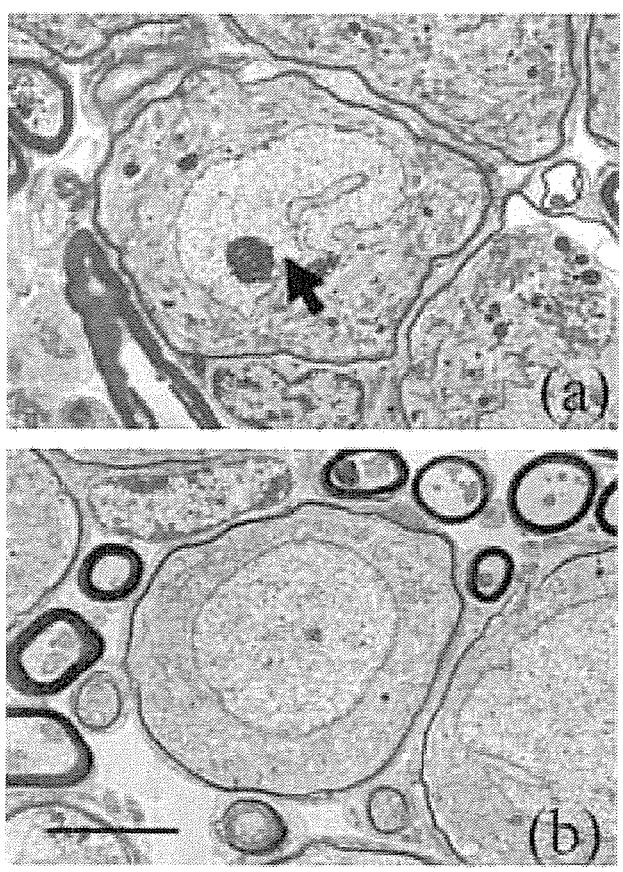

Summary of immunostaining for Bcl-xL

control	Day 1	Day 1 + gRb1
++	+	++ to +++

0: no staining
+: mild staining
++: moderate staining
+++: strong staining
+gRb1 : animals given ginsenoside Rb1
control: before the induction of ischemia
D1: 1 day after the induction of ischemia

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Neuroscience xx (2007) xxx

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

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AQ: 1

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.

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

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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 approved by the Ethics Committee at Ehime University Graduate School of Medicine and were conducted ac-

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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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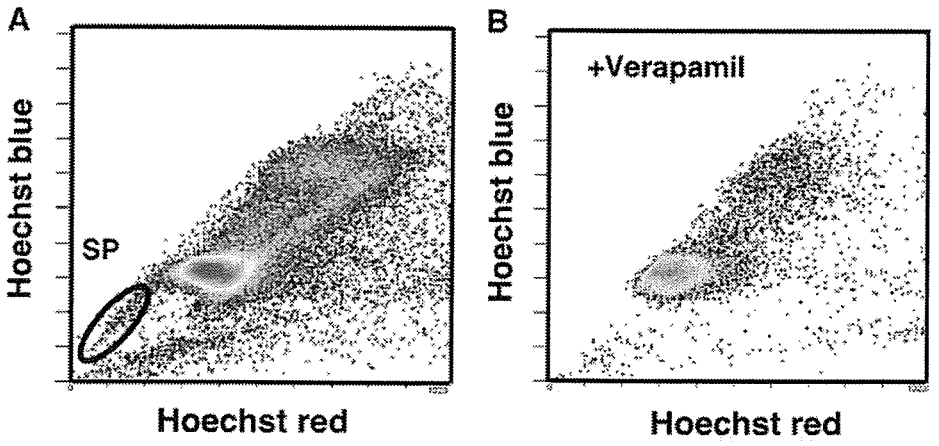

Fig. 1. Flow cytometric analysis of SP cells in Hoechst-stained bone marrow (BM). SP region of whole BM stained with Hoechst in the absence (A) and presence (B) of 50 μM verapamil. The region marked SP was sorted for transplantation experiments. Approximately 0.2–0.4% of cells fell into the SP gate.

cording to the Guidelines for Animal Experimentation at Ehime University Graduate School of Medicine. 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 the ischemic insult by sequential recording of ABR. Under halothane anesthesia, ABR was recorded using a signal processor (NEC Synax 1200, NEC Medical Systems, 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.

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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 with a green filter (BP 546, FT 580, LP 590) and a UV filter (BP 365, FT 395, LP 397). 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 the 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

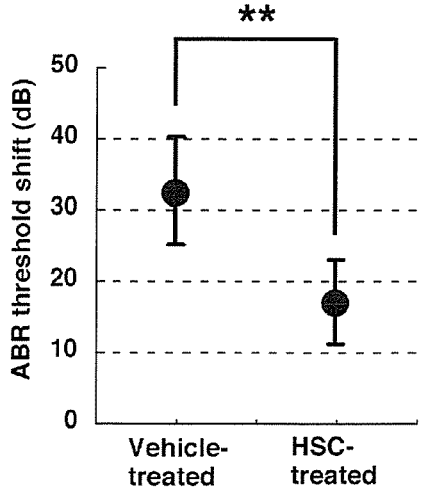

Fig. 2. ABR threshold at 4 days after cochlear ischemia. Pretreatment with HSCs (HSC-treated) significantly suppressed the elevation of threshold in comparison with the vehicle-treated control. The average ABR threshold shift on the vehicle- and HSC-treated sides (n=6 in each side) was analyzed using 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.

