(Wako Pure Chemicals, Osaka, Japan) and 1.5 g/l penicillin G (Wako Pure Chemicals), at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h.

Gentamicin application and insulin-like growth factor-1 protection assay

The aminoglycoside antibiotic gentamicin was used as an ototoxic agent. To reflect clinical situations, we applied IGF-1 after intoxication with gentamicin. Initially, utricle explants were cultured in medium containing 0.5 mM gentamicin (Nacalai Tesque, Inc., Kyoto, Japan) for 24 h. The explants were then transferred to medium containing recombinant human IGF-1 (Astellas, Tokyo, Japan) at concentrations of 0, 0.01, 0.1, or 1.0 μg/ml, with five to six utricles incubated at each concentration for another 48 h. We determined IGF-1 concentrations in the media according to our earlier observation of IGF-1 concentrations in the perilymph after its local application onto the round-window membrane using gelatin hydrogels [12]. The maximum concentration of IGF-1 in this study was equivalent to that in the perilymph after local application.

Four explants immediately after dissection and four explants immediately after incubation with gentamicin were obtained for the hair cell survival assay. In this assay, explants were fixed for 15 min with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Specimens were incubated with Alexa-Fluor 488-conjugated phalloidin (1 : 250; Invitrogen) to label F-actin, and then viewed with a TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). Quantitative analysis of the hair cell number in utricles was performed according to the method described earlier [5,8,13]. Initially, each utricle was observed under low magnification. Two frames ($100 \times 100\,\mu m^2$) were set in the center area (the central region in Fig. 1e). Additional frames were made around the two central frames, keeping within the sensory epithelium as much as possible. We then randomly chose two frames that were not adjacent to the center frames (the peripheral region in Fig. 1e). The hair cell numbers in each frame were counted, and the average of each region was used as the data for the specimen.

Differences in hair cell numbers among IGF-1 concentrations were statistically tested by analysis of variance with Scheffe's method. *P* values less than 0.05 were considered statistically significant.

Hair bundle of surviving hair cells

To examine the functional structures of the hair bundles in surviving hair cells, we performed immunostaining for trio-binding protein (TRIOBP) and espin, which are crucial for the MET function of hair cells. TRIOBP is an actin-bundling protein that is selectively located in the rootlet of hair bundles [14], and espin is an actin-bundling protein that colocalizes with F-actin in hair bundles [15]. The expression of these proteins in the hair bundles of utricle hair cells was examined in utricles immediately after dissection, immediately after 24-h incubation with gentamicin, and after culture with 1.0 μg/ml IGF-1 ($n = 4$ for each condition).

After fixation with 4% PFA, specimens were examined by immunohistochemistry. The primary antibodies were rabbit polyclonal antibodies against TRIOBP (1 : 1000; Shin-ichiro Kitajiri, Kyoto University, Japan) and espin (1 : 100; James Bartles, Northwestern University, Evanston, Illinois, USA), and the secondary antibody was Alexa-Fluor 594 donkey anti-rabbit immunoglobulin G (1 : 500; Invitrogen). At the end of the staining procedures, the specimens were incubated with Alexa-Fluor 488-conjugated phalloidin and viewed with a TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc.).

To evaluate the MET function of surviving hair cells, we carried out labeling with FM1-43FX dye (Invitrogen), which passes through MET channels at hair bundles. Explants immediately after dissection, immediately after 24-h incubation with gentamicin, and after culture with 1.0 μg/ml IGF-1 were used for this purpose. The explants were transferred to culture media supplemented with FM1-43 at a concentration of 5 μM for 10 s. During the incubation with FM1-43, we applied mechanical stimulation, during which frequency was 1–2 Hz and intensity was approximately 100 V, as described earlier [4]. After fixation with 4% PFA, the specimens were examined with a TCS-SP2 laser-scanning confocal microscope. Four independent assays were performed in each condition.

