and no fluorescence was visible after 24 h (FIGURE 1A–1C). By contrast, in mice treated with stealth-nano–rhodamine, red fluorescence dots were visible in the liver 15 min, 2 and 24 h after administration (FIGURE 1D–1F), with obvious accumulation of rhodamine fluorescence in the liver at 24 h. These results indicated a delay in the metabolism of rhodamine B encapsulated in PEG-coated PLA nanoparticles by the reticuloendothelial system in the liver.

In the cochlea, no rhodamine fluorescence was observed after the administration of free rhodamine B (FIGURE 1G–1I), as we had previously observed in normal guinea pigs [11]. By contrast, red fluorescence dots were observed in the cochleae of mice given stealth-nano–rhodamine at 15 min, 2 and 24 h after treatment (FIGURE 1J–1L). Rhodamine fluorescence was located in the stria vascularis and spiral prominence in all the cochlear turns, and in the cochlear modiolus, which correspond to the locations of blood vessels in the cochlea. These results suggested that encapsulating drugs in PEG-coated PLA nanoparticles could be an effective method for delivering drugs to the cochlea.

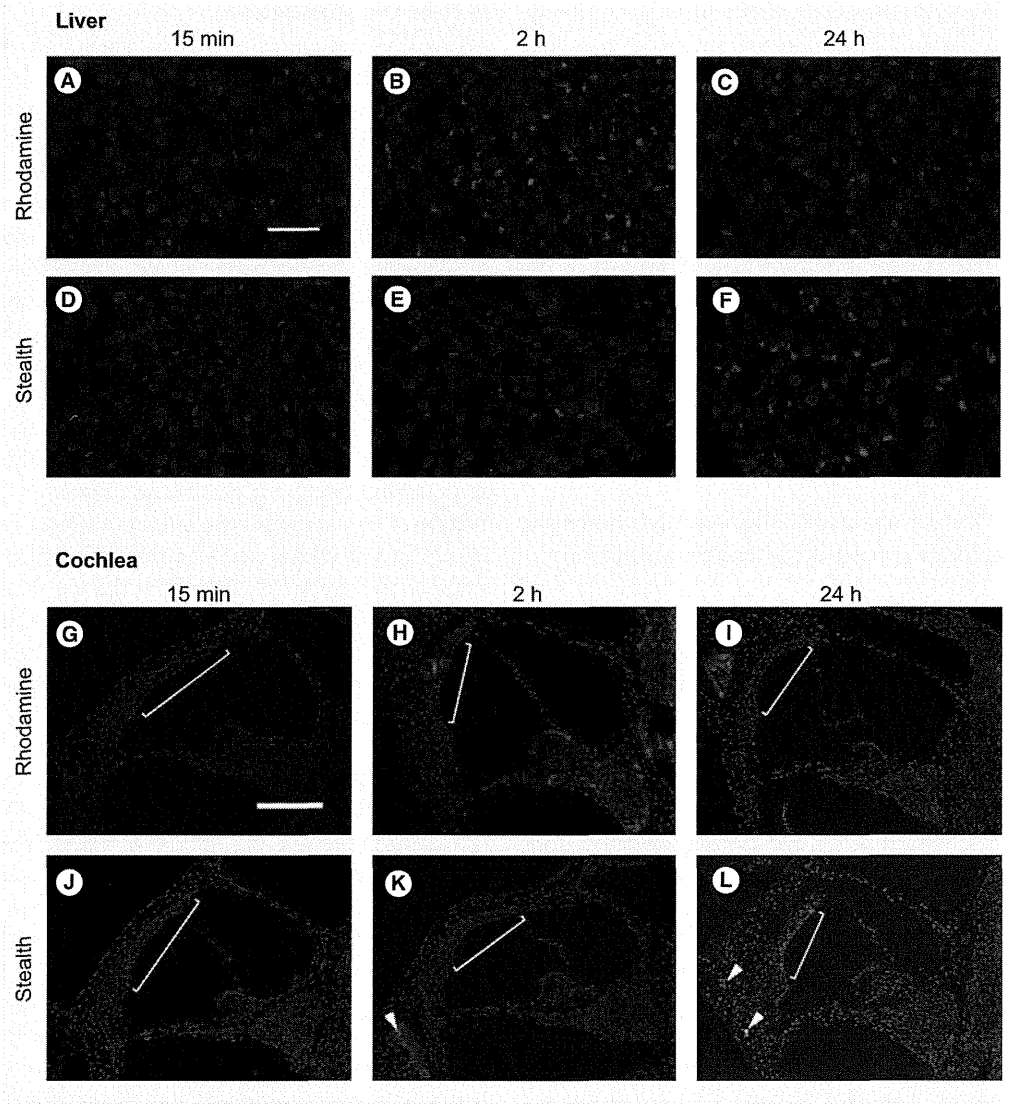

Figure 1. Rhodamine distribution in the liver and cochlea. In the liver in mice after application of free rhodamine **(A–C)**, numerous rhodamine fluorescence dots (red) were observed 2 h after application. In mice treated with stealth-nano–rhodamine **(D–F)**, rhodamine fluorescence was visible at each time point. After free rhodamine application **(G–I)**, no rhodamine fluorescence was detectable in cochleae. After application of stealth-nano–rhodamine **(J–L)**, rhodamine fluorescence was seen in the stria vascularis (brackets) and in the vessels in the bony wall of the cochlea (arrow heads) at each time point. Nuclei were stained with DAPI (blue). Bar in **(A)** represents 50 μm in **(A–F)**, and bar in **(G)** represent 200 μm in **(G–L)**.
DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

■ Betamethasone concentrations

We assessed betamethasone concentrations in the liver and cochlea at 1, 12 and 24 h after BP or stealth-nano–BP had been systemically administered to mice. Time-resolved fluoroimmunoassay analyses revealed significantly higher BP concentrations in the livers of mice given stealth-nano–BP than those given free BP, at each time point (Figure 2A). In addition, the BP concentrations did not significantly drop between 1 and 12 or 24 h in mice given stealth-nano–BP, while they did in mice given free BP (Figure 2A), supporting the hypothesis that encapsulating BP in PEG-coated PLA nanoparticles effectively enables the drug to escape metabolism by the reticuloendothelial system in the liver.

In the cochlea, BP was detectable by the time-resolved fluoroimmunoassay method in all the experimental groups. In the cochleae of mice treated with free BP, the concentrations were low (4.44 ± 1.69 ng/cochlea) even 1 h after BP application and dropped to extremely low concentrations after 12 h (0.07 ± 0.03 ng/cochlea) and 24 h (0.17 ± 0.14 ng/cochlea). By contrast, the BP concentrations measured in the cochleae of mice treated with stealth-nano–BP were much higher. The BP concentrations were 21.57 ± 2.55 ng/cochlea after 1 h, 10.45 ± 3.92 after 12 h and 9.69 ± 3.65 after 24 h, which were significantly higher than in mice treated with free BP at every time point (Figure 2B). In contrast to the liver, in the cochlea the BP concentration 12 h after stealth-nano–BP administration was significantly lower than 1 h after administration (Figure 2B). However, comparatively high BP concentrations were maintained between 12 and 24 h in these mice, with no significant difference between the BP concentrations at 12 and 24 h after treatment (Figure 2B). Even 24 h after stealth-nano–BP treatment, the BP concentration in the cochlea was significantly higher than at 1 h after free BP treatment. These results support the hypothesis that encapsulating BP in PEG-coated PLA nanoparticles is an effective strategy for sustained BP delivery to the cochlea.

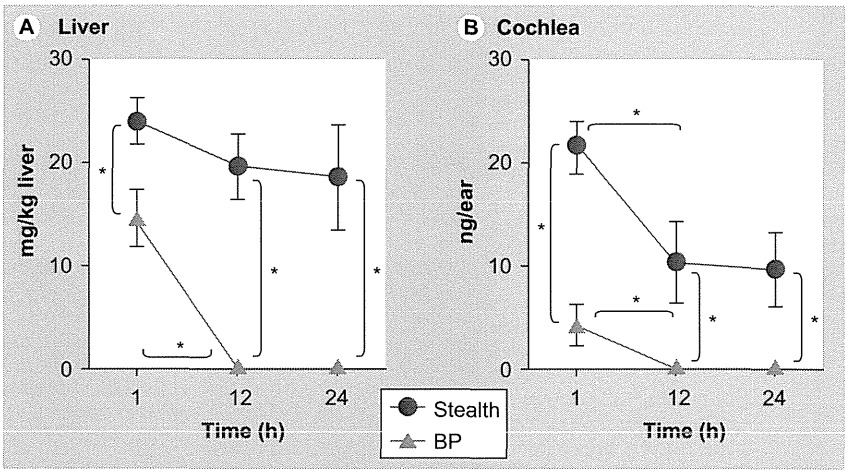

Figure 2. Betamethasone concentrations in the liver and cochlea. Tissue concentrations of betamethasone in the liver **(A)** and the cochlea **(B)** were determined at 1, 12 and 24 h after systemic application of free BP or stealth-nano–BP. Betamethasone concentrations after stealth-nano–BP application were significantly higher than those after free BP application in both the liver **(A)** and cochlea **(B)** at each time point. After BP injection, betamethasone concentration significantly dropped between 1 and 12 h. In the cochlea, samples treated with stealth-nano–BP also exhibited significant reduction of betamethasone levels between 1 and 12 h, but no significant reduction was observed between 12 and 24 h **(B)**. Asterisks indicate statistical significance with multiple t-tests with Bonferroni corrections. Bars represent one standard deviation.
BP: Betamethasone phosphate.

