lear fluid space in various experimental models, or suggested the presence of resident tissue macrophages in the inner ear, there has been some controversy on the distribution and phenotype of resident tissue macrophages in the inner ear.

A good understanding of the origins and distribution of the resident tissue macrophages in the inner ear, as well as the timing and context of their recruitment, will be essential to understanding the pathogenensis of inner ear immune disorders in which a loss of tissue homeostasis might result from dysfunction of resident tissue macrophages. The aims of the present study were to establish the distribution and phenotype of resident macrophages in the cochlea and to test the contribution of hematopoietic BM to the recruitment of cochlear macrophages.

BM chimeric mice were made by transplantation of hematopoietic stem cells (HSCs) from enhanced green fluorescent protein (EGFP)-transgenic mice into irradiated adult wild-type mice, and the distribution of BM-derived cells in the cochlea was traced. Immunohistochemistry was employed to determine the phenotype of BM-derived cells. The mobilization of cochlear macrophages was tested by using systemic application of macrophage colony-stimulating factor (M-CSF), the primary regulator of the activation of mononuclear phagocytes. We also examined the response of cochlear macrophages to local surgical invasion, used as a model for an acute, local, exogenous stress on the inner ear.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from Japan SLC, Inc (Shizuoka, Japan). EGFP transgenic mice [B6;C3-Tg(ACtb-EGFP)CX-FM139Osb] were used as a source of HSCs (Okabe et al., 1997). The animals were maintained in a specific pathogen-free microisolator environment in the Institute of Laboratory Animals, Kyoto University Graduate School of Medicine. All experimental protocols were approved by the Animal Research Committee, Kyoto University Graduate School of Medicine, and conducted in accordance with the NIH *Guide for the care and use of laboratory animals*.

BM Chimeric Mice

HSCs were collected as lineage marker (Lin)-negative, c-kit (CD117)-positive, stem cell antigen 1 (Sca1)-positive cells from the BM of EGFP transgenic mice via cell sorting on a FACS Vantage (Becton-Dickinson, San Jose, CA) as described previously (Yoshimoto et al., 2003). Briefly, whole BM cells were isolated from the femurs and tibiae of EGFP transgenic mice (8–12 weeks of age). BM mononuclear cells were labeled with a primary antibody cocktail (BD Pharmingen, San Diego, CA) for CD3 (145-2C11), B220/CD45R (RA3-6B2), Mac-1 (M1/70), Gr-1 (RB6-8C5), and TER119 (TR119). Lineage-depleted cells (Lin^- cells) were obtained by using auto-MACS (Militenyi Biotec, Bergish Gladbach, Germany). $Lin^-/c\text{-}kit^+/Sca1^+$ cells were collected by cell sorting on a FACS Vantage as HSCs using R-PE-conjugated anti-mouse Ly-6A/E (Sca-1; clone: E13-161.7; BD Pharmingen) and APC-conjugated anti-mouse CD117 (c-Kit; clone: 2B8; BD Pharmingen). C57BL/6 mice (n = 6, 10–12 weeks of age) were irradiated with 9.5 Gy gamma rays (Gamma Cell 40 Exactor; MDS Nordion Inc., Ottawa, Ontario, Canada) and each received 5×10^3 HSCs through the tail vein. At 1 (four ears from two animals), 2 (four ears from two animals), or 4 weeks (four ears from two animals) and 3 (6 ears from 6 animals), or 6 months (six ears from six animals) after transplantation, the temporal bones were dissected out under overdose anesthesia. Peripheral blood samples were also collected from the animals 3 months after transplantation to determine the chimeric ratio of blood cells.

Systemic Application of M-CSF

C57BL/6 (10 weeks of age, n = 5) mice received an intraperitoneal injection of M-CSF (Kyowa Hakko Kogyo, Tokyo, Japan) dissolved in physiological saline once per day for 7 consecutive days (total dose 7×10^5 units). The dose of M-CSF was equivalent to that used in a clinical setting, relative to body weight. On the day after M-CSF application, the temporal bones were collected and prepared for cryostat sections. Animals (n = 5) receiving physiological saline instead of M-CSF were used as controls.

Local Surgical Treatment

C57BL/6 mice (10 weeks of age) were injected with 3 μl physiological saline into the posterior semicircular canal (PSCC). This minimally invasive treatment was a modified protocol used in previous studies when administrating drugs (Lee et al., 2003; Nakagawa et al., 2003; Kim et al., 2005) or cells (Iguchi et al., 2003; Okano et al., 2006) into the inner ear of mice. With animals under general anesthesia with ketamine (75 mg/kg) and xylazine (9 mg/kg), a retroauricular incision was made in the left ear, and the PSCC was exposed. A small hole (approximately 180 μm in diameter) was made in the bony wall of the PSCC with a 26-G needle. A fused silica glass needle (170 μm outer diameter; EiCOM, Kyoto, Japan) was then inserted into the perilymphatic space of the PSCC, and the solution was injected at a rate of 1 μl/min for 3 min using a Micro Syringe Pump (EiCOM). The hole in the PSCC was plugged with connective tissue and covered with fibrin glue. On days 1 (n = 4), 7 (n = 5), and 28 (n = 5), the left temporal bones were collected and prepared as cryostat sections to study the density of Iba1-positive cells in the cochlea. Temporal bones collected from age-matched normal animals were used as preoperative controls (n = 5).

The auditory function of experimental animals was monitored by auditory brainstem response (ABR) recording as described previously (Shiga et al., 2005). ABRs were recorded before treatment and on days 1, 7, and 28 after surgery. Thresholds were determined for frequencies of 10, 20, and 40 kHz.

The mobilization of Iba1-positive cells from BM to the cochlea were also examined in BM chimeric mice (n = 8) that had received EGFP-labeled HSCs 3 months before. Four animals received a saline injection into the PSCC as described

above, and the cochleae were collected 7 days after surgery. The remaining four animals were preserved as controls, receiving no surgical treatment.

Immunohistochemistry

Under general anesthesia with ketamine and xylazine, animals were perfused intracardially with ice-cooled phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in phosphate buffer. The temporal bones were collected and immersed in the same fixative for 4 hr at 4°C. The samples were decalcified with 10% EDTA in PBS and cryoprotected with 30% sucrose. Specimens were prepared as cryostat sections (10 μm in thickness). Midmodiolar sections were provided for histological analyses.

Cryostat sections were immersed in blocking solution containing 10% goat serum for 30 min and incubated with a primary antibody at 4°C overnight. Characteristics of BM-derived cells were examined by immunostaining for leukocyte common antigen CD45; ionized calcium binding adapter molecule 1 (Iba1), which is specific for microglia/macrophages (Imai et al., 1996); microglia/macrophage-specific glycoprotein F4/80; and macrosialin CD68, which is specifically expressed by tissue macrophages. Immunohistochemistry for Ki67, a nuclear protein expressed in proliferating cells, was performed on the BM chimeric mice to determine the proliferation of BM-derived cells in situ. The primary antibodies used in this study were rat anti-mouse CD45 (1:50; 30-F11; BD Pharmingen), rabbit anti-Iba1 (1:1,000; Wako Pure Chemicals, Osaka, Japan), rat anti-mouse F4/80 (1:10; Cl:A3-1; Serotec, Oxford, United Kingdom), rat anti-mouse CD68 (1:1,000; FA-11; Serotec), and rabbit anti-Ki67 (1:200; SP6; Lab Vision, Fremont, CA). The localization of primary antibodies was visualized using secondary antibodies conjugated with Alexa Fluor 488, 555, or 633 (1:500; Molecular Probes, Eugene, OR). Nuclei were counterstained by 4′,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS; Molecular Probes). Negative controls lacked primary antibody labeling. Specimens were viewed with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) or a Leica TCS-SP2 confocal laser scanning microscope (Leica Microsystems, Tokyo, Japan) with a digital image-capture system.

Quantification

To determine the chimeric ratio in peripheral blood, smears of blood samples were made on slides. Total cells with nuclei were based on nuclear counts with DAPI staining observed with a fluorescence microscope. The ratio of EGFP-positive cells to the total number of cells was calculated. At least 200 nuclei were counted in each sample.

For the quantitatification of BM-derived cells or Iba1-positive cells, four sections were selected randomly from the 12 most midmodiolar sections for each experimental or control animal. To assess the distribution of BM-derived cells in the cochlea, the number of cells derived from engrafted HSCs in one midmodiolar section from base to apex was counted in six animals at 6 months after HSC transplantation. All BM-derived cells defined by coexpression of EGFP and DAPI within the cochlea were counted by two double-blinded examiners. The number of HSC-derived cells coexpressing Iba1, F4/80, CD45, or CD68 was also counted for examination of the phenotype of BM-derived cells. The ratio of EGFP-positive cells labeled with Iba1, F4/80, CD45, or CD68 to the total number of EGFP-positive cells was calculated. To study the replacement of Iba1-positive cells in the cochlea by engrafted EGFP-positive cells, the number of cells dual-labeled with EGFP and Iba1 in one midmodiolar section was counted. The ratio of the expression of EGFP to the total number of Iba1-positive cells was calculated in the cochleae harvested at 1, 2, or 4 weeks and 3, or 6 months after HSC transplantation. To investigate the dynamics of EGFP- or Iba1-positive cells in the cochlea, the density of EGFP- or Iba1-positive cells in SG was calculated by a modified method as described previously for evaluating the density of SG neurons (Shinohara et al., 2002). All EGFP- or Iba1-positive cells with nuclei stained for DAPI within each profile of Rosenthal's canal from the midbasal portion of the cochlea were counted. The outline of Rosenthal's canal profile was then traced under a brightfield image to generate the area of SG in Image J software (http://www.nist.gov/lispix/imlab/prelim/dnld.html). The density of EGFP- or Iba1-positive cells in SG was expressed as the cell number for an area of 10,000 μm^2. The density of EGFP- or Iba1-positive cells in SL of midbasal portion of the cochlea was also calculated by tracing the outline of the SL region occupied by type I-V fibrocytes and expressed as the cell number for an area of 10,000 μm^2. The number of total cells in the area of interest was determined by counting nuclei on DAPI-stained sections in Image J software. The number of proliferating macrophages in the cochlea was determined by counting the colocalization of Ki67 and DAPI. The ratio of EGFP-positive cells labeled with Ki67 to the total number of CD68-positive cells per section was also calculated.

