロゲル含 BDNF の内耳に対する保護作用も，内耳の感覚細胞，神経細胞の数をカウントすることにより確かめられ，内耳細胞の保護，再生に有用であることが推測された．

今後の展開

内耳の特に感覚細胞が障害されるとそれは回復不能であると考えられてきた．しかし，最近では障害を受けた感覚細胞を回復させる，また再生を促す物質が色々と報告されるようになってきた．一方，内耳という特殊な解剖学的構造，特殊なバリアー（blood cochlear barrier）などの存在により内耳に薬物を局所投与することは難しいとされてきた．また仮にうまく投与されても脳脊髄液方向に流出してしまうなど，効果を発揮するにはいたらない結果が報告されている．今回紹介した徐放システム，特に徐放性ハイドロゲルを用いた薬物投与方法，さらにはナノテクノロジーを利用したナノカプセルの利用，薬物徐放の可能性のある細胞の移植などは内耳障害への新しい治療方法への可能性を示すものとして期待される．

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内耳の再生医療

特集　Tissue engineeringをベースとした組織再生誘導治療

伊藤壽一*

Regeneration of the inner ear

This paper reviews the potential of cell transplantation to restore inner ear hair cells. Histological analysis revealed survival and incorporation of grafted stem cells in various portion of the inner, suggesting the ability of stem cells for differentiation into inner ear hair cells.

内耳の有毛細胞の障害が高度難聴や高度平衡機能障害を引き起こす．従来，哺乳類の内耳有毛細胞は，一度障害を受けると回復は困難であると考えられてきた．各種幹細胞を内耳に移植することにより，内耳有毛細胞を再生させることに成功した．さらに，DDSの技術を用い，内耳へ神経栄養因子などを投与することも可能になった．これらの方法を組み合わせることにより，障害を受けた内耳を再生させることは可能であると思われる．

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key words : inner ear, regeneration, stem cell, transplantation, drug delivery system

再生医学は障害を受けた器官・組織を修復し，機能も回復しようとするものであり，医学の分野では21世紀前半の最大のプロジェクトと考えられている．再生医学がすでに臨床応用されている分野もある．造血幹細胞を利用した骨髄移植や角膜移植など多岐にわたる．その他，再生医学により動物実験の段階ではすでに作製が可能なものに，血管，心筋細胞，骨組織，軟骨組織，ドーパミン産生神経細胞など多分野にわたるものを列挙することが出来る．

耳鼻咽喉科・頭頸部外科学の分野での再生医学は比較的最近研究がはじまったといえる．ヒトを含め哺乳類の感覚器細胞はこれまで，一度障害を受けると再生は困難であり，機能回復はしないと考えられてきた．そのため，耳科学の分野では，中耳までの伝音難聴は治療の対象になるが，内耳の障害，特に高度感音難聴や高度内耳障害による平衡機能障害などの，感覚細胞(有毛細胞)の障害に起因する疾患は有効な治療手段がなかった．内耳の有毛細胞も一度障害を受けると再生しないと考えられ，このことが特に内耳の有毛細胞の障害に起因することの多い感音難聴の治療の困難さの原因とされている．

しかし，哺乳類の中枢神経系でも再生能力が有することがわかってきた．再生能力はあり，障害を受けても再生しようとする徴候はみられるが，再生を妨げる因子が働き，結局は再生しないと説明されている．内耳の有毛細胞も障害を受けるとまったく再生しないと考えられてきたが，鳥類ではある程度の再生が認められ[1]，哺乳類でも前庭器の感覚細胞は多少再生するとの報告がみられるようになってきた[2]．

再生医学を内耳に応用する場合，現段階ではいくつかの方法があると考えられる．一つは“自発的再生の誘導”である．この自発的再生は発生，細胞死とも関連するが，内耳発生の分子機構を解明し，それを内耳障害の治療に応用しようとする試みである．

一方，筆者らはこのような自発的再生を促進する研究と並行して，“細胞移植”による内耳再生を試みた．具体的には種々の幹細胞を障害を受けた内耳に移植し，有毛細胞その他の細胞の再生に応用しようとする試みである．内耳障害を組織学的に観察すると，最も障害を受けやすいのは内耳有毛細胞である．まず，有毛細胞が障害を受けるが，初期の段階では有毛細胞以外の周囲環境(支持細胞なども含め)は，比較的正常に保たれている場合が多いと考えら