■ GR translocation in hair cells

We evaluated nuclear translocation of GRs after acoustic trauma to determine whether BP that had been systemically administered actually activates GR in hair cells or not. Immunostaining for GR demonstrated GR distribution in hair cells of cochleae 24 h after noise exposure. The majority of hair cells that were treated with stealth-nano–BP exhibited GR immunoreactivity in the nucleus and cytoplasm (Figure 3A & 3B), while in saline-treated cochleae, most of hair cells showed GR immunostaining only in the cytoplasm (Figure 3C & 3D). GR translocation rates in OHCs of stealth-nano–BP- and BP-treated cochleae were 72.22 ± 6.42% and 19.44 ± 16.67%, respectively. No OHCs of saline-treated cochleae showed GR translocation into the nuclei. Differences in GR translocation rate in OHCs between stealth-nano–BP- and BP-treated cochleae, and stealth-nano–BP- and saline-treated cochleae were statistically significant (Figure 3E). GR translocation rates in IHCs of stealth-nano–BP-, BP- and saline-treated cochleae were 83.33 ± 33.33%, 66.67 ± 47.14% and 41.67 ± 50.00%, respectively. No significant differences in GR translocation rates in IHCs among experimental groups were found (Figure 3E). These findings indicate that BP delivered from stealth-nano–BP actually activates GRs in hair cells.

■ Auditory function

We used conventional ABR recordings to monitor auditory function after exposure to traumatic noise. Time courses of the changes in ABR thresholds after drug treatment, at 8, 16 and 32 kHz, are shown in Figure 3. Drug treatment showed significant effects on the ABR thresholds at each frequency with two-way

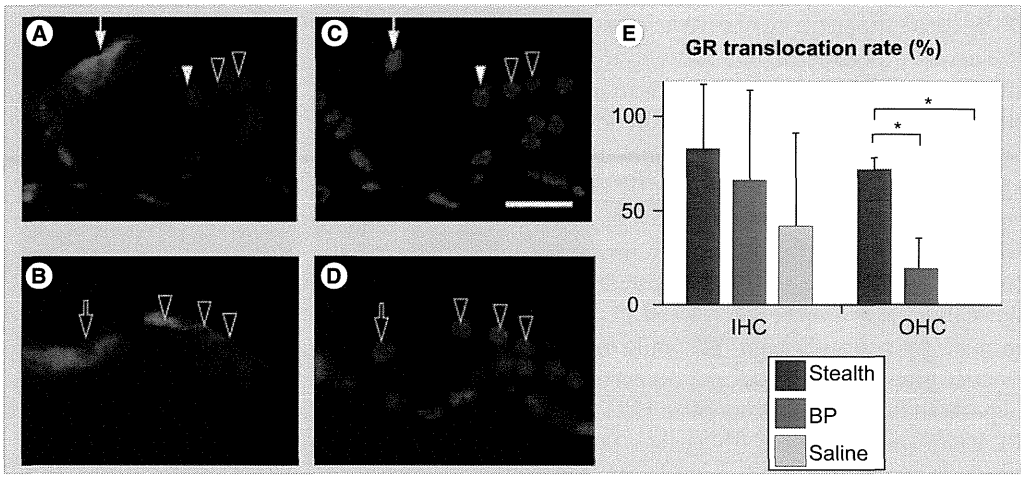

Figure 3. Nuclear translocation of glucocorticoid receptors in cochlear hair cells.
Immunostaining for GRs demonstrates the cellular location of GRs in hair cells **(A & B)** and the nuclear locations are demonstrated by DAPI staining **(C & D)**. Arrows indicate IHCs and arrowheads indicate OHCs **(A & B)**. White arrows or arrowheads show IHCs or OHCs with GR translocation into the nuclei, and white outlined arrows or arrowheads show nontranslocated IHCs or OHCs **(A & B)**. Bar in **(C)** represents 20 µm for **A–D**. GR translocation rates in IHC and OHC for cochleae treated with stealth-nano–BP (stealth), free BP (BP) or saline are shown in **(E)**. Significant differences in GR translocation rates are found between stealth and BP, and between stealth and saline in OHCs (* in **[E]**). Bars in **(E)** represent one standard deviation.
BP: Betamethasone phosphate; DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride; GR: Glucocorticoid receptor; IHC: Inner hair cell; OHC: Outer hair cell.

ANOVA. Multiple comparison analyses using the Bonferroni test identified specific, significant differences between the groups of mice given stealth-nano–BP or BP on day 14 after treatment at 8 kHz, on days 4, 7 and 14 at 16 kHz, and on days 7 and 14 after treatment at 32 kHz; specific significant differences were also identified between the groups of mice treated with stealth-nano–BP or saline on days 4 and 14 after treatment at 8 kHz, and on day 14 at 16 kHz. In contrast to stealth-nano–BP, BP treatment showed no significant effects on the attenuation of ABR thresholds compared with saline injection (Figure 4). These results showed that stealth-nano–BP had better therapeutic effects on noise-induced hearing impairment than free BP.

■ Hair cell protection

We examined the cochlear sensory epithelia histologically and counted the surviving hair cells in order to investigate the ability of stealth-nano–BP to protect these cells at a histological level. Phalloidin staining showed well-ordered rows of hair cells, three rows of OHCs and a single row of IHCs in cochlear epithelia from mice treated with stealth-nano–BP (Figure 5A), while a loss of both IHCs and OHCs was notable in both the apical and basal portions of cochleae from mice treated with BP or saline (Figure 5B & 5C). Quantitative assessments using one-way ANOVA demonstrated that drug treatment had significant effects on the numbers of IHCs in the apical and basal regions and on the number of OHCs in the apical region, but not in the basal region of the cochlea (Figure 5D & 5E). Tukey's multiple comparison tests showed significant differences in the number of IHCs in the apical region between mice treated with stealth-nano–BP and BP or saline and in the number of IHCs in the basal region between mice treated with stealth-nano–BP and saline (Figure 5D). There was also a significant difference in the number of OHCs in the apical region between mice treated with stealth-nano–BP and saline (Figure 5E). These results showed that stealth-nano–BP had a greater potential as a therapeutic to protect hair cells against noise-induced damage than free BP.

Discussion

Betamethasone phosphate is one of the most widely used steroids in the clinic for the treatment of acute SNHL, including noise-induced SNHL. In this study, we have demonstrated that a single injection of BP at a dose of approximately 2.5 mg/kg, which is clearly a much higher dose than is generally used in the clinic, had no significant effect on the function and the histology of mouse cochleae damaged by noise. Strikingly, the intracochlear concentration of BP after a free BP injection was incredibly low, even 1 h after the administration of such a high

dose, and despite the serum concentration of BP reaching a peak 1 h after systemic administration [16]. This suggested that single injections of BP at the doses used clinically may deliver very limited amounts of BP into the cochlea, and this may account for the poor effects of glucocorticoids seen in patients with acute SNHL. It is certainly consistent with the lack of effect on noise-induced trauma to the cochlea seen in this study after a single intravenous injection of BP. Previously, protective effects of glucocorticoids on noise-induced hearing loss have widely been investigated. However, the results vary depending on used administration routes (systemic or local) and doses, intensities of exposed noise or species of experimental animals. Among previous studies, two reports have been published using a similar experimental setting to that of the present study, in which mice are used as the experimental animals and glucocorticoid is systemically administered [17,18]. These two studies show significant protection of auditory function by systemic application of glucocorticoids; however, the dose of glucocorticoids applied or the severity of noise trauma in these studies are quite different from that used in the present study. We therefore consider that such differences may vary outcomes in hearing protection by glucocorticoids.

Nanoparticle technology has been included in potential strategies for improving delivery of therapeutic agents, including glucocorticoids to the cochlea. We previously studied the efficacy of a nonstealth type of nanoparticle for

