RWM under direct vision was shown in the Table 1. The transtympanic microendoscopic approach enabled visualization of the RWM in all the specimens (Fig. 3). In three specimens, the RWM was totally observed (Fig. 4a). We used the incuidostapedial joint as a landmark to identify the location of the round window niche and the tip of the microendoscope was safely oriented to the RWM. No hazardous events such as ossicular dislocation or disruption of the tympanic membrane occurred. In contrast to the transtympanic microendoscopic approach, a transtympanic approach using a microscope provided visualization of the RWM in only three specimens (Fig. 3). Even in those three specimens, the view of the RWM was very limited (Fig. 4c). In the other seven specimens, the RWM was not observed, as the overhang of the round window niche was an obstacle for visualization. The visibility of the RWM through the transtympanic microendoscopic approach was significantly superior to that through transtympanic microscopic approach (Fig. 3, $P < 0.01$, Wilcoxon matched-pair signed-rank test).

In all the specimens, the transmastoid approach provided an excellent view of the RWM using either microendoscope (Fig. 4b) or microscope (Fig. 4d). The transmastoid microendoscopic approach provided a wide view of the middle ear cavity; for instance more than 70% of the tympanic membrane was visible in nine (microendoscopic), and seven (microscopic) specimens.

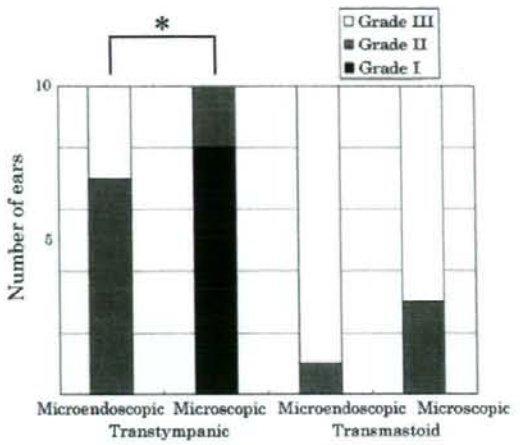

Fig. 3 The visibility of the RWM for four approaches. Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. The visibility through the transtympanic microendoscopic approach was better than that with transtympanic microscopic approach

Discussion

The present results demonstrate that a microendoscope provided a satisfactory view of the RWM through a transtympanic approach with only a 2-mm incision on the tympanic membrane. Although the transmastoid microscopic approach provides an excellent view and favorable access to the RWM, this approach requires mastoidectomy and is not adequate for local drug application for treatment of SNHL. In contrast, the transtympanic microendoscopic approach requires only a small fenestration in the tympanic membrane. Therefore, the transtympanic microendoscopic approach may be applicable for office-based treatment.

Conventional endoscopes with 30° provide good visualization of the RWM [9, 10]. However, endoscopes with attached CCD cameras are not easy to handle. In office-based usage, the endoscope is usually placed just outside of the tympanic membrane [11], and tools used for drug application can hinder the view. The outer diameter is 1.7 mm or larger, requiring larger myringotomy. In addition, use of a conventional endoscope for drug delivery onto the RWM requires another channel for drug application, resulting in

Table 1 The percentage of the visible area of the round window membrane using four approaches

No	Side	Transtympanic		Transmastoid	
		Microendoscope (%)	Microscope (%)	Microendoscope (%)	Microscope (%)
1	Left	80.2	0.0	91.6	70.1
2	Left	54.5	0.0	78.1	72.0
3	Left	78.8	23.0	87.3	79.6
4	Left	59.1	0.0	73.3	84.8
5	Left	48.2	14.6	94.8	71.6
6	Right	49.7	0.0	80.7	61.3
7	Right	79.9	0.0	87.6	75.7
8	Right	39.5	0.0	66.2	42.3
9	Right	62.0	20.1	84.9	83.2
10	Right	56.9	0.0	82.8	65.4

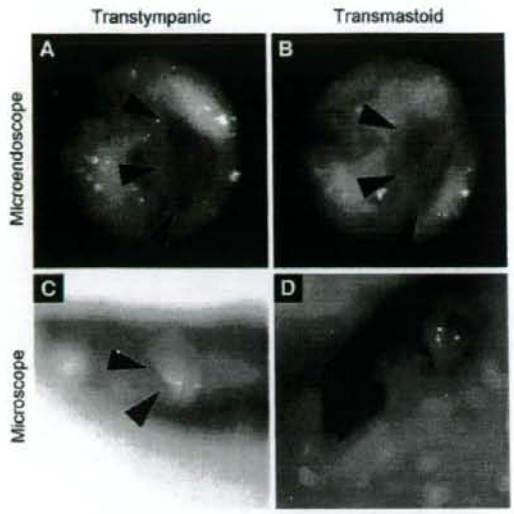

Fig. 4 The RWM of bone three observed through four approaches (*arrow heads*). The transtympanic microendoscopic approach (**a**), transmastoid microendoscopic approach (**b**), and transmastoid microscopic approach (**d**) provided good views. In the transtympanic microscopic approach (**c**), only a small part of the RWM was observed with the aid of a curved needle

increase of surgical invasion on the tympanic membrane. This means that enlargement of the size of tympanotomy or making additional tympanotomy site is necessary. Conventional microendoscopes are made for the inspection of the nasolacrymal ducts, and their tips are straight. The external auditory canal is S-shaped [12], and it is difficult to direct straight microendoscope to the RWM. The modified microendoscope used in the current study is quite smaller than conventional ones, and is connected to a CCD camera system via a cable. The curved tip fitted the external auditory canal. This configuration provides excellent handling of equipment for drug delivery. In addition, the microendoscope used in this study has a working channel that can be utilized for application of substrates onto the RWM.

The aim of the current study was to evaluate the accurate RWM drug application efficacy of a microendoscope with angles modified to ease RWM access. For clinical use of previously developed local drug delivery systems [3, 8], safe and stable visualization of the RWM through the tympanic membrane is necessary. In this manuscript, we compared the transtympanic microendoscopic approach with the transmastoid microscopic approach, since it is the most common procedure to access the RWM. The transmastoid microscopic approach is the most reliable approach for observation of the RWM, and additional removal of the round window niche enabled measurement of the total area of the RWM, which was indispensable for quantitative analysis in the present study. The view provided by a microendoscope is enough to deliver drugs or biomaterials incorporating drugs onto the RWM, although it is not satisfactory for precise surgical procedures. Previous studies have demonstrated the efficacy of biodegradable gelatin hydrogels for local application of brain-derived neurotrophic factor [6] and insulin-like growth factor 1 [7, 13]. The present findings resolve the problem of how to place a hydrogel onto the RWM in the clinic.

This study also found some drawbacks for this instrument. The resolution of the microendoscope is not as high as that of conventional microscopes, which may impede the differentiation of the false membrane from the RWM [14]. Sufficient understanding of the surgical anatomy of the middle ear is necessary for appropriate use of the microendoscope in drug delivery onto the RWM. However, we consider that refinement of the quality of view provided by microendoscopes may resolve this problem.

Conclusion

The transtympanic microendoscopic approach provided satisfactory visualization of the RWM through the tympanic membrane, indicating that the microendoscope is a useful tool for placing drugs or drug-containing materials onto the RWM.

Acknowledgements This study was supported by a Grant-in-Aid for Researches on Sensory and Communicative Disorders from the Japanese Ministry of Health, Labour and Welfare, by a Grant from Japanese Foundation for Research and Promotion of Endoscopy and by a Grant from Tinnitus Research Initiative.

Conflict of interest We do not have a financial relationship with the organization that sponsored the research.

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Journal of Neuroscience Research 86:3075–3085 (2008)

Potential of Embryonic Stem Cell-Derived Neurons for Synapse Formation With Auditory Hair Cells

Masahiro Matsumoto,[1] Takayuki Nakagawa,[1]* Ken Kojima,[1,2] Tatsunori Sakamoto,[1] Fumino Fujiyama,[3] and Juichi Ito[1]

[1]Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[2]Establishment of International COE for Integration of Transplantation Therapy and Regenerative Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[3]Department of Morphological Brain Science, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Recent studies have indicated that embryonic stem cells (ESCs) can be a source for the replacement of spiral ganglion neurons (SGNs), auditory primary neurons, and neurite projections from ESC-derived neurons to auditory sensory epithelia. However, the potential of ESC-derived neurons for synapse formation with auditory hair cells (HCs) has not been elucidated. The present study therefore aimed to examine the ability of ESC-derived neurons to form synaptic connections with HCs in vitro. Mouse ESC-derived neural progenitors expressing enhanced green fluorescence protein (EGFP) were cocultured with explants of cochlea sensory epithelia obtained from postnatal day 3 mice. After a 7-day culture, neurites of ESC-derived neurons predominantly elongated toward inner hair cells (IHCs), which play a crucial role in sound transmission to SGNs. Immunohistochemical analyses revealed the expression of synapsin 1 and synaptophysin in the nerve endings of ESC-derived neurons adjacent to IHCs, indicating the formation of synaptic connections. Transmission electron microscopy demonstrated synaptic contacts between nerve endings of ESC-derived neurons and IHCs. The present findings show that ESC-derived neurons can make synaptic connections with IHCs.