Statistcal Analysis

Statistical analyses were performed by using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer's test, for the analysis of ABR thresholds, alteration of Iba1-positive cells by EGFP-positive cells, and effect of local surgical stress. An unpaired *t*-test was used in other statistical analyses. $P < 0.05$ was considered statistically significant. All data are presented as the mean ± SE.

RESULTS

BM-Derived Cells Are Widely Distributed in the Spiral Ganglion and the Spiral Ligament

Six months after BM transplantation, numerous EGFP-positive cells were found widely within the cochlea of transplanted mice (Fig. 1a). A large population of EGFP-positive cells is located in the connective tissue of SL and auditory nervous system, including SG and acoustic nerve (AN; Fig. 1b–d). In SL, EGFP-positive cells were observed predominantly in its lower portion, corresponding to the type II and IV fibrocyte regions (Fig. 1b). In the auditory nervous system, EGFP-positive cells were observed along nerve fibers both in SG and

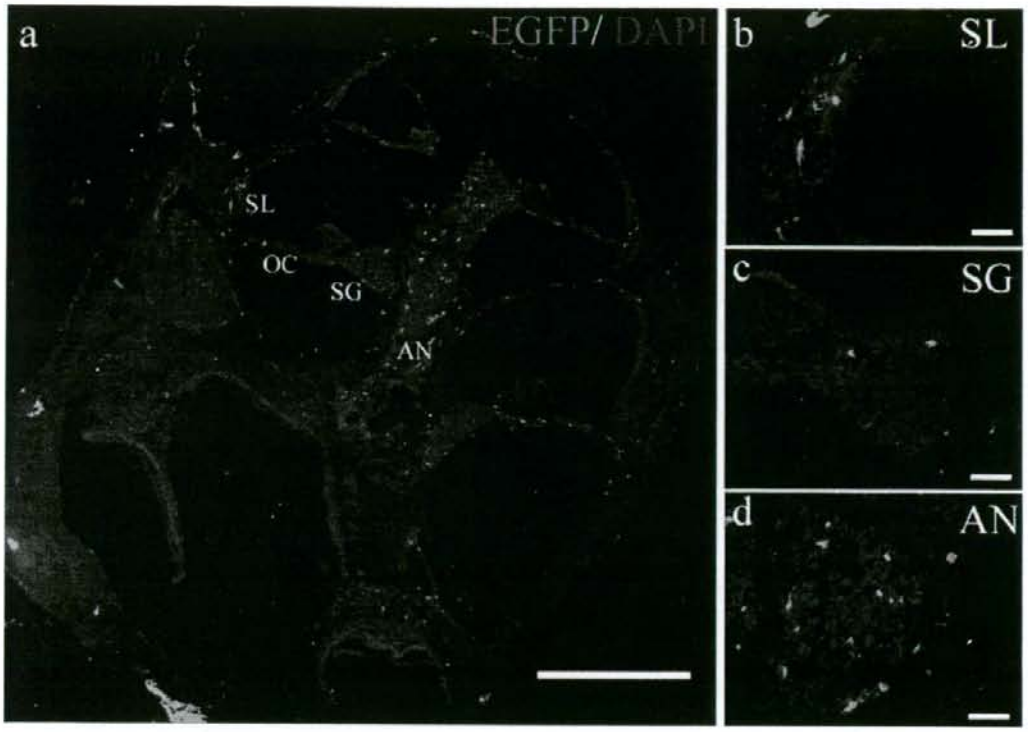

Fig. 1. Distribution of hematopoietic cell-derived cells in the cochlea. **a:** Distribution of hematopoietic cell-derived cells is shown in a midmodiolar section obtained from bone marrow (BM) chimeric mice 6 months after transplantation. BM-derived cells expressing EGFP were distributed from the base to the apex of the cochlea. Blue fluorescence shows nuclear staining with DAPI. **b–d:** In SL, EGFP-positive cells were observed predominantly in the lower portion of SL occupied by type II and IV fibrocytes (b). In the auditory nervous system, EGFP-positive cells were observed along nerve fibers in both SG (c) and cochlear modiolus (d). AN, auditory nerve; OC, organ of Corti; SG, spiral ganglion; SL, spiral ligament. Scale bars = 500 μm in a; 50 μm in b–d.

AN (Fig. 1c,d). In one midmodiolar section obtained at 6 months after transplantation, 90.3 ± 6.9 cells expressing EGFP were observed, 35.6% ± 3.1% of which were in the cochlear connective tissues of SL and the spiral limbus and 62.2% ± 3.0% in the cochlear nervous system, including SG and AN. The proportion of BM-derived cells to the total cells was 7.7% ± 0.9% in SL and 5.5% ± 0.9% in SG, which was compatible with the study by Lang et al. (2006). EGFP-positive cells were occasionally identified on the undersurface of the basilar membrane in the scala tympani and in the stria vascularis (data not shown). No EGFP-positive cells were observed within the cochlear sensory epithelium.

More Than 80% of BM-Derived Cells in the Cochlea Demonstrated the Phenotype of Macrophages

The following analyses of immunohistochemistry were carried out to characterize hematopoietic BM-derived cells in the adult mouse cochlea. Most EGFP-positive cells expressed F4/80 (Fig. 2a), Iba1 (Fig. 2b), or CD68 (Fig. 2c), indicating that cochlear HSC-derived cells have differentiated into the macrophage lineage. Cells dually labeled with EGFP and Iba1, EGFP, or F4/80 or with EGFP and CD68 were localized both in the cochlear connective tissue and the cochlear nervous system. The expression of Iba1 was found in 92.5% ± 3.1% of EGFP-positive cells, and F4/80 expression was observed in 87.3% ± 4.2%. Although more than 80% of BM-derived cells in the cochlea demonstrated the phenotype of macrophage, immunoreactivity for CD45, a common leukocyte antigen, was identified in only 7.4% ± 0.5% of EGFP-positive cells in the cochlea of BM chimeric mice. The number of cells doubly stained with Iba1 and CD45 was limited to one or two in one section (0.9% ± 0.3% of the total number of Iba1-positive BM-derived cells) except for cells in BM of the temporal bone. In contrast, CD68 expression was found in 81.7%

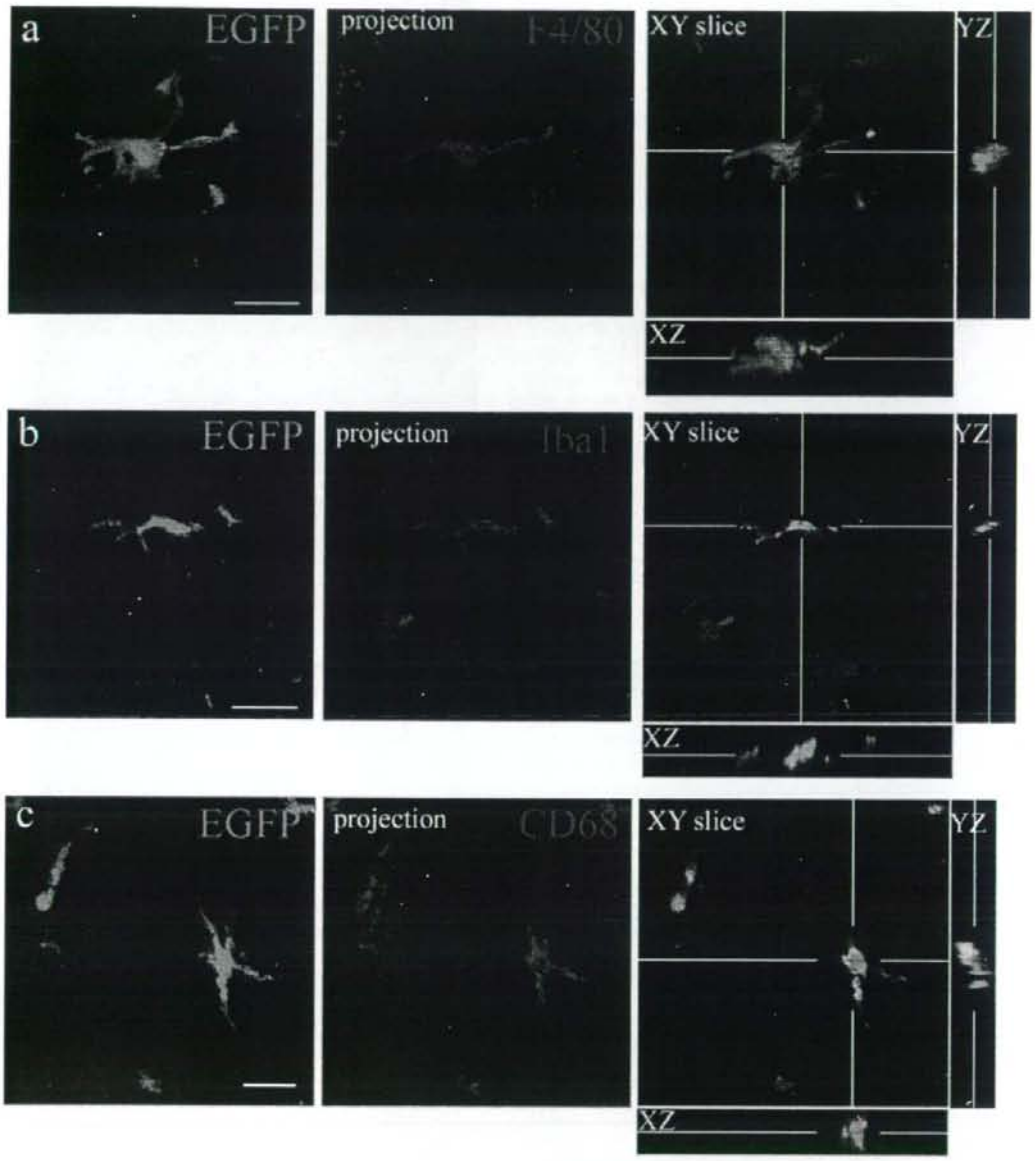

Fig. 2. Immunohistochemistry for F4/80, Iba1, and CD68 in the cochleae of bone marrow chimeric mice 6 months after transplantation. **a:** Photomicrographs obtained by confocal microscopy demonstrate colocalization of EGFP and F4/80 in the cells derived from transplanted HSCs within the spiral ganglion (SG). Immunoreactivity for F4/80 was frequently observed in HSC-derived cells in the lower part of spiral ligament (SL) and the SG. **b:** Iba1 expression was also found in HSC-derived cells in the SL and SG. With confocal microscopy, HSC-derived cells in SL are found to be dually labeled with EGFP and Iba1. **c:** CD68 was also colocalized in BM-derived cells expressing Iba1 in SL. Scale bars = 20 μm.