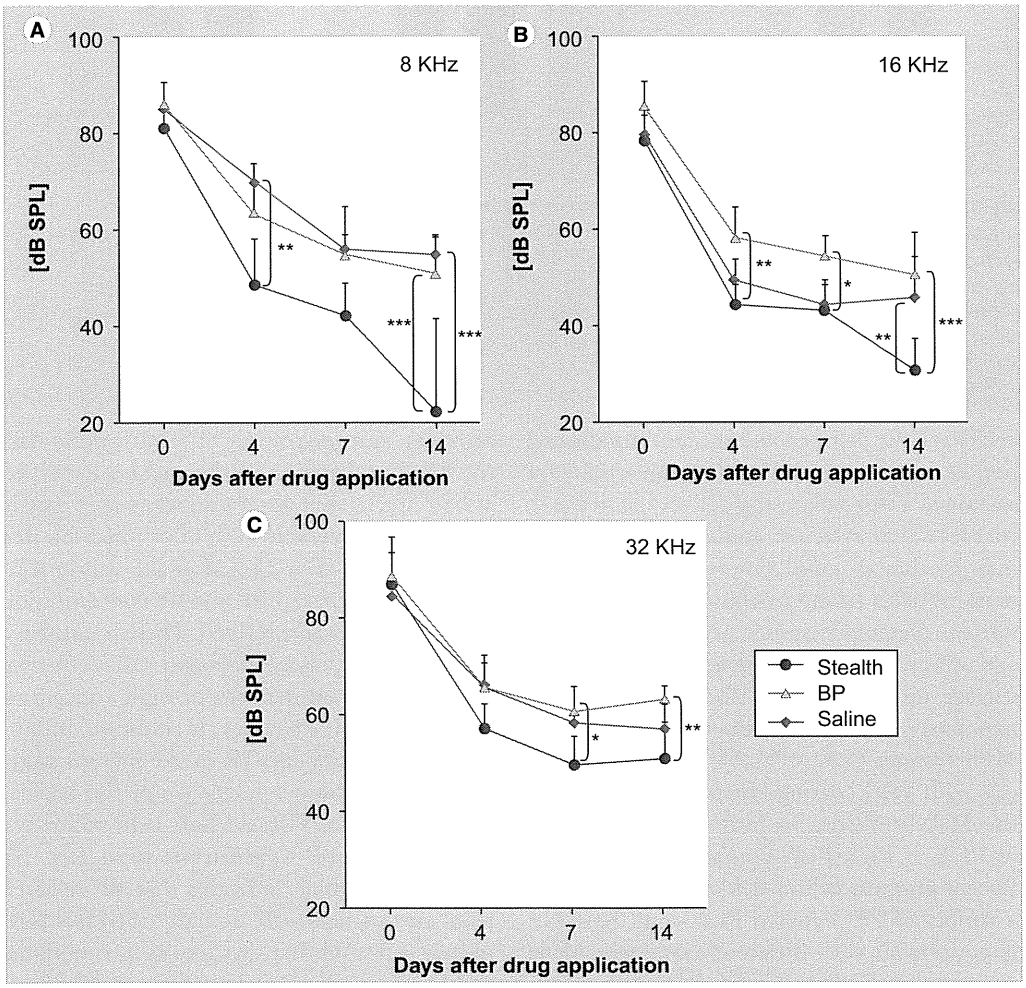

Figure 4. Auditory brain stem response thresholds following saline, free betamethasone phosphate or stealth-nano–betamethasone phosphate. Changes in ABR thresholds at **(A)** 8, **(B)** 16 and **(C)** 32 kHz were determined immediately after the treatment, and 4, 7 and 14 days after treatment. Drug treatment showed significant effects on the ABR thresholds at each frequency by two-way ANOVA. Multiple comparisons with Bonferroni corrections identified significantly better ABR threshold in mice given stealth-nano–BP compared with mice given BP or saline at multiple time points shown by *$p < 0.05$, **$p < 0.01$ or ***$p < 0.001$. Bars represent one standard deviation. ABR: Auditory brain stem response; BP: Betamethasone phosphate; SPL: Sound pressure level.

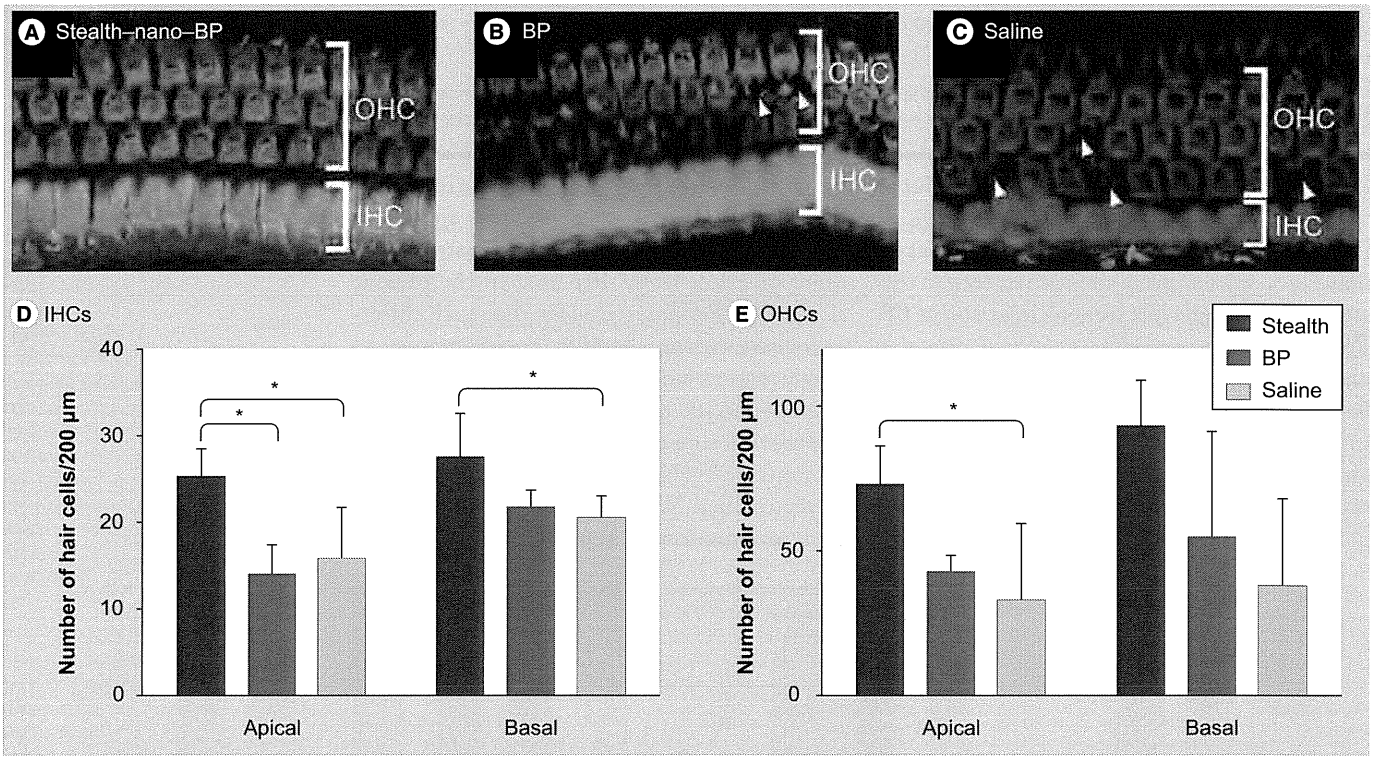

Figure 5. Stealth-nano–betamethasone phosphate attenuates noise-induced damage on cochlear hair cells. Phalloindin staining demonstrated well-maintained IHC and OHC in cochlear specimens of the basal portion treated with stealth-nano–BP **(A)**, while in cochleae treated with free BP **(B)** or saline **(C)** exhibited the loss of OHCs (arrowheads). Numbers of remaining IHCs/OHCs in 200 µm length in the apical and basal part of each cochlea were shown in **(D & E)**. Quantitative analyses showed that drug treatment had significant effects on the numbers of IHCs in the apical and the basal portions **(D)** and on the numbers of OHCs in the apical portion **(E)**. Significant differences in the number of IHCs/OHCs by one-way ANOVA with Tukey's multiple comparison tests are indicated by asterisks. Bars represent one standard deviation.
BP: Betamethasone phosphate; IHC: Inner hair cell; OHC: Outer hair cell.

cochlear drug delivery, and the results indicated limited capacity of nonstealth nanoparticles for sustained and/or targeting delivery of drugs to cochleae after systemic application [11], which may be due to rapid removal of nonstealth nanoparticles from circulation by the mononuclear phagocyte system (MPS) in the liver and spleen. PEG is an attractive material for surface modification of PLA or PLA/PLGA nanoparticles to reduce interactions with the MPS system. PLA or PLA/PLGA nanoparticles with PEG coating efficiently escape from the MPS resulting in prolonged circulation of PLA or PLA/PLGA nanoparticles [12–14]. Recent progress in this field enables preparing a variety of PEG-coated PLA or PLA/PLGA nanoparticles with different diameters, blend ratios and molecular weights [19]. Stealth-nano–BP used in the present study releases a half of loaded BPs for 36 days *in vitro* and their $T_{1/2}$ is 7.8 h after intravenous injection [19]. In the present study, a single injection of stealth-nano–BP resulted in high BP concentrations in the cochlea, which at 1 h after treatment were four-fold higher than for free BP. In addition, 12 h after the administration of stealth-nano–BP, the BP concentration in the cochlea remained at half of that seen after 1 h, whereas virtually no BP was detectable 12 h after injecting free BP. Moreover, even 24 h after the administration of stealth-nano–BP, the BP concentration seen after 12 h was sustained. These results have demonstrated that stealth-nano–BP is effective for the sustained delivery of high concentrations of BP to the cochlea. In addition, immunohistochemistry for GR in the present study demonstrates higher incidence of the nuclear translocation of GRs in hair cells following stealth-nano–BP application than free BP application, which indicates that BP released from stealth-nano–BP actually activates GRs in hair cells and that this results in functional and histological protection of the cochlea from the trauma resulting from loud noise.

