that the administration of gRb1 prevented apoptotic cell death signaling in SGCs and minimized injury resulting from cochlear ischemia.

Many substances and drug delivery systems specific to the inner ear are in development, with some under evaluation in animal experiments or clinical studies. Intravenous administration of gRb1 has been shown to rescue cortical neurons in ischemic injury [17]. Our findings suggest that gRb1 may be effective in the treatment of sensorineural hearing loss. Further research is required to reveal the protective effect of gRb1 against long-term damage to SGCs caused by cochlear ischemia.

We performed a histological examination of the cochleae to determine SGC degeneration caused by transient cochlear ischemia. Seven days after ischemia, the number of SGCs decreased in the basal turn. In addition, we investigated the neuroprotective effect of gRb1 against ischemic injury and our results demonstrated a significant protective effect against SGC degeneration via the suppression of the apoptotic cell death pathway. Therefore, gRb1 may be effective for the treatment of sensorineural hearing loss that eventually follows transient ischemia of the cochlea.

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HEMATOPOIETIC STEM CELLS PREVENT HAIR CELL DEATH AFTER TRANSIENT COCHLEAR ISCHEMIA THROUGH PARACRINE EFFECTS

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Abstract—Transplantation of hematopoietic stem cells (HSCs) is regarded to be a potential approach for promoting repair of damaged organs. Here, we investigated the influence of hematopoietic stem cells on progressive hair cell degeneration after transient cochlear ischemia in gerbils. Transient cochlear ischemia was produced by extracranial occlusion of the bilateral vertebral arteries just before their entry into the transverse foramen of the cervical vertebra. Intrascalar injection of HSCs prevented ischemia-induced hair cell degeneration and ameliorated hearing impairment. We also showed that the protein level of glial cell line-derived neurotrophic factor (GDNF) in the organ of Corti was upregulated after cochlear ischemia and that treatment with HSCs augmented this ischemia-induced upregulation of GDNF. A tracking study revealed that HSCs injected into the cochlea were retained in the perilymphatic space of the cochlea, although they neither transdifferentiated into cochlear cell types nor fused with the injured hair cells after ischemia, suggesting that HSCs had therapeutic potential possibly through paracrine effects. Thus, we propose HSCs as a potential new therapeutic strategy for hearing loss. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cochlear ischemia, hematopoietic stem cell, stem cell therapy, hearing loss, hair cell death, GDNF.

The prevalence of acquired hearing loss is very high. About 10% of the total population and more than one third of the population over 65 years suffer from debilitating hearing loss (Li et al., 2004). The most common type of hearing loss in adults is sensorineural hearing loss (SNHL). In the majority of cases, SNHL is permanent and typically associated with loss of sensory hair cells in the organ of Corti. Humans are born with a complement of about 16,000 sensory hair cells and 30,000 auditory neurons in each ear. Sensory hair cells and auditory neurons do not regenerate throughout life, and loss of these cells is irreversible and cumulative. At present, the only therapeutic intervention for patients with profound SNHL is a cochlear implant that electrically stimulates residual primary auditory neurons. In many cases, a cochlear prosthesis and associated speech processor can restore accurate speech reception to a person who otherwise has little or no auditory sensitivity. For the last two decades, cochlear implants have been in common clinical use. Following the loss of sensory hair cells, however, the auditory neurons undergo secondary degeneration. Evidence from animal studies indicates that ongoing degeneration of auditory neurons has the potential to compromise the efficacy of a cochlear implant (Shepherd et al., 2004). From the clinical perspective, there are likely to be benefits if sensory hair cells can be rescued.

Recent advances in stem cell biology have provided hope that stem cell therapy will come closer to regenerating sensory hair cells in humans. A major advance in the prospects for the use of stem cells to restore normal hearing comes with the recent discovery that hair cells can be generated *ex vivo* from embryonic stem (ES) cells, adult inner ear stem cells and neural stem cells (Li et al., 2003a,b; Tateya et al., 2003). These stem cells are pluripotent, such that all cell types in the inner ear can be derived from them. Furthermore, stem cells can secrete several kinds of trophic factors. There is increasing evidence that stem cells can promote host neural repair in part by secreting diffusible molecules such as growth factors (Mahmood et al., 2004). These findings suggest that stem-cell-based treatment regimens could be applicable to the damaged inner ear as future clinical applications. Previously we have shown that neural stem cells can prevent ischemia-induced inner hair cell (IHC) loss and ameliorate hearing impairment. Among the several types of stem cells, we propose that hematopoietic stem cells (HSCs) are one of the best candidates for stem cell therapy in clinical practice, because autologous transplantation can not only eliminate the need to find suitable donors, but can also avoid the problems of immunological incompatibility and ethical concerns. In this study, we explored the feasibility of HSC transplantation as therapy for hearing loss.

EXPERIMENTAL PROCEDURES

All experiments were conducted according to the U.S. National Institutes of Health Guide for the Care and Use of Laboratory

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Abbreviations: ABR, auditory brainstem response; Ang1, angiopoietin-1; BDNF, brain-derived neurotrophic factor; DMEM, Dulbecco's modified Eagle's medium; EPO, erythropoietin; FCS, fetal calf serum; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; HSC, hematopoietic stem cell; IHC, inner hair cell; OHC, outer hair cell; PI, propidium iodide; SDS, sodium dodecylsulfate; SNHL, sensorineural hearing loss; SP, side population.

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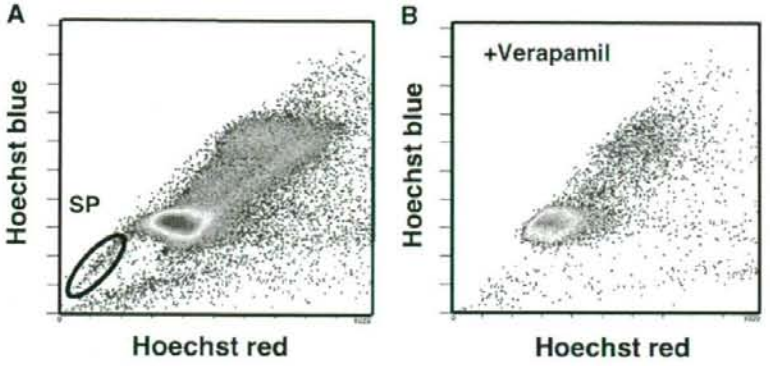

Fig. 1. Flow cytometric analysis of SP cells in Hoechst-stained bone marrow (BM). SP region of whole BM stained with Hoechst in the absence (A) and presence (B) of 50 μM verapamil. The region marked SP was sorted for transplantation experiments. Approximately 0.2–0.4% of cells fell into the SP gate.

Animals (NIH Publication No. 80-23) and were approved by the Ethics Committee at Ehime University Graduate School of Medicine. All efforts were made to minimize the number of animals used and their suffering. Animals were housed in an animal room with a temperature of 21–23 °C and a 12-h light/dark cycle (light on: 7 a.m. to 7 p.m.). Animals were allowed access to food and water *ad libitum* until the end of the experiment.

Induction of transient cochlear ischemia

Adult male Mongolian gerbils weighing 60–80 g were used in this study. Following the methods of Hata et al. (1993), transient cochlear ischemia was induced by temporarily occluding bilateral vertebral arteries in the neck, since they lack the posterior cerebral communicating arteries and the labyrinthine arteries are nourished solely by the vertebro-basilar system. Anesthesia was induced with 3% halothane in a 7:3 mixture of nitrous oxide and oxygen, and maintained with 1% halothane. An anterior midline cervical incision was made, and bilateral vertebral arteries were exposed just before their entry into the transverse foramen of the cervical vertebra. Then, a 4-0 silk suture was loosely looped around each vertebral artery. The animals were orotracheally intubated, and artificially ventilated to prevent systemic anoxia. The tidal volume was set at 1 ml and the ventilation rate at 70/min. Ischemia was induced in both cochleae by pulling the ligatures with 5 g weights. After 15 min of ischemia, the sutures were removed to allow recirculation, which was confirmed by observation with an operating microscope. Some gerbils were sham-operated as control animals, where bilateral vertebral arteries were exposed but no arterial occlusion took place. Rectal temperature was maintained at 37 °C with a heat lamp during the surgical procedure.