± 5.0% of EGFP-positive cells, and 87.3% ± 2.6% of Iba1-positive cells coexpressed CD68, which also demonstrated that BM-derived cells expressing Iba1 were of macrophage lineage.

Observation of BM-derived cells expressing Iba1 in the cochlea with a confocal microscope revealed a specific morphological feature that was characterized by a spindle shape with several ramified processes, a characteristic morphological feature of macrophages (Fig. 2a–c). In addition to the immunohistochemistry phenotype, BM-derived cells expressing Iba1 in the cochlea morophologically followed microglias that are referred to as resident tissue macrophages in the CNS.

Systemic Application of M-CSF Increased the Number of Iba1-Positive Cells in the Cochlea

To characterize Iba1-positive cells in the cochlea, we next examined the mobilization of Iba1-positive cells using systemic application of M-CSF, the primary regulator of activation of mononuclear phagocytes in wild-type C57BL/6 mice. Iba1-positive cells in both SL and SG were observed more densely in M-CSF-treated mice (Fig. 3a,b) than in controls (Fig. 3c,d). The density of Iba1-positive cells in SL of the middle turn in the controls was 1.46 ± 0.22 (cells/10^4 μm^2), which increased to 2.54 ± 0.45 after M-CSF treatment (Fig. 3e), although the difference was not significant. By contrast, a significant increase was identified in the density of Iba1-positive cells in SG (from 1.39 ± 0.18 to 2.95 ± 0.3; Fig. 3e). These data revealed that Iba1-positive cells in the cochlea are under the control of M-CSF signaling. In addition to the findings of phenotype and morphology, we have demonstrated that BM-derived cells labeled with Iba1 have a quality of resident tissue macrophage in the cochlea.

Cochlear Macrophages Gradually Turn Over for More Than 6 Months

The percentage of chimerism in the peripheral blood at 3 months after BM transplantation was 82.8% ± 3.6%, indicating that hematopoietic reconstitution was performed successfully at this time point. The chimeric ratio observed in the present study was compatible with previous reports on BM chimeric mice (Yoshimoto et al., 2003; Lang et al., 2006). Sequential observation of Iba1-positive cells in the cochlea of transplanted mice demonstrated that cochlear macrophages labeled with Iba1 survived systemic irradiation and were gradually replaced by EGFP-positive cells derived from transplanted HSCs. One or two weeks after HSC transplantation, no EGFP-positive cells were found within cochlear tissues. By contrast, at 4 weeks after transplantation, expression of EGFP was found in 15.4% ± 6.6% of Iba1-positive cells in the cochlea. The ratio for EGFP expression in Iba1-positive cells increased remarkably to 64.9% ± 8.1% in cochlear specimens obtained at 3 months (12 weeks) after transplantation, then reached 84.1% ± 1.6% at 6 months (24 weeks) after transplantation. The differences in the ratio for EGFP expression in Iba1-positive cells were significant at 3 and 6 months compared with 1, 2, or 4 weeks (Fig. 4). The distribution of Iba1-positive cells in the cochlea was identical at each time point (data not shown). The density of Iba1-positive cells in SL was 2.39 ± 0.32 (cells/10^4 μm^2) at

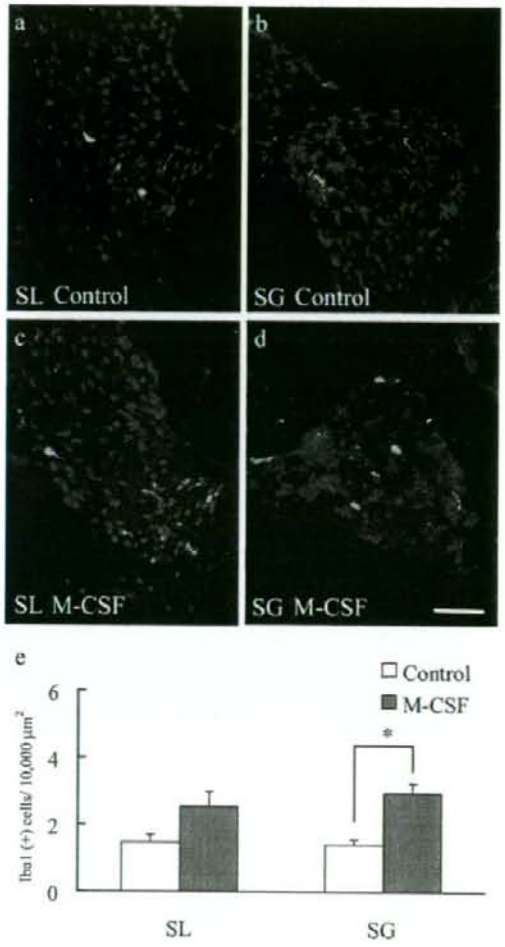

Fig. 3. Systemic application of macrophage colony-stimulating factor increases the density of Iba-1-positive cells in the spiral ligament (SL) and spiral ganglion (SG). **a–d:** Several Iba1-positive cells were found in the SL (c) and the SG (d) following systemic application of macrophage colony-stimulating factor (M-CSF), although few cells expressing Iba1 were observed in control specimens (a,b). **e:** Densities of Iba1-positive cells (cells/10^4 μm^2) in SG of M-CSF-treated cochleae were significantly higher than those of control cochleae (*P = 0.002, unpaired t-test), although no significant difference is found in SL (P = 0.06). Bars represent standard errors. Scale bar = 50 μm.

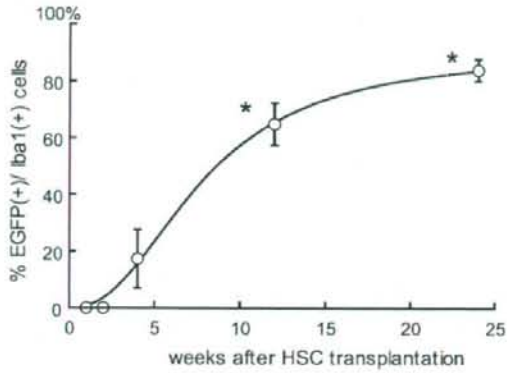

Fig. 4. Sequential analysis of chimeric ratios for Iba1-positive cells following transplantation of hematopoietic stem cells derived from GFP mice. The ratios for EGFP expression in Iba1-positive cells of the whole cochlea are shown at 1, 2, and 4 weeks and 3 (12 weeks) and 6 (24 weeks) months after transplantation. The graph demonstrates a gradual increase in the ratio of EGFP expression in Iba1-positive cells, indicating replacement of native Iba1-positive cells by EGFP-positive cells derived from engrafted hematopoietic stem cells. The ratio at 3 or 6 months is significantly higher than that at 1, 2, or 4 weeks (*$P < 0.001$, ANOVA with Tukey-Kramer's test). Bars show standard errors.

1 week, 2.67 ± 0.07 at 2 weeks, 3.02 ± 0.42 at 4 weeks, 3.25 ± 0.18 at 3 months, and 3.31 ± 0.11 at 6 months after BM transplantation. The density of Iba1-positive cells in SG was 1.35 ± 0.10 (cells/10^4 μm^2) at 1 week, 1.73 ± 0.37 at 2 weeks, 1.22 ± 0.15 at 4 weeks, 1.93 ± 0.32 at 3 months, and 2.26 ± 0.14 at 6 months after BM transplantation. There were no significant differences in the density of Iba1-positive cells in SL among the experimental groups. However, the increase with age in the density of Iba1-positive cells in SG was statistically significant between 1 week and 6 months and between 4 weeks and 6 months. These findings indicate that cochlear macrophages are not eliminated by systemic irradiation and gradually turn over for more than 6 months in nondamaged cochlea.

Cochlear Macrophages Increased in Response to Local Surgical Stress

To examine the reaction of cochlear macrophages to an acute, local, exogenous stress on the cochlea, surgical invasion with application of physiological saline via PSCC was performed on both wild-type and BM chimeric mice. ABR recoding was performed to determine the extent of functional damage following treatment. Significant elevation of ABR thresholds was observed on day 1 after local treatment, whereas no elevation was found on days 7 and 28 at all frequencies (Fig. 5a), indicating that the surgical stress used in the present study caused temporary damage to the cochlea.

Iba1-positive cells were increased temporarily in response to local surgical stress in SL and SG (Fig. 5b–e). The density of Iba1-positive cells in SL exhibited an immediate response to the treatment on day 1 after surgery (3.96 ± 0.16 cells/10^4 μm^2 vs. 1.62 ± 0.18, preoperative), significantly increased on day 7 after surgery (5.42 ± 0.59), and decreased on day 28 (2.90 ± 0.41; Fig. 5f). No significant difference was found in the densities of Iba1-positive cells between before surgery, on day 1, or on day 28. A temporary increase was also found in the density of Iba1-positive cells in SG on day 1 (1.94 ± 0.06) and day 7 (2.66 ± 0.33; Fig. 5f) compared with densities of 1.20 ± 0.09 preoperatively and 1.33 ± 0.16 on day 28. The differences between preoperative values and day 7 and between days 7 and 28 were statistically significant.