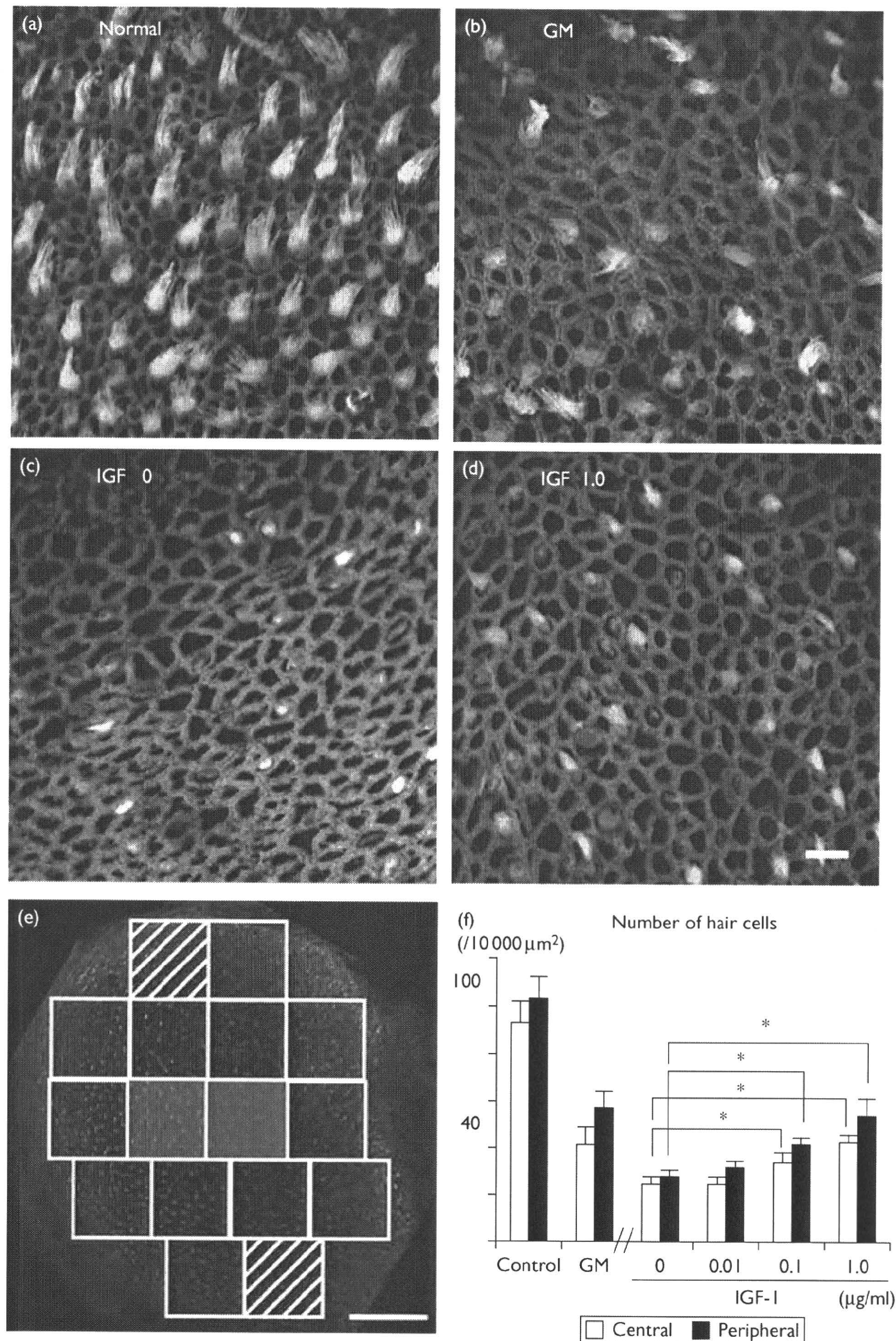
Results

Insulin-like growth factor-1 protection assay

In comparison with normal utricles that were fixed immediately after dissection (Fig. 1a), incubation with 0.5 mM gentamicin for 24 h caused approximately one-half of all hair cells to be lost (Fig. 1b) in both the central and peripheral regions. After an additional 48-h culture with no extra IGF-1 supplementation, extended loss of hair cells occurred in both central (Fig. 1c) and peripheral regions. Supplementation of the culture medium with 0.1 or 1.0 μg/ml IGF-1 resulted in increased survival of hair cells in these regions (Fig. 1d).