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れる．単純に考えれば，有毛細胞を再生することが出来れば，内耳機能も回復するのではないかという発想である．

細胞移殖による内耳再生医療に関し，当初は有毛細胞の再生を念頭において研究をはじめたが，このような方法が他の付随的な結果を生み出すことが判明した．動物の内耳に移植した，特に神経幹細胞の多くのものがグリア系の細胞に分化し，神経栄養因子を産生する可能性が認められた[3]．神経栄養因子のなかには，有毛細胞の障害に対し，保護作用を有するものがあり，内耳に移植した神経幹細胞が，結果的に障害を受けた有毛細胞の機能を回復させる可能性を示唆する所見が得られた．

他の成果は，蝸牛軸の方向に移植した幹細胞が神経細胞に分化したことである．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，人工内耳手術を行ってもらせん神経節細胞が障害を受けていれば，信号を中枢に送ることは出来ず，また送る信号が限られ，結果的には良好な聞き取りが得られない．特に内耳奇形例で，らせん神経節細胞の障害が推定される場合は，人工内耳手術後の言葉の聞き取りも不良な例が多い[4]．幹細胞移植により，らせん神経節細胞が再生し，内耳有毛細胞や脳幹の蝸牛神経核細胞に神経連絡をつくることが出来れば，人工内耳での聞き取りも飛躍的に増大すると期待される．

内耳障害の現状

現在，わが国には補聴器も使用できない高度難聴者および聾者が数10万人存在し，また，補聴器によりかろうじて聴覚が得られる難聴者を加えると，高度聴覚障害者の数は数100万人に達すると推測される．また，内耳障害に起因する平衡機能障害者はその総数が把握できないほどである．高度難聴者のコミュニケーション手段は聴覚以外の方法 ― 筆談，手話などに頼っている．

このような高度難聴の原因は，内耳および中枢聴覚路の老化，騒音による障害，抗生物質や抗がん剤などの種々の薬物による障害など多岐にわたる．いずれの原因にせよ，これらの高度感音難聴に対する有効な治療手段はほとんどない．感音難聴の原因はさまざまであるが，病態としては大部分が内耳の有毛細胞の障害である．有毛細胞の再生が可能となれば，一度喪失した聴覚の再獲得も可能と思われる．

従来，内耳有毛細胞を含め，哺乳類の中枢神経系は，一度障害を受けると再生は困難とされてきた．しかし，最近では障害を受けた哺乳類の中枢神経系でも条件さえよければ再生する系もあるという報告がある．さらに，胚性幹細胞や神経幹細胞の分離が可能となり，神経移植のドナーとして利用し，障害を受けた中枢神経系の再生を試みる報告もある．各種神経成長因子を中枢神経系に投与し，障害を受けた中枢神経系が修復されたとする報告もある．

内耳再生医療の目的は，各種神経成長因子や幹細胞を利用して，障害を受けた内耳有毛細胞や中枢聴覚路の再生を試みることである．

内耳障害の病態

内耳障害の病態を考えるに，最も障害を受けやすいのが内耳の有毛細胞であり，さらに有毛細胞からの信号を中枢に伝えるらせん神経節細胞，また内耳のイオン組成を調節するといわれる血管条細胞の障害も内耳機能低下をきたす．このなかでも最も重要なものは有毛細胞であり，障害を受けた有毛細胞が再生すれば失われた内耳機能も回復するのではないかと考えられる．

従来，鳥類，哺乳類では，内耳有毛細胞が形成されるのは発生の過程に限られ，出生後は内耳有毛細胞が一度傷害されると再生することはないとされてきた．しかし，1980年代後半に，鳥類では前庭の有毛細胞は常に再生をしており[5]，聴覚系の有毛細胞も再生されることが明らかにされた[6]．哺乳類においても，蝸牛における有毛細胞の再生はいまだに証明されていないものの，前庭の有毛細胞は再生することが明らかにされている[2,7]．