Key words: embryonic stem cell; hair cell; cochlea; transplantation; spiral ganglion neuron

Sensorineural hearing loss (SNHL) is one of the most common disabilities in our society today, but, because mammalian inner ear cells have limited regenerative activity, treatment options are limited to cochlear implants and the use of hearing aids. Spiral ganglion neurons (SGNs) located in the bony axis (modiolus) of the cochlea are bipolar auditory primary neurons that make synaptic connections with auditory hair cells (HCs) and the cochlear nucleus in the brainstem. They consist of two types of neurons: type I neurons making synaptic connections with inner hair cells (IHCs) that play a predominant role in the transduction of sound stimuli to neural stimuli, and type II neurons making synaptic connections with outer hair cells (OHCs) that assist in controlling the sensitivity of regions of the auditory epithelia to specific sound stimuli (Spoendlin, 1987; Rubel and Fritzsch, 2002). The loss of SGNs therefore compromises auditory function. SGN degeneration resulting in SNHL occurs during the aging process (Keithley et al., 1989), and can also be caused by several ototoxic agents (Lee et al., 2003) or acoustic trauma (Pujol and Puel, 1999). It compromises the clinical benefits of cochlear implants (Nadol et al., 1989), which rely on direct stimulation of SGNs to improve impaired hearing.

Recent investigations have indicated the potential for SGN regeneration using cell transplantation into the cochlea. Several candidates are available as a source of transplant; previous studies have demonstrated the survival and neurite outgrowth of neural stem cells (Tamura et al., 2004; Hu et al., 2005b) and dorsal ganglion neurons (Hu et al., 2005a) after transplantation into the cochlea. Bone marrow-stromal cells have also shown the capacity for settlement and differentiation into neuronal cells after engraftment into the cochlea (Naito et al., 2004). More recently, the survival of bone marrow stromal cell-derived neurons in the cochlear modiolus has been reported (Matsuoka et al., 2006). Embryonic stem cells (ESCs) are another possible transplant source with the ability to dif-

Contract grant sponsor: Ministry of Education, Science, Sports, Culture and Technology of Japan.

*Correspondence to: Takayuki Nakagawa, MD, PhD, Departments of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, Kyoto, 606-8507 Japan. E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

Received 6 September 2007; Revised 25 January 2008; Accepted 11 March 2008

Published online 9 June 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21754

ferentiate into neurons, and several neural-inducing methods have been established for this cell type. ESC-derived neural progenitors have a high potential for survival and differentiation into neurons after transplantation into the cochlear modiolus where they were previously shown to massively elongate their neurites toward the peripheral and central auditory systems, reaching host SGNs and the osseous spiral lamina, which is located between SGNs and auditory sensory epithelia (Okano et al., 2005; Corrales et al., 2006). In addition, transplantation of ESC-derived neurons into the cochlear modiolus contributes to the recovery of SGN functions, as monitored by electrically evoked auditory brainstem responses (Okano et al., 2005). In vitro analyses such as coculture studies of ESC-derived neural progenitors with cochlear or vestibular sensory epithelia have demonstrated their differentiation into bipolar neurons (Coleman et al., 2007) and neurite outgrowth that attaches to sensory HCs and expresses synaptophysin at the attachment site (Kim et al., 2005; Matsumoto et al., 2005).

Although there was no direct evidence of synapses establishment, there was functional evidence to indicate the establishment of functioning neural connection (Okano et al., 2005). The aim of the present study was therefore to determine the synapse formation ability of ESC-derived neurons with auditory HCs. In the cochlea, there are the afferent synapses between the peripheral fibers of spiral ganglion neurons and sensory HCs (Sobkowicz, 1992) and the efferent synapses, both axosomatic with the sensory HCs and axodendritic with the peripheral afferents (Sobkowicz et al., 1997, 2002). We cocultured mouse ESC-derived neural progenitors with explants of mouse auditory sensory epithelia and morphologically examined the neural connections that arose between ESC-derived neurons and HCs.

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. All experimental procedures were performed in accordance with the NIH *Guide for the care and use of laboratory animals*.

ESCs and Neural Induction

We used the mouse ESCs G4-2 (generously donated by Dr Hitoshi Niwa of Riken CDB, Kobe, Japan), derived from the E14tg2a ESC line (Hooper et al., 1987) and carrying the EGFP gene driven by a ubiquitous strong promoter (CAG promoter), because our previous experiments demonstrated the potential of neurons derived from this cell line for neurite outgrowth toward inner ear HCs (Kim et al., 2005; Matsumoto et al., 2005). Stromal cell-inducing activity (SDIA) was used for the neural induction of ESCs (Kawasaki et al., 2000). Briefly, ESCs were cultured to form differentiated colonies on a feeder layer of PA6 stromal cells derived from newborn mouse calvaria (RCB1127; RIKEN Cell Bank, Japan) in Glasgow's modified Eagle's medium (GMEM; Invitrogen, Carlsbad, CA) supplemented with 5% knockout serum replacement (KSR; Invitrogen), 1 mM pyruvate (Sigma, St. Louis, MO), 0.1 mM nonessential amino acids (Invitrogen), and 0.2 mM 2-mercaptoethanol (2-ME; Wako, Osaka, Japan). Colonies that formed on the PA6 monolayer after 6 days of culture were isolated by incubation with collagenase B (Roche Diagnostics, Tokyo, Japan) for 5 min at room temperature (RT).

Isolation of Auditory Epithelia and Coculture

Postnatal day (P) 3 ICR mice were deeply anesthetized with diethyl ether and decapitated. The temporal bones were dissected, and the cochleae were freed from surrounding tissues in 0.01 M phosphate-buffered saline (PBS) at pH 7.4, supplemented with 0.2% glucose. After removal of cochlear lateral walls, auditory epithelia were dissected from the cochlear modiolus. SGNs were carefully removed from the explants using fine forceps as described previously (Matsumoto et al., 2005), resulting in complete removal of neural elements from explant cultures (Fig. 1). Auditory epithelia were explanted intact on a sterile membrane (Falcon BD Biosciences, San Jose, CA) into MEM (Gibco, Grand Island, NY) supplemented with 0.3% glucose in 24-well culture plates. Cultures were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hr. The cultured explants were then provided for coculture with ESC-derived neural progenitors. Explants including SGNs were excluded from analysis. Finally, 20 explants were provided for coculture with SDIA-treated ESCs, and an additional four explants cultured alone in the same medium were used as controls.

Cultured auditory epithelia were transferred into GMEM supplemented with 5% KSR, 1 mM pyruvate, 0.1 mM nonessential amino acids, and 0.2 mM 2-ME. Cell suspensions of SDIA-treated ESCs (2 μl, 1×10^4 cells/μl GMEM) were placed inside cultured auditory epithelia, where SGNs are normally located, using a Hamilton syringe (Hamilton, Reno, NV). The medium was changed daily, and cultures were maintained for 7 days.

Tissue Preparation

Histological analysis was performed on whole mounts or cryostat sections. After the culture period, whole mounts (cocultures n = 5; controls n = 2) were fixed in 4% paraformaldehyde in PBS for 15 min at RT. Cryostat sections (cocultures n = 15; controls n = 2) were fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in PBS for 30 min and then cryprotected by immersion in 30% sucrose in PBS at 4°C overnight. Specimens were mounted in OCT compound (Tissue-Tek, Sakura, Tokyo, Japan) and cut at 15 μm using a cryostat.