Two possible mechanisms are aroused for high concentrations of BP in cochleae following administration of stealth-nano–BP. One is an increase of blood circulation time of BP-loaded nanoparticles by the stealth effect, escaping of stealth-nano–BP from the MPS resulting in

an increase of blood circulation time of BP. Another is the accumulation of stealth-nano–BP in the cochlear capillaries. Our previous study using nonstealth nanoparticles encapsulating rhodamine showed limited delivery of nonstealth nanoparticles into the cochlear capillaries even 10 min after application, with virtually no rhodamine fluorescence identified in cochleae 2 h after application [11]. In contrast to nonstealth nanoparticles, histological findings using PEG-coated PLA nanoparticles loaded with rhodamine demonstrate the presence of PEG-coated PLA nanoparticles in the cochlear capillaries even 24 h after systemic application. We therefore consider that polymeric nanoparticles are capable of accumulating in the cochlear capillaries under pathological conditions, if sufficient amounts of nanoparticles are supplied in circulation. In addition, noise trauma is known to induce a decrease of the cochlear blood flow velocity [20], which could be a possible mechanism for prolonged presence of PEG-coated PLA nanoparticles. Some previous studies have indicated that noise trauma damages a blood–labyrinth barrier resulting in an increase of drug entry into cochlear tissues [21,22]. Conversely, another study demonstrates no significant change in the blood–labyrinth barrier after noise trauma [23]. In present findings, no rhodamine fluorescence was identified in cochlear specimens treated with PEG-coated PLA nanoparticles except for the locations corresponding to those of cochlear vascular spaces, suggesting that PEG-coated PLA nanoparticles used in the present study were not able to pass a blood–labyrinth barrier even after intense noise exposure.

Our results have shown that a single injection of stealth-nano–BP protected auditory function and cochlear sensory hair cells from noise trauma. We consider that this is likely to be due to the sustained delivery of BP at high concentrations to the cochlea achieved by using stealth-nanoparticle technology. In the clinical treatments of acute SNHL, daily injections of BP for 5–10 days are given, but this generally results in unsatisfactory outcomes in terms of hearing recovery. Our measurements of cochlear BP concentrations in mice, presented here, show that free BP disappeared from the cochlea within 12 h, while stealth-nano–BP could maintain higher BP concentrations in the cochlea for 24 h than were achieved after 1 h using free BP injections. All together, our results strongly suggest that the clinical application of stealth-nano–BP may improve hearing recovery in patients with acute SNHL, which would contribute to an improved quality of life for these patients. In the near future, we will investigate the efficacy of stealth-nano–BP in other acute SNHL models and the risk of adverse effects that may be associated with their use in preclinical studies.

Conclusion & future perspective

To examine the efficacy of stealth nanoparticles encapsulating BP for the treatment of noise-induced SNHL, we have carried out pharmacokinetic, functional and histological analyses in a mouse model of noise-induced SNHL. Pharmacokinetic experiments showed the efficacy of stealth nanoparticles for sustained drug delivery to the cochlea. Functional and histological analyses of noise-damaged cochleae

Executive summary

- To examine the efficacy of stealth nanoparticles encapsulating betamethasone phosphate (BP) for the treatment of noise-induced sensorineural hearing loss, pharmacokinetic, functional and histological analyses were performed using a mouse model of noise-induced sensorineural hearing loss.
- Histological analyses of rhodamine distribution demonstrated the efficacy of encapsulating a drug in stealth nanoparticles for delivery to the cochlea.
- The pharmacokinetics of BP showed that encapsulating BP in stealth nanoparticles, compared to administering free BP, is an effective strategy for sustained BP delivery to the cochlea.
- Immunohistochemistry for glucocorticoid receptor demonstrated a significant increase of glucocorticoid receptor nuclear translocation in outer hair cells of cochleae treated with stealth nanoparticles encapsulating BP than those with free BP.
- Monitoring auditory function using auditory brain stem responses demonstrated a significant therapeutic potential for stealth nanoparticles encapsulating BP for noise-induced hearing loss.
- Functional analyses using auditory brain stem response demonstrated that high doses of BP given as single injections had no therapeutic effects on the attenuation of noise-induced hearing loss.
- Histological analyses of cochlear hair cells demonstrated that the systemic application of stealth nanoparticles encapsulating BP significantly promoted hair cell survival after exposure to traumatic noise.
- A single injection of a high dose of free BP had no protective effects on cochlear hair cells exposed to loud noises.
- These results show that encapsulating BP in stealth nanoparticles could greatly enhance the therapeutic efficacy of BP in acute sensorineural hearing loss.

demonstrated significant amelioration of noise-induced damage after the systemic application of stealth nanoparticles encapsulating BP. These findings encourage us to perform preclinical studies with stealth nanoparticles encapsulating BP for the treatment of acute SNHL.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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RESEARCH ARTICLE Open Access

Topical insulin-like growth factor 1 treatment using gelatin hydrogels for glucocorticoid-resistant sudden sensorineural hearing loss: a prospective clinical trial

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Abstract

Background: Sudden sensorineural hearing loss (SSHL) is a common condition in which patients lose the hearing in one ear within 3 days. Systemic glucocorticoid treatments have been used as standard therapy for SSHL; however, about 20% of patients do not respond. We tested the safety and efficacy of topical insulin-like growth factor 1 (IGF1) application using gelatin hydrogels as a treatment for SSHL.

Methods: Patients with SSHL that showed no recovery to systemic glucocorticoid administration were recruited. We applied gelatin hydrogels, impregnated with recombinant human IGF1, into the middle ear. The primary outcome measure was the proportion of patients showing hearing improvement 12 weeks after the test treatment. The secondary outcome measures were the proportion of patients showing improvement at 24 weeks and the incidence of adverse events. The null hypothesis was that 33% of patients would show hearing improvement, as was reported for a historical control after hyperbaric oxygen therapy.

Results: In total, 25 patients received the test treatment at a median of 23 days (range 15-32) after the onset of SSHL, between 2007 and 2009. At 12 weeks after the test treatment, 48% (95% CI 28% to 69%; $P = 0.086$) of patients showed hearing improvement, and the proportion increased to 56% (95% CI 35% to 76%; $P = 0.015$) at 24 weeks. No serious adverse events were observed.

Conclusions: Topical IGF1 application using gelatin hydrogels is well tolerated and may be efficacious for hearing recovery in patients with SSHL that is resistant to systemic glucocorticoids.

Background

Sudden sensorineural hearing loss (SSHL) is a condition in which an individual experiences hearing loss of at least 30 dB over at least three test frequencies in one ear within a period of 3 days [1]. Some patients recover completely without medical intervention, often within the first 3 days. Others get better slowly over a 1-week or 2-week period, which is known as 'spontaneous recovery' [1]. Although a good recovery is likely, 15% of patients with SSHL experience hearing loss that worsens over time. Approximately 40,000 new cases of SSHL occur each year in the US [1], and 35,000 patients with SSHL consult a doctor each year in Japan [2]. SSHL can affect anyone; however, for reasons that so far remain unknown, it is most often reported in people aged between 30 and 60 years. The most common therapy for SSHL is the systemic application of glucocorticoids. Unfortunately, about 20% of patients do not respond to this treatment [3].

Based on these findings, researchers have sought alternative therapeutic options for SSHL. Protecting auditory hair cells and primary neurons from irreversible

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degeneration is a practical strategy, as inner ear cells have limited regeneration capacity [4]. Recent improvements in our understanding of the role of growth factors in the maintenance of mature peripheral auditory systems have led to numerous attempts to define ways to reduce auditory hair cell and neuron degeneration, which have indicated that some growth factors have potential for the treatment of SSHL [5-8]. However, growth factors have not yet been used for this purpose in a clinical setting, as several obstacles have hindered their progress. Safe and effective methods for the sustained delivery of growth factors to the inner ear need to be developed to facilitate their clinical application. As a solution to this problem, we used gelatin hydrogels as a vehicle to deliver growth factors to the inner ear [9]. Gelatin hydrogels consist of gelatin polymers that are electrostatically complexed with growth factors [10]. The growth factors are released by the enzymatic degradation of the gelatin polymers after application. Our focus was on insulin-like growth factor 1 (IGF1), which has been approved for clinical application. We conducted a series of animal experiments, which revealed that topical IGF1 application via gelatin hydrogels significantly improved hearing by protecting auditory hair cells against damage caused by intense noise exposure [11] or ischaemic injury [12]. Moreover, no adverse events were observed in animals following the local application of IGF1 via gelatin hydrogels [11].

Here, we report on a prospective clinical trial of topical IGF1 application through gelatin hydrogels for the treatment of glucocorticoid-resistant SSHL, which was intended to provide preliminary estimates of variables for generating hypotheses for more specific studies using randomised trials when appropriate. Systemic glucocorticoid application has been regarded as a primary treatment of choice for SSHL. We recruited patients with SSHL that showed no recovery to systemic glucocorticoid administration as subjects in the present study.

Methods

Patients

Patients were eligible for inclusion in the study if they met the following conditions: they had been diagnosed between December 2007 and July 2009 at the Department of Otolaryngology, Head and Neck Surgery of Kyoto University Hospital, Japan as having definite or probable SSHL within 29 days of onset; they presented with an abnormality in evoked otoacoustic emission, which indicated dysfunction of the auditory hair cells; no recovery was determined according to the criteria for hearing improvement as set by the Sudden Deafness Research Committee of the Japanese Ministry of Health, Labour and Welfare in 1984 [13] (Table 1) more than 7 days after systemic glucocorticoid treatment; and they were aged over 20 years. We excluded patients with active chronic otitis media, acute otitis media, otitis media with effusion or dysfunction of the auditory tube, a history of previous treatments (except for systemic application of glucocorticoids or prostaglandin E1), malignant tumours, severe liver dysfunction (aspartate aminotransferase (AST) >100 IU/L and alanine aminotransferase (ALT) >100 IU/L), uncontrolled diabetes (haemoglobin A1C (HbA1c) >10%), pituitary or adrenal dysfunction, severe systemic illness that affected life expectancy, a history of severe drug allergy, or a history of alcohol or drug dependence within the past 1 year, and pregnant or lactating women. Magnetic resonance imaging (MRI) was performed on all patients to rule out acoustic neurinoma.