内耳有毛細胞の自発的再生

内耳の特に有毛細胞の自発再生を考える際，以下の三つの機構があげられる．

① 有毛細胞の周囲にある支持細胞が細胞分裂に

つづく細胞増殖を起こし，有毛細胞が形成される．

② 支持細胞が性質を変えて有毛細胞に分化転換する．

③ 完全に死に至っていない有毛細胞が自己修復する．

各国の内耳再生を研究する施設の多くは，この自発的再生を促進しようとする研究を行っている．

鳥類を用いた実験では，有毛細胞の再生の過程でDNAの複製が支持細胞に認められることから，支持細胞あるいはそのなかの一部の細胞の非対称的な有糸分裂により，有毛細胞が再生するとされていた[8]．しかし，哺乳類においては，障害後の有毛細胞の再生の過程でDNAの複製を確認できる細胞が認められるものの，その数は非常に少なく有毛細胞の再生を担うほどではないと考えられている[9]．

有糸分裂を介さない内耳有毛細胞の再生の機構の一つとして，支持細胞が直接有毛細胞に変わる分化転換が，両生類，鳥類，哺乳類においていわれている．その根拠は形態学的なもので，上皮の基底面から頂上面に至るまで，細胞体が存在する支持細胞の特徴と，微絨毛を有するという未熟な有毛細胞の特徴を兼ね備えた細胞が観察されるというものである．分化転換による内耳再生の利点として，有糸分裂によるものと比較して短期間での再生が可能である点が考えられている．

これらに対し，内耳障害後の哺乳類における有毛細胞の再生を担っているのは，完全死に至っていない有毛細胞による自己修復であるという考えがある．自発的な内耳有毛細胞の再生を誘導する因子についての研究は，成長因子やホルモンなどの細胞外から作用を及ぼす外因性の物質が中心であったが，分子生物学の発展やさまざまな遺伝子の発見により，内耳有毛細胞再生を促す細胞内のメカニズムや内耳感覚上皮細胞の動態が解明されようとしている．さらなる解析が進めば，従来不可能とされていた内耳有毛細胞再生の誘導を促す手法の発見・開発につながると思われる．

図1　神経幹細胞の内耳移植

ラット内耳に神経幹細胞を移植後の蝸牛上皮．感覚上皮の上(太矢印)に移植細胞が巣状に生着している．一部の細胞(細矢印)は蝸牛の感覚上皮内に入り込んで有毛細胞に置き換わっているように見える．

細胞移植による内耳再生

内耳有毛細胞の再生を考えた場合，自発再生を促すことも一つの方法であるが，実際にはまだ困難な面が多い．そこで筆者らは細胞移植の方法を用い，内耳再生を試みた．

内耳障害に対し，細胞移植による治療を目指す際，克服しなくてはいけないいくつかの課題がある．最大の課題は移植材料の開発である．移植材料には幹細胞を利用することを考えているが，幹細胞にもいくつかの種類・段階がある．どの幹細胞を用いるべきなのかが問題となる．移植細胞の候補にあがるのは，胚性幹細胞(ES細胞)，神経幹細胞，内耳幹細胞，間葉系幹細胞である．

ES細胞はすべての組織・器官のもとになる細胞であり，全能細胞ともいわれる．ES細胞から神経細胞に誘導するおおよその方法が確立されているが，内耳有毛細胞への誘導に関してはまったくまだ研究がなされていない．ES細胞を内耳への移植材料として用いて，果たして内耳の環境のなかで分化の方向に向かうかは疑問視される．内耳が外胚葉由来であることを考えると，ES細胞を外胚葉方向に誘導した細胞を使用するのも一つの方法である．