We also examined the effect of local surgical stress in BM chimeric mice that had been transplanted with HSCs labeled with EGFP 3 months before, to test the mobilization of Iba1-positive cell from BM to the cochlea. The density of cells dually labeled with EGFP and Iba1 studied in SL at day 7 after treatment was 4.01 ± 0.21 (cells/10^4 μm^2) in the operated group and 2.54 ± 0.39 in nonoperated BM chimeric mice, and that in SG was 1.88 ± 0.52 in the operated group and 1.42 ± 0.21 in the nonoperated group. The difference in the density of EGFP-positive cells in SL between the two groups was statistically significant, whereas that in SG was not significant.

Immunohistochemistry for Ki67 was performed before and at days 1 andf 7 after surgical treatment, to test whether the increase in cochlear macrophages following surgical treatment was due to proliferation in situ. On day 1 following treatment, 1.75 ± 0.85 cells/section were found to be dually labeled with Ki67 and CD68 (1.74 ± 0.71% in total number of cells positive for CD68). On day 7 following treatment, 2.25 ± 0.75 were dually labeled with Ki67 and CD68 (2.49% ± 0.81%), in contrast to 1.00 ± 0.40 in untreated mice (1.53% ± 0.51%). All Ki67-positive cells in the three groups were found in the lower part of SL. However, no statistically significant difference was observed in the number of Ki67-positive cells among the three groups. These findings indicated that the increase in cochlear macrophages was due mainly to the migration of macrophages from the circulation into the cochlea. However, it was also revealed that the proliferation of macrophages in situ is one of the possible sources for cochlear macrophages.

DISCUSSION

The present study revealed that BM-derived cells are supplied continuously to the cochlea even in the adult mouse and demonstrated that the predominant phenotype of macrophages involved expression of CD68, F4/80, or Iba1 in SL and SG. Our study on the morphology, immunohisotochemical phenotype, and reactivity to M-CSF provides evidence that hematopoi-

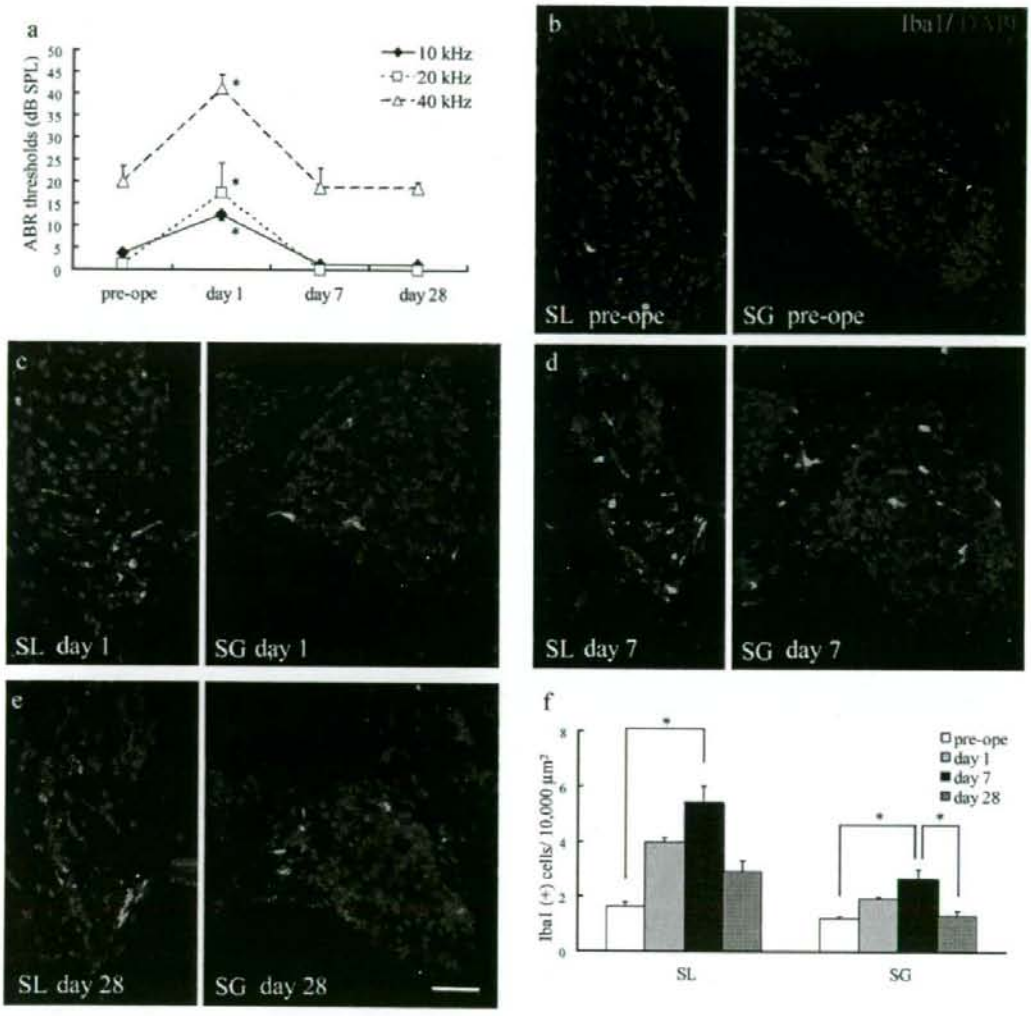

Fig. 5. Effects of local surgical stress on densities of Iba1-positive cells in the spiral ligament (SL) and spiral ganglion (SG). **a:** Local surgical stress causes significant elevation of ABR thresholds at each frequency on day 1 after treatment (*$P < 0.001$, ANOVA with Tukey-Kramer's test), and the elevation recovered on days 7 and 28 at all the frequencies tested. **b–e:** Immunostaining for Iba1 in cochlear specimens obtained preoperatively (pre-ope; b) and on day 1 (c), day 7 (d), and day 28 (e) after treatment. Increase in Iba1-positive cells was observed in the SL and SG of cochleae obtained on days 1 and 7. **f:** The density of Iba1-positive cells (cells/10^4 μm^2) on day 7 was significantly higher than that in preoperative specimens in both SL (*$P = 0.003$) and SG (*$P = 0.002$) or day 28 in SG. Bars show standard errors. Scale bars = 50 μm.

etic BM-derived cells expressing Iba1 are constitutively present as resident tissue macrophages in the cochlea. The examination of the recruitment of cochlear macrophages demonstrated that these cells exhibited slow turnover for several months during steady-state conditions and quickly increased in response to local surgical stress.

Although earlier studies have demonstrated infiltration of inflammatory cells, including macrophages, into the cochlea following inner ear injury (Fredelius, 1988; Bhave et al., 1998; Hirose et al., 2005; Tornabene et al., 2006; Ladrech et al., 2007), two recent studies have suggested the presence of resident tissue macrophages in the inner ear.

Lang et al. (2006) reported that BM-derived cells are constitutively present and widely distributed in the cochlea in the same manner demonstrated in the present study. Although they concluded negatively that only 5% of BM-derived cells differentiated into CD45R-positive macrophages, the rate for CD45 expression in BM-derived cells is also compatible with the present study. The authors instead emphasized that histological analysis of the cochleae following HSCs revealed the contribution of BM-derived cells to fibrocytes or mesenchymal cells in the inner ear. However, the specific ratios for immunoreactivity of Na, K-ATPase, or the Na-K-Cl transporter in the BM-derived cells were not determined in their study. Moreover, there is a discrepancy in the ratio for the expression of F4/80 between the study by Lang et al. (2006) and the present study, although both studies used HSC-transplanted mice that exhibited similar chimeric ratios in peripheral blood and SL cells. One possible explanation for this discrepancy is a difference in methods for immunohistochemistry. We used cryostat sections, whereas Lang et al. used paraffin-embedded sections. Our study demonstrated that more than 80% of BM-derived cells in SL and SG show the phenotype of macrophages and that resident tissue macrophages in the cochlea during steady-state conditions are present in a larger number than reported previously.

Another report was made by Hirose et al. (2005) that CD45-positive mononuclear phagocytes are present in the SL of nonnoise-exposed $CX3CR1^{GFP/GFP}$ transgenic mice, and these cells are also labeled with Iba1 or CD68, which is consistent with the results of the present study. However, the density of CD45-positive cells in the cochlea was quite different between the wild-type and $CX3CR1^{GFP/GFP}$ transgenic mice used in their study, although the number of BM-derived cells expressing CD45 in the cochlea demonstrated in the present study was identical to that of CD45-positive cells in the cochlea of nonnoise-exposed wild-type mice presented by Hirose et al. (2005). The most controversial point is whether it is appropriate to assume that macrophages observed in the cochlea can be described as "microglia-like" cells. Although the authors failed to describe the distribution of macrophages in the auditory nervous system including the SG and cochlear modiolus during steady-state conditions, they distinguish the infiltrating macrophages observed after noise exposure from microglias in the CNS by the morphology and the potentiality of exchanging from the vascular space. The proliferation in situ is thought to be one of the main sources of microglia in adults, but some studies have reported that BM-derived cells can enter the CNS and populate the microglial cell compartment (Lawson et al., 1992; Corti et al., 2002; Hess et al., 2004; Simard and Rivest, 2004; Malm et al., 2005). Moreover, previous studies in the CNS using flow cytometry or immunohistochemistry defined a profile of characterization of microglial cells corresponding to the following phenotype: $CD68^+$, CD45 low, $CD11b^+$, CD11c high, and MHC class II^+ (Guillemin and Brew, 2004; Floden and Combs, 2007). Our results on cochlear resident macrophages "$CD68^+$, $Iba1^+$, and $CD45^-$" were compatible with the immunochemical definition of microglias as reported above. In addition, the cochlear resident macrophages were observed in the auditory nervous system as well as in the connective tissue of SL in the present study. Because there is a considerable heterogeneity in the phenotypes of the macrophage lineage, and because resident tissue macrophages share several antigens with infiltrating macrophages (Guillemin and Brew, 2004), further studies should be carried out to define a profile of characterization on both resident and infiltrating macrophages in the cochlea.