This study was single arm, non-randomised and open. Placebo applications and blinding were not used, as it was anticipated that they would have reduced compliance.

The primary outcome measure was the proportion of patients showing hearing improvement, which was defined as better than slight recovery according to the criteria shown in Table 1, 12 weeks after the test treatment. The secondary outcome measures were the proportion of patients showing hearing improvement 24 weeks after the test treatment and the incidence of adverse events during the observation period.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki and its amendments, and approved by the Ethical Committee of the Graduate School of Medicine, Kyoto University (registered number, C165). Each patient gave written, informed consent to participate in this study.

Trial registration

This trial was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) on 6 December 2007 under trial registration number UMIN-CTRR000000936.

Procedures

The test treatment was performed within 4 days of registration. Gelatin hydrogels were made from porcine skin gelatin (Nitta Gelatin Inc., Osaka, Japan) in a clean room at the Department of Pharmacy, Kyoto University Hospital, according to the method described previously [14], and were preserved at temperatures below 4°C before use. Procedures for topical IGF1 treatment were performed in the Day-Surgery Unit of Kyoto University Hospital. Mecasermin (recombinant human IGF1 (Somazon), 10 mg injection; Astellas Pharma Inc., Tokyo, Japan) was dissolved in physiological saline at a final concentration of 10 mg/ml. A 30 μl sample of

Table 1 Criteria for hearing improvement determined by the Sudden Deafness Research Committee of the Japanese Ministry of Health, Labour and Welfare in 1984

Improvement	Criteria
Complete recovery	Recovery of a hearing level within 20 dB at all five frequencies tested (0.25, 0.5, 1.0, 2.0 and 4.0 kHz) or recovery to the same level as the opposite side in pure tone auditometry
Marked recovery	More than 30 dB recovery in the mean hearing level at the five frequencies tested
Slight recovery	Recovery of 10 to 29 dB in the mean hearing level at the five frequencies tested
No recovery	Less recovery than 10 dB in the mean hearing level at the five frequencies tested

mecasermin solution was mixed with 3 mg of gelatin hydrogels 60 min before application. The hydrogel containing 300 μg of mecasermin was placed in the round-window niche of the middle ear following tympanostomy under local anaesthesia with 1% lidocaine. A single application was used. Patients were hospitalised for 4 days after the surgical procedure, and their general and local conditions were examined at the outpatient clinic of the Department of Otolaryngology, Head and Neck Surgery, Kyoto University Hospital, for 24 weeks after the test treatment. Pure-tone audiometry and evoked otoacoustic emission were measured on the day of registration, at 3 days after the test treatment, and at 1, 2, 4, 12 and 24 weeks after the test treatment. During the observation period, all adverse events were recorded.

Statistical analysis

The threshold improvement (33%, 66/199) was based on a historical control of hyperbaric oxygen therapy (19 times in total; range 5-55) for 199 patients with glucocorticoid-resistant SSHL at Kyoto University Hospital between October 2000 and September 2006 [15]. The null hypothesis was that the proportion of patients with hearing improvement at 12 or 24 weeks after the test treatment would be equivalent to the proportion of patients with hearing improvement reported in a historical control administered hyperbaric oxygen therapy. The sample size was based on binominal distribution with a one-sided significance level of 0.05 and a power of 0.90 (expected proportion of 63%). The required sample size was 25 after considering 10% (3 samples) of patients who would be excluded from the analysis. The null hypothesis was rejected at the 0.05 level of probability (one-sided) based on a binominal distribution. Statistical analyses were performed using SAS v.9.2 (SAS Institute Inc. Cary, NC, USA).

Results

In all, 26 patients fulfilled the inclusion criteria, 1 of whom was excluded before the test treatment because of a diagnosis of functional hearing loss. In total, 25 patients (13 women and 12 men) were treated in accordance with the study protocol, and data for assessment of the primary and secondary outcomes were available for all patients. The median age at registration was 49 years (range 23-72 years). Comorbidities were found in 22 of the 25 patients (88%), and 18 of the 25 patients (72%) had a history of previous diseases. None of the comorbidities or previous diseases presented were directly associated with SSHL. None of the patients had family histories of SSHL. All 25 patients complained of associated symptoms: 22 (88%) complained of tinnitus, 19 (76%) had a feeling of ear fullness and 14 (56%) complained of dizziness. The median interval between the onset of SSHL and the initiation of the test treatment was 23 days (range 15-32 days). The mean hearing level at registration was 81.2 dB (95% confidence interval (CI), 71.2 to 91.1).

A summary of the hearing recovery according to pure-tone audiometry for all of the patients is shown in Table 2. At 12 weeks after the test treatment, 48% (95% CI 28% to 69%; $P = 0.086$) of the patients showed hearing improvement. The null hypothesis for the primary outcome was not rejected. Of the 25 patients, 0 showed complete recovery, 1 (4%) showed marked recovery, 11 (44%) showed slight recovery and 13 (52%) showed no recovery at 12 weeks. None of the patients who were treated more than 26 days after the onset of SSHL showed hearing improvement. At 24 weeks after the test treatment, the proportion of patients showing hearing improvement was 56% (95% CI 35% to 76%; $P = 0.015$), showing that the null hypothesis was rejected for the data at 24 weeks. Of the 25 patients, none showed complete recovery, 1 (4%) showed marked recovery, 13 (52%) showed slight recovery, and 11 (44%) showed no recovery. Two patients showed a hearing improvement of less than 10 dB at 12 weeks after the treatment, but an improvement of 10 dB at 24 weeks.

No serious adverse events associated with the test treatment occurred, although any adverse events were recorded in all of 25 patients to be evaluated. Adverse events with an incidence rate of more than 20% included dizziness (44%), nausea (24%), otitis externa (32%), common cold (20%) and otitis media (28%). All adverse events disappeared within the observation period. Except for two patients, the dizziness appeared either on the day of local IGF1 application or on the next day, and continued for a mean of 6.4 days (range

Table 2 Hearing recovery according to pure-tone audiometry

				Averaged hearing level (dB)			Hearing improvement	
Patient	Age	Gender	Days from onset	Before registration	12 weeks	24 weeks	12 weeks	24 weeks
1	54	M	19	88	77	75	SR	SR
2	36	F	31	62	55	60	NR	NR
3	46	M	21	107	81	86	SR	SR
4	29	F	24	107	95	95	SR	SR
5	38	M	19	65	64	62	NR	NR
6	72	M	29	98	97	97	NR	NR
7	49	M	17	111	105	105	NR	NR
8	49	F	26	47	42	42	NR	NR
9	55	M	21	104	78	75	SR	SR
10	55	F	29	52	57	57	NR	NR
11	60	F	27	37	33	32	NR	NR
12	35	F	21	76	68	66	NR	SR
13	59	M	23	90	79	78	SR	SR
14	58	M	32	60	81	77	NR	NR
15	60	F	26	63	40	39	SR	SR
16	36	M	19	56	51	46	NR	SR
17	33	F	18	88	88	87	NR	NR
18	61	F	25	92	72	74	SR	SR
19	42	F	15	111	89	92	SR	SR
20	23	F	18	79	22	18	MR	MR
21	45	F	26	95	82	77	SR	SR
22	45	M	28	87	84	85	NR	NR
23	60	F	23	108	84	86	SR	SR
24	26	M	20	109	92	86	SR	SR
25	55	M	21	37	34	35	NR	NR

Average hearing level was the mean hearing level according to pure-tone audiometry at the five frequencies tested (0.25, 0.5, 1.0, 2.0 and 4.0 kHz). Hearing improvement was determined by the criteria shown in Table 1.

MR = marked recovery; NR = no recovery; SR = slight recovery.

1-20 days). In all patients, the dizziness appeared after the test treatment. In one patient, dizziness appeared 2 months after the test treatment and continued for 4 months. In another patient, dizziness appeared 7 days after the application and disappeared 2 days later. Otitis media was found in 7 of the 25 (28%) patients, and was cured within a mean of 9.4 days (range 2-17 days). Exacerbation of tinnitus appeared in two patients at 29 and 33 days after the test treatment, respectively. None of the patients showed residual perforation of the tympanic membrane or additional hearing loss over 10 dB.

Discussion

Hearing loss is common, affecting about 5% to 6% of the population of the USA [1]. SSHL is one of the most common clinical conditions encountered by otolaryngologists, although it is less common than age-related hearing loss. National surveys have demonstrated the incidence of SSHL to be 5-30 per 100,000 per year [2,16,17]. Systemic application of glucocorticoids has been used as a standard therapy, although the supporting evidence is weak. Although systemic glucocorticoid application results in hearing recovery in some patients with SSHL, approximately 20% show no recovery [3]. Alternative therapeutic treatment options for SSHL have thus been eagerly sought. Against this background, we began developing topical IGF1 treatments using gelatin hydrogels in animal models [5,11,12], followed by a clinical trial to investigate their safety and efficacy for use in patients with SSHL. Some studies have indicated that SSHL develops when the inner ear does not receive a sufficient oxygen supply [18]. Consequently, hyperbaric oxygen treatment has been used as an alternative option for the treatment of SSHL [19,20]. At Kyoto University Hospital, hyperbaric oxygen therapy has been used as a secondary treatment of choice for glucocorticoid-resistant SSHL [14]. We thus used the proportion of patients with glucocorticoid-resistant SSHL showing hearing

recovery following hyperbaric oxygen therapy as a historical control.