神経幹細胞はすでに実験的に内耳で生着し，一部内耳有毛細胞に分化することが確かめられており(**図1**)[10]，今後も移植材料としての可能性は高い．

これらの幹細胞にくらべ，内耳幹細胞が実際に存在し，分離できれば内耳感覚細胞への分化という意

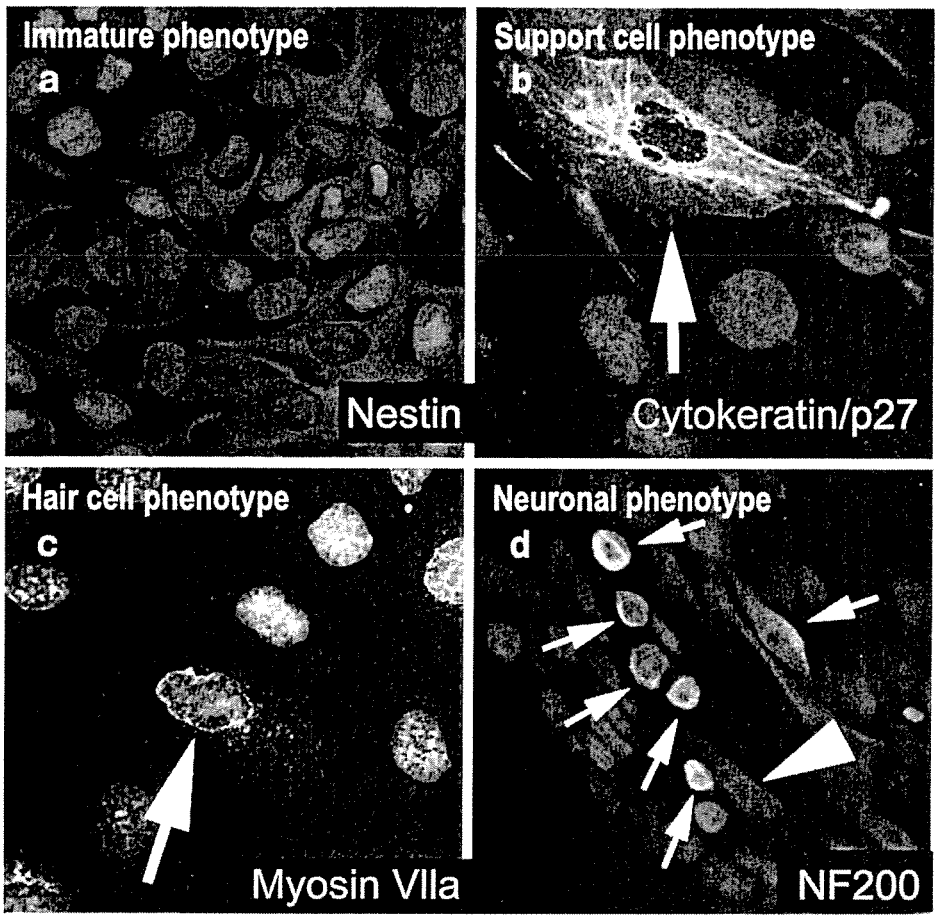

図2　内耳感覚器前駆細胞

内耳の原基細胞由来の細胞株1005は旺盛な増殖を示している培養状態では神経幹細胞のマーカー蛋白であるネスチンを高率に発現し(a)，分化傾向になる培養状態では支持細胞(サイトケラチン，$p27^{Kip1}$)(b)，有毛細胞(ミオシンⅦa)(c)，神経細胞(ニューロフィラメント200)(d)，のマーカー蛋白を発現する．

味では最も期待が持たれるものである．筆者らは，ラット胎児の耳の原器である耳胞から内耳前駆細胞と考えるものを分離培養することに成功した(図2)[11]．この内耳前駆細胞の内耳有毛細胞への分化についても検討する価値がある．

以上の幹細胞は，いずれも内耳への細胞移植の有力なドナー候補となりうるが，ES細胞を使用するにせよ，胎児由来の細胞を使用するにせよ，倫理的な問題を解決しなくてはならない．このような幹細胞にくらべ，自己由来の間葉系幹細胞は倫理的な問題は少ない．間葉系幹細胞は骨髄から採取し，自己のものを利用することが可能であるからである．間葉系幹細胞が内耳有毛細胞に誘導できれば，比較的近い将来の臨床応用の可能性も考えられる．これらの内耳への細胞移植概念図を図3に示す．

以下に各幹細胞を用いた内耳細胞移植研究の概要または結果を示す．

1．胚性幹細胞

胚性幹細胞(ES細胞)ES細胞は，初期胚中の全能性幹細胞と同様に，個体を構成するすべての細胞に分化する能力を保持し，無制限に増やすことが出来る．

ヒトES細胞に対する期待は大きいが，臨床応用を考えたときには，第一に第3者の細胞であるから免疫抑制剤が必要，第二に奇形腫の可能性，第三に発生早期の細胞は得やすいが発生後期の細胞を得るのが難しいなどの問題がある．