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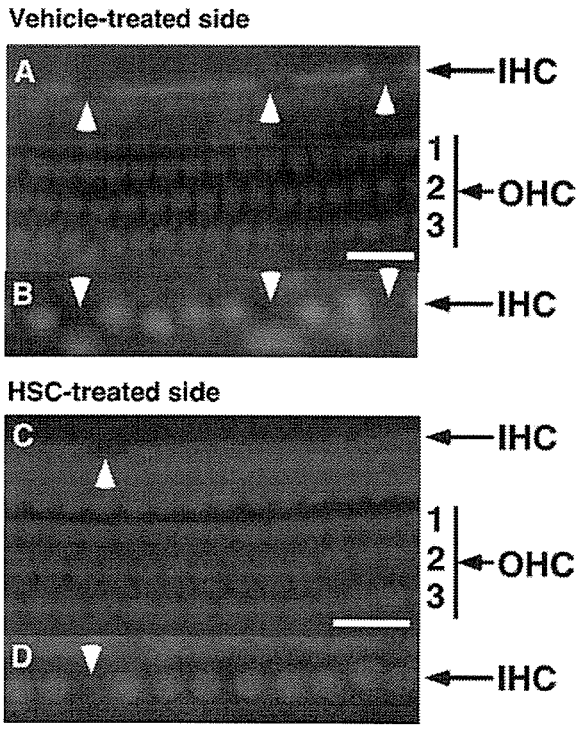

Fig. 3. Surface structure of organ of Corti at 4 days after cochlear ischemia. Representative fluorescence images of the organ of Corti stained with rhodamine-phalloidin (A and C) and Hoechst 33342 (B and D). Gerbils were subjected to cochlear ischemia for 4 days. The organs of Corti were obtained from 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. Arrows 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

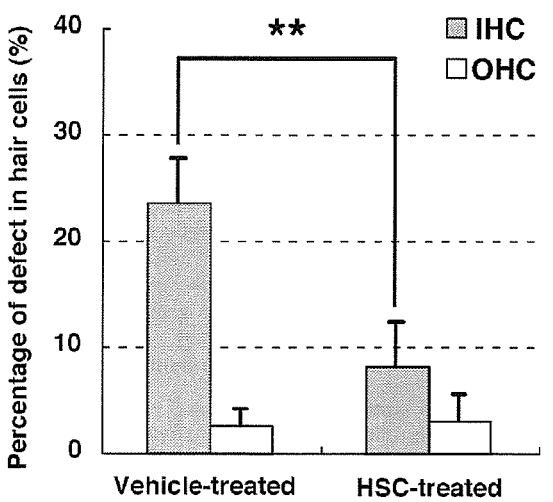

Fig. 4. Percentages of defects in IHCs and OHCs at 4 days after cochlear ischemia. Pretreatment with HSCs significantly reduced IHC damage at 4 days after cochlear ischemia. On the HSC-treated side (n=6), the proportion of deficits in IHCs was lower than that on the vehicle-treated side (n=6). On the 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.

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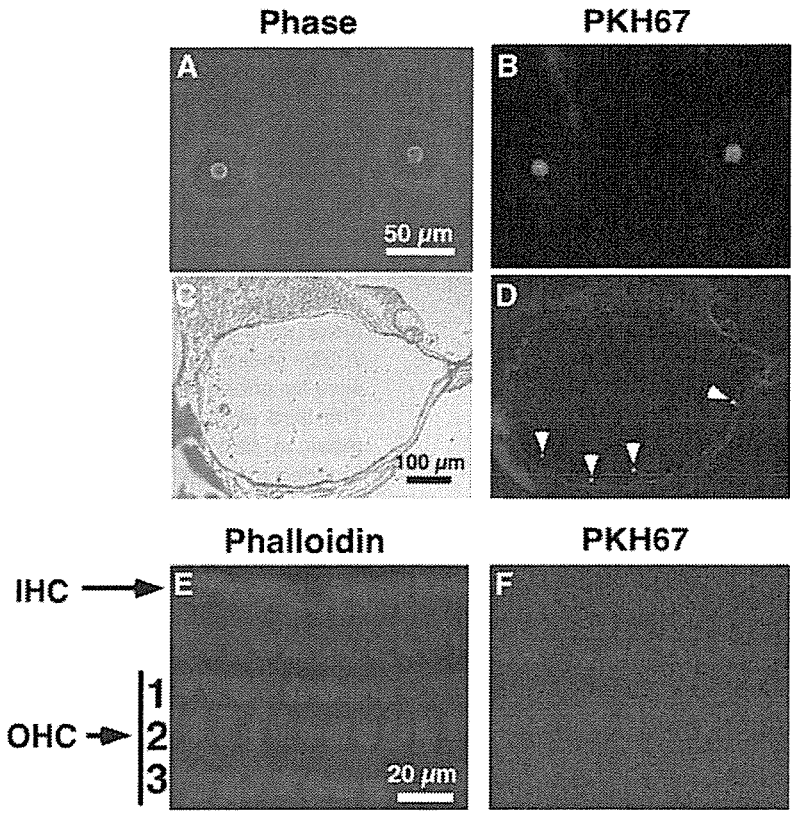

Fig. 5. PKH67 labeling. HSCs were labeled with PKH67 (B: green). Gerbils were treated with PKH67-labeled HSCs. One day later, animals were subjected to cochlear ischemia for 15 min. Four days after ischemia, the temporal bones were dissected out, fixed, decalcified and cut into 10-μm-thick cryostat sections 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; Roehm and Hansen, 2005). 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 (unpublished observa-