Isolation of HSCs

Bone marrow specimens were extracted from the tibias and femurs of 6–12-week-old gerbils. The bone marrow cells were suspended at 10^6 cells/ml in pre-warmed Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS). HSCs were isolated by the method described by Goodell et al. (1996). In brief, the bone marrow cells were stained with 5 μg/ml Hoechst 33342 (Sigma Chemical Co., St. Louis, MO, USA) for 90 min at 37 °C. Analysis and sorting were executed with an EPICS ALTRA flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). Hoechst dye was excited with a UV laser at 333.4–363.8 nm. Two wavelengths, obtained by using a 450 BP filter (Hoechst Blue) and a 675 EFLP optical filter (Hoechst Red), were used to measure its fluorescence. Propidium iodide (PI) fluorescence was also measured at 675 EFLP (having been excited at 350 nm). Cells stained with PI were seen on the far right of Hoechst red (675 EFLP) and excluded. The addition of PI allowed exclusion of dead cells and did not affect the Hoechst staining profile. Both Hoechst blue and red fluorescence are shown on a linear scale. The gating on forward and side scatter was not rigorous, and excluded only erythrocytes and debris. The side population (SP) sorting gates were defined on the flow cytometer using Hoechst red and blue axes to exclude dead cells, erythrocytes (no Hoechst stain), and debris. After collecting 10^5 events within this live gate, the SP population could be clearly identified and defined and was considered as HSCs (Goodell et al., 1996). The gate established on this population is shown in Fig. 1A.

Administration of HSCs

One day before the induction of transient cochlear ischemia, the gerbils received HSCs (2×10^3 cells/μl in DMEM, total 4 μl) in their left cochleae. The right cochlea of each animal was treated with vehicle (DMEM; total 4 μl) and used as vehicle control. Under halothane anesthesia, the otic bulla was opened through a retroauricular approach and the round window was exposed. A 0.15-mm-diameter glass microtube was inserted into the scala tympani through the round window with a micromanipulator. HSCs or vehicle were infused at a flow rate of 1 μl/min for 4 min using a microinfusion pump.

Recording of auditory brainstem response (ABR)

Hearing of the animal was assessed before and 4 days after ischemic insult by sequential recording of ABR. Under halothane anesthesia, ABR was recorded using a signal processor (NEC Synax 1200, NEC Medical Systems, Tokyo, Japan). Stimulus sound was led to the ear canal via a tiny polypropylene tube; thus each ear was stimulated separately. Recording needle electrode was placed at the vertex and retroauricle. As the animal did not tolerate long-term anesthesia, ABR was recorded only to 8000-Hz tone burst (0.5 ms rise/fall time and 10 ms duration). Cochlear region corresponding to the tone of this frequency was proved most vulnerable to ischemic injury, according to our previous study (Hakuba et al., 2003b). Responses to 300 consecutive stimuli were averaged, and the threshold of ABR was determined by measuring the responses in 5 dB steps.

Evaluation of hair cell loss

The degree of hair cell loss was assessed by staining the cochlea with rhodamine-phalloidin and Hoechst 33342. Rhodamine-phalloidin is appropriate for observation of cell architecture and Hoechst 33342 for their nuclei. Four days after ischemia, the organs of Corti were dissected out by means of a surface preparation and were stained with rhodamine-phalloidin (Hakuba et al., 2003b). Fluorescence was detected using an Olympus BX60 microscope (Olympus, Tokyo, Japan) with a green filter and a UV filter. The numbers of intact and dead hair cells were counted in the basal turn of the cochlea, and the ratio of intact to dead hair cells was calculated.

Western blot analysis

After deep anesthesia with an i.p. injection of sodium pentobarbital (0.1 g/kg), the otic bulla (wet weight 10 mg) was removed and transferred to ice-cold PBS. The samples were homogenized in microcentrifuge tubes containing 100 μl lysis buffer (0.5% sodium dodecylsulfate (SDS), 0.5% Triton-X, 100 μM phenylmethane sulfonyl fluoride, 20 μM Tris–HCl pH 8.0). The homogenates were sonicated on ice and centrifuged at 13,000 r.p.m. for 10 min at 4 °C. The protein content in the supernatant was determined using a BCA protein assay kit (Pierce, Rockland, IL, USA) with bovine serum albumin as a standard. The supernatant was mixed with sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol and 0.001% Bromophenol Blue) to a final protein concentration of 1 mg/ml. The samples were boiled for 5 min. Equal amounts of protein (15 μg/lane) were resolved by SDS-PAGE electrophoresis, transferred onto a nitrocellulose membrane, and immunoblotted with an antibody against glial cell line-derived neurotrophic factor (GDNF) (sc-328, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Densitometric analysis of scanned bands was performed to quantify the GDNF protein levels in the samples. The integrated optical density was obtained using a NIH Image program (National Institutes of Health, Bethesda, MD, USA). The data were normalized to internal standards (vehicle-treated control) on each gel and expressed in percentage.

PKH67 staining

The sorted cells were labeled using a fluorescent membrane dye, PKH67 (Sigma), which excites at a wavelength of 496 nm and emits at 520 nm. According to the manufacturer's instructions, samples were stained with PKH67 at room temperature for 10 min. Staining was stopped by addition of four volumes of DMEM containing 10% FCS. The cells were collected by centrifugation (1500 r.p.m., 10 min, 4 °C), and washed twice with DMEM.

Tissue preparation for short-term cellular tracking

The gerbils were treated with PKH-labeled HSCs as described above. Four days after ischemic insult, they were deeply anesthetized intraperitoneally with a lethal dose of sodium pentobarbital (0.5 g/kg), and perfused intracardially with saline, followed by 4% paraformaldehyde in PBS. The temporal bones were removed and fixed in the same fixative at 4 °C for 4 h. In some animals, the fixed temporal bones were decalcified with 0.1 M EDTA for 24 h at 4 °C and 10-μm-thick cryostat sections of the temporal bone were prepared. The sections were then mounted on 3-aminopropyl triethoxysaline (APS)-coated slide glasses. In other animals, the organs of Corti in the fixed temporal bones were dissected out by means of a surface preparation, stained with rhodamine-phalloidin and mounted on slide glasses as described above. The sections were viewed with an Olympus BX60 fluorescence microscope.

Statistical analysis

All values are presented as mean±S.D. The changes in ABR threshold between the vehicle-treated side and HSC-treated side were analyzed using two-tailed Mann-Whitney *U* test. All other statistical significances were tested by one way ANOVA followed by Bonferroni's multiple comparison test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

ABR threshold shift

We initially evaluated the sequential changes in hearing by ABR in six gerbils. ABR threshold to 8000 Hz tone burst was approximately 30 dB SPL in normal animals, which was significantly elevated by ischemic insult. Hearing deterioration was prevented by pre-ischemic transplantation of HSCs (Fig. 2). Four days after ischemia, the average increase in the ABR threshold on the vehicle-treated side was 32.5±7.6 [mean±S.D.] dB. In contrast, the average increase in ABR threshold on the HSC-treated side was 16.9±5.9 dB. These results suggested that treatment with HSCs ameliorated the ischemia-induced hearing impairment.