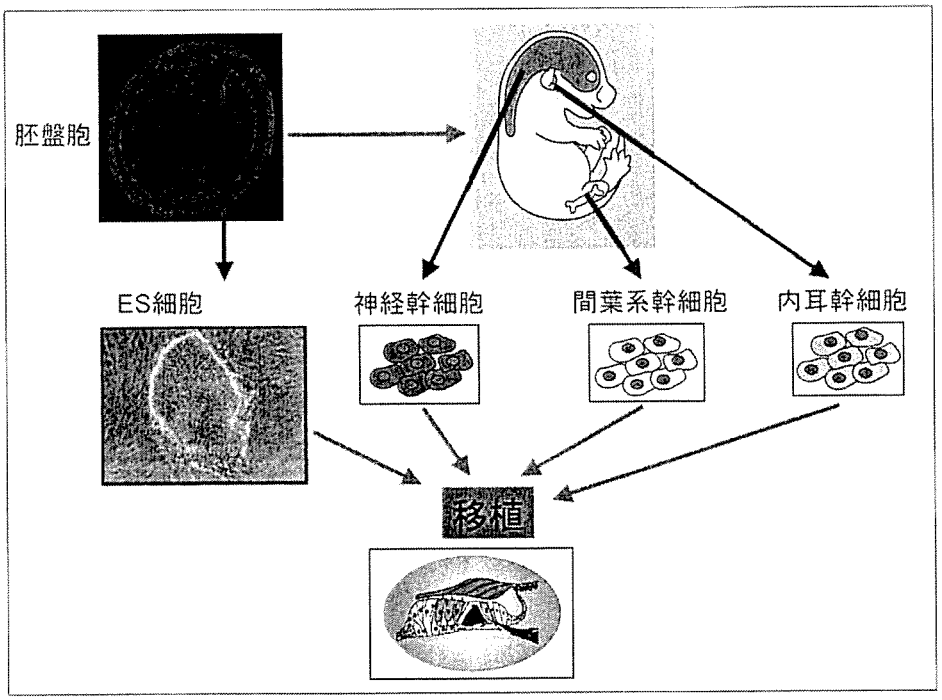

図3　内耳細胞移植の概念図

技術的な問題以外に倫理面の問題が大きく，特にヒトの場合，どの段階から生命を持った人間として扱うべきか，ES細胞樹立に必要な胚をどのようにして入手するか，遺伝子操作を行ってよいのか，など，議論の対象となる点は多い．

2. ES誘導細胞

ES細胞は，生体のすべての細胞に分化する能力を持つ細胞であるが，ある細胞への分化を選択的に誘導したり，選別したりする方法はわずかな細胞種でしか知られていない．内耳有毛細胞も，もちろんその誘導方法はまったく明らかになっていないが，蝸牛管が皮膚外胚葉のなかに形成される耳プラコードに由来することを考えると，外胚葉への誘導をES細胞から内耳有毛細胞への誘導の第1段階と考えるのが妥当である．

ES細胞の分化誘導は胚葉体を経由した誘導する方法と，経由しないで間質細胞と共培養して誘導する方法にわけられる．ES細胞を間質系細胞株上(PA6)で培養することで，ES細胞から胚葉体を経ず，直接，皮膚外胚葉の細胞を誘導することが可能である[12]．

このSDIA法とよばれる方法は，ラット胎児の頭蓋骨から得られた細胞(PA6)とES細胞の共培養によって効率よく神経分化を誘導できる方法で，マウスだけでなく，サルES細胞でも同じ方法が適応できる．また，この培養液中にBMP-4を添加するだけで，皮膚外胚葉へも誘導できる．このことから，SDIA法で誘導されたES細胞は，ある時期には皮膚・神経の共通の祖先である未分化外胚葉に相当する分化状態を経ていることが予想される．

筆者らの研究では，SDIA法によって外胚葉方向に誘導されたES細胞をマウスの内耳に移植したところ，らせん神経節細胞を再生することが可能であることを明らかにした(未発表)．

3. 神経幹細胞

筆者らは神経幹細胞の内耳への移植実験を行った[10]．新生ラット(生後2～3日)の内耳に海馬由来の神経幹細胞を移植し，投与2～4週間後の移植した細胞の様子を観察した．その結果，蝸牛の鼓室階，前庭階，蝸牛管の内部，管腔壁に移植細胞が一塊となって見いだされ，神経幹細胞が移植後数週間たっても生着しつづけることがわかった．また，一