Many studies on resident tissue macrophages in other organs have been performed with BM chimeric mice. In terms of replacement of resident tissue macrophages by BM, donor-derived cells are reported rapidly to populate the liver with Kupffer cells, resident macrophages in the liver, within 3 weeks, and donor Kupffer cells in liver transplants are replaced with similar kinetics (Naito et al., 1997). Microglias in the CNS exhibit longer turnover period than cochlear resident macrophages. At 12 months following HSC transplantation, 40% of microglias have been replaced by hematopoietic cells (Hess et al., 2004; Simard and Rivest, 2004). In contrast, Xu et al. (2007) reported that EGFP-positive BM-derived cells infiltrate normal retina in significant numbers at 8 weeks after BM transplantation and that by 6 months all retinal microglia/macrophges were replaced by BM-derived EGFP-positive cells, the turnover rate of which is very similar to that of the inner ear in the present findings. From the viewpoint of turnover rates, cochlear resident macrophages have characteristics as resident macrophages in the retina.

Although BM chimeric mice are a powerful tool for the analysis of the dynamics of BM-derived cells, there are some limitations. The results of BM chimeric mice are obtained under extraordinary conditions; systemic lethal irradiation and following BM transplantation. It is also technically difficult to set an ideal negative control, with irradiation but without BM transplantation. Moreover, it is difficult to exclude completely the possibility that the results observed in this study are caused by irradiation-induced damage. However, despite these limitations in studies using BM chimeric mice, our results provided some new insights into the origin and distribution of cochlear resident macrophages and the possible supply of cochlear macrophages by replenishment with BM-derived cells.

Recent studies have demonstrated multiple key functions of resident tissue macrophages not only in phagocytosis of foreign bodies or senescent cells but also in the production and secretion of cytokines and the regulation of specific immune responses (Gordon and Taylor, 2005). In the CNS, microglias have both neurotrophic and neurotoxic properties (Kreutzberg, 1996; Moore and Thanos, 1996; Streit, 1996) and play an effector role in both innate and adaptive immune responses, allowing the CNS to respond rapidly and

efficiently to a wide range of pathogens (Olson and Miller, 2004). Although it has not been elucidated whether the infiltrating macrophages play beneficial or harmful roles in the maintenance of auditory function, pharmacological intervention in the infiltration of macrophages may be a possible strategy for treatment of SNHL. In conclusion, the resident cochlear macrophages have potential as a therapeutic target by means of controlling their ability of phagocytosis, migration, or release of cyokines in the pathology of inner ear immune disorders.

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総　説

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「第109回日本耳鼻咽喉科学会総会シンポジウム」

内耳疾患の治療をめざして—基礎研究の最前線

薬物の経正円窓投与

中川　隆之

京都大学大学院医学研究科
耳鼻咽喉科頭頸部外科

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

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

はじめに

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

新規治療法開発に対する期待感は，耳鼻咽喉科医が日常の外来で強く感じていることではないかと思われる．このような背景から，内耳再生など聴覚再生を目的とした研究が活発に行われており，一般市民の期待も高い．

ひとくちで聴覚障害の新しい治療といっても，感音難聴の原因は多様であり，症例ごとに病態も異なる．感音難聴の治療法開発への戦略を考えるにあたり，感音難聴の進行度に応じた治療法開発を想定することは，現実的対策を考えるにあたり有効な手段ではないかと考える．障害の原因や部位（例えば，有毛細胞障害なのか，血管条障害なのか）など病態に応じた治療を開発することが理想的であるが，臨床の現場では病態が特定できる感音難聴はむしろまれである．しかし，時間的，聴力喪失レベルに応じた進行度であれば，多くの耳鼻咽喉科医がイメージしやすいのではないだろうか．このような感音難聴進行度に対応して，最も妥当ではないかと考えられる治療的戦略を想定してみた（図1）．感音難聴のごく初期には，予防的な治療法が現実的な手段と考えられる．これには，生活指導など幅広い対応が包括され，老人性難聴や騒音性難聴が対象疾患として想定される．予防という見地から，細胞移植や遺伝子治療より，薬物内服など非侵襲的なアプローチが望ましい．最近では，ダイエットやサプリメント摂取の有効性を示唆するような報告も散見される[1,2]．次の段階は，明らかな感音難聴が発症した比較的早い段階，例えば突発性難聴，遅発性内リンパ水腫，ウイルス性難聴が想定される．自覚症状が出現して間もない老人性難聴も含めることができるかもしれない．この段階の第一選択は，やはり薬物治療になるのではないだろうか．全身投与で有効性が期待できれば理想的だが，ごく限られた選択肢しかないのが現状であることは先述した．聴力障害が固定した段階に対しては，聴力喪失の程度，聴力型に応じ，補聴器，人工中耳，人工内耳，脳幹インプラントなどが選択される．これら電子器機の進歩にも目覚ましいものがあり，また装着性やデザインなどにも大きな改善が進みつつある．しかし，補聴器や人工内耳を装用している患者が聴力回復の可能性を求めて，外来を受診することはめずらしくなく，治療法の有無について相談された経験がある耳鼻咽喉科医も少なくないのではないかと思う．すなわち，進捗は著しいともいえるが，必ずしも患者は満足していないのが現状ではないだろうか．これらの電子器機の有効性が期待できない場合，あるいは，これらの電子器機に替わる手段として，細胞移植や遺伝子導入による内耳再生が期待されている[3,4]．

この総説では，感音難聴に対する薬物治療，すなわち，感音難聴発症直後，あるいは急性期といえる段階に対する薬物治療の開発について，特に，内耳への薬物局所投与についての最近の基礎的研究の進捗状況と臨床応用の取り組みについて紹介する．まず，内耳薬物投与システム開発に関する基礎的研究について述べ，臨床試験に進むためのステップ，そして臨床試験の進捗状況について紹介する．最後に，今後の展望について述べたい．

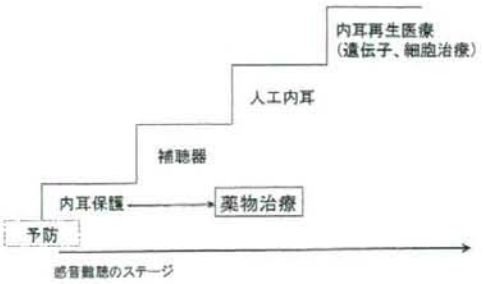

図1　感音難聴治療のストラテジー

内耳薬物投与システムに求められる条件

われわれは，臨床応用を念頭において内耳薬物投与システム開発に着手した．内耳薬物投与システムは，局所投与により効率よく内耳に薬物を到達させようとするものであるが，薬物の局所投与という方法は，決して新しいものではなく，30年以上前から鼓室投与として用いられている方法である．しかしながら，局所投与の有効性について，これまでに詳細な臨床的な検討は行われておらず，最近米国でステロイドの鼓室内注入の大規模な臨床試験が展開されていることが，本年のMid Winter Research Meeting of the Association for Research in Otolaryngology（ARO）で報告されていた[5]．鼓室内投与は簡単な方法であり，手技自体の安全性は高い．しかし，内耳への薬物動態を考えた場合，ばらつきが大きく，1回投与では内耳に薬物が認められる時間はごく限られており，内耳保護効果を期待することはできず，なんらかの持続投与を行うための工夫が必要となる[6]．われわれが研究を開始した当初，いくつかのデバイスが内耳への持続的な薬物投与を目的として，臨床に供されていた．ひとつは，Silverstein のMicroWickというシステムである[7]．この方法はきわめて単純なもので，鼓膜切開，チューブ留置を行い，このチューブに細い綿棒のようなものを通し，正円窓窩に留置するというものである．薬物は通常の点耳という形で投与される．シンプルなシステムであることから，現在でも用いられているが，薬物の徐放という機能は全くなく，正円窓への投与の確実性にも疑問が残る．他には，埋め込み型浸透圧ポンプが臨床に供されていた．鼓室形成術の要領で外耳道

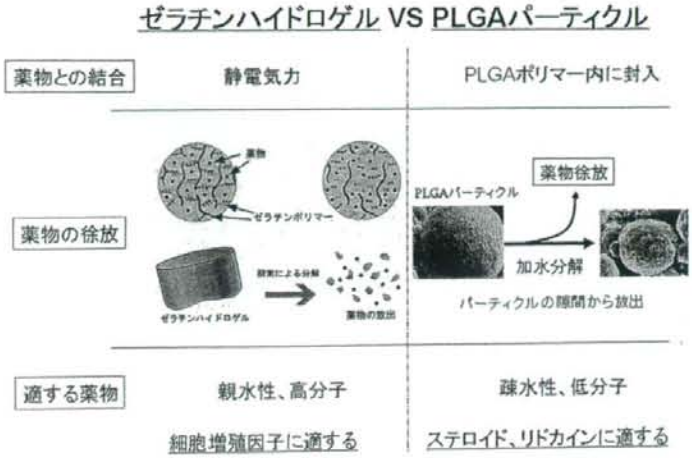

図2　バイオマテリアルを用いた薬物徐放

皮膚を挙上し，ポンプ先端部を正円窓窩に留置し，チューブは外耳道皮下を通し，体外にリザーバーを置くというものである．実際，ステロイド局所投与に用いられ，有効性を示唆する報告がなされているが[8]，広く普及するには至らず，販売は停止している．投与できる薬物はリザーバーに一定期間入れておいても活性を失わないものではなくてはならないし，鼓室形成術に準ずる外科的侵襲を要する上に，治療終了後にデバイスを取り除く必要がある．このシステムは，外科的侵襲が問題であったが，薬物の徐放という点では優れたシステムであったといえる．

以上から，われわれは，開発すべき内耳薬物投与システムに求められる条件として，1）簡便かつ安全性の高い方法であること，ただし，2）耳鼻咽喉科医としての特色が活かせる，3）薬物が効果を発揮する期間，適切な量の薬物を内耳に徐放することができる，を想定し，研究開発に臨むこととした．