Quantitative analyses revealed statistically significant increases in hair cell numbers in explants treated with 0.1 or 1.0 μg/ml IGF-1 in both central and peripheral regions (Fig. 1f). Notably, supplementation with 1.0 μg/ml IGF-1 largely inhibited the hair cell loss that occurred during an additional 48-h culture after gentamicin exposure. These findings indicate that IGF-1 has protective effects on vestibular hair cells against gentamicin toxicity. However, no increase in hair cell numbers was observed in IGF-1-treated specimens in comparison with those in explants immediately after incubation with gentamicin (Fig. 1f), suggesting that little or no hair cell regeneration was induced by IGF-1.

Fig. 1

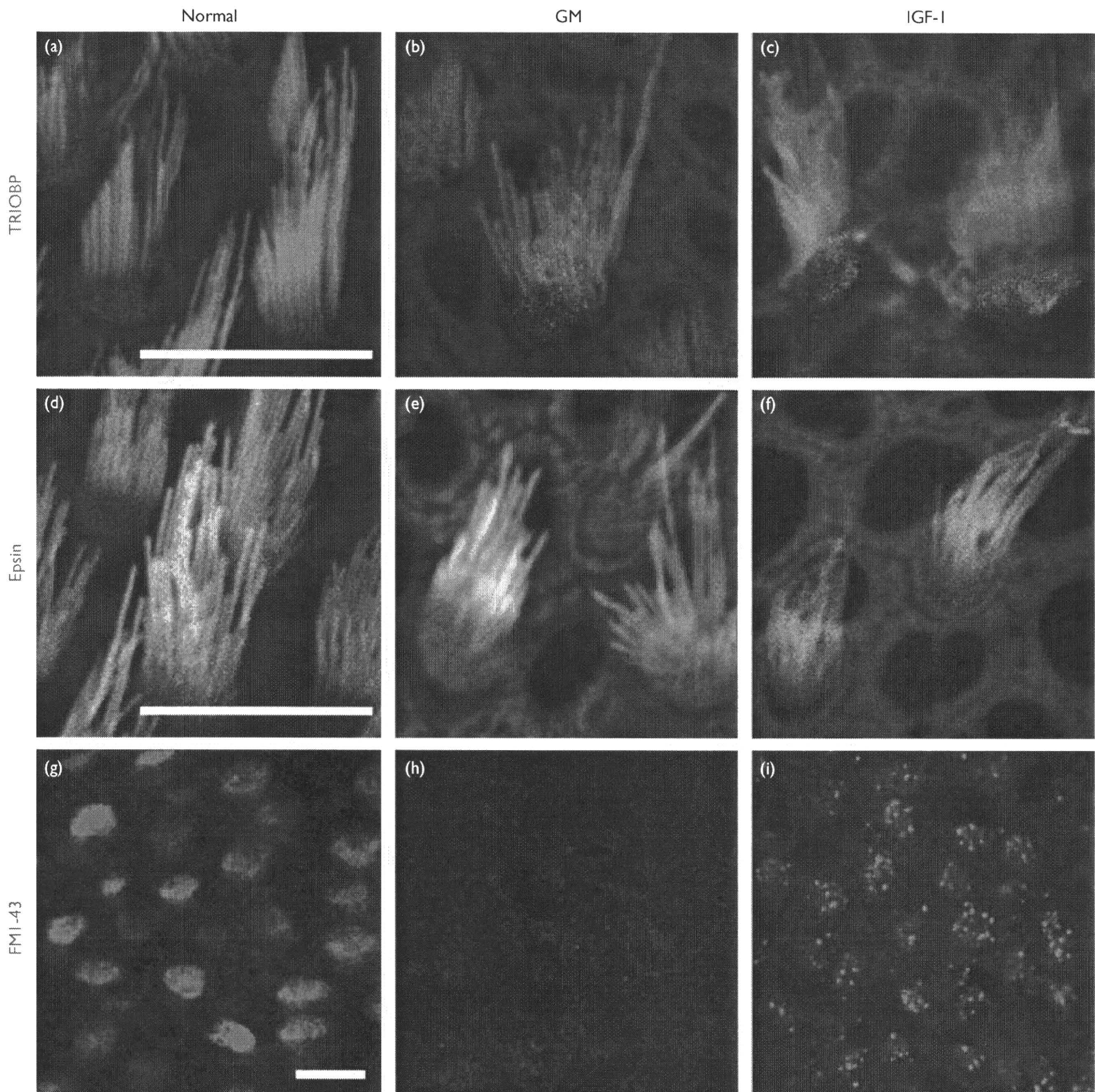


Surface images of the central regions in utricle explants immediately after dissection (a), incubation with 0.5 mM gentamicin (GM) for 24 h (b), following additional 48-h culture with no extra insulin-like growth factor 1 (IGF-1) (c) or with 1.0 μg/ml IGF-1 (d). Scale bar in (d) represents 10 μm for (a–d). In (e), grey squares represent the central regions in a sensory epithelium, and shaded portions show the peripheral regions. Scale bar represents 100 μm. Quantitative analyses (f) revealed severe loss of hair cells after 24-h incubation with GM in comparison with controls, which were immediately after dissection. Significant increases in hair cell numbers (* $P < 0.05$) were found in explants treated with 0.1 or 1.0 μg/ml IGF-1 in both central and peripheral regions.

Hair bundle of surviving hair cells

In normal utricles, TRIOBP was distributed in the rootlet of hair bundles (Fig. 2a), and colocalization of espin and F-actin was also identified (Fig. 2d). In utricles, immediately after gentamicin exposure, the expression of TRIOBP was still observed in the rootlet of hair bundles (Fig. 2b). All surviving hair cells exhibited espin expression in hair bundles in a similar distribution to normal hair cells (Fig. 2e). In utricles treated with 1.0 μg/ml IGF-1, the hair bundles of surviving hair cells exhibited a similar distribution of TRIOBP (Fig. 2c) to utricles immediately after dissection or after gentamicin exposure. The