Morphological study

Previously, we reported that cochlear ischemia for 15 min resulted in progressive IHC loss by 4 days after ischemia, while a little outer hair cell (OHC) loss was observed (Watanabe et al., 2001). We also reported that this progressive IHC loss was closely related to hearing impairment evaluated by ABR (Watanabe et al., 2001). Hence, we next investigated the effects of HSC transplantation on ischemia-induced IHC loss. Hair cell loss was identified at

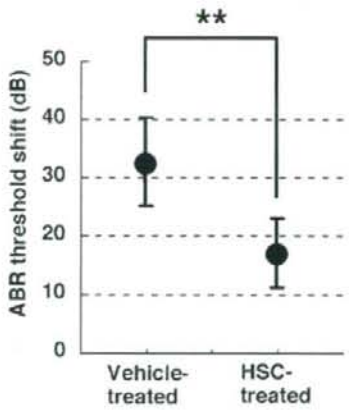

Fig. 2. ABR threshold at 4 days after cochlear ischemia. Pretreatment with HSCs (HSC-treated) significantly suppressed the elevation of threshold in comparison with the vehicle-treated control. The average ABR threshold shift on the vehicle- and HSC-treated sides (n=6 in each side) was analyzed using two-tailed Mann-Whitney *U* test. A *P* value less than 0.05 was considered statistically significant. Double asterisk indicates statistical significance (P<0.01). All values are presented as mean±S.D.

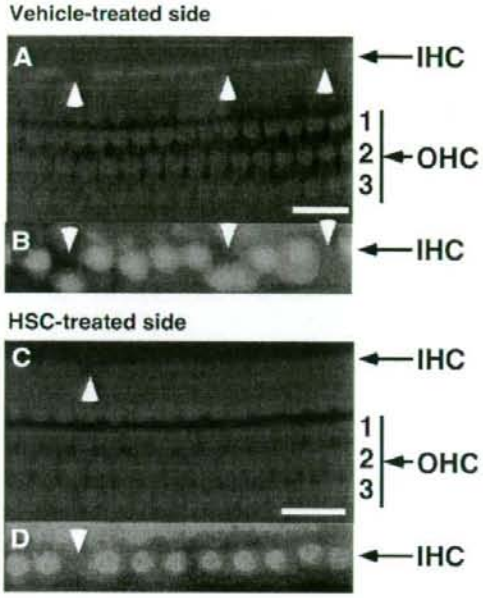

Fig. 3. Surface structure of organ of Corti at 4 days after cochlear ischemia. Representative fluorescence images of the organ of Corti stained with rhodamine-phalloidin (A and C) and Hoechst 33342 (B and D). Gerbils were subjected to cochlear ischemia for 4 days. The organs of Corti were obtained from the otic bullae on the vehicle-treated side (A and B) and HSC-treated side (C and D). There are three rows of OHCs and a single row of IHCs. Fluorescence microscopy revealed fewer deficits in IHCs on the HSC-treated side than on the vehicle-treated side. Scale bar=20 μm. Arrowheads indicate deficits in IHCs.

4 days after ischemia while only a small hair cell loss was identified in normal animals (Fig. 3). It is apparent that the stereocilia of hair cells on the vehicle-control side disappeared sporadically. In contrast, the hair cell loss was ameliorated in the specimen obtained from the HSC-treated side. The percentages of hair cell loss are summarized in Fig. 4. In each group (n=6), cell loss was more prominent in IHCs than in OHCs. In IHCs, the percentage of cell loss was 23.6±4.0% on the vehicle-treated side and 8.2±4.0% on the HSC-treated side. The difference was statistically significant (P<0.01). In OHCs, the percentage of cell loss was 2.5±1.4% on the vehicle-treated side and 2.9±2.4% on the HSC-treated side, representing no significant difference. These results were consistent with a greater change of ABR threshold in the vehicle-treated control, compared with that in the HSC-treated group.

Fate of HSCs injected into organ of Corti

We next investigated whether the HSCs transdifferentiated into cochlear cell types or fused with the injured hair cells after cochlear ischemia. To confirm the fate of HSCs, we used PKH67 for short-term tracking *in vivo*. PKH has been used for cellular tracking (Punzel et al., 2001), and this dye has been demonstrated to be stable on the surface of quiescent cells for periods exceeding 3 weeks, does not compromise cellular viability, and does not impair the capacity of stem cells to reconstitute hematopoiesis in myeloablated recipients (Askenasy and Farkas, 2002). By using this dye (Fig. 5A and B), tracking of the HSCs injected into the cochleae was performed at 4 days after ischemia. In a 10-μm-thick cryostat section, transplanted cells were predominantly located in the perilymphatic space of the cochlea (Fig. 5C and D). In sections of a cochlear surface preparation that were washed with PBS several times, no PKH-labeled cells were observed in hair cells and supporting cells of the organ of Corti (Fig. 5E and F). These results suggested that transplanted HSCs were retained within the perilymphatic space of the cochlea, but neither transdifferentiated into cochlear cells nor fused with the injured hair cells.

Induction of trophic factor after cochlear ischemia

To gain an insight into the mechanisms underlying IHC survival, we investigated the changes in the expression of GDNF, brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF)1, FGF2, angiopoietin-1 (Ang1), neurotrophin-3 (NT3) and erythropoietin (EPO). Among them, only GDNF protein expression was markedly upregulated by treatment with HSCs after cochlear ischemia. As shown in Fig. 6A, a single protein band of the expected size (approximately 35 kDa) for GDNF was detected by Western blot with a GDNF-specific primary antibody. No band was detected when the blots were incubated without primary antibody (data not shown). Five independent experiments were carried out and the results of densitometric

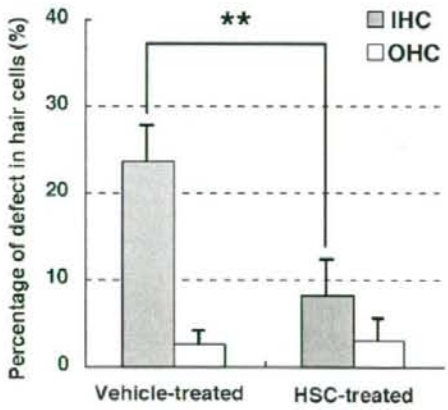

Fig. 4. Percentages of defects in IHCs and OHCs at 4 days after cochlear ischemia. Pretreatment with HSCs significantly reduced IHC damage at 4 days after cochlear ischemia. On the HSC-treated side (n=6), the proportion of deficits in IHCs was lower than that on the vehicle-treated side (n=6). On the other hand, there was no statistically significant difference in the amount of OHC loss between the HSC-treated side and vehicle-treated side. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test. A P value less than 0.05 was considered statistically significant. Double asterisk indicates statistical significance (P<0.01). All values are presented as mean±S.D.

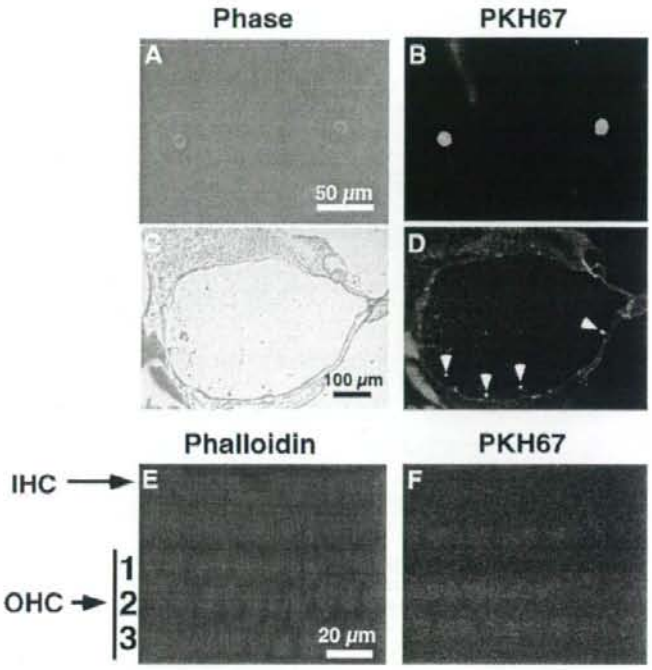

Fig. 5. PKH67 labeling. HSCs were labeled with PKH67 (B: green). Gerbils were treated with PKH67-labeled HSCs. One day later, animals were subjected to cochlear ischemia for 15 min. Four days after ischemia, the temporal bones were dissected out, fixed, decalcified and cut into 10-μm-thick cryostat sections at −20 °C. PKH67-positive cells were located in the perilymphatic space of the cochlea (D: green, arrowheads). In addition, other animals were treated with PKH67-labeled HSCs, and the temporal bones were dissected out and fixed. Then, the organs of Corti were dissected out by means of a surface preparation and visualized with rhodamine-phalloidin (E: red). No PKH-labeled cells were observed in hair cells, supporting cells, and other types of cochlear cells throughout the organ of Corti (F). Scale bars=50 μm (A and B), 100 μm (C and D), 20 μm (E and F).

analysis are shown in Fig. 6B. In sham-operated animals, there was no significant difference in the level of GDNF protein between the vehicle-treated control and HSC-treated group. In contrast, the level of GDNF protein was significantly increased at 4 days after cochlear ischemia. The increase of the GDNF protein level was more prominent in the HSC-treated group than in the vehicle-treated control. These results revealed that ischemia-induced GDNF expression was augmented by treatment with HSCs.