バイオマテリアルを用いた薬物徐放

ドラッグデリバリーは，再生医学や組織工学の分野で注目されている研究テーマであり，世界で活発な研究が行われている．ドラッグデリバリー開発の中心的な研究テーマがバイオマテリアルを用いた薬物徐放である．われわれは，ドラッグデリバリー分野の研究成果を内耳に応用することにより，薬物徐放という問題は解決できるのではないかと考えた．バイオマテリアルによる薬物徐放について，簡単に説明を加えると，最も広く知られている方法としてシリコンからの薬物徐放がある[9]．気管支喘息治療目的の気管支拡張薬の徐放製剤として広く用いられているし，禁煙補助目的のニコチン徐放パッチや癌性疼痛治療のためのフェンタニル徐放パッチもこの方法を用いた経皮的な薬物徐放システムである．感音難聴治療研究でも，内耳への薬物徐放にシリコンを用いた研究が報告されているが[10]，薬物徐放後もシリコンは中耳に残存するという問題がある．複数回投与が必要な場合には必ず取り出さなければならない．また，シリコンで徐放できる薬物は，種類が限定されている．従って，内耳へバイオマテリアルを用いた薬物徐放の応用を考える場合，生体内で分解されるバイオマテリアルを使用することが望ましい．また，多くの薬物の内耳への徐放を行い，治療効果を検証するためには，できるだけ多くの製剤に用いることが可能な薬物徐放システムを開発する必要がある．

生体分解性のバイオマテリアルとして，薬物徐放に用いられている材料として，ポリ乳酸やポリグリコール乳酸などの合成材料とゼラチンやヒアルロン酸などの天然材料がある．ポリ乳酸やポリグリコール乳酸は，吸収糸の材料としてすでに広く臨床で用いられている．ゼラチンやヒアルロン酸も種々の用途で医療材料として用いられており，生体への安全性が確認されている材料といえる．これらの材料には，それぞれに適した薬物があり，それぞれの特徴を生かした内耳薬物投与を考える必要がある（図2）．ポリ乳酸やポリグリコール乳酸を用いる場合，アセトンなどにポリ乳酸やポリグリコール乳酸と薬物を溶解，混合し，マイクロあるいはナノパーティクルを精製する[11]．これらのパーティクルが分解される過程で薬物が徐放される．従って，脂溶性で低分子，安定性の高い薬物がこの方法に適する．一方，ゼラチンを用

いる場合，あらかじめ陽性もしくは陰性に荷電させたゼラチンポリマーを作製し，薬物と静電気的に結合させ，ゼラチンポリマーの加水分解に伴い薬物が徐放される[12]．従って，ゼラチンポリマーには，水溶性で高分子な薬物が適している．われわれは，タンパクやペプチド製剤の徐放にゼラチンポリマーを用い，疎水性，低分子化合物の徐放にポリグリコール乳酸を用いることで，内耳障害治療を標的とした薬物の多くをカバーできる内耳薬物投与システムが開発できると考え，この2つのマテリアルを用いた内耳薬物投与システムを開発することとした．

ゼラチンハイドロゲルを用いた内耳薬物投与

ゼラチンハイドロゲルは，ゼラチンを化学的に重合させたゼラチンポリマーから構成されている．薬物徐放に用いるゼラチンポリマーは，静電的結合により薬物と結合するが，現在陰性あるいは陽性荷電する薬物に対するゼラチンハイドロゲルが1種類ずつ臨床応用可能な段階にある．実際には，種々の等電点のゼラチンを用いることにより，静電結合の特性はある程度変化させることが可能となる．ゼラチンは体内ではコラゲナーゼなどの酵素により加水分解され，薬物が徐放される仕組みとなっている[12]．ゼラチンポリマーの加水分解の速度は，ゼラチンを化学重合させる程度をコントロールすることにより変化させることができるので，薬物を徐放する時間もある程度の範囲で制御可能となる．

われわれは，まず脳由来神経栄養因子（BDNF）を投与薬物として選択し，蝸牛のラセン神経節細胞に対する保護効果を検討することとした．BDNFは，ラセン神経節細胞の発生，生存に深く関与していることが知られており，最近では聴覚刺激の中枢への伝達調整にも関与していることが示されている[13]．さらに，埋め込み型ポンプや遺伝子導入を用いた実験などで，すでにラセン神経節細胞に対する保護効果が示されていた[14][15]．従って，ゼラチンハイドロゲルの内耳への薬物投与に対する有効性を調べる実験モデルとして最も適切ではないかと考えた．過去の報告に準じて，耳毒性薬物全身投与により蝸牛有毛細胞を喪失させ，2次的なラセン神経節細胞変性が誘導されるモデルを用いた．第一に蝸牛外リンパへのBDNF徐放について調べたところ，1週間以上の徐放が可能であることが判明した[16]．次に，ラセン神経節細胞の組織学的，機能的な保護効果について調べたところ，ゼラチンハイドロゲルによるBDNF投与により，ラセン神経節細胞の減少が抑制され，機能が保持されることが電気刺激聴性脳幹反応にて示された（図3）[16]．つまり，ゼラチンハイドロゲルは，内耳への神経栄養因子や細胞成長因子の投与に応用できることが示されたわけである．

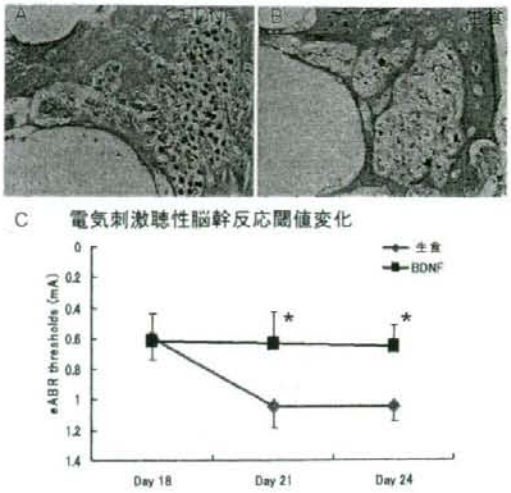

図3　ゼラチンハイドロゲルを用いたBDNF投与によるラセン神経節細胞保護効果
BDNFを局所投与された蝸牛では，ラセン神経節細胞が多く認められるが（A），コントロールした生食投与を受けた蝸牛では，ラセン神経節細胞がほとんど消失している（B）．電気刺激聴性脳幹反応の閾値もBDNF投与を受けた蝸牛では低下が認められないが，生食投与を受けた蝸牛では経時的に低下している（C）．

次に用いた薬物は，インスリン様細胞成長因子1（IGF1）という細胞成長因子である．IGF1を選択した理由は，1）日本および米国で既に市販されている薬物であったこと，2）内耳の発生や保護効果を示唆する基礎的な研究結果があったということが挙げられ，臨床応用に最も近い細胞成長因子と考えられたからである．IGF1の内耳に対する保護効果は十分には調べられているとはいえなかったため，まず効果が期待しやすい条件，すなわち音響外傷前に薬物投与を行った．すると，IGF1を含浸させたゼラチンハイドロゲルを正円窓膜上に留置することにより，音響外傷から蝸牛有毛細胞を組織学的に保護することができ，恒久的な聴覚閾値上昇をほぼ完全に防御することができた[17]．この結果を受け，臨床試験を実施するための非臨床試験としての実験を行った．治療的効果を調べるために，薬物投与は難聴発症後とし，急性高度難聴を対象とした臨床試験を想定し，音響外傷モデルに加え，内耳虚血モデルでの有効性も同時に検討した．なお，虚血モデル解析は愛媛大学耳鼻咽

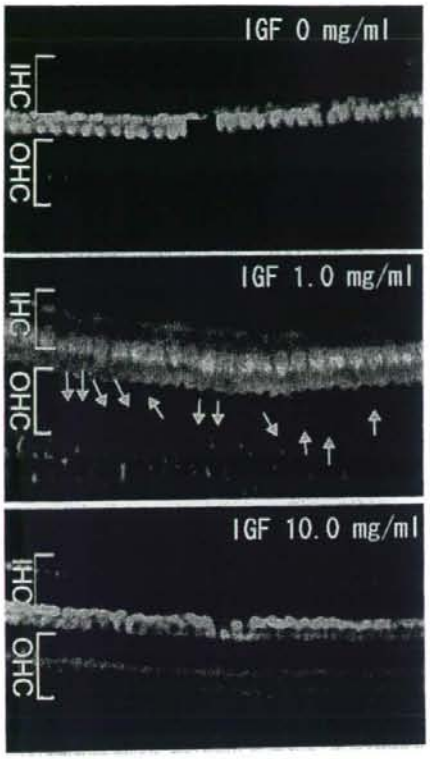

図4　ゼラチンハイドロゲルを用いたIGF1投与による蝸牛有毛細胞保護効果
音響外傷後，IGF1を含まない生食を投与された蝸牛では，ほとんどの外有毛細胞（OHC）が消失しているが，IGF1投与量が増加するに従い，残存している外有毛細胞数が増えている．内有毛細胞（IHC）には大きな変化は認められない．（文献18）より改変）

喉科羽藤直人先生が中心となって行った．結果，音響外傷，内耳虚血の両モデルともに感音難聴を有意に抑制することができ，組織学的にも蝸牛有毛細胞生存促進効果が確認された（図4）[18][19]．さらに，中耳炎などの有害事象が起こらないことも確認された．

ゼラチンハイドロゲル・IGF1治療の臨床応用

IGF1は既に販売されている薬物であり，ゼラチンハイドロゲルも血管再生などの臨床試験ですでに院内製剤として使用されていたこと[20]，さらに薬物投与方法が外用に相当することから，臨床応用への問題点は少ないと思われたが，プロトコル作成開始から倫理委員会承認までおよそ1年を要した．臨床試験実施までには解決すべき問題として，いくつかの課題があった．ひとつは，ヒトでの投与手技の確立である．薬物側の安全性が高いことから，投与手技が有害事象の要因となる可能性が高い因子と考え，安全性に留意した方法の開発を意図した．また，正円窓窩に正確にゼラチンハイドロゲルを留置することが，治療法の有効性に重要な因子となることから，確実しかも低侵襲かつ簡単な方法が必要となる．ヒト側頭骨標本（耳介，外耳道つき）を用いて，手術用顕微鏡，超細径内視鏡を用いて，外来で施行可能な方法を検討した．われわれが用いた超細径内視鏡は外径が1mm以下であり，2mmの鼓膜切開をおけば鼓室内のかなりの範囲が観察できる．鼓膜後下象限に2mm程度の切開をおき，超細径内視鏡を挿入すると，確実に正円窓窩を確認することができ（図5），手術用顕微鏡（外来処置用顕微鏡）下での操作を併用することにより，安全にしかも容易に正円窓窩にハイドロゲルが留置できることが分かった[21]．