Fig. 2



Expression of trio-binding protein (TRIOBP) and espin in hair bundles of hair cells and FM1-43 labeling of functional hair cells. Green fluorescence in (a–f) shows F-actin labeling with phalloidin. TROBP expression (red fluorescence in a–c) was found in the rootlet of hair bundles in explants immediately after dissection (Normal), incubation with 0.5 mM gentamicin for 24 h (GM), and following additional 48-h culture with 1.0 μg/ml insulin-like growth factor 1 (IGF-1). Espin (red fluorescence in d–f) colocalized with F-actin in hair bundle in each condition. In normal (g) and IGF-1 treated explants (i), FM1-43 labeling (red fluorescence in g–i) was identified, whereas virtually no labeling was found in explants immediately after incubation with GM (h). Scale bar in (a) represents 10 μm for (a–c), that in (d) represents 10 μm for (d–f), and that in (g) represents 10 μm for (g–i).

expression of espin in hair bundles was also observed in all surviving hair cells (Fig. 2f). These findings show that surviving hair cells treated with IGF-1 maintain the expression of TRIOBP and espin in hair bundles during the culture period after gentamicin incubation, suggesting that IGF-1 contributes to the protection of vestibular hair cell functionality.

FM1-43 dye is frequently used to evaluate MET channels in hair cells, and clearly labeled the hair cells in normal utricles in this study (Fig. 2g). In contrast, utricles immediately after gentamicin exposure showed no FM1-43 labeling in surviving hair cells (Fig. 2h), which might have been because of the blockage of MET channels by gentamicin [16,17]. Following the additional 48-h culture with 1.0 μg/ml IGF-1, the FM1-43 labeling in surviving hair cells recovered (Fig. 2i). Together with TRIOBP and espin immunohistochemistry findings, these results suggest that the surviving hair cells that were rescued by IGF-1 application retained their functionality.