Immunohistochemistry

Whole-mount specimens were doubly immunostained for myosin VIIa and βIII tubulin to identify the location of HCs in the auditory epithelia and ESC-derived neurons and their neurites, respectively. The latter location was also determined by EGFP expression. We focused on neurite extension

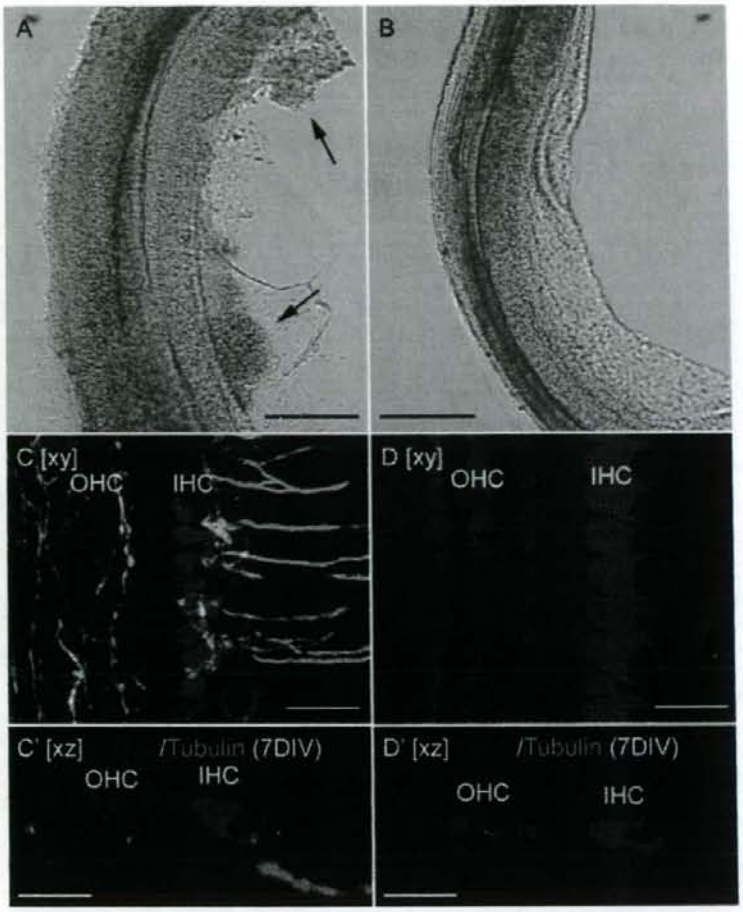

Fig. 1. Explants of auditory epithelia with or without spiral ganglion neurons. On 7 days in vitro (DIV), an explant of the auditory epithelium with spiral ganglion neurons (**A**) exhibits the presence of neurites and nerve endings expressing βIII tubulin (blue) adjacent to hair cells (red; **C:** xy projection; **C′:** xz projection). Arrows in A indicate the remaining spiral ganglion neurons. An explant of the auditory epithelia after elimination of spiral ganglion neurons (**B**) demonstrates three rows of outer hair cells and a single low of inner hair cells expressing myosin VIIa (red) and no neural elements in the auditory epithelium (**D:** xy projection; **D′:** xz projection). Myo, myosin VIIa; tubulin, βIII tubulin; IHC, inner hair cell; OHC, outer hair cell; xy, xy projection image; xz, xz projection image; 7DIV, 7 days in vitro. Scale bars = 200 μm in A,B; 20 μm in C,C′,D,D′.

of ESC-derived neurons and the relationship between HCs and their extended neurites.

Immunohistochemistry for myosin VIIa, βIII tubulin, synapsin 1, synaptophysin, growth-associated protein 43 (GAP43), C-terminal binding protein 2 (ctbp2)/ribeye, and calretinin was carried out on cryostat sections. Synapsin 1 and synaptophysin were used as markers for synaptic vesicles and GAP43 to identify growing neurites. Ctbp2/ribeye was used as a specific marker for synapse ribbons and calretinin as a maker for mature SGNs and their neurites. We focused on the expression of these marker proteins in the nerve endings of ESC-derived neurons that were attached to HCs. Cochlear specimens obtained from P4 (before hearing onset), P12 (onset of hearing), and adult (10-week-old) mice were also stained for synapsin 1 and synaptophysin to correlate the expression patterns of these proteins with the development of mouse auditory function. Cochlear specimens obtained from adult mice were used as controls for ctbp2/ribeye or calretinin.

Whole mounts and cryostat sections were permeabilized and blocked with 10% normal goat serum (Chemicon, Teme-

cula, CA) in PBS-T (PBS containing 0.02% Triton X) for 30 min at RT, followed by overnight incubation at 4°C in the presence of primary antibodies diluted in an appropriate blocking solution. On the following day, sections were washed three times in PBS-T and incubated in diluted secondary antibody for 1 hr at RT. The primary antibodies used were rabbit antimyosin VIIa polyclonal antibody (1:250; Proteus BioSciences, Ramona, CA), mouse anti-βIII tubulin monoclonal antibody (1:250; Covance Research Products, Berkeley, CA), mouse antisynapsin 1 monoclonal antibody (1:50; BD Biosciences Pharmingen, San Diego, CA), mouse antisynaptophysin monoclonal antibody (1:100; BD Biosciences Pharmingen), mouse anti-GAP43 monoclonal antibody (1:200; Chemicon), mouse anti-ctbp2/ribeye monoclonal antibody (1:50; BD Biosciences Pharmingen), and rabbit anti-calretinin polyclonal antibody (1:250; Chemicon). The secondary antibodies used were Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-mouse IgG (1:500; Molecular Probes, Eugene, OR). Fluorescent images were obtained with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems Inc., Wetzlar, Germany).

The ratio of synapsin 1, synaptophysin, or GAP43 expression in nerve endings of ESC-derived neurons adjacent to IHCs was quantitatively assessed by counting the number of nerve endings positive for each marker in three randomly selected cryostat sections from each specimen. The ratio was determined by dividing the number of positive nerve endings by the total number of nerve endings. Values are given as mean ± SD.

Transmission Electron Microscopy

Cryostat sections adjacent to the section in which synaptophysin-positive nerve endings of ESC-derived neurons attached to IHCs were stained by immunohistochemistry using an anti-GFP rabbit polyclonal primary antibody (diluted 1:500 in PBS; Molecular Probes). GFP immunoreactivity was visualized using a Vectastain ABC kit and DAB substrate kit (Vector Laboratories, Burlingame, CA). Specimens were then postfixed with 1% OsO_4 in phosphate buffer for 90 min at 4°C. After dehydration in a graded ethanol series, specimens were embedded in Epon-812 and cut into 80-nm ultrathin sections. The sections were stained with lead citrate and viewed with a transmission electron microscope (TEM; H-7000, Hitachi, Tokyo, Japan).

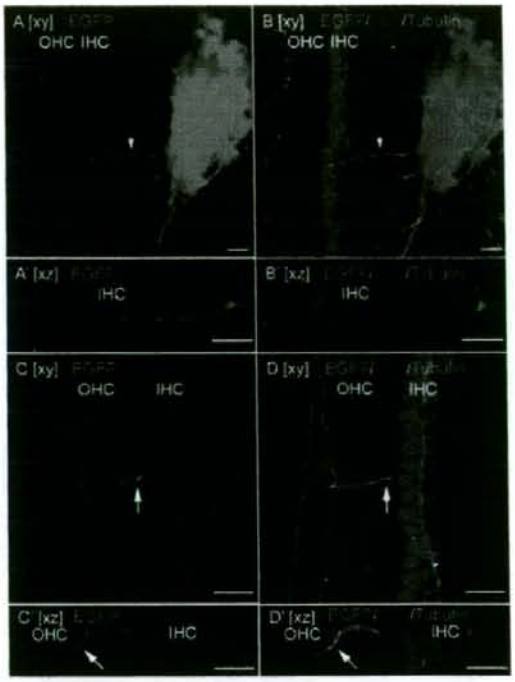

Fig. 2. Embryonic stem cell-derived neurons project neurites to auditory hair cells. A–D show xy projections and A′–D′ show xz projections of cocultures with embryonic stem cell (ESC)-derived neurons and auditory epithelia on 7 days in vitro (DIV). **A,B:** ESC-derived neurons labeled with EGFP (green) and βIII tubulin (blue) extend many processes toward IHCs (red). **A′,B′:** EGFP- and βIII tubulin-expressing fibers (arrowhead in A,B) extend beneath the auditory epithelium and attach to the basal portion of IHCs. **C,D:** ESC-derived neurons project a few processes toward OHCs like cross-tunnel fibers (arrow in C,D). **C′,D′:** ESC-derived neurites attach to the basal portion of OHCs (arrow in C′,D′). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa; tubulin, βIII tubulin; IHC, inner hair cell; OHC, outer hair cell. Scale bars = 20 μm.

RESULTS

Projection of eurites From ESC-Derived Neurons to HCs

Auditory epithelia explants were cocultured with ESC-derived neural progenitors labeled with EGFP for 7 days. After this, βIII tubulin expression was detected in ESC-derived cells, as previously observed (Kim et al., 2005; Matsumoto et al., 2005), indicating that ESC-derived neural progenitors had differentiated into neurons. Seven days after plating, ESC-derived neurons formed colonies inside of a single row of IHCs and projected their neurites toward IHCs of the auditory epithelia (Fig. 2A,B). EGFP-expressing fibers were shown to extend from ESC-derived neurons beneath the auditory epithelium and attach to the basal portion of IHCs (Fig. 2A′,2B′), where nerve fibers and nerve endings from type I SGNs also attach in vivo conditions. Some EGFP-labeled neurites were observed around OHCs (Fig. 2C,D). Three of twenty cocultured specimens contained ESC-derived neurites running through the auditory epithelium like the cross-tunnel fibers (Fig. 2C′,D′). These findings demonstrate that ESC-derived neurons have the ability to project neurites to auditory HCs and that these neurites exhibit the same distribution as SGN-derived nerve fibers.