Here, we report hearing recovery according to pure-tone audiometry and incidence of adverse events following topical IGF1 application using gelatin hydrogels in patients with SSHL enrolled in a single arm, non-randomised and open trial. Topical IGF1 treatment resulted in hearing recovery in approximately half of the patients with SSHL that had not responded to systemic glucocorticoid application, although the null hypothesis was rejected at 24 weeks after the test treatment but not at 12 weeks. In addition, no serious adverse events were observed during the 24-week observation period. The results indicated that the topical IGF1 application using gelatin hydrogels was safe, and had equivalent or superior efficiency to the hyperbaric oxygen therapy that was used as a historical control; this suggests that the efficacy of topical IGF1 application using gelatin hydrogels for SSHL that is resistant to systemic glucocorticoid treatments should be evaluated using randomised clinical trials.

Spontaneous recovery occurs in 40% to 65% of patients with SSHL [21,22], which makes it difficult to examine the exact therapeutic effects of interventions. It is therefore important either to eliminate patients with spontaneous recovery from such trials or to include a placebo control. In the present study, the test treatment was initiated in all patients more than 14 days (mean 23 days; range 15-32 days) after the onset of SSHL. In most cases, spontaneous recovery occurs within 14 days of onset [21]. We therefore consider spontaneous recovery to have had a negligible influence on the present results.

As a secondary treatment of choice for SSHL, intratympanic injection of glucocorticoids has gained considerable attention, because it seems to deliver a high concentration of glucocorticoids to the inner ear [23]. In addition, local application can reduce the total amount of glucocorticoids that needs to be applied, leading to a reduced risk of adverse events [24]. However, this approach remains controversial, because the criteria used to judge its efficacy differ in the literature. Haynes *et al.* [25] reviewed the literature on the intratympanic injection of glucocorticoids for SSHL after the failure of systemic treatment, and re-estimated the hearing recovery based on their own criteria, according to which a 20 dB improvement as indicated by pure-tone audiometry or a 20% improvement in discrimination was considered to be a successful therapeutic intervention. The recovery rates according to their criteria were 0% to 40%. When these criteria for successful intervention were applied to the data from the present study, the recovery rate was 24%, suggesting that the efficacy of topical IGF1 treatment using gelatin hydrogels might be equivalent to that of the intratympanic injection of glucocorticoids. We therefore recommend that the efficacy of topical IGF1 treatment using gelatin hydrogels should be evaluated in a randomised clinical trial, and its effectiveness for SSHL should be compared with that of the intratympanic injection of glucocorticoids.

Conclusions

The present results indicate the safety and efficacy of the use of topical IGF1 treatment using gelatin hydrogels for SSHL resistant to systemic glucocorticoid treatments. A double-blinded, randomised clinical study could clarify these findings. However, there are ethical obstacles to the use of double-blinded, randomised clinical trials for SSHL. For instance, the time from the onset of SSHL to the start of treatment has been regarded as important for the outcome, with prompt treatment preventing the development of irreversible auditory pathological changes. In addition, systemic glucocorticoid treatments have widely been accepted as a standard therapy for SSHL, and have led to improvement in some patients [26]. Hence, there would be ethical difficulties in not offering patients treatment with systemic glucocorticoids. Moreover, topical IGF1 application using gelatin hydrogels requires the use of surgical procedures, which would make it difficult to test in a double-blinded study. Therefore, as a next step, we will conduct a randomised clinical trial to compare the efficacy of topical IGF1 treatment using gelatin hydrogels with that of the intratympanic injection of glucocorticoids in patients with SSHL that is resistant to systemic glucocorticoids; it is hoped that this might clarify the efficacy of topical IGF1 treatment using gelatin hydrogels.

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Authors' contributions

TN, RO, SaT and JI planned the study. TS, HH, YSK and NM performed surgical treatment and collected the data. KH, KO, AY, KI, MY and YT prepared the gelatin hydrogels. SaT, ShT and HT analysed the data. TN wrote the article. JI edited the article.

Competing interests
The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Inner ear drug delivery system from the clinical point of view

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Abstract

Conclusion: Three types of inner ear drug delivery systems (DDS) that were ready to be applied in clinics were developed. *Objectives:* To develop clinically applicable inner ear DDS for the treatment of inner ear disorders. *Methods:* Inner ear DDS using clinically applicable materials were developed and evaluated. *Results:* The systemic application of stealth-type nanoparticles encapsulating betamethasone provided superior therapeutic results for the treatment of noise-induced hearing loss compared with the systemic application of betamethasone in mice. Microparticles made of biodegradable polymer (poly (lactic/glycolic) acid, PLGA) encapsulating lidocaine were placed on the round window membrane of guinea pigs, and resulted in reasonable concentrations of lidocaine in the cochlea without serious adverse effects. The phase I/IIa clinical trial of the application of insulin-like growth factor-1 (IGF-1) in combination with gelatin hydrogel on the round window membrane was conducted, recruiting patients with acute sensorineural hearing loss after the failure of systemic application of steroids.

Keywords: *Sensorineural hearing loss, tinnitus, biodegradable polymer, gelatin hydrogel, betamethasone, insulin-like growth factor 1, lidocaine*

Introduction

Sensitive sensors in the inner ear, hair cells, are mechanically protected in the bony capsule. The unique high potassium environment required for hair cells to work is actively maintained in the endolymph, which is sealed by tight junctions. The blood–labyrinthine barrier [1] is partly composed of tight junctions and also a system to protect these delicate cells from agents that may cause damage. However, these isolation systems make inner ear diseases difficult to be treated. Direct access into the inner ear is difficult because of the bony capsule. The blood flow of the inner ear is accordingly limited; 1/10 000 of cardiac output in rodents and 1/1 000 000 in humans [2]. It is difficult to deliver systemically applied therapeutic agents into the inner ear because of this limited blood flow and the existence of the blood–labyrinthine barrier [3]. Specific strategies to deliver therapeutic agents into the inner ear are required to overcome this difficulty.

The purpose of a drug delivery system (DDS) is to deliver a drug to a specific site in a specific time and release pattern [4]. Several types of inner ear-specific DDS have been developed, most of which use the round window (RW) as a route to deliver the agent into the inner ear, because the RW is a unique structure in that the inner ear is not covered with bone but sealed with a RW membrane (RWM). One well studied example of inner ear DDS is RWμCath™ (DURECT™ Co., Cupertino, USA), which utilizes the catheter tip placed on the RWM to deliver the therapeutic agent. Plontke et al. [5] conducted a clinical trial using this device. Patients with acute

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sensorineural hearing loss and insufficient recovery after systemic glucocorticoid treatment were included in that study, and significantly better improvement of hearing after a continuous intratympanic delivery of glucocorticoid via RWμCath was observed. This device would be applicable to other therapeutic agents; however, major surgery is required before and after the drug application, and more seriously, it is impossible to use this device because this has been commercially discontinued.

It is obviously mandatory to develop clinically applicable and available inner ear DDS. Here we show our approaches to realize this aim. The first is the systemic approach to deliver drugs to the inner ear more effectively. The other two involve local drug delivery via the RWM.

Material and methods

Inner ear DDS via systemic application – stealth-type nanoparticles

It would be more useful and its clinical application would be wider if a systemically applied therapeutic agent could be delivered selectively into the inner ear; however, to date, there is no reported system available to achieve this aim. Instead, we tried to improve the utilization of drugs in the inner ear. We used stealth-type nanoparticles for this purpose, which are made of biodegradable polymer, poly lactic acid (PLA), with polyethylene glycol coating (Figure 1A). Stealth-Nano-Steroid, stealth-type nanoparticles containing betamethasone, have been shown to accumulate preferentially in artificially inflamed joints as a model of rheumatoid arthritis and to reduce inflammation [6]. We first tested the distribution of stealth-type nanoparticles in the inner ear. In terms of clinical application, PLA is widely used as absorbable surgical threads, pins, screws and facial injectables (Sculptra®). Also, polyethylene glycol (PEG) is frequently used to modify the molecular weight, size and solubility of therapeutic drugs. These factors support the clinical safety of Stealth-Nano-Steroid.

Inner ear drug delivery via the round window

Intratympanic injection has been used as a method to realize inner ear treatment to deliver aminoglycosides, steroids and other therapeutic drugs [7]. However, the pharmacokinetics of intratympanically applied drugs are not stable because of the dynamic environment of the tympanic cavity; e.g. liquid in the tympanic cavity is easily ejected into pharynx by swallowing. To stabilize drug delivery via the RWM into the inner ear, we used microparticles made of biodegradable polymer and gelatin hydrogel. These slow releasing materials are placed on the RWM, and as these degrade, encapsulated therapeutic molecules diffuse into the inner ear.

Local application using PLGA microparticles

While tinnitus is a common symptom among patients with hearing impairment, no specific therapeutic strategy has been established. Lidocaine is known to be effective via intratympanic application [8,9]. However, it has been an unacceptable option because of its short effective duration (up to several hours) and serious vertigo after the application due to inner ear anesthesia [10]. We designed the inner ear DDS to reduce the concentration in the inner ear and elongate the release of lidocaine [11]. Poly (lactic/glycolic) acid (PLGA) is another commonly used biodegradable polymer. PLGA microparticles encapsulating lidocaine (Figure 1B) were applied on the RWM of guinea pigs and the lidocaine concentrations in the cochlea were measured at various time points.