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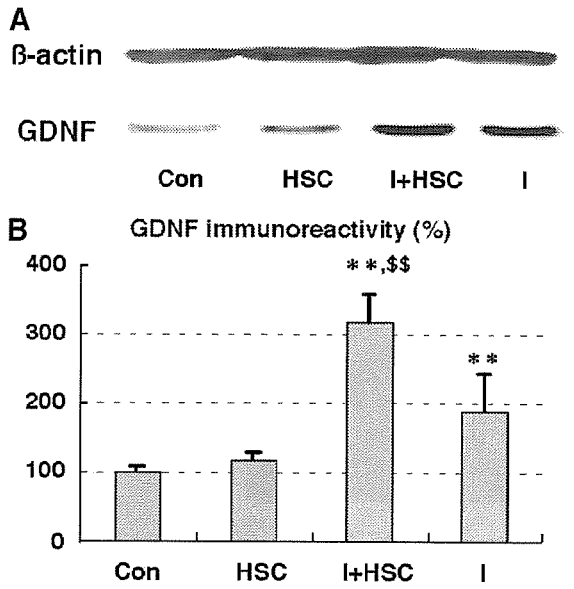

Fig. 6. Western blot analyses of GDNF in gerbil cochlea on the vehicle-treated and HSC-treated side. Samples were derived from the cochlear on the vehicle-treated side (Con) and HSC-treated side (HSC) in sham-operated gerbils, and on the vehicle-treated side (I) and HSC-treated side (I+HSC) in gerbils 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.

AQ: 6

tions), 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 (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

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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 exo 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.

UNCITED REFERENCE

Seidman et al., 1999.

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DDSを用いた感覚器領域における再生医療

Regenerative medicine using DDS in sensory organs

Keywords

ドラッグデリバリーシステム
感音難聴
視力障害

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Summary

Drug delivery to the cochlea is hampered by blood-inner ear barrier together with its dense bony capsule. The round window is a possible access to deliver drugs effectively into the cochlea. Intratympanic injections and osmotic mini-pump has been utilized to get access to the round window membrane (RWM). Gelatin hydrogel immersed with brain derived-neurotrophic factor (BDNF) or IGF1 is successfully applied to the RWM to protect spiral ganglion neurons or cochlear hair cells. PLGA nano-particles are shown to penetrate round window membrane, and are expected to provide sustained-release in the cochlea. Drug delivery to the retina is also a challenge due to the existence of blood-retina barrier. Photo dynamic therapy and intraocular implants are clinically utilized methods to overcome this difficulty. Iontophoresis using drug immersed hydrogel as a contact electrode effectively brings drugs into vitreous. PLGA nano-particles with pigment epithelium-delived factor protected the retina from ischemic injury. PLGA nano-particles may also be used as vehicles to transfect cells with plasmid DNA. Pegaptanib, an RNA aptamer which inhibit vascular endothelium-delived growth factor, is used for ocular vascular disease. PLGA nano-particles will also be used for better sustained-release of pegaptanib.

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はじめに

耳や眼は外界と接した臓器であり，アクセスは一見容易に思えるが，全身投与でも局所投与でも薬剤がターゲットとなる細胞に到達するまでにはさまざまな阻害要因があり，効率的な薬物投与は決して容易ではないため，これまでもDrug Delivery System (DDS) 的な工夫がなされてきた。本稿では，この領域で使われているDDSを紹介し，その再生医療としての応用について述べる。

感音難聴に対するDDS

感音難聴は非常に頻度の高い身体障害で，その原因としては，音響外傷，耳毒性薬剤，遺伝子異常，老化，メニエール病を含む内リンパ水腫関連の疾患などが挙げられるが，多くは蝸牛の障害が難聴を引き起こしている。空気

の疎密波として鼓膜に到達した音は，3つの耳小骨を介して蝸牛に伝えられ，蝸牛内では感覚上皮が振動する。感覚上皮に存在する感覚細胞（有毛細胞）では，振動が神経伝達物質の放出を引き起こし，一次感覚ニューロンであるらせん神経節細胞を興奮させ，中枢へと伝達される（図1）。多くの感音難聴ではこの有毛細胞やらせん神経が傷害されているため，治療の際には蝸牛がターゲットとなる。感音難聴に対して臨床的に実際に行われている治療はステロイドやビタミン剤，循環改善薬などの全身投与（経口，または経静脈）であるが，効果のある症例は限られている。感音難聴が治りにくい第一の理由は，いったん傷害された有毛細胞やらせん神経節細胞が再生しないことがまず挙げられるが，もう一つの理由が蝸牛への薬剤到達の難しさである。蝸牛を含めた内耳は非常に密な骨に囲まれており，血流も限られていること[1]，さらに，「血液ー脳関門」と同様の「血液ー内耳関門」[2]が存在することが蝸牛への薬物到達を難しくしている。ただ，蝸牛には正円窓という小さな開窓部があって，薄い正円窓膜が蝸牛を満たす外リンパ液をシールしているため，薬物投与のルートとしては可能性がある。ただし，膜を損傷して外リンパ瘻を起こすと難聴やめまいを引き起こし，またもう1枚の偽性膜が存在するために確実に正円窓膜上に投与できない例も多いといわれており，取り扱いに注意を要する。鼓膜を穿刺してあるいは鼓膜穿孔があればそこから薬液を鼓室内に充満させる方法（鼓室内投与法）は，簡便であるが，薬物は耳管を通じて鼻腔へ容易に排出されるため，蝸牛への到達の程度も決して高くはなく，その効果を持続させることも難しい[3][4]。マイクロカテーテルを正円窓膜近傍に留置する方法も報告されていて，より確実に正円窓に向けて薬物を持続投与できる。突発性難聴に対するステロイド投与法として臨床的に有効であることも示されているが[5][6]，マイクロカテーテル留置には中耳手術と同程度の侵襲が必要であり，普及するには至っていない。