DISCUSSION

In the present study, we used an animal model of transient cochlear ischemia induced by extracranial occlusion of the bilateral vertebral arteries in gerbils. This animal model has been described as a brain stem ischemia model. By using this animal model, selective vulnerability to ischemia in the brain stem was closely observed (Hata et al., 1993). This brainstem ischemia model has the following advantages: (1) it avoids intracranial injury, (2) it produces severe reproducible brainstem ischemia, and (3) it allows reperfusion. We also showed that reversibility of the ABR after reperfusion was correlated with ischemic lesions in the acoustic relay nuclei in the brainstem (Hata et al., 1998). Because the inner ear is supplied by the labyrinthine artery from the basilar artery, Hakuba et al. (1997) first introduced this animal model as a cochlear ischemia model, and showed progressive IHC loss up to 4 days after cochlear ischemia (Hakuba et al., 2000; Watanabe et al., 2001). Our series of studies showed that this progressive IHC degeneration was induced by the ischemia-induced increase of glutamate concentration in the perilymph, activation of AMPA/kainate receptors on the presynaptic membrane of IHCs, and the subsequent accumulation of intracellular Ca^{2+} in IHCs, leading to cell death (Hakuba et al., 2003a; Hyodo et al., 2001; Maetani et al., 2003; Morizane et al., 2005; Taniguchi et al., 2002). Impairment of cochlear blood flow is thought to play an important role in the etiology of sudden deafness, presbycusis and noise-induced hearing loss (Nakashima et al., 2003; Seidman et al., 1999). Because of the fact that 90–95% of afferent sensory neurons synapse on IHCs and only 5% of neurons synapse on OHCs (Spoendlin, 1967), IHCs are thought to be the main mechanosensory cells that transform mechanical stimuli into neuronal signals (Brandt et al., 2003). In this ischemia model, the mean inter-peaked latency between waves I and V of ABR was not changed (K. Fujita, unpublished

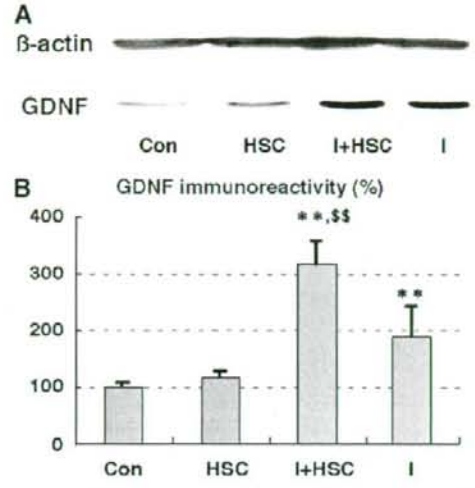

Fig. 6. Western blot analyses of GDNF in gerbil cochlea on the vehicle-treated and HSC-treated side. Samples were derived from the cochlear on the vehicle-treated side (Con) and HSC-treated side (HSC) in sham-operated gerbils, and on the vehicle-treated side (I) and HSC-treated side (I+HSC) in gerbils subjected to cochlear ischemia. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's multiple comparison test. A P value less than 0.05 was considered statistically significant. ** Indicates significantly greater than vehicle-treated control (Con) ($P<0.01$). $^{\$\$}$ Indicates significantly greater than vehicle-treated group with cochlear ischemia (I) ($P<0.01$). Data were obtained from five independent experiments. All values are presented as mean±S.D.

observations), suggesting that main ischemic lesion was not located within the brain stem. Furthermore, IHC loss started 1 day after ischemia and peaked at 4 days after ischemia, whereas neuronal loss in the spiral ganglion started at 4 days after ischemia and peaked at 7 days after ischemia. These data suggested that ischemia-induced IHC loss resulted in the secondary degeneration of the spiral ganglion neurons (K. Fujita, unpublished observations). In fact we showed that progressive IHC loss was closely related to hearing impairment evaluated by ABR (Watanabe et al., 2001). In the present study, we clearly showed that treatment with HSCs ameliorated this progressive IHC damage and prevented a shift in the ABR threshold after transient cochlear ischemia in gerbils.

The precise mechanism by which intrascalar injection of HSCs prevented ischemia-induced progressive IHC damage is unclear. There are several possibilities to enable functional recovery by treatment with HSCs. One explanation is that HSCs can induce the endogenous cochlear cells to proliferate and differentiate into hair cells to rescue or restore hearing loss. Several studies have shown that both neural stem cells and inner ear stem cells have the ability to differentiate into different inner ear cell types *in vivo* (Tateya et al., 2003) or *in vitro* (Li et al., 2003b). Recent studies have shown that HSCs are capable of transdifferentiating into a variety of nonhematopoietic lineages in multiple organs (Masson et al., 2004). Another explanation is that HSCs can fuse with damaged hair cells and restore their function. A Cre/lox recombination system to identify transplanted cells indicated that bone marrow–derived stem cells fused with hepatocytes in the liver, with Purkinje neurons in the brain, and with cardiac muscle in the heart, resulting in the formation of multinucleated cells (Alvarez-Dolado et al., 2003). After transplantation of bone marrow from female wild-type mice into male fumarylacetoacetate hydrolase knockout mice, analysis of DNA from the tertiary recipients revealed that hepatocytes derived from bone marrow arose from cell fusion and not by transdifferentiation of HSCs (Wang et al., 2003). These reports suggested that cell fusion is responsible for phenotypic changes of HSCs into the target cells. The third explanation is that HSCs can promote hair cell repair in part by secreting trophic factors. It has been reported that production of trophic factors from stem cells can confer resistance to disease, or promote the survival, migration, and differentiation of endogenous precursors (Chopp and Li, 2002). Stem cell transplantation may be linked to the up-regulation of trophic factors (Mahmood et al., 2004). These reports suggest the possibility that stem cells can also play a part in promoting functional recovery by means other than cell replacement. In fact, bone marrow stem cells and neural stem cells are also known to secrete interleukins and neurotrophic factors (NGF, BDNF, and GDNF) (Crigler et al., 2006; Mahmood et al., 2004). Furthermore, HSCs were reported to secrete growth factors with neurotrophic properties, such as Ang1 (Takakura et al., 2000). In the present study, no PKH-labeled HSCs were observed throughout the organ of Corti. PKH-labeled HSCs were predominantly located in the perilymphatic space of the cochlea. These findings revealed that HSCs could survive in the perilymphatic space in the cochlea, and that at least in our experimental conditions, HSCs were not incorporated into hair cells, supporting cells, and other types of cells in the organ of Corti through cell fusion or transdifferentiation. For these reasons, the third explanation may be preferable, although further cellular and molecular biological investigations are required to clarify its mechanism.