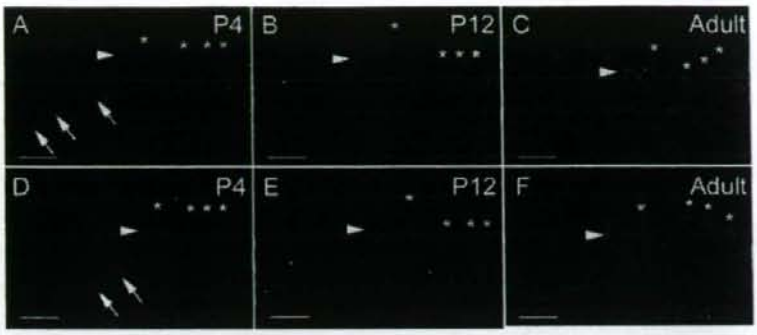

Fig. 3. Synapsin 1 and synaptophysin expression in auditory epithelia of postnatal day (P) 4, P12, and adult mice. **A:** In P4 mice, immunoreactivity for synapsin 1 is observed in the nerve fibers (arrows) and nerve endings adjacent to inner hair cells (arrowhead). **B,C:** In P12 and adult mice, the distribution of synapsin 1 immunoreactivity is limited to the nerve endings adjacent to inner hair cells (arrowhead). **D:** P4 mice exhibit synaptophysin immunoreactivity in the nerve fibers (arrows) and nerve endings adjacent to inner hair cells (arrowhead) and in the cytoplasm of hair cells. **E,F:** In P12 and adult auditory epithelia, synaptophysin expression is accumulated in the nerve endings adjacent to inner hair cells (arrowhead). Cellular nuclei are stained with DAPI (blue). Asterisks indicate the location of hair cells. Synapto, synaptophysin. Scale bars = 20 μm.

Expression of Synapsin 1, Synaptophysin, GAP43, Ctbp2/Ribeye, and Calretinin in ESC-Derived Neurons

To investigate the synapse formation of ESC-derived neurites with HCs, we examined the expression of the synaptic proteins synapsin 1, which is expressed in small synaptic vesicles containing neurotransmitters (De Camilli et al., 1983a,b; Huttner et al., 1983; Navone et al., 1984), and synaptophysin, which is an intrinsic membrane protein of small synaptic vesicles (Wiedenmann and Franke, 1985; Navone et al., 1986; Sudhof et al., 1987) under normal conditions. In auditory epithelia of P4 mice, before the onset of hearing, synapsin 1 immunoreactivity was observed in the nerve fibers and nerve endings adjacent to HCs (Fig. 3A). In P12 mice, at the onset of auditory function, and in adult mice, the distribution of synapsin 1 immunoreactivity was limited to the nerve endings adjacent to HCs (Fig. 3B,C). P4 mice exhibited synaptophysin immunoreactivity in nerve fibers and nerve endings adjacent to IHCs and in the cytoplasm of HCs (Fig. 3D), whereas, in P12 and adult auditory epithelia, synaptophysin expression was observed in the nerve endings adjacent to HCs (Fig. 3E,F).

In cocultured specimens, the expression of synapsin 1 was identified in the nerve endings of ESC-derived neurons adjacent to the base of IHCs (Fig. 4), equivalent to the case in P12 mice. Synaptophysin immunoreactivity was found in the nerve endings of ESC-derived neurons adjacent to the base of IHCs and in the cytoplasm of IHCs (Fig. 5), equivalent to P4 mice. We quantified the ratio of the expression of synapsin 1 or synaptophysin in nerve endings of ESC-derived neurons adjacent to the base of IHCs as 90.5% ± 16.2% and 95.2% ± 12.6%, respectively. These findings indicate active synapse formation between ESC-derived neurons and IHCs in cocultured specimens. We also examined GAP43 immunoreactivity, which reflects neurite elongation and synaptic sprouting (Meiri et al., 1986; Skene et al., 1986; Skene, 1989). GAP43 expression was found in 81.0% ± 17.7% of the nerve endings of ESC-derived neurons adjacent to the base of IHCs and the nerve fibers (Fig. 6).

We also performed immunohistochemistry for ctbp2/ribeye and calretinin to examine the types or maturation status of synaptic contacts between IHCs and ESC-derived neurons. We identified expression of calretinin in SGNs, their nerve fibers, and the HCs in adult mouse cochleae (Fig. 7). In cocultured specimens, the expression of calretinin was not observed in ESC-derived nerve fibers and endings, although HCs were positive (Fig. 7). The expression of ctbp2/ribeye was found in both the nucleus and the base of cytoplasm of IHCs in adult mouse cochleae (Fig. 7). In cocultured specimens, ctbp2/ribeye expression was also observed in the nuclei and the base of cytoplasm of IHCs, although few ctbp2/ribeye-positive dots were identified in IHC cytoplasm (Fig. 7).

Transmission Electron Microscopy

To investigate synaptic contacts between ESC-derived neurons and IHCs, we carried out TEM analysis of cryostat sections next to those containing the nerve endings of ESC-derived neurons expressing both EGFP and synaptophysin (Fig. 8A). EGFP expression was visualized by DAB staining, which was identified as electronically dense regions (Fig. 8B). High magnification revealed vesiculated nerve endings of ESC-derived neurons and their contacts with IHCs (Fig. 8C) as well as a parallel apposition of the two membranes with electronically dense material (Fig. 8D) showing the existence of

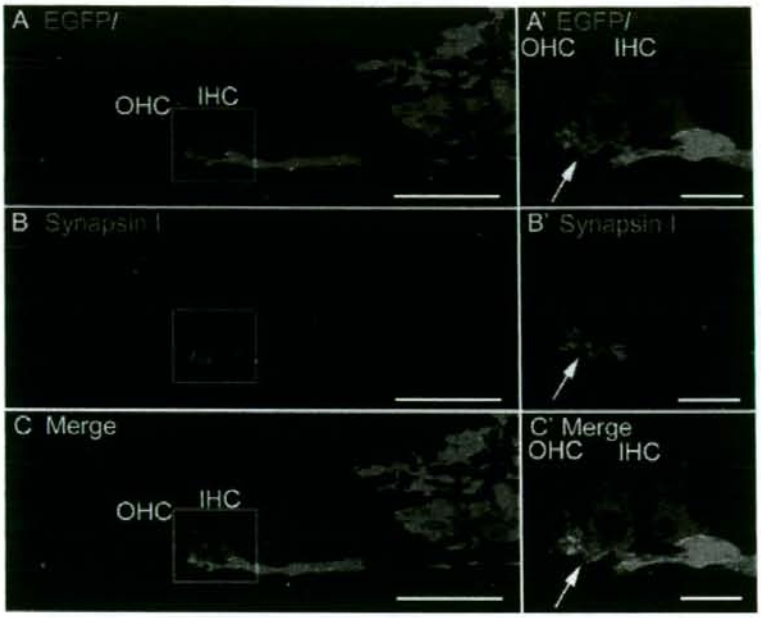

Fig. 4. Synapsin 1 expression in the nerve ending of embryonic stem cell-derived neurons. A–C: Low-magnification photographs of coculture with ESC-derived neurons and auditory epithelia on 7 days in vitro (DIV). A′–C′: High magnification of the boxes shown in A–C, respectively. **A:** ESC-derived neurons labeled with EGFP (green) elongate their neurites toward IHCs (red). **A′:** ESC-derived nerve endings attach to the base of IHCs (arrow). **B,B′:** The expression of synapsin 1 (blue) is accumulated in the nerve endings (arrow), not nerve fibers and soma of ESC-derived neurons. **C,C′:** Merged image. The expressions of EGFP (green) and synapsin 1 (blue) overlap in the nerve endings of ESC-derived neurons (arrow). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa; IHCs, inner hair cells; OHCs, outer hair cells. Scale bars = 50 μm in A–C; 10 μm in A′–C′.

synaptic contacts. However, synapse ribbons, which are included in characteristics for afferent synaptic contacts between IHCs and SGN dendrites (Sobkowicz, 1992; Sobkowicz et al., 1997), were not observed in the base of IHCs attaching to ESC-derived nerve endings.

DISCUSSION

Our ultimate goal is to regenerate auditory HCs and SGNs and improve functional auditory systems after the degeneration of inner ears. Previous studies on human temporal bones have indicated that the loss of SGNs can cause SNHL (Schuknecht, 1993; Nelson and Hinojosa, 2001), and other studies have shown that cell transplantation is a realistic potential strategy for the replacement of SGNs (Naito et al., 2004; Tamura et al., 2004; Hu et al., 2005a,b; Okano et al., 2005; Corrales et al., 2006; Matsuoka et al., 2006).