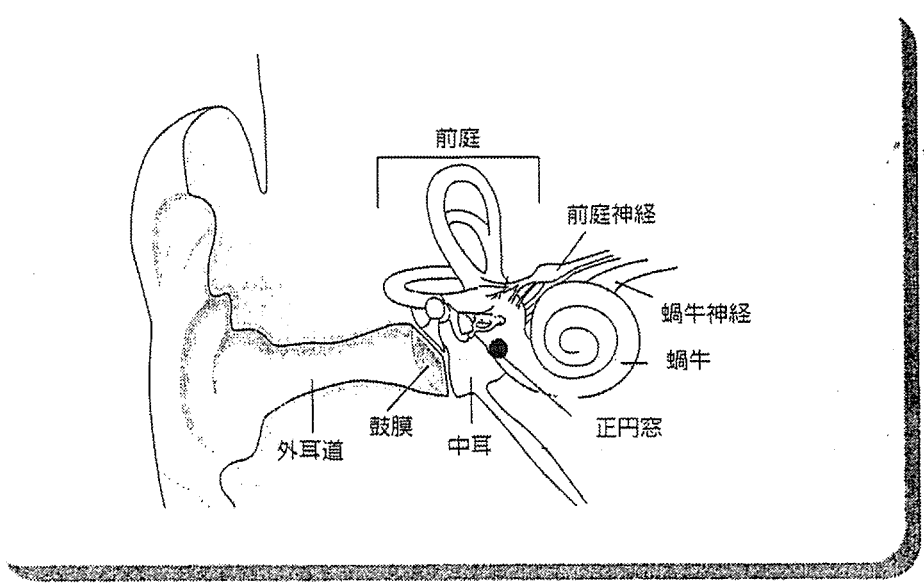

図1　外耳・中耳・内耳

ハイドロゲル

ハイドロゲルとは，ゼラチンやコラーゲン，ヒアルロン酸，アルギン酸などの親水性高分子化合物を架橋してできるゼリー状物質の総称である。特に，カチオン化，あるいはアニオン化したゼラチンを用いたハイドロゲル（ゼラチンハイドロゲル）は，静電気的に薬物と結合し，生体内で加水分解されるに従って薬物を徐放することが知られている。結合が静電気的であるため薬物を修飾する必要がなく，その架橋の程度を変えることで分解速度を制御できるので，DDS基材としては使いやすい。

Endoら[7]は，脳由来神経栄養因子（BDNF）を浸潤させたハイドロゲルをモルモットの正円窓膜上に留置して，蝸牛におけるその効果を検証した。外リンパ液中のBDNF濃度を調べると，1週間以上にわたってBDNFは徐放されており，また，らせん神経節細胞に対する保護効果が組織学的，機能的に確認された。また，Iwaiら[8]は

IGF1を含ませたゼラチンハイドロゲルを正円窓膜上に留置して，音響外傷による感音難聴に対する予防効果を検討した。その結果，機能的には難聴の防止効果が，組織学的には蝸牛有毛細胞の細胞死を防御する効果があることを確認した。現在我々はゼラチンハイドロゲルを用いたIGF1投与による急性高度難聴治療の臨床応用を準備中である。

生体適合性プラスチックによるナノパーティクル

PLGA（copoly lactic acid/glycolic acid）は，手術用吸収糸や創傷被覆膜として医療で広く用いられている生体適合性高分子である。材料である乳酸とグリコール酸の比率を変えることで分解を遅延することができ，生体内で加水分解されたあとの単量体は無害である。最近の微粒化技術の進歩によって，幅広い薬剤をPLGAナノパーティクル化できるようになり，新しいDDS基材として注目されている。

Tamuraら[9]は，PLGAナノパーティクルが正円窓膜を超えて蝸牛まで到達できるかどうかを調べた。ローダミン含有PLGAナノパーティクルを蝸牛の正円窓膜上に留置して24時間後の蝸牛内の分布を組織学的に調べると，ローダミン含有PLGAナノパーティクルは蝸牛の正円窓に近い基底回転から頂回転までの広い範囲にわたって存在しており，PLGAナノパーティクルは正円窓膜を通過して，蝸牛の外リンパ液中を拡散することがわかった。ハイドロゲルでは薬物はいったん徐放されてから拡散してターゲットへと輸送されていくが，ナノパーティクルの場合はパーティクルのままターゲットに輸送され，その場で徐放するので，薬物の安定性やターゲティング性能などの点で有利である。突発性難聴に用いられているステロイドやメニエール病の治療に用いられるアミノ配当体，さらに前述のBDNFやIGF1などをPLGAナノパーティクル化して用いれば，全身的な副作用などを回避しながら，蝸牛のみに高濃度かつ長期間，薬物を維持することができるので，今後の有効性の検証・臨床応用が待たれる。

視力障害に対するDDS

重篤な視力障害の原因として頻度の高いものは，糖尿病網膜症，緑内障，加齢黄斑変性症，網膜色素変性症などがあげられるが，その主な障害部位は網膜・視神経である（図2）。網膜において幹細胞の存在が明らかになっているが，それが傷害された網膜を再生させるという報告はいまだなく，細胞障害を受けると修復されることは困難であるということを意味する。眼球は強膜，結膜による強固なバリアーや涙によるクリアランスのほかに，「血液ー網膜関門」[10]が存在するため，点眼や全身投与を行っても，眼球後方への薬物投与の効率は低い。