It is well known that several kind of trophic factors including GDNF play a crucial role in the survival of sensory hair cells and auditory neurons (Gillespie and Shepherd, 2005; Roehm and Hansen, 2005). Because the protection by HSCs against ischemia-induced hair cell damage appeared to occur through their paracrine effects, we evaluated the ischemia-induced alterations of trophic factors (i.e. FGF1, FGF2, BDNF, NT-3, EPO, Ang1 and GDNF) in the cochlea. Consequently, we revealed that only GDNF expression was upregulated after cochlear ischemia, and this ischemia-induced GDNF expression was augmented by treatment with HSCs. GDNF belongs to the transforming growth factor-β superfamily and was discovered to be a potent neurotrophic factor for midbrain dopaminergic neurons (Lin et al., 1993). GDNF was reported to confer protection to neurons during various types of injury to the nervous system *in vitro* and *in vivo* (Li et al., 1995; Wang et al., 2002). A survival-promoting effect of GDNF on inner ear hair cells against ototoxicity has been reported *in vivo* (Kuang et al., 1999). In our previous report,

we also showed that adenovirus-mediated overexpression of GDNF significantly prevented progressive IHC degeneration after cochlear ischemia in gerbils (Hakuba et al., 2003b). In accordance with these previous reports, we showed that HSCs had the potential to upregulate the GDNF protein level in the organ of Corti after ischemia, suggesting protective effects of GDNF against ischemia-induced hair cell damage. In normal adult rodent, GDNF expression is observed in IHCs (Ylikoski et al., 1998) and the level of GDNF in IHCs was upregulated after noise exposure (Nam et al., 2000). We, then, speculate that cochlear ischemia can upregulate the GDNF level in IHCs and HSCs can modulate the GDNF level in the organ of Corti after ischemia, although further histochemical investigation must be required to confirm this assumption.

CONCLUSION

In conclusion, our study clearly showed that intrascalar injection of HSCs prevented a shift in the ABR threshold and attenuated the progressive IHC damage after cochlear ischemia. In addition, injected HSCs had the potential to upregulate the protein level of GDNF in the organ of Corti after cochlear ischemia. At present, there are some difficulties for the clinical use of HSCs because we can get only a small amount of HSCs from the bone marrow and the technique for proliferating HSCs *ex vivo* is not established yet. Moreover, long-term effects of HSC transplantation are not fully elucidated and unexpected adverse effects such as malignancy and inappropriate immuno-response are not negligible. However, these data suggest that HSC transplantation could be useful in the treatment of SNHL.

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Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea

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Recent studies using explant cultures have demonstrated that pharmacological inhibition of Notch signaling by γ-secretase inhibitors generates supernumerary hair cells in embryonic or neonatal cochleae. The aim of this study was to examine the effects of such pharmacological inhibition on mature auditory epithelia *in vivo*. Normal adult guinea pig auditory epithelia exhibited weak or no immunoreactivity for Notch1 and Jagged1, whereas ototoxic treatment caused the upregulation of these molecules in damaged auditory epithelia. Local application of a γ-secretase inhibitor in damaged cochleae generated ectopic hair cells in mature auditory epithelia. These findings indicate that pharmacological inhibition of Notch signaling is a possible strategy for hair cell regeneration in adult auditory epithelia. *NeuroReport* 18:1911–1914 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: γ-secretase inhibitor, hair cell, hearing loss, inner ear, regeneration

Introduction

Hearing impairment is a common disability in industrialized countries. Hearing depends largely on the presence of hair cells (HCs) in the cochlea, defects of which result in hearing loss or deafness. HC regeneration in mammals occurs naturally over a limited time period during development [1], and many attempts have been made to restore HCs in adult mammals. Recent studies have demonstrated the existence of stem cell-like cells in the mature auditory epithelium that have the potential to differentiate into HCs [2,3]. Notch signaling plays a critical role during auditory epithelium development [4–6]. Even after maturation, viral overexpression of the Notch signaling transcription factor Atoh1 induces transdifferentiation of supporting cells (SCs) into HCs [7–9].

Atoh1 expression is regulated by the expression of Hes1 and Hes5 in Notch signaling [10]. In general, the binding of Notch ligands to their receptors induces the release of Notch intracellular domains from the plasma membrane to the nucleus, resulting in the activation of RBP-J in the nucleus [11]. This induces Hes1 and Hes5 transcription, which, in turn, suppresses Atoh1 expression. Therefore, suppression of Hes1 and Hes5 expression by inhibition of Notch signaling causes an increase in Atoh1 expression [12].

Gamma secretase plays a crucial role in the proteolytic release of Notch intracellular domains from the cytoplasmic membrane. Thus, the pharmacological inhibition of this process results in the inhibition of Notch signaling [12]. Recent studies using explant cultures of auditory epithelia have demonstrated that a γ-secretase inhibitor increases HC numbers in the auditory epithelia of embryonic [13] and newborn mice [14]. These findings prompted us to investigate the potential for pharmacological inhibition of Notch signaling in inducing HC proliferation in mature auditory epithelia.

Our working hypothesis was that pharmacological inhibition of Notch signaling induces the transdifferentiation of SCs into HCs in the damaged cochleae of adult animals. We therefore examined the effects of this pharmacological inhibition in the damaged auditory epithelia of adult guinea pigs *in vivo*.

Materials and methods

Experimental animals

Hartley strain guinea pigs weighing 350–400 g were purchased from Japan SLC Inc. (Hamamatsu, Japan). The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all of the experimental protocols. Animal care was supervised by the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Ototoxic treatment

The animals were deafened bilaterally with a single intramuscular injection of kanamycin sulfate (KM; 500 mg/kg; Wako Pure Chemical Industries Ltd, Osaka, Japan), followed 2 h later by an intravenous injection of ethacrynic acid (EA; 50 mg/kg; Wako Pure Chemical Industries Ltd). Measurements of auditory function were performed by recording auditory brain-stem responses at

frequencies of 4, 8 and 16 kHz on day 2 or 3 after ototoxic treatment. Animals that exhibited greater than 90 dB sound pressure level of auditory brain-stem response thresholds at all frequencies were used in the following experiments.

Immunohistochemistry for Notch1 and Jagged1

To evaluate Notch signaling activation, we determined the expression level of Notch1 and Jagged1 in auditory epithelia. Cochlear specimens from guinea pig embryos (embryonic day 50; n=4), normal adult guinea pigs (n=4) and adult guinea pigs on days 2 (n=4), 4 (n=4) and 7 (n=4) following ototoxic treatment were fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; pH 7.4) at 4°C for 12 h. After decalcification with 0.1 M ethylenediamine tetra-acetic acid for 14 days at 4°C, 10-μm thick cryostat sections were prepared. Four midmodiolus sections were chosen from each cochlea, and antigen retrieval was accomplished by boiling the slides for 5 min in 0.01 M citrate buffer (pH 6.0). Slides were incubated with antimyosin VIIa rabbit polyclonal antibody (1:500; Proteus Bioscience Inc., Romana, California, USA), anti-Notch1 goat polyclonal antibody (1:200; Santa Cruz Biotechnology Inc., California, USA) and anti-Jagged1 goat polyclonal antibody (1:200; Santa Cruz Biotechnology Inc.) as primary antibodies, followed by Alexa Fluor 555 donkey antirabbit immunoglobulin G (IgG; 1:500; Molecular Probes, Eugen, Oregon, USA) and Alexa Fluor 488 donkey antigoat IgG (1:500, Molecular Probes) secondary antibodies. Nuclei were counterstained with 4′,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS; Molecular Probes). Cochlear specimens obtained from mice at postnatal day 2 (P2) were used as positive controls for Notch1 and Jagged1. Nonspecific labeling was tested by omitting the primary antibody from the staining procedures. The specimens were viewed with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

Pharmacological inhibition of Notch signaling

On day 4 after ototoxic treatment, the γ-secretase inhibitor MDL28170 (MDL; Sigma-Aldrich, St Louis, Michigan, USA) was locally applied to the perilymph. Under ketamine (75 mg/kg) and xylazine (9 mg/kg) general anesthesia, a silicone tube (180 μm in outer diameter) connected to a micro-osmotic mini pump (pumping 0.25 μl/h for 14 days; Alzet, Cupertino, California, USA) was inserted into the scala tympani of the basal turn of the left cochlea of 15 guinea pigs. MDL was dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS to give a final concentration of 1 mM containing 0.3% DMSO. The MDL solution was continuously injected through a micro-osmotic pump into the left cochleae of seven deafened and four normal animals, whereas the corresponding right cochleae received no local drug application (n=7). An additional four deafened animals received PBS containing 0.3% DMSO in the left cochlea instead of MDL solution. On day 14 after local drug application, the temporal bones were collected and immersed in 4% paraformaldehyde in PBS at 4°C for 12 h. After decalcification with 0.1 M ethylenediamine tetra-acetic acid for 14 days at 4°C, the cochleae were subjected to histological analysis of whole mounts.