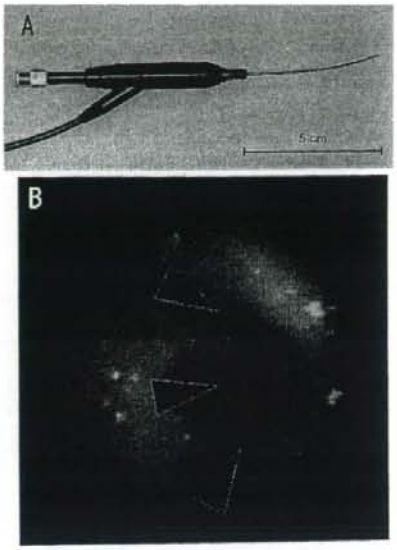

図5　超細経内視鏡（A）と経鼓膜的に超細経内視鏡により観察した正円窓（B）．矢頭は正円窓膜を示す．（文献21）から改変）

われわれにとって最大の課題は，臨床試験のデザインであった．科学的（臨床統計学的に正しい）かつ倫理的に考慮されたデザインが必要となる．しかし，いくら理想的なデザインであっても，症例のリクルートが行えないデザインでは臨床試験として成立しない．厳密なデザインでありながら，倫理的配慮に富み，なおかつ必要な症例数を集めることができる臨床試験ということになる．難しい課題である．また，当然のことながら基礎的な研究結果に立脚し，効果が期待できる症例を対象とし

適応基準

登録時に下記の規準を全て満たす患者を対象とする。

1)	純音聴力検査および耳鼻咽喉科的診察にて突発性難聴診断基準の突発性難聴確実例または疑い例と診断されている	はい	いいえ
2)	誘発耳音響放射検査にて蝸牛有毛細胞障害が示されている	はい	いいえ
3)	ステロイド治療の開始日から1週間以上後の有効性判定において、不変と判定されている	はい	いいえ
4)	急性高度難聴の発症後30日未満である	はい	いいえ
5)	同意取得時において、年齢が20歳以上である	はい	いいえ
6)	耳鼻咽喉科外来に通院可能な全身状態である	はい	いいえ

除外基準

登録時に下記の除外規準に1つでも当てはまる患者は対象としない。

1)	活動性の慢性中耳炎、急性中耳炎、滲出性中耳炎および炎症所見が存在するあるいは耳管機能障害が存在する	いいえ	はい
2)	既にステロイド以外の感音難聴の治療として、パトロキソビンの全身投与、プロスタグランディンE1および1製剤の全身投与、高気圧酸素療法を実施している	いいえ	はい
3)	現在治療が必要な悪性新生物を有する	いいえ	はい
4)	悪性腫瘍の治療後5年以上経過しているが治癒もしくは寛解状態が保たれていない	いいえ	はい
5)	重篤な肝障害を有する（AST>100, ALT >100）	いいえ	はい
6)	コントロール不良の糖尿病を有する（HbA1cが10を超えるもの）	いいえ	はい
7)	下垂体機能不全、副腎機能不全の治療中である	いいえ	はい
8)	生命予後が不良の合併症を有する	いいえ	はい
9)	妊婦、授乳婦および妊娠の可能性（意思）のある女性である	いいえ	はい
10)	重度の薬剤アレルギーの既往を有する	いいえ	はい
11)	過去1年以内にアルコールもしくは薬物依存の既往がある	いいえ	はい

図6　第Ⅰ−Ⅱ相臨床試験「急性高度難聴症例に対する生体吸収性徐放ゲルを用いたリコンビナント・ヒト・インスリン様細胞成長因子１内耳投与による感音難聴治療の検討」適応基準と除外基準

なければならない．通常の薬物の臨床試験では，第一段階として安全性のみを検討する第Ⅰ相という臨床試験が健常者を対象として行われるが，今回は鼓膜切開を要することなどから，安全性と少数例での治療効果を調べる第Ⅰ−Ⅱ相臨床試験としてのデザインを行った．対象は，突発性難聴を含める急性高度難聴とし，厚労省班研究の突発性難聴診断基準での確実例および疑い例とした．本研究課題で行った動物実験でも音響外傷および内耳虚血を行ってから早いタイミングで治療的処置を行っているが，他の同様の実験でも薬物投与を早期に開始することが良好な治療効果に結びつくことが示唆されている．つまり，突発性難聴発症から早期に治療を開始した方が有効率は高まることが期待される．しかし，確実なエビデンスはないがステロイドの全身投与が一般的な治療法として，世界で広く用いられている．すなわち，ある程度の有効性が期待できると推察される既存の治療法が存在するともいえる．いいかえると，すべての突発性難聴症例で効果がある程度分かっている治療法を受ける機会を奪うことはできないということになる．このような背景から，今回の臨床試験では，ステロイド全身投与が無効であった急性高度難聴症例を対象とし，ただし，発症後30日未満という条件を設けた．有効性を考えると２週間以内が妥当だと感じられたが，症例のリクルートを考え，30日未満とした．次の問題は，症例数の設定である．症例数を設定するためには，統計学的な仮説が必要となる．つまり，ハイドロゲルによるIGF1局所投与をステロイド無効の急性高度難聴例に行った場合，何%で有効となることが予想されるかを事前に設定しなければならない．そこで，これまでに京都大学でステロイド無効例に行われてきた治療法である高気圧酸素療法の臨床統計を行った．2000年から2006年に高気圧酸素療法を行ったステロイド無効突発性難聴症例は199例あり，この内63例に回復以上の治療効果を認めた．従って，回復以上は約33%となる．ハイドロゲルによるIGF1局所投与の回復以上の期待値を63%として，αエラー0.05（片側），βエラー0.1とすると，二項分布に基づく必要適格例数は22例となるため，約10%の不適格例を見込んで目標登録症例数を25例とした．症例登録の適応基準と除外基準を図6に示す[22]．エンドポイント（この試験から何がわかるか）は，1）ゼラチンハイドロゲルによるIGF1局所投与がステロイド無効急性高度難聴例（発症後30日未満）に対してどの程度の有効性が期待できるのか，2）有害事象はどの程度発生するのかを明らかにすることになる．2008年７月現在，12例の登録が終了している．京都大学医学部附属病院には，探索医療センターという基礎から臨床への橋渡し研究を行う機関があり，われわれ

の臨床試験も同センターの検証部の協力のもとにデザインを行い，実際の臨床試験における登録や経過観察では臨床部のサポートを受けて行っている．詳細な結果は公開できないが，やはり治療を行うまでの期間が長いと有効性は乏しい印象を受けている．現在のところ，問題となる有害事象は発生していない．

ポリグリコール乳酸を用いた内耳薬物投与

ゼラチンハイドロゲルは静電気力により薬物と結合することから，親水性でサイズの大きな分子に適する．疎水性，低分子な薬物については，ゼラチンハイドロゲルは十分な結合力を発揮できないため，薬物は短時間で放出されてしまい，徐放はできない．例えば，ゼラチンハイドロゲルと同じゼラチンポリマーであるゼルフォームなどを用いた場合も同様の理由で徐放はできない．われわれは，疎水性あるいは低分子化合物を徐放する手段として，ポリグリコール乳酸を用いたパーティクル形成を用いている．簡単にいうと薬物を封じ込んだ小さなパーティクルを作り，ポリグリコール乳酸が加水分解されるに伴い形成されるパーティクルのクラック（ひび）から薬物が放出される仕組みである．この手法は，全身投与する製剤としても用いることができる点がゼラチンハイドロゲルを用いた徐放と大きく異なる．現在，われわれはいくつかの薬物の徐放についてポリグリコール乳酸を用いた方法を用いて検討しているが，ここでは耳鳴り抑制を目的としたリドカインの徐放について紹介する．

リドカインは最も広く用いられている局所麻酔薬であるが，不整脈治療として全身投与でも用いられている．リドカインの点滴静注が耳鳴り抑制に有効なことは広く知られており，最も効果的な薬物のひとつである．また，鼓室内投与での有効性を示す報告も散見される[23]．リドカイン全身投与は常に厳重な副作用の監視の必要があり，局所投与ではめまいなどの前庭症状が問題となる．リドカインによる耳鳴り治療については，検討すべき課題が多く残されているが，作用時間が短いことも大きな問題の一つである．適切な濃度のリドカインを長期的に蝸牛に供給することができれば，耳鳴りの長期的な抑制，緩和が可能となる可能性がある．そこで，われわれはリドカイン含有ポリグリコール乳酸マイクロパーティクルを作製し，その徐放特性を生体内外で調べた．ポリグリコール乳酸では，ナノスケールのパーティクルを作ることも可能であり，ナノパーティクルは正円窓膜を通過し，蝸牛内での徐放が可能であることを既に報告しているが[24]，長期的な徐放を目的とした今回の検討では，直径100μmと5μmの大小2つのサイズのマイクロパーティクルを作製し，リン酸緩衝液中での徐放動態を調べた．5μmのパーティクルでは，最初の5日間で約50%のリドカインが放出され，残りが2週間かけて徐放されることが分かった．100μmのパーティクルでは，最初の1日で約60%のリドカインが放出され，残りは4週間かけて徐放されることが示された．最初にある程度の量のリドカインが放出され，長期に濃度が維持される徐放動態が望ましいと考え，100μmのパーティクルを使用して，生体内での徐放解析を行った．結果，2週間蝸牛外リンパ中にリドカインを検出することが可能であり，かなり長期の徐放が可能であることが分かった[25]．リドカイン含有パーティクル局所投与による中耳，内耳の明らかな傷害は認められていない．以上の結果から，ポリグリコール乳酸は内耳へのリドカイン徐放に有効なバイオマテリアルであることが示唆された．