Local application using gelatin hydrogels

Gelatin is a natural polymer composed mainly of collagen. By crosslinking with glutaraldehyde, gelatin forms hydrogel. The isoelectric point of gelatin can be modified to yield either a negatively charged acidic gelatin or a positively charged basic gelatin at physiological pH. This allows specific design so that electrostatic interaction takes place between a charged bioactive molecule (e.g. proteins and plasmid DNAs) and gelatin. The crosslinking density of gelatin hydrogels affects their degradation rate. Accordingly, gelatin hydrogels can be used as a delivery vehicle for the controlled release of bioactive molecules [12] (Figure 1C).

Various growth factors including brain-derived neurotrophic factor (BDNF) [13], hepatocyte growth factor (HGF) [14] and insulin-like growth factor 1 (IGF-1) have been placed on the RWM of the cochlea in combination with gelatin hydrogel to test the possibility of their use as therapeutic agents for the treatment of hearing impairment in rodents. Among them, IGF-1 has been shown to be protective [15] and therapeutic [16] against noise-induced inner ear damage, and therapeutic against ischaemic inner ear damage [17]. In addition, recombinant human IGF-1 (rhIGF-1, Mecasermin®, Astellas Pharma Inc., Tokyo, Japan) is commercially available as an orphan drug for

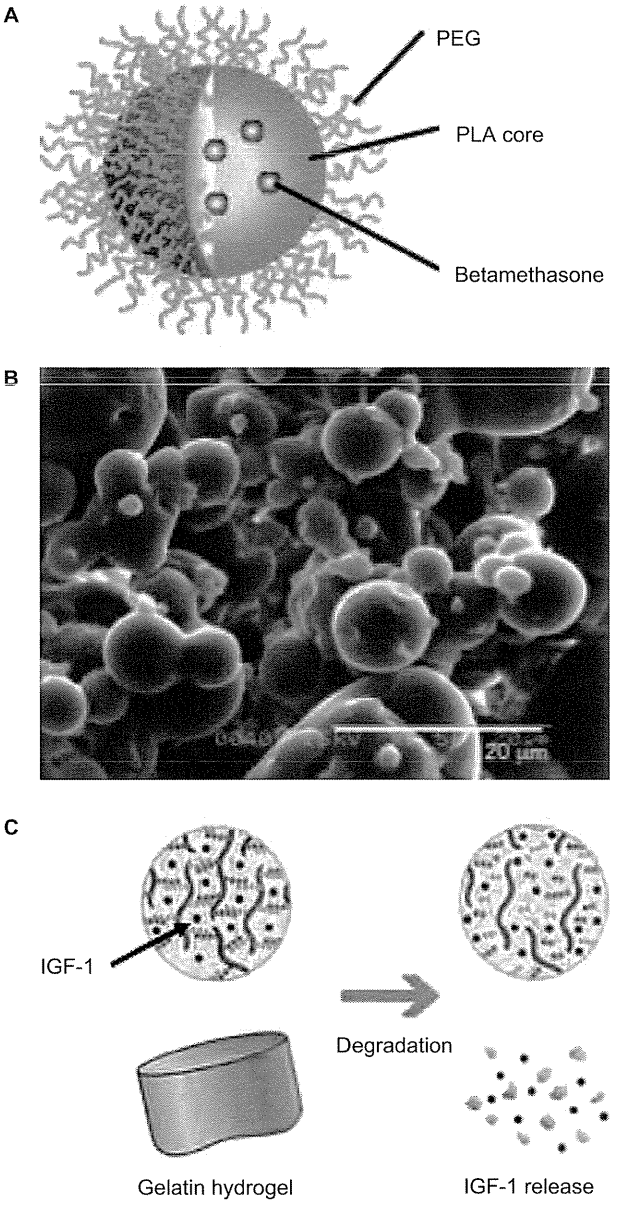

Figure 1. (A) Schematic illustration of a stealth-type poly lactic acid (PLA) nanoparticle with polyethylene glycol (PEG) coating and encapsulated betamethasone. (B) Scanning electron microscopic view of poly (lactic/glycolic) acid (PLGA) microparticles encapsulating lidocaine. (C) Schematic illustration of a gelatin hydrogel drug delivery system (DDS). Target molecules (IGF-1) entrapped in the crosslinked gelatin polymer are gradually released from the polymer matrix as gelatin hydrogel degrades.

the treatment of a type of juvenile growth failure, a certain type of diabetes mellitus and dwarfness.

Against this background, we conducted and have finished a phase I/IIa clinical trial to examine the safety and efficacy of local IGF-1 application via the RWM using gelatin hydrogel for patients with acute sensorineural hearing loss (UMIN000000936). Subjects are patients with acute sensorineural hearing loss, (1) with abnormality in evoked otoacoustic emission, (2) within 29 days after the onset of hearing loss, (3) determined as non-responders to systemic steroid application, and (4) age over 20 years. Major exclusion criteria are (1) presenting active middle ear abnormality, (2) history of previous other treatments including systemic application of batroxobin, prostaglandin I, and hyperbaric oxygen therapy, except for systemic steroid application. Each registered patient received a tympanotomy and the RW niche was inspected with a thin endoscope. Gelatin hydrogel combined with recombinant human IGF-1 (rhIGF-1) was placed on the RWM. Average hearing levels and adverse events were followed up for 24 weeks.

Results and Discussion

Inner ear DDS via systemic application – stealth-type nanoparticles

The systemic application of conventional nanoparticles made of PLGA without PEG coating did not lead to distribution in the inner ear [18]. On the other hand, stealth nanoparticles encapsulating rhodamine B distributed to the inner ear. Systemic application of Stealth-Nano-Steroid after the noise-induced hearing loss showed higher concentrations of betamethasone in the inner ear, and better recovery of hearing compared with the simple systemic application of betamethasone (in print).

Local application using PLGA microparticles

When PLGA microparticles encapsulating lidocaine (Figure 1B) were applied on the RWM of guinea pigs and the lidocaine concentrations in the cochlea were measured at various time points, the highest concentrations were observed on day 3. Nystagmus was not induced by this procedure. Hearing thresholds determined by auditory brainstem responses showed only temporal elevation on day 7. Inflammatory responses in the middle and inner ear were not observed except for minor mucosal thickening and lymphatic cell infiltrations. These results suggest a high possibility for the clinical application of these particles for the treatment of tinnitus without causing serious adverse effects [11]. Animal experiments to show the effectiveness of these particles are difficult because tinnitus is a subjective symptom; however, there are a number of animal models to evaluate tinnitus in rodents [19,20]. We are investigating the effects on the reduction of tinnitus in rodents, and at the same time, a clinical trial is planned.

Local application using gelatin hydrogels

With this method, average hearing levels were comparable to hyperbaric oxygen therapy, which we usually use as a rescue after the failure of systemic steroid therapy. No serious related adverse events were observed. Details of the results will be published separately.

Conclusions

We have developed several DDS that can be used for the treatment of inner ear diseases. All the materials described above were selected from those that are already used in clinics to facilitate clinical applications. These strategies will become templates to realize clinical application of other candidate agents for the treatment of inner ear diseases. We would like to focus more on the demonstration of clinical usefulness of these inner ear DDS.

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ORIGINAL ARTICLE

Distribution of bone marrow-derived cells in the vestibular end organs and the endolymphatic sac

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Abstract

Conclusion: Bone marrow-derived cells (BMDCs) are constitutively present in the vestibular end organs and in the endolymphatic sac, and may play a role in the maintenance of inner ear homeostasis. *Objectives:* The aim was to examine the distribution and characteristics of BMDCs in the vestibular end organs and in the endolymphatic sac. *Methods:* Bone marrow-chimeric mice were generated by bone marrow transplantation from mice genetically labeled with enhanced green fluorescent protein to C57 Bl/6 mice to visualize BMDCs. Three months after bone marrow transplantation, inner ear specimens were processed for histochemical analyses. *Results:* BMDCs were widely distributed in the vestibular end organs and the endolymphatic sac, whereas there were differences in the phenotype and the distribution between the vestibular end organs and the endolymphatic sac. A subpopulation of BMDCs in the vestibular end organs expressed antigen-presenting protein MHC class II. Moreover, the density of BMDCs in the vestibular end organs increased in response to local mechanical stress.

Keywords: *Hematopoietic stem cell, transplantation, immune response, macrophage*

Introduction

The first description of Meniere's disease (MD) was made in 1861, and since then many hypotheses have been postulated as to its etiopathology. Although convincing evidence for any of these hypotheses is still under debate, to date, MD has been defined as an inner ear disorder of unknown causes where endolymphatic hydrops represents the anatomical correlate. Since it is well known that some MD patients show remarkable recovery from fluctuating and repeated sensorineural hearing loss or vertigo by systemic application of corticosteroid, the immunological pathogenesis of MD has been claimed by several authors for more than two decades [1,2].