滲出性加齢黄斑変性症は，脈絡膜に新生血管が生じ，出血，網膜剥離，浮腫などが生じ，病変が中心窩に及ぶと重大な視力低下をきたす疾患である。これに対して，光線力学的療法（photo dynamic therapy：PDT）が行われている。PDTは，新生血管集積性の光反応薬剤を全身投与した後，病

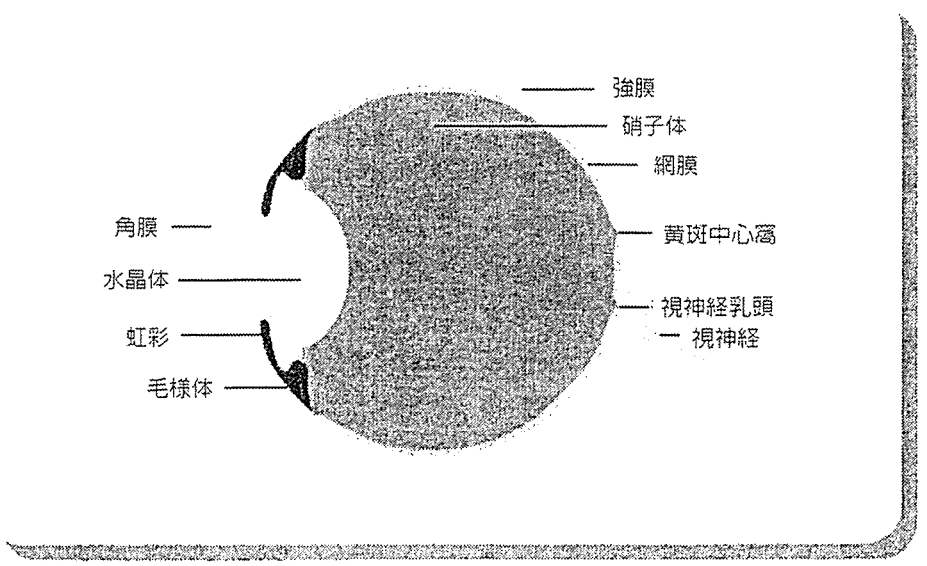

図2　眼球断面図

変部分である網膜の脈絡膜に弱いレーザーを照射することで局所の血管を閉塞させるという，ターゲティングDDSである。

また，日本国内では未承認であるが，ステロイド徐放インプラント（Retisert®：フルオシノロンをペレット状にしてポリビニルアルコールとシリコンで被覆して徐放化）[11]や抗ウイルス薬徐放インプラント（Viterasert®：ガンシクロビルペレットをポリビニルアルコールとエチレン酢酸ビニル共重合体で被覆して徐放化）[12]といった，眼球内に埋め込む形の徐放剤が実用化されている。

ハイドロゲル，PLGAナノパーティクルによる徐放

ポリヒドロキシエチルメタクリレート（HEMA）は，コンタクトレンズの材料として1960年代から用いられている高分子素材である。Eljarrat-Binstockら[13][14]はゲンタマイシンで膨潤させたHEMAハイドロゲルを角膜表面に，あるいはHEMAにエチレングリコールジメタクリレート（EGDMA）を組み合わせたハイドロゲルを強膜面にあて，1分程度通電することで房水，あるいは硝子体に効率的にゲンタマイシンを輸送できることを示した。このようなイオントフォレーシスと呼ばれる方法は，耳鼻咽喉科臨床的には局所麻酔薬を鼓膜に効率よく到達させるために古くから頻用されているが，眼球後方の薬物到達が困難な領域へのDDSとしても，簡便で有効な方法である。

Liら[15]は，網膜血管新生を抑制する作用をもつ色素上皮由来因子（pigment epithelium-delived factor：PEDF）をPLGAナノパーティクルに封入し，網膜虚血モデルマウスの眼球内に投与した。PEDF封入PLGAナノパーティクルを使うことで，PEDF単体で投与するよりも長期にわたって網膜神経節細胞と内網状層の保護できることを示した。

核酸導入

*In vivo*で用いられている遺伝子導入法は，ウイルスベクターによるものがほとんどである。ウイルスベクターによるものは遺伝子導入効率は高いが，ウイルス蛋白に対する免疫反応，増殖能をもったウイルス混在の可能性が排除できないこと，ベクターがゲノムに挿入される場合には他の遺伝子に影響する可能性があることなどが問題になる。非ウイルスベクター法として，プラスミドDNAとカチオン性脂質との複合体（リポプレックス）を用いるリポフェクションと，プラスミドDNAとカチオン性ポリマーとの複合体（ポリプレックス）を用いるポリフェクションなどが用いられている。現状ではリポフェクションのほうが頻用されているが，ポリフェクションは①均一で安定な複合体形成，②遺伝子導入率が高い，③血清の影響を受けやすいなどの利点がある[16]。前述のように，PLGAナノパーティクルが正円窓から蝸牛に導入できることから，同じ方法で蝸牛への遺伝子導入ができると期待される。

米国では，加齢性黄斑変性症などによる毛細血管新生を抑制して視力障害の進行を抑える効果をねらって，血管内皮増殖因子（VEGF）抑制性のRNAアプタマー（pegaptanib，Macugen®）が使用されており[17]，日本でも臨床試験が行われている。アプタマーとは標的蛋白と特異的に結合するようにデザインされた核酸分子であるが，pegaptanibの場合，核酸残基の置換，40kDaのポリエチレングリコール（PEG）との結合によって，硝子体液中での安定性と反応特異性を向上させた[18]。これをPLGAマイクロスフェア化することでさらなる徐放効果をねらう試みも行われている[19]。