今後の展望

現在行っているゼラチンハイドロゲルを用いたIGF1局所投与による急性高度感音難聴治療の臨床試験を完了し，次のステップ，多施設での有効性の解析に進めることが第一の目標となる．ゼラチンハイドロゲルは，多くの細胞成長因子や神経栄養因子の徐放に用いることができるので，今後臨床での使用が可能である，あるいは近い将来に可能となる製剤の感音難聴治療への可能性を多角的に検討していきたい．ポリグリコール乳酸による薬物徐放では，まずリドカイン徐放による耳鳴り抑制に関する臨床試験開始が目標となる．現在，ステロイドの徐放についても検討しており，臨床応用を考慮した基礎的研究を展開中である．今回紹介させて頂いた内耳薬物投与システムを内耳再生医療に応用することも重要なミッションと考えている．われわれは，ノッチ情報伝達系阻害薬が内耳有毛細胞再生に応用できる可能性を示唆する所見を得ている[26]．このような薬物の内耳への徐放や内耳細胞移植治療の支持療法としての内耳薬物投与が内耳再生に関連する研究課題となる．

耳鼻咽喉科臨床医として内耳基礎研究を行っていると，特に留学を経験されている先生方は感じられるようだが，欧米の内耳研究者との間に時間的，予算的ハンディを感じることがある．そして，「日本で臨床しながら，研究してもだめだ」という声をしばしば耳にする．しかし，今回紹介させて頂いた一連のトランスレーショナル（橋渡し）研究は，基礎的研究ができる臨床医が最も有利な分野ではないだろうか．基礎的研究から得られた着想を実際に自分の手で臨床まで持っていく，逆に臨床の現場で感じるジレンマを解消するためのプロジェクトを立ち上げることもできる．一方，多くの他分野の研究者の協力がなければ，内耳薬物投与システムの開発研究，

そして臨床応用を行うことはできなかったことも事実である．ゼラチンハイドロゲル，ポリグリコール乳酸マイクロパーティクル作製では，京都大学再生医学研究所の田畑泰彦教授のグループにお世話になり，東京慈恵会医大DDS研究所の故・水島　裕教授，檜垣　恵教授には，ナノパーティクルに関連する研究でご協力頂いた．リドカイン濃度測定では武庫川女子大学薬学部岡村　昇教授のお世話になった．臨床試験は，京都大学医学部附属病院薬剤部および探索医療センターの協力のもとに現在施行中である．関係各位にこの場をお借りして深謝いたします．

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Steroid and antiviral treatment for Bell's palsy

Bell's palsy is an acute peripheral unilateral facial paralysis of unknown cause. It is the most common acute facial paralysis, with an incidence of 20–30 per 100 000 people annually. In our series of 3385 patients with acute facial paralysis over the past 30 years, Bell's palsy accounted for 2097 cases (62%). PCR usually identifies fragments of DNA of herpes simplex virus in clinical samples from patients with Bell's palsy. These fragments were detected more often in saliva from patients with Bell's palsy than in saliva from healthy volunteers.[1] Such DNA fragments were also identified in the endoneural fluid of the facial nerve during facial-nerve decompression surgery in 11 of 14 patients (79%) with Bell's palsy.[2] Thus we speculate that reactivation of herpes simplex virus might lead to Bell's palsy: herpes simplex virus is involved in about 31–79% of cases of Bell's palsy.[1–3]

Ramsay-Hunt syndrome is the second most common acute facial paralysis, and is caused by reactivation of latent varicella zoster virus. It is associated with zoster oticus and is often complicated by vestibulocochlear dysfunction. The diagnosis of acute facial paralysis is sometimes complicated by the presence of so-called zoster without vesicles (zoster sine herpete). In the absence of vestibulocochlear dysfunction or zoster in the auricle, such cases are clinically diagnosed as Bell's palsy. Serological and PCR studies show that the prevalence of zoster sine herpete in Bell's palsy ranges from 8% to 28%.[4,5] Therefore although herpes simplex virus is a major cause of Bell's palsy, varicella zoster virus and other unknown causes might also be important (figure).

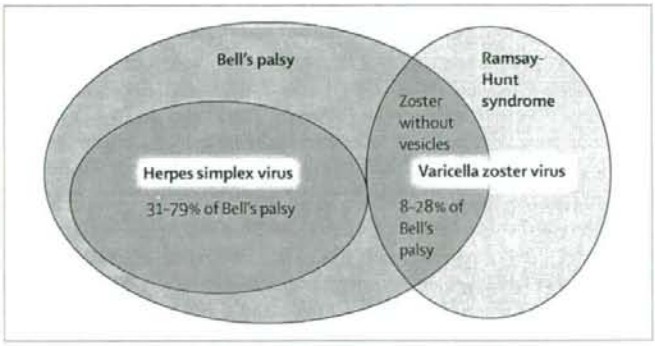

Figure: Involvement of herpes simplex and varicella zoster viruses in acute facial palsy

Steroids and adjunctive antiviral drugs might improve outcome for patients with Bell's palsy. In 2007, two randomised placebo-controlled trials assessed the role of antiviral drugs but gave conflicting findings.[6,7] In a single-blind study in 296 patients, we compared valaciclovir (1000 mg a day for 5 days) plus prednisolone (60 mg for the first 3 days, tapered thereafter) with placebo plus prednisolone.[6] Complete recovery was significantly higher (p=0·045, 95% CI 0·47–0·60) in the valaciclovir group than in the placebo group (110 of 114 [96·5%] patients vs 96 of 107 [89·7%] patients, respectively. By contrast, Sullivan and colleagues[7] noted that 92·7% of 124 patients treated with aciclovir (2000 mg a day) plus prednisolone (50 mg a day) for 10 days had complete recovery compared with 96·1% of 127 patients treated with placebo plus prednisolone for 10 days (p=0·28, 95% CI 0·42–0·55). The researchers concluded that an antiviral was not beneficial in improving the prognosis of facial paralysis.

Systematic reviews[8,9] suggest that oral steroids are safe and effective in the treatment of Bell's palsy. Steroids have many side-effects, including anti-inflammatory effects and the suppression of immune reactions. Regardless of the cause, the pathological condition underlying Bell's

palsy involves inflammatory oedema and entrapment neuropathy in the narrow bony facial canal. Therefore treatment with steroids can be advocated regardless of the cause of acute facial paralysis. Antiviral drugs, such as aciclovir, valaciclovir, and famciclovir (the last is only available in some countries), are nucleotide analogues that interfere with DNA polymerase, specific to herpes simplex virus and varicella zoster virus, and inhibit viral replication. The doses differ between viruses—ie, 1000 mg valaciclovir or 1000 mg aciclovir per day for 5 days for herpes simplex virus and 3000 mg valaciclovir or 4000 mg aciclovir per day for 7 days for varicella zoster virus. The timing also differs between antiviral drugs and steroids. Because antivirals cannot destroy viruses, but rather only prevent further viral replication, such drugs should be administered within 3 days of the onset of facial palsy.[10] By contrast, steroids are effective over a longer period and work when neural oedema peaks 7–10 days after onset.[11]

The apparently conflicting findings between our study[6] and the Scottish study[7] might have been caused by several factors. Because 1000 mg valaciclovir is insufficient to treat varicella zoster virus, we used serological and PCR studies to exclude patients infected with varicella zoster virus. By contrast, Sullivan and colleagues[7] did not assess virological status, and so patients infected with varicella zoster virus might have been included in their study, because 2000 mg acyclovir is also insufficient to treat this virus. Gilden and Tyler[12] noted that the severity of facial palsy affects the prognosis of the paralysis. The patients in Sullivan's study seemed to have milder facial palsy than our patients, because the mean House-Brackmann score for their patients was 3·6 of 6 (the most severe palsy scores as 6), whereas our patients had a mean score of 4·3 of 6. This difference in the scores is consistent with the different populations: our study was in tertiary medical centres whereas Sullivan recruited patients in primary care. Additionally, the methods used to assess paralysis differed between the two studies. We assessed facial paralysis by recording voluntary facial movements, whereas Sullivan used four photographs for assessment, which might have masked small functional defects. Furthermore, we used valaciclovir, a prodrug of aciclovir with higher antiviral activity than aciclovir. These factors might have led to the different conclusions about efficacy.

The treatment strategy for Bell's palsy is to accelerate recovery to prevent further worsening of facial palsy in patients who are only mildly affected, and to avoid sequelae, such as synkinesis, contracture, and facial asymmetry, in more severe cases. The most important issue in the treatment of acute facial palsy is the existence of infection with varicella zoster virus in Bell's palsy. Additionally, Ramsay-Hunt syndrome can be misdiagnosed as Bell's palsy when a patient does not show zoster or vestibulocochlear dysfunction. In our study[13] zoster oticus appeared well after the onset of paralysis in 34% of patients with Ramsay-Hunt syndrome. Because varicella zoster virus is more virulent and causes more severe facial paralysis than herpes simplex virus, differential diagnosis is important to select the appropriate treatment: varicella zoster virus needs higher doses of antiviral than herpes simplex virus. Increasing the dose of antiviral for all patients seems unwise, because higher doses can increase the risk of side-effects, such as acute renal failure and neurological disorders. Higher doses also increase the cost of drug treatment. Various attempts have been made for the prompt detection of DNA fragments of varicella zoster virus by real-time PCR, for example, from the auricular scratch exudates. However, the sensitivity of this test is not high enough at present.

There are no established guidelines for the treatment of Bell's palsy. On the basis of the current published work, we believe that Bell's palsy should be treated by the following protocol. In moderate paralysis, oral administration of prednisolone is sufficient, because such patients usually show excellent prognosis. In severe paralysis, treatment should differ by the timing of initial medication. When treatment starts within 3 days of the onset of paralysis, we prescribe valaciclovir in addition to prednisolone, because of the high incidence of viral infection. In most cases, 1000 mg per day of valaciclovir is given for 5 days, whereas a higher dose of valaciclovir (ie, 3000 mg per day for 7 days) is needed in patients who have the preceding symptoms of reactivation of varicella zoster virus, such as severe auricular pain, stiff neck, and reddish auricle. When treatment starts more than 4 days after the onset of paralysis, we prescribe prednisolone, but antiviral drugs are not used. We believe that the key point in improving the prognosis of Bell's palsy is to diagnose infection with herpes