In regeneration of the auditory afferent system, the formation of new synaptic contacts between IHCs and regenerated SGNs is crucial for its functionality. To investigate this, we have established an explant culture model in which SGNs are selectively eliminated by using fine forceps and HCs are well maintained (Matsumoto et al., 2005). The procedure causes no damage to auditory epithelia and does not disrupt the arrangement of HC rows. By contrast, alternative elimination methods, such as the use of β-bungarotoxin that binds the potassium channels, have the potential to destroy HCs (Fuchs, 1996; Herkert et al., 2001; Shakhman et al., 2003; Martinez-Monedero et al., 2006). Our mechanical elimination method is preferable for in vitro experiments that examine the activity of engrafted cells for neurite outgrowth and the formation of new synaptic contacts with HCs.

The present findings demonstrate that ESC-derived neurons located at the corresponding position of SGNs used appropriate routes to extend their neurites, which were correctly terminated at the base of HCs. In the current study, ESC-derived neural progenitors were cocultured with explants of auditory epithelia just after dennervation. Thus, the routes in which nerve fibers of SGNs had existed may not be scarred at the time point for starting coculture. In addition, previous studies have indicated that HCs have the potential to induce neurite outgrowth from neurons (Kawamoto et al., 2003;

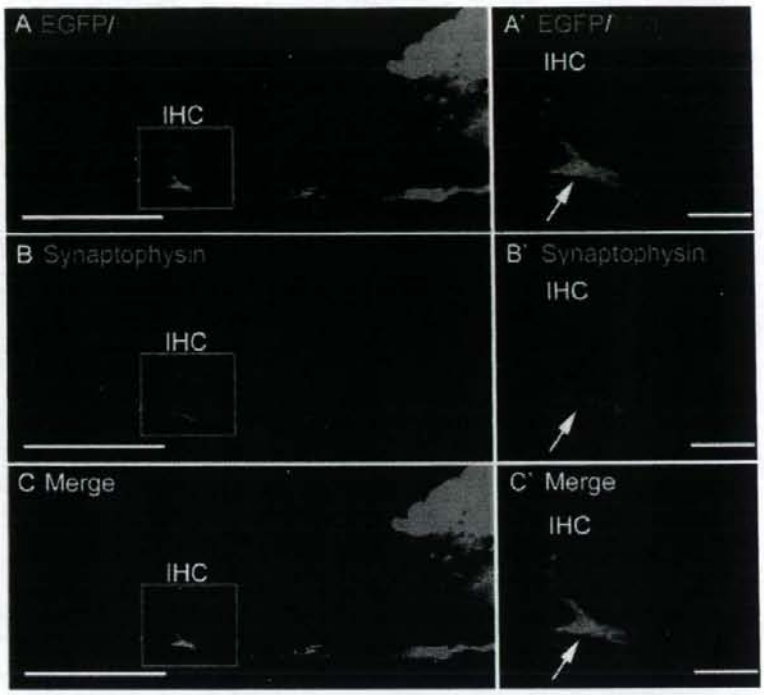

Fig. 5. Synaptophysin expression in the nerve ending of embryonic stem cell-derived neurons. A–C: Low-magnification photographs of coculture with ESC-derived neurons and auditory epithelia on 7 days in vitro (DIV). A′–C′: High magnification of the boxed shown in A–C, respectively. **A:** ESC-derived neurons labeled with EGFP (green) elongate their neurites toward IHCs (red). **A′:** ESC-derived nerve endings attach to the base of IHCs (arrow). **B,B′:** Synaptophysin immunoreactivity (blue) is observed in the nerve endings of ESC-derived neurons adjacent to the base of IHCs (arrow) and in the cytoplasm of IHCs, not nerve fibers and soma of ESC-derived neurons. **C,C′:** Merged image. The expressions of EGFP (green) and synaptophysin (blue) overlap in the nerve endings of ESC-derived neurons (arrow). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa. Scale bars = 50 μm in A–C; 10 μm in A′–C′.

Kondo et al., 2005; Matsumoto et al., 2005; Martinez-Monedero et al., 2006). Therefore, ESC-derived neurons may elongate neurites through the route taken by host auditory nerves under the guidance of HCs. However, the details of HC guidance cues are still unclear and will have to be elucidated prior to the development of cell therapy for functional restoration of the auditory system.

Immunohistochemical analyses in the present study revealed the active formation of synaptic contacts between ESC-derived neurons and IHCs. The distribution of synapsin 1 immunoreactivity was identical to that in the auditory epithelia of P12 mice, at the onset of hearing, and showed an accumulation in the nerve endings of ESC-derived neurons adjacent to IHCs. Moreover, the distribution of synaptophysin immunoreactivity in cocultured specimens was similar to that of P4 mice. In addition, the nerve endings and neurites of ESC-derived neurons exhibited GAP43 immunoreactivity, indicating active neurite outgrowth and synaptic sprouting of ESC-derived neurons (Knipper et al., 1995). Immunohistochemistry for ctbp2/ribeye indicated the presence of synaptic ribbons in the basal pole of IHCs adjacent to ESC-derived nerve endings, similar to immature synaptic contacts between IHCs and afferent nerve fibers (Sobkowicz, 1992). Such immunohistochemical findings suggest active synapse formation between ESC-derived neurons and IHCs.

In the present study, TEM analysis revealed contacts between vesiculated nerve endings from ESC-derived neurons and IHC membranes, as well as synaptic densities in the membrane between IHCs and nerve endings of ESC-derived neurons. This represents the first evidence of synapse formation between ESC-derived neurons and IHCs. In normal cochleae of mature mice, afferent synapse contacts at the base of IHCs are characterized by the presence of synaptic ribbons and by a postsynaptic membrane density on the dendrite (Sobkowicz, 1992; Sobkowicz et al., 1997). However, in TEM analysis, no synaptic ribbons were found in the base of

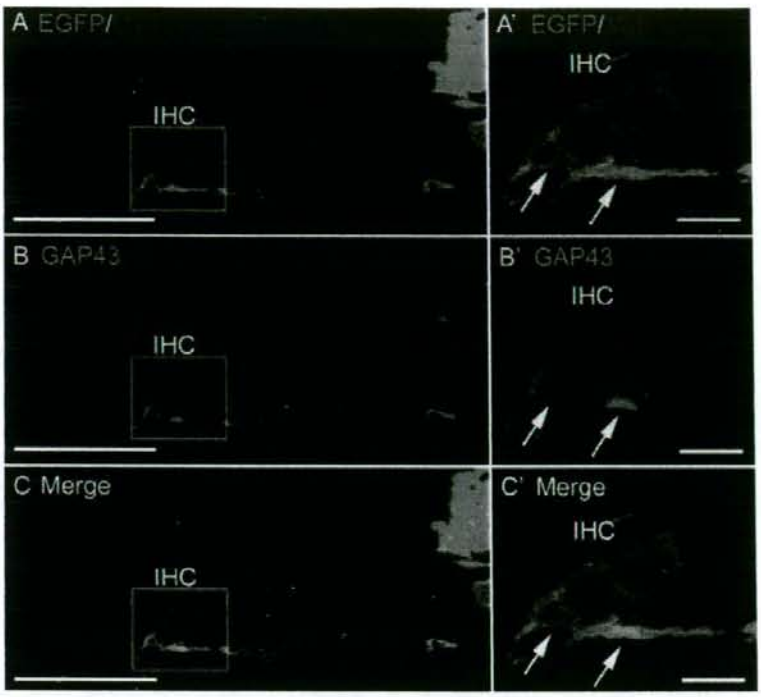

Fig. 6. GAP43 expression in the nerve ending of embryonic stem cell-derived neurons. A–C: Low-magnification photographs of coculture with ESC-derived neurons and auditory epithelia on 7 days in vitro (DIV). A′–C′: High magnification of the boxes shown in A–C, respectively. **A:** ESC-derived neurons labeled with EGFP (green) elongate their neurites toward IHCs (red). **A′:** ESC-derived nerve endings attach to the base of IHCs (arrows). **B,B′:** GAP43 immunoreactivity (blue) is found in the nerve endings of ESC-derived neurons adjacent to the base of IHCs (arrows) and the nerve fibers. **C,C′:** Merged image. The expressions of EGFP (green) and GAP43 (blue) overlap in the nerve endings of ESC-derived neurons (arrows) and the nerve fibers. EGFP, enhanced green fluorescent protein; Myo, myosin VIIa. Scale bars = 50 μm in A–C; 10 μm in A′–C′.