Cochlear specimens were permeabilized in 0.2% Triton X in PBS for 30 min at room temperature. Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin was performed to determine the location of the HCs. After immunostaining for myosin VIIa, the specimens were stained with fluorescein-phalloidin (1:400; Molecular Probes), and viewed with a Leica TCS SP2 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). Quantitative analysis for numbers of ectopic myosin VIIa-positive cells and inner HCs (IHCs) was performed. Cochlear specimens treated with systemic KM and EA application followed by local MDL application and those treated with KM and EA alone were used. The numbers of ectopic myosin VIIa-positive cells and IHCs were counted in a 0.2-mm long region of the midbasal portion at 7-mm distance from the hook portion. The difference in numbers of ectopic myosin VIIa-positive cells or IHCs was examined by the unpaired t-test. Values of P less than 0.05 were considered statistically significant. Values are expressed as mean ± standard error of the mean (SEM).

Results

Immunohistochemistry for Notch1 and Jagged1

The expression of Notch1 and Jagged1 was identified in the auditory epithelium of guinea pig embryos, as previously reported in mouse embryos [15]. Strong expression of Notch1 was observed in SCs, and moderate expression was detected in the greater epithelial ridge, which is located between the spiral limbus and IHCs of immature cochleae (Fig. 1a). The expression of Jagged1 was distributed throughout the auditory epithelium of guinea pig embryos, similar to that of Notch1 (Fig. 1e). By contrast, with embryonic cochleae, the auditory epithelium of normal adult guinea pigs exhibited virtually no immunoreactivity for Notch1 or Jagged1 (Fig. 1b, f).

On days 2 and 4 after the systemic application of KM and EA, immunostaining for myosin VIIa demonstrated the loss of outer HCs (OHCs). Immunoreactivity for Notch1 was found in the inner sulcus cells, which are located between the spiral limbus and the IHCs of mature cochleae (Fig. 1c). We observed weak expression of Notch1 in the SCs of other regions of the auditory epithelium. Immunoreactivity for Jagged1 was also identified in the inner sulcus cells and SCs of the organ of Corti (Fig. 1g). Weak or no expression of Notch1 or Jagged1 was observed in the auditory epithelia on day 7 after ototoxic treatment (Fig. 1d, h).

Pharmacological inhibition of Notch signaling

Normal animals treated with local MDL application exhibited no loss of HCs, and no ectopic myosin VIIa-positive cells were seen in their auditory epithelia. Cochlear specimens treated with systemic KM and EA application followed by local DMSO application exhibited total OHC loss in each turn of the cochleae, and partial loss of IHCs in the basal turn similarly to those treated with KM and EA alone (Fig. 2a). Total OHC loss and partial IHC loss were also observed in cochlear specimens treated with systemic KM and EA application followed by local MDL application (Fig. 2b, c). No significant difference in the number of IHCs was found between cochlear specimens treated with KM and EA followed by MDL treatment and those with KM and EA alone (Fig. 2d).

No ectopic myosin VIIa-positive cells were found in the basal portion of cochleae treated with KM and EA alone (Fig. 2a) or followed by DMSO application, whereas in those treated with KM and EA followed by MDL application,

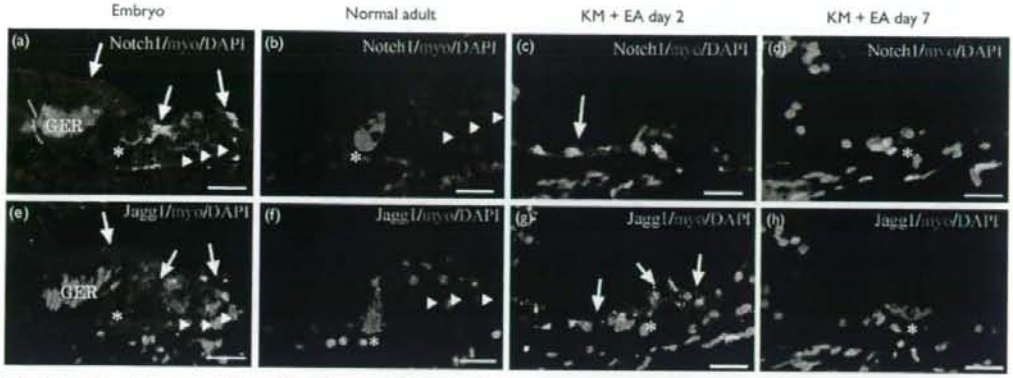

Fig. 1 Expression of Notch1 and Jagged1 in auditory epithelia of embryonic and adult auditory epithelia with or without ototoxic treatment. The embryonic auditory epithelium exhibits immunoreactivity for Notch1 and Jagged1 (Jagg1) in the greater epithelial ridge (GER) and the supporting cells (arrows in a, e), whereas neither expression is found in normal adult epithelia (b, f). The adult auditory epithelium on day 2 after kanamycin and ethacrynic acid application (KM + EA day 2) exhibits immunoreaction for Notch1 in the inner sulcus cells (arrow in c), and that for Jagg1 in the inner sulcus cells and the supporting cells (arrows in g), while neither expression is found in that obtained on day 7 (d, h). Red fluorescence shows immunoreactivity for myosin VIIa (myo), and blue shows DAPI. The locations of inner hair cells are indicated by asterisks and those of outer hair cells are indicated by arrowheads. Bars represent 25 μm. DAPI, 4′,6-diamidino,2-phenylindole dihydrochloride.

ectopic myosin VIIa-positive cells were identified in the inner sulcus region of the basal turn of cochleae (Fig. 2b, c). Ectopic myosin VIIa-positive cells were found in four of seven experimental animals. The mean and SEM for numbers of ectopic myosin VIIa-positive cells was 0.71 and ±0.29 (cells/200 μm). The difference in numbers of ectopic myosin VIIa-positive cells between specimens with MDL treatment and those KM and EA alone was statistically significant (P=0.028, Fig. 2d).

Discussion

In this study, we examined the activation of Notch signaling in adult and embryonic guinea pig auditory epithelia, with or without systemic KM and EA application. Notch1 and Jagged1 immunohistochemistry indicated a quiescent state of Notch signaling in the nonaffected auditory epithelia of adult guinea pigs and the activation of Notch signaling in damaged auditory epithelia. Strong immunoreactivity for Notch1 and Jagged1 was found in the auditory epithelia on days 2 and 4 after systemic KM and EA application. Immunoreactivity for these molecules then returned to the levels seen in normal adult tissues on day 7. These findings indicate that transient activation of Notch signaling might occur in adult auditory epithelia after systemic KM and EA application.

The nonaffected auditory epithelia of adult guinea pigs exhibited no ectopic myosin VIIa-positive cells following local MDL application, which supported the present immunohistochemical findings for Notch1 and Jagged1. By contrast, local MDL application to cochleae on day 4 after systemic KM and EA treatment, which demonstrated Notch1 and Jagged1 expression, induced the appearance of ectopic myosin VIIa-positive cells in the auditory epithelia. Notch1 and Jagged1 expression was found in the inner sulcus region of damaged auditory epithelia, and a limited number of ectopic myosin VIIa-positive cells were identified in this region. These findings indicate that pharmacological inhibition of Notch signaling by a γ-secretase inhibitor generates the appearance of ectopic HCs in adult auditory epithelia.