おわりに

感覚器領域においても，昨今の再生医学研究の成果で，これまで機能回復が難しかった病態に対して治療に用いることのできる多数の因子が同定されつつあり，それとともに，それぞれに適したDDSの開発も進みつつあり，近い将来の臨床応用が期待される。さらに我々は，骨髄由来幹細胞を末梢血管から導入するとマクロファージ様の細胞が内耳に集積することに着目して，これを利用したDDSを模索している。

辞

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Drug delivery systems for the treatment of sensorineural hearing loss

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Abstract
Sensorineural hearing loss is one of the most common disabilities in our society. Experimentally, many candidates for therapeutic molecules have been discovered. However, the lack of safe and effective methods for drug delivery to the cochlea has been a considerable obstacle to clinical application. Local application of therapeutic molecules into the cochlea has been used in clinic and in animal experiments. Advances in pharmacological technology provide various drug delivery systems via biomaterials, which can be utilized for local drug delivery to the cochlea. Recent studies in the field of otology have demonstrated the potential of synthetic and natural biomaterials for local drug delivery to the cochlea. Although problems still remain to be resolved for clinical application, introduction into clinical practice of these controlled-release systems may be reasonable because of their certain advantages over previous methods.

Keywords: *Drug delivery, topical application, hearing loss, inner ear, biodegradable material*

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in industrial countries. Excessive noise, ototoxic drugs, genetic disorders and aging can all initiate SNHL. Endolymphatic hydrops-associated diseases including Meniere's disease also cause 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. Despite the high prevalence of SNHL in our society, therapeutic strategies for the treatment of SNHL today are limited to hearing aids and cochlear implants. These therapeutic tools do not provide complete restoration of hearing ability, although they have significant clinical benefits. Based on such backgrounds, many attempts have been made to provide alternative means of biological therapy, which have identified a number of candidates for therapeutic molecules. Experimentally, protective effects of neurotrophins have been demonstrated [1,2], and inhibitors of apoptosis and glutamate antagonists have also shown the ability to promote hair cell survival [3–5]. Recently, local application of genes by virus vectors was shown to induce hair cell regeneration in the mammalian auditory epithelium [6,7], and silencing the mutant gene by RNA interference restored hearing loss in a genetic mouse model [8].

These therapeutic strategies are attractive and promising for restoring SNHL. However, clinical application is still limited. The problem of how to deliver therapeutic molecules to the inner ear has been a considerable obstacle to the development of treatments for SNHL. The systemic application of drugs carries the risk of unwanted side effects. In addition, the blood–inner ear barrier, which inhibits the transport of therapeutic molecules from the serum to the inner ear, represents a fundamental obstacle to systemic application [9]. The inner ear tissues are isolated from the surrounding organs by a bony construction, which allows the topical introduction of therapeutic molecules by local application. Therefore, development of strategies for local delivery into the inner ear is crucial for developing clinical therapies based on the experimental findings.

Previous methods for local application

Substances are applied intratympanically under the premise that they will enter the scala tympani (ST)

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through the round window membrane (RWM) and then be distributed throughout the inner ear fluids. The idea of a topical application of medicine to the inner ear is not new. Decades ago local anesthetics and aminoglycosides were applied through the tympanic membrane into the tympanic cavity to treat inner ear disorders [10–12]. Intratympanic injections have been used for local application of aminoglycosides or steroids in the therapy of Meniere's disease and sudden hearing loss. There are a number of clinical reports showing the efficacy of intratympanic injections of these drugs (reviewed by Salt et al. [13]). However, it is very difficult to predict the amounts of drugs that reach the inner ear fluid space. Some reports have indicated that this method led to varying results in the therapy of Meniere's disease [14–16]. An intratympanic injection is a simple and easy method; however, controlled and sustained release of drugs cannot be achieved by this method.

Recent animal studies have indicated the efficacy of growth factors, neurotrophins [1,2], antioxidants [5], and apoptosis inhibitors [3,4], which are locally applied to the inner ear, for otoprotection. Sustained treatment of inner ears by local viral gene transfer represents sufficient protection of inner ears from noise, drug toxicity, and reperfusion injury [17–21]. While basic studies have represented the benefits of local treatment with these substances, no cases have been approved for clinical application. Adenoviral vectors or adeno-associated viral vectors are being used most widely today for cochlear gene transfer. Despite their high efficiency for transfection, availability of high titers, or ease of production, they do not integrate into the genome, leading to transient expression, and their use potentially initiates an immune response resulting in destruction of recipient cochlear cells.

A controlled release system, in which the rate of release is determined by the design of the device, is required for certain biological effects of therapeutic molecules and elimination of unwanted side effects. For this purpose, implantable osmotic mini-pumps have been used for inner ear drug delivery in animal experiments [2,22]. This method, however, requires surgical treatment in the middle and inner ear, which may limit its clinical application. Previously, clinical efficacy of an implantable mini-pump, which delivers drugs via diffusion across the round window, has been described [23]. However, this technique has not been widely used in a clinical setting, because it requires surgical invasiveness almost equal to tympanoplasty. There remains intense interest in the development of safe and effective drug delivery systems for the inner ear, with a number of groups working on intracochlear catheter-based application systems. One approach has been to combine drug delivery with an existing device, such as by incorporating a drug delivery cannula into a cochlear implant electrode [24].