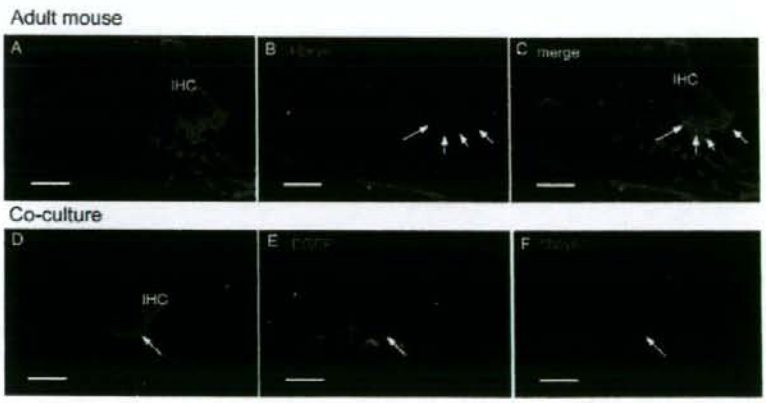

Fig. 7. Expression of ctbp2/ribeye and calretinin in adult mouse cochleae and cocultured specimens. **A–C:** In the adult mouse cochlea, ctbp2/ribeye expression (blue) is found in the nuclei and the base pole of the IHC (arrows), and calretinin expression (red) is observed in hair cells and afferent nerve fibers. **D–F:** In cocultured specimens, immunoreactivity for ctbp2/ribeye is found in the nucleus and in the basal pole of the IHC (arrow), and ESC-derived neurites expressing EGFP (green) are negative for calretinin. On the other hand, the IHC is positive for calretinin. Scale bars = 10 μm.

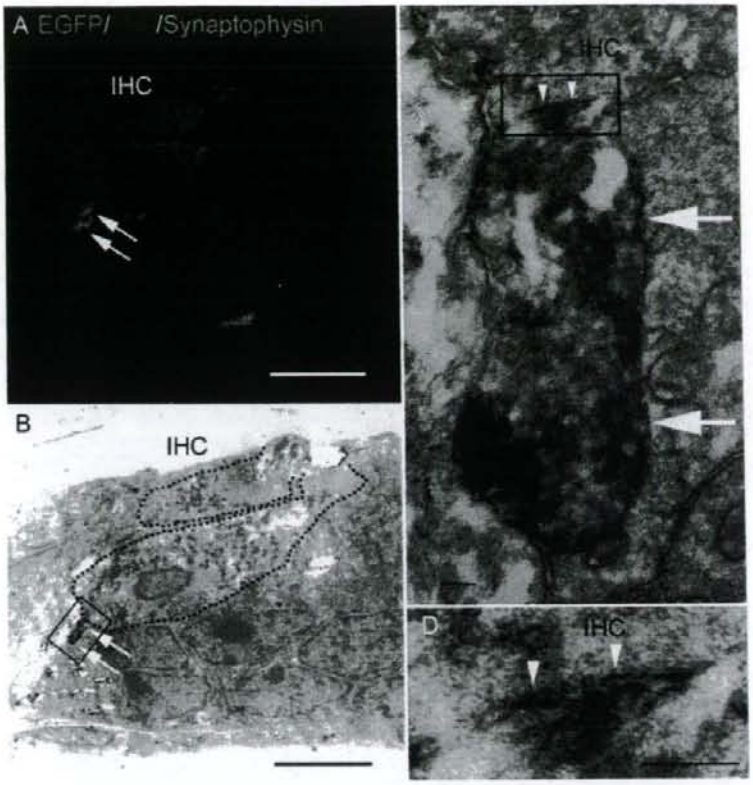

Fig. 8. Ultramicrostructure of contacts between inner hair cells and nerve endings of embryonic stem cell-derived neurons. **A:** Synaptophysin-positive nerve endings (blue) of ESC-derived neurons expressing EGFP (green) attach to the basal portion of the IHC (red; arrows). **B:** An EGFP-positive nerve ending visualized by DAB staining, which is identified as electronically dense regions, is found at the base of the IHC (arrows). **C:** A high magnification of the box shown in B demonstrates the nerve ending of ESC-derived neurons as a vesiculated nerve ending (arrows) and their contact with the membrane of the IHC (arrowheads). **D:** In a higher magnification of the box shown in C, this region exhibits a parallel apposition of the two membranes, with an electronically dense material between the nerve endings of ESC-derived neurons and the IHC (arrowheads). EGFP, enhanced green fluorescent protein; Myo, myosin VIIa; IHC, inner hair cell. Scale bars = 10 μm in A,B; 0.1 μm in C,D.

IHCs adjacent to ESC-derived nerve endings. Therefore, the demonstration that the ECS-derived neurons may be used for replacement of SGNs requires additional experiments.

The neurites of ESC-derived neurons adjacent to IHCs share some properties of presynaptic fibers, because synapsin 1 and synaptophysin are markers for small synaptic vesicles, which are usually located in the presynaptic compartments. In addition, the neurites of ESC-derived neurons were negative for calretinin, which is expressed in mature SGN and their neurites (Dechesne et al., 1994). However, previous studies have observed expression of synapsin 1 and synaptophysin in afferent growth cones (De Camilli et al., 1988). Immature afferent dendrites of SGNs are lack of calretinin expression (Dechesne et al., 1994). Moreover, recent findings using cocultures of auditory epithelia explants and primary SGN cultures found that the processes from SGNs adjacent to HCs express markers for presynaptic fibers (Martinez-Monedero et al., 2006). In addition, coincident with synaptophysin and GAP-43 immunoreactivity in SGNs, outer spiral bundle fibers (OSB) exhibit immunoreactivity for synaptophysin on P2 but not P4 (Knipper et al., 1995), suggesting the localization of synaptophysin in the afferent growth cones of immature SGNs. Morphologically, immature efferent fibers are practically indistinguishable from those of afferent processes (Sobkowicz, 1992). Therefore, determining the type of synaptic contacts between ESC-derived neurons and IHCs will require further examinations.

CONCLUSIONS

The present findings demonstrate that ESC-derived neurons project neurites toward HCs in the auditory epithelium and have the ability to form new synaptic contacts with IHCs. In future experiments, we will investigate the maturation process of ESC-derived neurons after the formation of synaptic contacts with HCs and elucidate HC-derived guidance cues for neurite projection.

ACKNOWLEDGMENTS

The authors thank Marlies Knipper, Takayuki Okano, Yayoi S. Kikkawa, and Tetsuji Sekiya for critical reviews of this work; Hitoshi Niwa for providing mouse ESCs; Yoshiki Sasai for providing PA6 cells; and Makio Fujioka, Akemi Saito, Yoko Nishiyama, and Tomoyo Namura for their technical expertise.

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Local Drug Delivery to Inner Ear for Treatment of Hearing Loss

Takayuki Nakagawa* and Juichi Ito

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Abstract: Sensorineural hearing loss (SNHL) is one of the most common disabilities in our society. Experimentally, many candidates for use as therapeutic molecules have been discovered. However, a considerable obstacle to clinical application is the lack of an effective method for drug delivery to the cochlea. In order to overcome this obstacle, there needs to be development of a local cochlear drug delivery system. Advances in pharmacological technology have provided various drug delivery systems that use biomaterials, and which can be utilized for local drug delivery to the cochlea. Indeed, recent studies have demonstrated the potential of synthetic and natural biomaterials for local drug delivery to the cochlea, indicating that the clinical application of such local drug delivery systems could be used in the near future for therapeutic treatments. Recent progress in cell therapy research also offers a novel drug delivery method for the cochlea. In addition, transplantation of stem cells into the cochlea has been demonstrated to provide protective effects for the auditory function. Transplantation of genetically engineered cells has also resulted in the sustained delivery of aimed therapeutic molecules within the inner ear. Although problems involving clinical application still need to be resolved, these drug delivery systems for the inner ear may hold the future therapeutic options for treatment of SNHL.

Key Words: Drug delivery system, cochlea, biodegradable polymer, cell transplantation, gene therapy.

THERAPEUTIC TARGETS FOR TREATMENT OF HEARING LOSS

Sensorineural hearing loss (SNHL) is one of the most prevalent disabilities in our society. Sound stimuli are received by auditory hair cells (HCs) in the bony, snail-shaped cochlea, followed by transduction of the sound stimuli by the HCs to neural signals. Spiral ganglion neurons (SGNs), which are auditory primary neurons, are located in the central bony axis of the cochlea and responsible for transmitting auditory signals to the central auditory system. Excessive noise, ototoxic drugs, genetic disorders and aging all contribute to the causes of SNHL. Severe to profound SNHL affects 1 in 1000 newborns, and another 1 in 2000 children before they reach adulthood. About 60% of individuals older than 70 years will manifest SNHL. Previous studies on human temporal bones have demonstrated that the loss of HCs and/or SGNs is a major cause of SNHL [1]. Protecting HCs and SGNs from irreversible degeneration is therefore a primary objective due to the limited regeneration capacity of these cells. Acute SNHL sometimes responds to drug treatment; however, there are no therapeutic options for chronic SNHL except for hearing aids and cochlear implants, which are small devices that are surgically implanted into the cochlea in order to stimulate SGNs. However, the success of cochlear implants depends on the remaining SGNs and with their loss, this severely compromises the efficacy of this technique. HCs and SGNs are therefore the major targets for the treatment of SNHL.

WHY IS LOCAL DRUG DELIVERY REQUIRED FOR THE INNER EAR?