The pathogenesis of immune-mediated inner ear disorders remains unknown, although autoantibodies, autoreactive T cells, and immune-complex deposition have been suggested [3–5]. As diagnostic biopsy of the human inner ear is not feasible, this may be a major obstacle when studying the etiology of inner ear disorders. Using experimental animals, Rask-Andersen reported the existence of macrophages and lymphocytes in the endolymphatic sac in 1980 [6]. Whereas immunocompetent cells have been thought not to exist in the normal inner ear except for the endolymphatic sac [7], several studies have recently reported the presence of immunocompetent cells in the cochlea [8–10]. However, there is still some distance between the clinical entity and the basic knowledge of anatomy and immunology obtained so far. It is unclear how the antigens in the inner ear are presented, or how the immune reaction is initiated in the inner ear. Moreover, little is known about the existence of immunocompetent cells in the vestibular end organs.

The aim of the present study was to examine the presence of immunocompetent cells in the vestibular end organs and the endolymphatic sac. We studied the distribution and phenotypes of

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bone marrow-derived cells (BMDCs) with green fluorescence in the vestibular end organs and the endolymphatic sac using bone marrow-chimeric mice. The results proved that BMDCs exist as macrophages in the stroma underneath the vestibular sensory epithelia and in the epithelial cell layer of the endolymphatic sac.

Material and methods

Animals

Male C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Enhanced green fluorescent protein (EGFP)-transgenic mice (B6;C3-Tg (ACtb-EGFP)CX-FM139Osb) [11] were used as a source of hematopoietic cells (HSCs), which share genetic background with C57BL/6 mice. The animals were maintained in a specific pathogen-free environment in the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. All experimental protocols were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Bone marrow-chimeric mice

HSCs were collected as Lin (Lineage marker)-negative, c-kit (CD117)-positive, and Sca1 (Stem cell antigen 1)-positive cells from bone marrows of EGFP-transgenic mice using cell sorting on a FACS Vantage (Becton-Dickinson, San Jose, CA, USA) as described previously [12]. Briefly, whole bone marrow cells were isolated from the femurs and tibiae of EGFP-transgenic mice (8–12 weeks of age). Bone marrow mononuclear cells were labeled with a primary antibody cocktail (BD Pharmingen, San Diego, CA, USA) 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 using auto MACS (Militenyi Biotec, Bergish Gladbach, Germany). $Lin^-/c\text{-}kit^+/Sca1^+$ cells were collected by cell sorting 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 = 14, 10–12 weeks of age) were irradiated with 9.5 Gy gamma rays (Gamma Cell 40 Exactor, MDS Nordion Inc., Ottawa, ON, Canada) and received 5×10^3 cells of HSCs through the tail vein. Three months after transplantation, the temporal bones were dissected out under overdose of anesthetics.

Mechanical stress to the inner ear

A saline injection into the posterior semicircular canal (PSCC) was adopted as a model for mechanical stress to the inner ear, using bone marrow-chimeric mice (n = 8) that had been transplanted EGFP-labeled HSCs 3 months before surgical treatment, as described previously [9]. 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 diameter) was made in the bony wall of the PSCC using 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 3 μl of physiological saline was injected toward the common crus of the semicircular canal at the rate of 1 μl min for 3 min using a Micro Syringe Pump (EiCOM). Four of the animals received a saline injection and the inner ears were collected 7 days after surgery. The remaining four animals were preserved as controls receiving no surgical treatment.

Immunohistochemistry

Under general anesthesia with overdoses of ketamine and xylazine, animals were perfused intracardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in phosphate buffer. The temporal bones were collected and immersed in the same fixative for 4 h at 4°C. The samples were decalcified with 10% ethylenediaminetetraacetic acid in PBS and cryo-protected with 30% sucrose. Specimens were prepared as cryostat sections (10 μm in thickness) and 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.

The characteristics of BMDCs were examined by immunohistochemistry for a leukocyte common antigen CD45, and ionized calcium binding adapter molecule 1 (Iba1), which is specific for microglia/macrophages [13]. The primary antibodies used in this study were: rat anti-mouse CD45 (1:50, BD PharMingen), rabbit anti-Iba1 (1:1000, Wako Pure Chemicals, Osaka, Japan), rat anti-major histocompatibility complex (MHC) class II (1:5, Serotec, Oxford, UK), rat anti-mouse E-cadherin (1:1000, TaKaRa, Shiga, Japan). The localization of primary antibodies was visualized using secondary antibodies

conjugated with Alexa Fluor 488, 555 or 633 (1:500, Molecular Probes). Nuclei were counterstained by 4',6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS, Molecular Probes, Eugene, OR, USA). Brain and spleen specimens obtained from normal adult C57BL/6 mice were used as positive controls for the expression of CD45, Iba1, or MHC class II. 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.

Quantitative analyses

To determine the ratio of EGFP-positive cells for the number of total cells in the vestibular stroma, every fourth section mounted on a glass slide was counter-stained with DAPI. Three sections were randomly selected from the sections containing vestibular end organs for each animal and used for quantitative analysis of BMDCs. EGFP-positive cells and the total stromal cells in a fixed area (100 ×100 μm) just beneath the vestibular epithelia of saccule, utricle, and ampullas were counted using Image/J software (http://rsbweb.nih.gov/ij/index.html), and the ratio of EGFP-positive cells for the number of total cells was calculated per section. All data are presented as the mean ± standard error. The unpaired t test was performed for statistical analyses of difference among the groups and a p value < 0.05 was considered statistically significant.

Results

Distribution of BMDCs

Substantial numbers of BMDCs expressing EGFP were found in the vestibular end organs of bone marrow-chimeric mice. The cells were observed in the utricle (Figure 1A), the saccule (Figure 1B), the ampulla in the anterior semicircular canal (Figure 1C), and the endolymphatic sac (Figure 1D). The number of BMDCs in each section was 1.9 ± 0.1 cells/10 000 μm^2 in the vestibular end organs, and 13.0 ± 2.7 cells/section in the endolymphatic sac. The ratio of BMDCs to the total nuclei in the vestibular stroma amounted to 5.0 ± 0.3%, while that in the endolymphatic sac amounted to 9.9 ± 0.9%. While the BMDCs in the vestibular end organs were observed in the stroma beneath the epithelia (Figure 1E), substantial numbers of the BMDCs were migrated into the epithelial cell layer

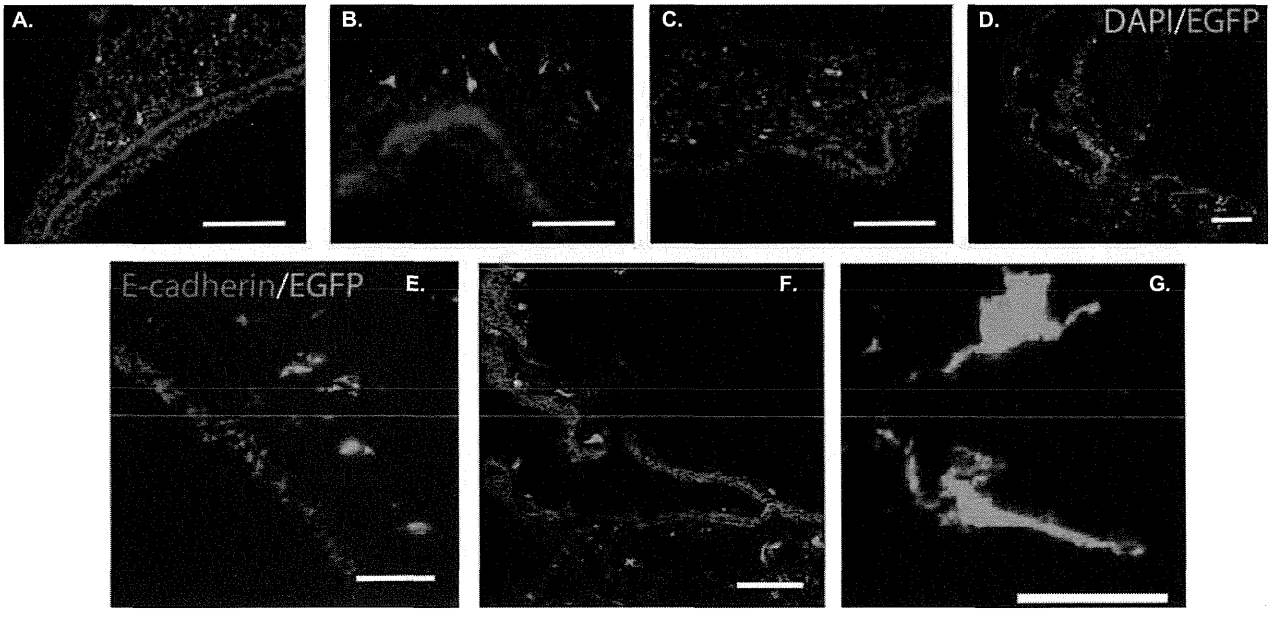

Figure 1. Distribution and morphology of bone marrow-derived cells (BMDCs) in the vestibular end organ and in the endolymphatic sac. EGFP-positive cells (green) are observed in the stroma of the utricle (A), the saccule (B), the ampulla of the anterior semicircular canal (C), and the endolymphatic sac (D). While the BMDCs in the vestibular end organ are observed in the stroma beneath the epithelia (E), substantial numbers of BMDCs are located in the epithelial cell layer of the endolymphatic sac (F). Epithelial cells are visualized with E-cadherin (magenta, in E and F). Most of the BMDCs have a spindle shape with ramified processes (G). Scale bars: 100 μm (A–C), 200 μm (D), 50 μm (E and F), 20 μm (G).