Candidates for therapeutic molecules for the treatment of SNHL are being discovered. It is therefore necessary to develop appropriate strategies for local delivery of therapeutic molecules. For clinical application, safe, effective, and direct methods for delivery of therapeutic molecules to the inner ear need to be developed.

Controlled-release systems

In the past decade, pharmaceutical technologists have paid increasing attention to the controlled or sustained release technology via biomaterials for the delivery of drugs in order to avoid side effects and achieve sufficient drug levels in tissues. Such technology is utilized not only for drug delivery but also for gene delivery [25]. In an effort to develop controlled-release systems, a variety of methods using synthetic and natural materials have arisen. Recent publications have reported on the use of controlled-release systems for local drug delivery to the inner ear. Two synthetic materials, siloxane-based polymers [26] and poly lactic/glycolic acid (PLGA) polymers [27], and one natural material, gelatin-hydrogels [28,29], have been used for this purpose. Although these materials have been included in biomaterials for controlled-release systems, mechanisms for loading and releasing drugs apparently differ among these materials (Figure 1). In siloxane-based polymer systems, the drug dissolves in the polymer and then moves by diffusion [30]. For PLGA polymers, the drug is encapsulated in PLGA polymers and then released by hydrolysis of PLGA [31]. In gelatin-based release systems, the drug binds to gelatin carriers by polyion complexation and is released by enzymatic hydrolysis of gelatin polymers [32].

Siloxane-based polymers

Siloxane-based polymers have been used for years in medical applications in contact with the human body. Silicone-transdermal patches have been widely used in clinic. The drug release in this system is controlled by its diffusion through the silicone network [30]. The releasing rate in this system 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. [26] have utilized this system for local application of beclomethasone into cochlear fluids. A silicone-microimplant was placed onto the RWM

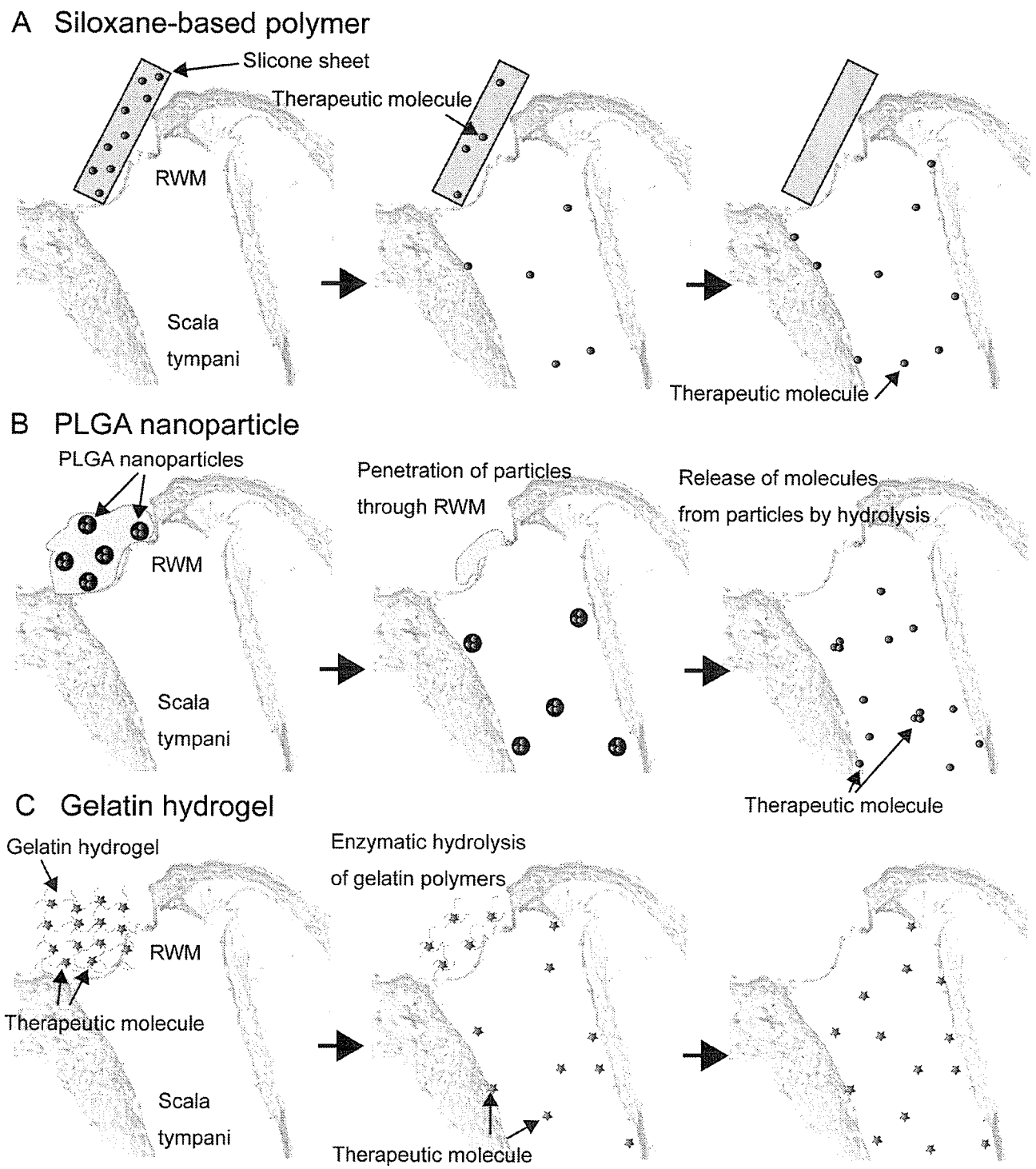

Figure 1. Mechanisms for controlled release of therapeutic molecules from biopolymers. (A) Therapeutic molecules dissolved in siloxane-based polymers move into the scala tympani by diffusion. A silicone sheet remains on the round window membrane (RWM). (B) Poly lactic/glycolic acid (PLGA) nanoparticles containing therapeutic molecules penetrate through the RWM. Therapeutic molecules are released from nanoparticles by their hydrolysis. (C) Therapeutic molecules bind to gelatin carriers by polyion complexation and are released by enzymatic hydrolysis of gelatin polymers.