Based on the backgrounds described above, studies are being conducted with the hopes of providing an alternative means of biological therapy. Thus far, research has identified a number of candidates for use as therapeutic molecules. Experimentally, protective effects of neurotrophins have been demonstrated [2,3], and inhibitors of apoptosis and glutamate antagonists have also been shown to have the ability to promote HC survival [4-6]. Recently, it has been found that local application of genes by virus vectors induces HC regeneration in the mammalian auditory epithelium [7,8], and additionally, by silencing the mutant gene *via* RNA interference, can restore hearing loss in the genetic mouse model [9]. These therapeutic strategies are attractive and promising for the restoration of SNHL. However, clinical application is still quite limited. The problem of how to deliver such therapeutic molecules to the inner ear has been a considerable obstacle in the development of treatments for SNHL. One of the reasons for the difficulty of drug delivery involves the limited blood flow to the cochlea [10]. In addition, the blood–inner ear barrier, which inhibits the transport of drugs from serum to the inner ear, represents a fundamental obstacle to the use of systemic applications [11]. The inner ear tissues are isolated from the surrounding organs by a bony construction, which allows for the topical introduction of drugs or genes. Based on these considerations, local application has generally been the preferred method for drug administration to the inner ear. The sustained delivery of therapeutic molecules is also critical for the efficient treatment of the cochlea, as bioactive molecules usually require a period of minutes or hours over which they produce their pharmacological actions. Consequently, a number of researchers are currently working to solve these problems and develop methods for the local direct application of these molecules into the cochlea [12].

STRATEGIES FOR LOCAL DRUG DELIVERY

The cochlea is connected to the tympanic cavity by the round window membrane (RWM). When substances are applied intratympanically, the assumption is that they will enter the scala tympani through the RWM and then be dis-

*Address correspondence to this author at the Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, 606-8507 Kyoto, Japan; Tel: +81-75-751-3346; Fax: +81-75-751-7225;
E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

1574-8855/08 $55.00+.00

tributed throughout the cochlear fluids. The idea of using a topical application of medicine to the inner ear is not new, as local anesthetics and aminoglycosides were applied decades ago, with the compounds passing through the tympanic membrane into the tympanic cavity during the treatment of the inner ear disorders [13,14]. Intratympanic injections have been used for local application of aminoglycosides or steroids during therapy for Ménière's disease and sudden hearing loss. There are a number of clinical reports showing the efficacy of intratympanic injections of these drugs [see review in reference 12]. However, it is very difficult to predict the amounts of drugs that will actually reach the cochlear fluid space. Some reports have indicated that this method can lead to varying results during therapeutic treatment of Ménière's disease [15-17]. While intratympanic injection is a simple and easy method to perform, unfortunately, a controlled and sustained release of drugs cannot be achieved using this method. The pharmacokinetics of drug entry into cochlear fluids is crucial to determine the efficacy of the method for drug delivery into cochlear fluids [12]. Salt and Plontke have indicated importance of sustained delivery of drugs on the RWM by means of perilymph sampling from various regions of the cochlea [18] and computer simulation [19].

Implantable mini-pumps have also frequently been used for local drug delivery to the cochlea in animal experiments [20]. Several clinical reports have described the efficacy of local glucocorticoid application when using a semi-implantable mini-pump [21,22]. However, the use of an implantable mini-pump has not been widely adopted, given the need for surgical procedures similar to tympanoplasty that must be done in order to place the mini-pump. The use of a local viral gene transfer as a sustained treatment of the inner ear can provide sufficient protection from noise, drug toxicity and re-perfusion injury [23-28]. Today, adenoviral vectors or adeno-associated viral vectors are the most widely used for cochlear gene transfer, because of the high efficiency for the transfection, the availability of high titers, and the ease of production. However, their use can potentially initiate an immune response that results in the destruction of the recipient's cochlear cells.

The use of biomaterials for local drug delivery has recently gained attention as an alternative to the implantable mini-pumps or gene transfer using virus vectors. In general, biodegradable polymers containing therapeutic molecules are placed on the RWM, with the therapeutic molecules released into the cochlear fluids from the polymers in a controlled manner *via* the RWM [12,28].

DRUG DELIVERY *VIA* BIOMATERIALS

In the past decade, pharmaceutical technologists have paid increasing attention to controlled or sustained release technology using biomaterials for the delivery of drugs in order to avoid side effects and achieve sufficient drug levels in tissues. In an effort to develop a controlled-release system, a variety of methods using synthetic and natural materials have been undertaken. Recent publications have reported the use of a controlled-release system for local drug delivery to the inner ear. Two synthetic materials, siloxane-based polymers [29] and polylactic/glycolic acid (PLGA) polymers [30], and several natural materials, which include hyaluronic acid [31] and gelatin [32-34], have been used for this purpose.

Siloxane-based polymers have been used for years in medical applications that involve contact with the human body. In the clinic, silicone-transdermal patches have been widely used. In this system, drug release is controlled by its diffusion through the silicone network [35]. The actual release rate is determined by the composition of the polymer. This system is particularly suitable for application of lipophilic and low-molecular weight molecules. Arnold *et al.* [29] have utilized this system for local application of beclomethasone into the cochlear fluids. When using this system, the silicone microimplant remains on the RWM, although it does not induce functional and histological damage in the cochlea. Therefore, repeated treatments require that there is extirpation of the material used during the procedure.

Encapsulating bioactive molecules in PLGA or polylactic acid (PLA) particles has been also used as a method of controlled-release application. Water-insoluble, low-molecular weight agents have been encapsulated in PLGA or PLA microparticles and nanoparticles [36,37]. PLGA and PLA are familiar substances to surgeons, as they are the materials that make up absorbable sutures. Tamura *et al.* [30] examined the potential of PLGA nanoparticles for drug delivery to the cochlea using guinea pigs. To evaluate the use of PLGA nanoparticles (140 to 180 nm in diameter) in the cochlea, rhodamine, which is a red fluorescent dye, was encapsulated and then following local application onto the RWM, its overall distribution was evaluated. PLGA nanoparticles containing rhodamine were observed in the cochlea, indicating that PLGA nanoparticles can penetrate through the RWM. Rhodamine is released from PLGA nanoparticles after penetration of the particles through the RWM. Compared to a silicone microimplant, PLGA nanoparticles have the advantage of being able to be repeatedly applied, as the PLGA is dissolved by hydrolysis. However, there is a limitation with regard to the variation of the drugs that can be applied, since the process of encapsulation in the PLGA particles requires that compounds must be dissolved in acetone. Therefore, this method is not suitable for the delivery of proteins or peptides.

GELATIN HYDROGEL

Gelatin is a commonly used natural polymer that is derived from collagen. In the clinic, gelatin polymers have been widely used as hemostats. Recently, gelatin-based controlled-release systems have been developed [38]. During the fabrication process, the isoelectric point of gelatin can be modified to yield either a negatively charged acidic gelatin or a positively charged basic gelatin. This allows for electrostatic interactions to take place between charged therapeutic molecules and gelatin of the opposite charge, leading to the formation of polyion complexes. The significance of such a system is that it provides the ability for application of water-soluble, comparatively high-molecular weight proteins and peptides. Additionally, this method is also capable of being used for the delivery of plasmid DNA [39]. In this system, therapeutic molecules are released by enzymatic degradation of gelatin polymers, for which the rates can be determined based on the crosslinking density of the gelatin polymers.

The potential use of the gelatin hydrogel system was initially investigated for cochlear delivery of brain-derived neurotrophic factor (BDNF) [32]. BDNF plays a crucial role in the development of the inner ears [38] and in the maintenance of the auditory function [41]. In addition, previous studies have demonstrated the effects of local BDNF application when using an osmotic mini pump [3] or adenovirus [26]. We measured BDNF concentrations in the cochlear fluid after placing a gelatin hydrogel that contained this agent onto the RWM [32]. The results revealed a sustained delivery of BDNF into the cochlear fluid *via* the hydrogel over a seven-day period. The functional and histological protection of the SGNs by BDNF that was applied through the gelatin hydrogel was then examined using a guinea pig model of SGN degeneration. The measurement of electrically evoked auditory-brainstem responses, which reflect SGN function, demonstrated that BDNF delivered *via* gelatin hydrogels was able to significantly reduce the threshold elevation [32]. Histological analysis demonstrated an increased survival of SGNs due to BDNF application through gelatin hydrogels. These findings indicate that gelatin hydrogel can be utilized for drug delivery to the cochlea.

Subsequently, we examined the efficacy of cochlear delivery of insulin-like growth factor-1 (IGF1) for the protection of auditory HCs against acoustic trauma [33]. IGF1 is a mitogenic peptide that plays essential roles in the regulation of growth and development in the inner ear [42]. In addition, previous studies on the inner ear have suggested the possibility of inner ear protection by IGF1 [43,44]. Moreover, recombinant human IGF-1 (rhIGF1) has already been approved for clinical use. Therefore, we selected rhIGF1 as a suitable trophic factor for local cochlear application using a gelatin hydrogel. Local rhIGF1 application through the gelatin hydrogel prior to noise exposure has been shown to efficiently protect the hearing from noise trauma. Additionally, histological analysis also revealed that local rhIGF-1 treatment ameliorated the loss of HCs [33].