In this study, we applied an ototoxic treatment that was used previously in a study of Atoh1 overexpression by virus vectors [9]; we also introduced a γ-secretase inhibitor at the time point when the virus inoculation occurred in the previous report [9]. Atoh1 overexpression by virus vectors induced the generation of a number of new HCs in various portions of the auditory epithelia, whereas local MDL application resulted in a limited number of ectopic HCs in the inner sulcus region. In addition, viral overexpression of Atoh1 generated some ectopic HCs in normal adult guinea pig cochleae [8]. Atoh1 might have had a direct effect on the transdifferentiation of SCs into HCs, whereas the pharmacological inhibition of Notch signaling indirectly induced an increase in Atoh1 expression, which might explain the difference in effect on ectopic HCs.Atoh1 overexpression in auditory epithelia, however, requires viral vectors with their associated risk of toxicity, whereas pharmacological inhibition of Notch signaling requires only local drug application to the cochlea. We therefore consider pharmacological inhibition of Notch signaling to be preferable for clinical applications, although its effect on the generation of new HCs is limited.

In this study, the expression of Notch1 and Jagged1 and the generation of ectopic HC-immunophenotypes were observed in the inner sulcus region of the auditory epithelia. The greater epithelial ridge of embryonic and neonatal auditory sensory epithelia corresponds to the inner sulcus region in mature auditory epithelia, and is necessary for the normal development of the auditory epithelium [16]. Several cell-culture studies have demonstrated the existence of progenitor cells, even in adult auditory epithelia, with the potential to differentiate into HCs [2,3,17]. In addition, Malgrange *et al.* [17] reported on the preservation of such progenitor cells in the inner sulcus region of adult animals, suggesting that the inner sulcus cells might be important for the restoration of adult auditory epithelia. Future investigations should elucidate the unique characteristics of these

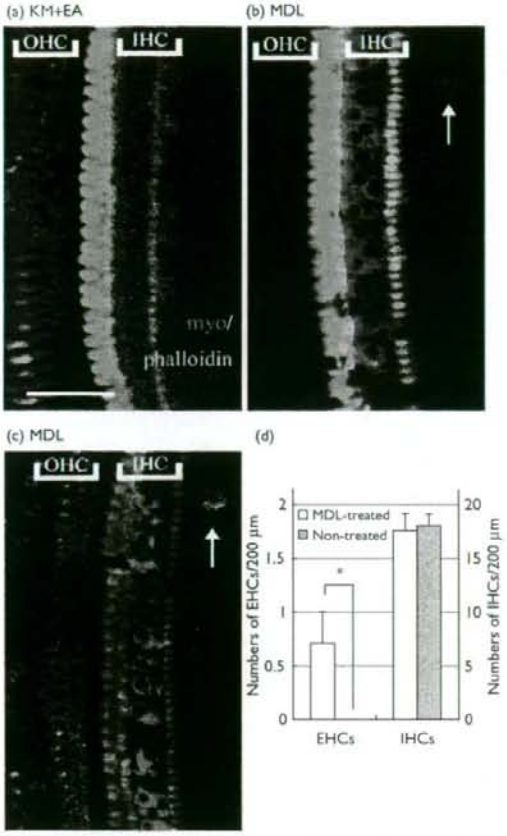

Fig. 2 Ectopic hair cell-immunophenotypes in damaged auditory epithelia. An auditory epithelium affected by kanamycin (KM) and ethacrynic acid (EA) exhibits no ectopic myosin VIIa (myo)-positive cells (a). In auditory epithelia treated with systemic KM and EA treatment followed by local MDL application, ectopic myo-positive cells are found in the inner sulcus region (arrows in b, c). IHC indicates the location of inner hair cells and OHC indicates that of outer hair cells. Bar represent 50 μm. The numbers of ectopic hair cells (EHCs) and inner hair cells (IHCs) in the midbasal portion of MDL-treated and nontreated cochleae following KM and EA application are shown in d. The difference in EHC numbers between two groups was significant (*P=0.028 in d). No significant difference in IHC numbers was found. Bars represent SEM.

cells in relation to the temporary activation of Notch signaling, which will contribute to the optimization of the pharmacological inhibition of Notch signaling.

Conclusion

The present findings demonstrate that pharmacological inhibition of Notch signaling has the potential to generate ectopic HCs in the damaged auditory epithelia of adult guinea pigs. The functionality of these ectopic HC immunophenotypes, however, remains to be determined. In addition, an increase in the number of new HCs will be necessary for functional recovery. We therefore aim to determine the optimal conditions for the pharmacological inhibition of Notch signaling in our future work.

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

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Hypothesis: Local application of recombinant human insulin-like growth factor 1 (rhIGF-1) via a biodegradable hydrogel after onset of noise-induced hearing loss (NIHL) can attenuate functional and histologic damage.
Background: The biodegradable gelatin hydrogel makes a complex with drugs by static electric charges and releases drugs by degradation of gelatin polymers. We previously demonstrated the efficacy of local rhIGF-1 application via hydrogels before noise exposure for prevention of NIHL.
Methods: First, we used an enzyme-linked immunosorbent assay to measure human IGF-1 concentrations in the cochlear fluid after placing a hydrogel containing rhIGF-1 onto the round window membrane of guinea pigs. Second, the functionality and the histology of guinea pig cochleae treated with local rhIGF-1 application at different concentrations after noise exposure were examined. Control animals were treated with a hydrogel immersed in physiologic saline alone.
Results: The results revealed sustained delivery of rhIGF-1 into the cochlear fluid via the hydrogel. The measurement of auditory brainstem responses demonstrated that local rhIGF-1 treatment significantly reduced the threshold elevation from noise. Histologic analysis exhibited increased survival of outer hair cells by local rhIGF-1 application through the hydrogel.
Conclusion: These findings indicate that local rhIGF-1 treatment via gelatin hydrogels is effective for treatment of NIHL.
Key Words: Biomaterial—Drug delivery—Growth factor—Noise trauma—Round window.
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Sensorineural hearing loss (SNHL) is one of the most common disabilities, but therapeutic options are limited to hearing aids and cochlear implants. Many investigations have therefore examined novel therapeutic molecules for SNHL and have discovered several agents exerting therapeutic activity against SNHL. Despite such progress in basic research, translation of these basic findings into clinical use is very rare. The lack of safe and effective methods for drug delivery to the cochlea has formed a considerable obstacle to clinical applications. Systemically applied drugs have great difficulty reaching cochlear cells because of 1) the blood-labyrinth barrier (1) acting as an obstacle to the transfer of drugs from serum to cochlear cells and 2) the limited blood flow to the cochlea (2). The development of local drug delivery systems to the cochlea thus remains crucial for the clinical application of basic findings in this field.

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

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

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

MATERIALS AND METHODS

Experimental Animals

Twenty-six adult female Hartley guinea pigs weighing 250 to 300 g (Japan SLC, Hamamatsu, Japan) served as experimental animals. Animal care was conducted under the supervision of the Institute of Laboratory Animals at the Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Biodegradable Gelatin Hydrogel

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

In Vivo IGF-1 Release Profile

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

Noise Exposure and Drug Application

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

Functional Analysis

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

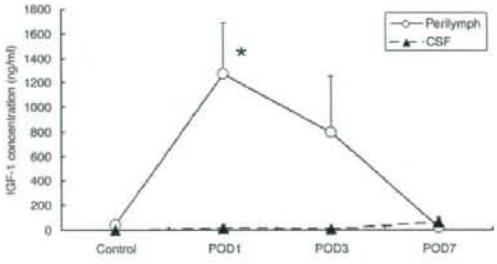

FIG. 1. Graph showing concentrations of human IGF-1 in cochlear perilymph and CSF. A significant increase in concentration of IGF-1 is found on postoperative Day 1 for perilymph (*$p < 0.05$), whereas no significant changes are observed in concentrations of IGF-1 in CSF. Bars represent SEM. POD indicates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