of guinea pigs. Liquid chromatography demonstrated release of beclomethasone from the silicone-microimplant into cochlear fluids. In this system, a silicone-microimplant remains on the RWM (Figure 1A), although it does not induce functional and histological damage in the cochlea. Therefore, repeated treatment requires extirpation of the material that had been used previously. In addition, only a limited number of molecules can be used in this system.

PLGA nanoparticles

Encapsulating bioactive molecules in PLGA or polylactic acid (PLA) particles has been used as a method for controlled-release application. Water-insoluble, low-molecular weight agents were encapsulated in PLGA or PLA microparticles and nanoparticles, and provided for clinical use [33,34]. However, recent advances in this field enable encapsulation of water-soluble, low-molecular weight agents in PLGA nanoparticles [31]. Tamura et al. [27] have examined the potential of PLGA nanoparticles for drug delivery to the cochlea using guinea pigs. The distribution of PLGA nanoparticles encapsulating rhodamine (140–180 nm in diameter) in the cochlea following local application onto the RWM was evaluated. PLGA nanoparticles containing rhodamine were observed in the cochlea, indicating that PLGA nanoparticles can penetrate through the RWM. Rhodamine will be released from PLGA nanoparticles after penetration of PLGA nanoparticles through the RWM (Figure 1B). On the other hand, systemic application of PLGA nanoparticles has no significant effects on sustained, targeted delivery of rhodamine into the cochlea. These findings indicate that encapsulating therapeutic molecules in PLGA nanoparticles is suitable for local drug delivery to the cochlea.

In comparison with a silicone-microimplant, PLGA nanoparticles have advances including the ability of repeated application, because PLGA is dissolved by hydrolysis. Various therapeutic molecules for inner ear diseases can be encapsulated in PLGA nanoparticles, and applied as intratympanic drugs. The efficacy of encapsulating betamethasone phosphate in PLGA nanoparticles has already been confirmed using animal models for rheumatoid arthritis and autoimmune uveoretinitis [35,36]. Local gentamicin application has been used for the control of intractable vertigo in Meniere's disease [14–16]. PLGA nanoparticles can be utilized for controlled release of gentamicin. However, PLGA nanoparticles are not suitable for delivery of proteins or peptides. Hence, this system cannot use for controlled delivery of neurotrophins or growth factors.

Gelatin hydrogel

Gelatin is a commonly used natural polymer that is derived from collagen. Gelfoam, which is prepared from porcine-skin gelatin, has been used for drug delivery to the cochlea [37]. Recently, gelatin-based controlled-release systems have been developed [32]. The isoelectric point of gelatin can be modified during the fabrication process to yield either a negatively charged acidic gelatin or a positively charged basic gelatin, which allows electrostatic interactions to take place between charged therapeutic molecules and gelatin of the opposite charge, forming polyion complexes. The significance of this system is the ability for application of proteins and plasmid DNA. Previous reports have demonstrated its efficacy for controlled release of various growth factors or plasmid DNA in other fields [25,38,39]. In this system, therapeutic molecules are released by enzymatic degradation of gelatin (Figure 1C), the rates of which are determined by the crosslinking density of gelatin hydrogels.

Endo et al. [28] have demonstrated sustained release of brain-derived neurotrophic factors (BDNFs) into cochlear fluids by a gelatin hydrogel. BDNF concentrations in the cochlear fluid after placing a hydrogel containing this agent onto the RWM of guinea pigs were measured by enzyme-liked immunosorbent assay (ELISA), which reveals sustained delivery of BDNF into the cochlear fluid via the hydrogel. In addition, local BDNF delivery using a gelatin hydrogel sufficiently protects spiral ganglion neurons in functionality and histology. More recently, Iwai et al. (29) have described significant protection of auditory hair cells from noise trauma in rats using local application of insulin-like growth factor I via gelatin hydrogels. These findings demonstrate that the gelatin-based controlled-release system is a useful method for sustained delivery of neurotrophins and growth factors into the cochlea. Repeated applications using this system are possible. This system has several advances in comparison with the other two controlled-release systems: (1) easy loading of therapeutic molecules into biopolymers, (2) it is applicable for delivery of proteins, peptides, or plasmid DNA. These advances are favorable for the treatment of SNHL, because the efficacy of neurotrophins or growth factors and the potential of gene therapy for treatment of SNHL have been demonstrated.

Conclusions for clinical application

The results in experimental studies using controlled-release systems are preferable; however, the delivery protocol in humans is likely to differ from that in animal experiments. The distribution of drugs applied in the cochlear fluid space depends on dispersal diffusion, which is influenced by the length and volume of the cochlear fluid space [13]. In addition, the round window niche membrane covers the round window niche in 57% of human subjects [40]. Therefore, it is necessary to remove tissues overlaying the RWM for drug penetration through the RWM in some cases. However, introduction into

clinical practice of these controlled-release systems may be reasonable since they have certain advantages over previous methods and implantable devices.

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