Our ultimate goal is the clinical use of a local rhIGF1 application using gelatin hydrogel as a therapeutic option for the treatment of SNHL. Therefore, we examined whether post-traumatic application of rhIGF1 to the cochlea *via* gelatin hydrogels could attenuate noise-induced hearing loss. The results demonstrated that functional and histological efficacy of local rhIGF1 treatment on the attenuation of noise-induced hearing loss occurred in a dose-dependent manner [34]. We also measured IGF1 concentrations in the cochlear fluid, cerebrospinal fluid (CSF) and serum after placing a gelatin hydrogel containing rhIGF1 onto the RWM of guinea pigs. The results demonstrated that there was sustained delivery of rhIGF1 into the cochlear fluid, in addition to no alterations of the IGF1 levels in CSF and serum [34]. There were also no adverse effects due to local rhIGF1 treatment found in any of the experimental animals. These findings document both the effectiveness and the safety of local rhIGF1 treatment using gelatin hydrogels for noise-induced hearing loss.

CELL TRANSPLANTATION

Chronic SNHL is usually incurable because of the loss of HCs and SGNs, and which at the present time is irreversible. Therefore, an alternative means of biological therapy, including cell therapy is required. Indeed, recent studies have indicated that cell therapy could be utilized to regenerate HCs [45] and SGNs [46]. In contrast, cell transplantation is an alternative that can be used as a method for drug delivery where the transplanted cells for this purpose have the ability to survive and generate therapeutic agents. Several stem cells have been reported to have the ability to secrete trophic factors [47-49]. Cell transplantation has been used as a means of delivering peptides or proteins into the central nervous system, demonstrating its viable use as a delivery vehicle for therapeutic molecules [50,51].

Iguchi *et al.* have reported on the ability of neural stem cell-derived cells being used for the production of BDNF and glial cell line-derived neurotrophic factor (GDNF) after engraftment into the cochlea [47]. In addition, transplantation of neural stem cells into the cochlea has the potential of being able to attenuate HC damages due to transient ischemia of the cochlea [48]. Bone marrow derived cells also have the potential for secreting trophic factors. Implantation of bone marrow stromal cells has been reported to contribute to functional recovery of the brain [52] and spinal cord [53] by means of producing trophic factors. Furthermore, previous studies have revealed the potential of bone marrow derived cells surviving in the cochlea [54,55]. Yoshida *et al.* have demonstrated a significant increase in the protein level of GDNF in cochlear specimens and the prevention of HC death due to transient cochlear ischemia by engraftment of hematopoietic stem cells [49]. These findings indicate that cell transplantation into the cochlea may be a novel strategy for treatment of SNHL by providing a means for local application of trophic factors within the cochlea.

Transplantation of cells that have been genetically manipulated ex vivo has been used as a means of delivering peptides or proteins into the central nervous system [56-58]. In comparison with the stem cell transplantation that has been described above, this strategy has an advantage in that aimed gene-encoded products are applicable. In addition, use of non-viral vectors for ex vivo gene transfer potentially could resolve the problem of viral vector toxicity in cochlear gene therapy. Therefore, we conducted an examination of the efficacy of cell-gene delivery in the application of therapeutic molecules into the cochlea [59]. NIH3T3 cells were chosen as a delivery vehicle for the gene. NIH3T3 cells are a well-established fibroblast cell line, thus, it is easy to optimize conditions for gene transfer and to select gene-expressing cells for use *in vitro*. In addition, such fibroblasts are available from various human sources, which may be advantageous for extending future clinical investigations. NIH3T3 cells were transfected with the BDNF gene using lipofection, with the cells expressing the BDNF gene being selected for use. We examined the potential for transplanting transfected NIH3T3 cells into the inner ear of the mouse. Immunohistochemistry and Western blotting demonstrated the survival of the grafted cells within the cochlea, and a BDNF-specific enzyme-linked immunosorbent assay revealed a significant increase in BDNF production in the inner ear following cell transplantations [59]. These findings indicate that cell-gene delivery with non-viral vectors may be applicable for the local, sustained delivery of therapeutic

molecules into the cochlea. Cell-gene delivery of therapeutic molecules into the inner ear is suitable for protection of inner ear cells against gradually progressive degeneration. Presbycusis, which is an age-related hearing loss, may also need to be included as one of the targets for cell-gene therapy. BDNF application *via* cell-gene delivery could be an effective strategy for survival promotion of SGNs in cases involving cochlear implants, which require the opening of the cochlea for the purpose of inserting an electrode.

CONCLUSIONS

The lack of effective methods for drug delivery to the cochlea has been a considerable obstacle with regard to developing novel therapeutic strategies for SNHL. However, recent findings in studies examining drug delivery systems using biomaterials and cell therapy demonstrate the efficacy of these strategies for cochlear drug delivery, which in the future may contribute to the establishment of novel therapeutic strategies for SNHL.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid for Regenerative Medicine Realization, a Grant-in-Aid for Scientific Research and a Grant from the 21st Century COE program from the Ministry of Education, Science, Sports, Culture and Technology of Japan, and by a Grant-in-Aid for Researches on Sensory and Communicative Disorders from the Japanese Ministry of Health, Labour and Welfare.

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Received: August 21, 2007 Revised: January 02, 2008 Accepted: January 11, 2008

総　説

日耳鼻 111：655-663，2008

「第109回日本耳鼻咽喉科学会総会シンポジウム」

内耳疾患の治療をめざして—基礎研究の最前線

薬物の経正円窓投与

中川　隆之

京都大学大学院医学研究科
耳鼻咽喉科頭頸部外科

感音難聴は，最も頻度の高い身体障害であり，新しい治療法開発に対する難聴者の期待は高い．この20年間に人工内耳など電子器機デバイス領域では新しい治療法の開発があるが，薬物療法を中心とした生物学的な治療法開発は基礎的研究にとどまっている．感音難聴治療開発に関連する研究成果にも目覚ましいものがあるが，臨床応用にはいくつかの解決すべき問題が残されている．そのひとつに，いかにして内耳に薬物を到達させるかという問題がある．簡便かつ安全に，内耳に持続的に薬物を供給することができれば，いくつかの内耳基礎研究成果は臨床応用されることが期待できる．われわれは，この問題に対する解決策として，生体吸収性素材を用いた内耳薬物投与システムを開発した．治療薬を徐放する生体吸収性素材を中耳正円窓に留置し，内耳に薬物を徐放しようとするものである．親水性の高分子（タンパクやペプチド）に適した薬物徐放の材料としてゼラチンポリマー，疎水性，低分子の薬物（ステロイドやリドカイン）を徐放する材料としてポリグリコール乳酸に着目し，内耳への薬物徐放に関する有効性を調べるために，いくつかの動物実験を行った．結果，ゼラチンポリマーは神経栄養因子や細胞増殖因子を内耳に徐放することができ，治療的効果を発揮することが示された．ポリグリコール乳酸を用いる方法では，耳鳴り抑制を目的としたリドカインの蝸牛内への徐放に成功した．ゼラチンポリマーを用いた内耳へのインスリン様細胞成長因子１投与は，京都大学大学院医学研究科の医の倫理委員会の承認を経て，ステロイド無効急性高度難聴例に対する第Ⅰ-Ⅱ相臨床試験を行っている．今後，臨床試験をさらに進めると同時に，内耳再生を標的とした治療薬の内耳局所投与に関連する基礎的研究開発を進めていき，新たな感音難聴治療法を日常臨床に１日も早く提供したい．

キーワード：感音難聴，正円窓，ゼラチン，薬物徐放，臨床試験
Keywords：Sensorineural hearing loss, Round window, Gelatin, sustained release, Clinical trial

はじめに

感音難聴は，最も頻度の高い身体障害のひとつである．身体障害者レベルの高度難聴者は約36万人あり，65歳以上の高齢者の60%にはなんらかの感音難聴が存在するとされている．しかしながら，一旦喪失した聴力を元に戻す方法はない．聴力の再生は，高度難聴者においては音のない世界から音のある世界の獲得を意味し，中等度難聴者にとっても社会生活を送る上で大きな福音となることは論を待たない．現在，高度難聴者に対しては，人工内耳が広く用いられるようになり，対費用効果の高い治療法として評価されている．人工内耳で得られる聴覚は，自然な聴覚とはかなり異なるものであるが，その有益性が高く評価されているということは，聴覚障害が生活の質に与える影響がいかに大きいものかを意味している．現状では，一旦固定した聴覚障害に対する治療としては，補聴器や人工内耳などの電子器機に頼らざるをえない．急性高度難聴を含めても感音難聴に対する有効な治療法が乏しいこと，この事実に対する患者の失望，