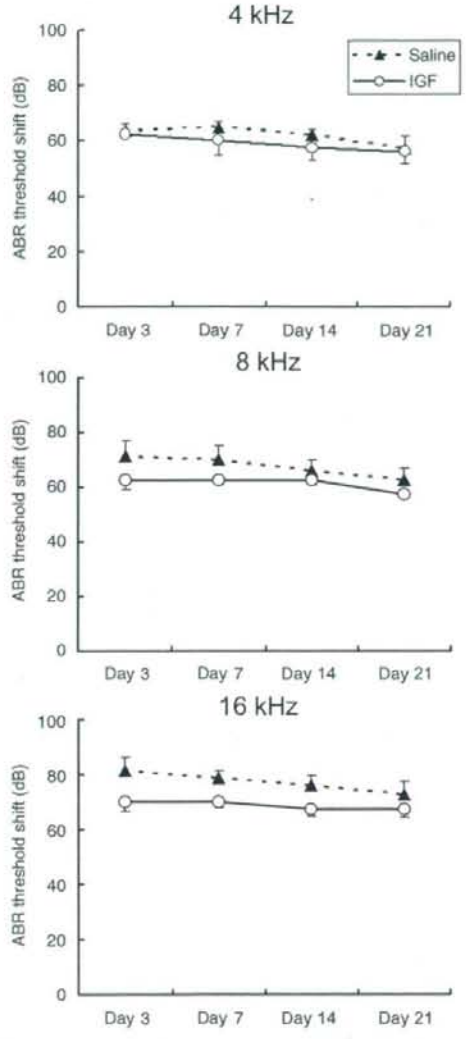

FIG. 2. Graphs showing time courses of alterations in threshold shifts of ABRs in cochleae treated with 1-μg/μl rhIGF-1 and cochleae treated with saline at frequencies of 4, 8, and 16 kHz. No significant differences are present in ABR threshold shifts between rhIGF-1– and saline-treated cochleae at any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

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

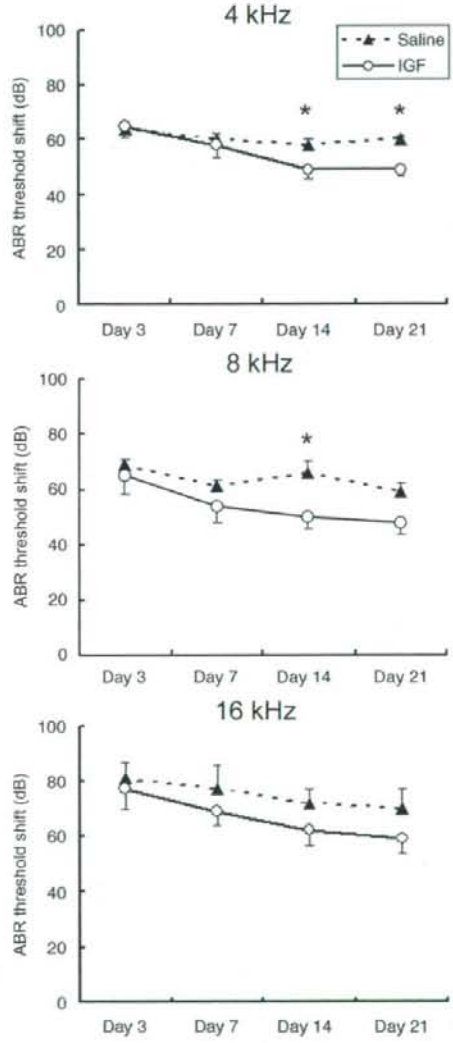

FIG. 3. Graph showing time courses of alterations in threshold shifts of ABRs in cochleae treated with 10-μg/μL rhIGF-1 and cochleae treated with saline at frequencies of 4, 8, and 16 kHz. The differences in ABR threshold shifts at 4 kHz between rhIGF-1– and saline-treated cochleae on Days 14 and 21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

Histologic Analysis

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

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

Adverse Effects

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

Statistics

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

RESULTS

In Vivo IGF-1 Release Profile

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

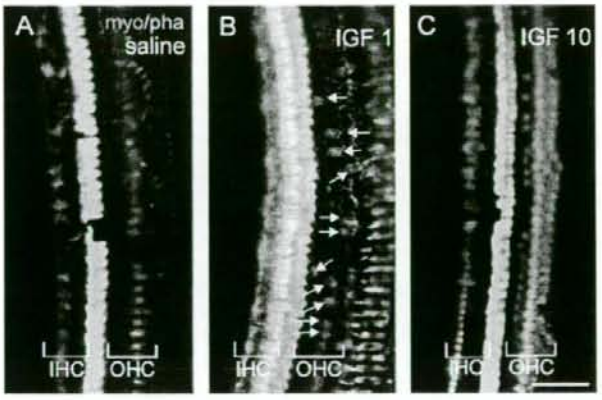

FIG. 4. Image showing immunostaining for myosin VIIa (*myo* [*red*]) and F-actin labeling with phalloidin (*pha* [*green*]) of cochlear sensory epithelia in the basal portion of cochleae. Severe loss of OHCs is observed in the saline-treated cochlea (*A*). Surviving OHCs in the specimen treated with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

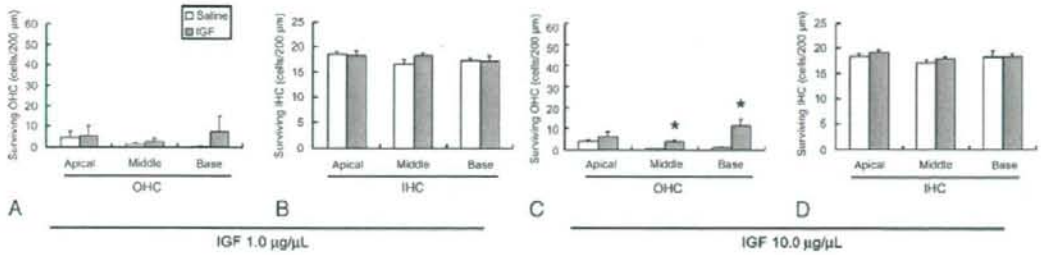

FIG. 5. Graphs showing numbers of surviving inner and outer hair cells in apical, middle, and basal portions of cochleae. No significant differences in numbers of OHCs (*A*) or IHCs (*B*) are found between saline- and 1-μg/μl rhIGF-1–treated cochleae. Differences in numbers of surviving OHCs between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

Auditory Function

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

Histologic Damage

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

Incidence of Adverse Effects

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

DISCUSSION

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

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

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

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

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

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特報 第43回 2006年度 ベルツ賞受賞論文

2等賞

内耳障害への再生医学的アプローチ

伊藤壽一[*1,**1] 中川隆之[*1] 山本典生[*2]

Summary

Inner ear disorders including sensorineural hearing loss is one of the most common disabilities in our society, but treatment options are currently limited to cochlear implants and hearing aids. The major reason for this is limited capacity for regeneration of the mammalian inner ear. We have sought alternative means of biological therapy for inner ear diseases via three different approaches, 1) cell therapy, 2) promotion of spontaneous regenerative activity and 3) development of drug delivery systems (DDS) for inner ears.

As for cell therapy, the first attempts to examine the feasibility of cell therapy in the treatment of inner ear disorders have been performed using neural stem cells (NSCs), making NSC transplantation for the restoration of inner ear cells a potentially viable treatment. Further studies have indicated the high potential of embryonic stem cells for restoration of spiral ganglion neurons in rodents and primates. Results from studies using bone marrow-derived cells suggest their possible use for restoration of spiral ganglions and gap junction systems in the cochlea. Cell transplantation has also been demonstrated as a strategy for gene delivery into the inner ear without use of virus vectors.

There are three possible strategies for hair cell regeneration in the inner ear, induction of proliferation of progenitor cells, transdifferentiation of supporting cells to hair cells and promotion of self-repair of damaged hair cells. Studies for induction of cell proliferation have indicated involvement of p27kip1 and skp2, beta-catenin and E-cadherin in mechanisms of regulation of cell proliferation in sensory epithelia. Pharmacological inhibition of Notch signaling has been demonstrated as a strategy for transdifferentiation of supporting cells to hair cells after birth. Results from studies using organotypic cultures demonstrate that functional hair cells can be regenerated through the process of self-repair.

We have attempted to develop DDS for inner ears, because lack of safe and effective

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