Discussion

IGF-1 is a mitogenic peptide that plays essential roles in the regulation of the growth and development of the inner ear [9]. Earlier, we showed the efficacy of local IGF-1 application on the round-window membrane using gelatin hydrogels for the protection of auditory hair cells against damage induced by noise [12,18] or ischemia reperfusion [19] in animal models. On the basis of these findings, we performed a prospective clinical trial, which indicated the clinical efficacy of local IGF-1 application using gelatin hydrogels for sudden sensorineural hearing loss that was resistant to systemic glucocorticoid treatment [20].

Local IGF-1 treatment could be effective for peripheral vestibular diseases that involve hair cell loss. Unlike cochlear hair cells, mammalian vestibular hair cells have the capacity for regeneration [1–5], and IGF-1 could contribute to this [7,8], and to the protection of vestibular hair cells [11,21]. The IGF-1 concentrations used in this study were adjusted to match the concentrations in the perilymph that can be achieved using local IGF-1 application onto the round-window membrane with gelatin hydrogels [12]. In addition, IGF-1 application was initiated after gentamicin intoxication in this study to reflect clinical situations.

Quantitative analyses of the numbers of surviving hair cells showed that 24-h exposure to 0.5 mM gentamicin caused extensive loss of hair cells in explant cultures of neonate mouse utricles. An additional 48-h culture with neither gentamicin nor IGF-1 resulted in extended hair cell loss in utricle cultures. However, IGF-1 application at a concentration of 0.1 or 1.0 μg/ml efficiently rescued hair cells from such postexposure effects of gentamicin. Notably, 1.0 μg/ml IGF-1 largely inhibited hair-cell loss because of these effects, suggesting that local IGF-1 application using gelatin hydrogels could reasonably be expected to protect vestibular hair cells *in vivo*.

In contrast, no significant increase in hair cell numbers was induced by IGF-1 application in this study, indicating that few or no hair cells were newly produced in utricle explants during the observation period. Recently, Kawamoto *et al.* [5] investigated the capacity of mouse utricles for hair cell regeneration after gentamicin treatment *in vivo*, and found that new hair cells appeared 3 weeks after gentamicin treatment. Taura *et al.* [4] showed the emergence of new hair bundles 13 days after gentamicin intoxication in explant cultures of rat utricles. The culture period used in this study might be too short for evaluating the regeneration of hair bundles.

This study examined the functionality of surviving hair cells. Morphologically, the hair bundles in surviving hair cells well maintained the expression patterns of TRIOBP and espin. In addition, FM1-43 labeling was also observed in utricles that were treated with IGF-1, indicating the maintenance of hair cell function. These findings strongly suggest that hair cells rescued by IGF-1 treatment retain their functionality.

Conclusion

These findings suggest that IGF-1 has the ability to protect vestibular hair cells against aminoglycoside toxicity at concentrations that are close to in-vivo conditions, even after ototoxic insults. This encourages us to investigate protective effects of IGF-1 for vestibular hair cells *in vivo*.

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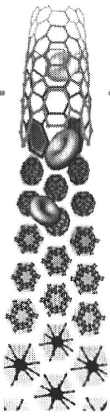

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Stealth-nanoparticle strategy for enhancing the efficacy of steroids in mice with noise-induced hearing loss

Aims: This study aimed to investigate the efficacy of encapsulating steroids, which is a primary choice for the treatment of sensorineural hearing loss, in polyethylene glycol-coated polylactic acid nanoparticles for drug delivery to the cochlea. **Materials & methods:** We prepared polyethylene glycol-coated polylactic acid nanoparticles encapsulating rhodamine or betamethasone phosphate (BP), and administered them systemically to CBA/N mice previously exposed to intense noise. We assessed nanoparticle distribution using rhodamine fluorescence, BP concentrations in tissues, nuclear translocation of glucocorticoid receptors and the function and histology of the mouse cochleae. **Results & conclusion:** Polyethylene glycol-coated polylactic acid nanoparticles delivered BP to cochleae over a sustained period, resulting in significant reductions in histological and functional damage to cochleae and indicating the potential therapeutic benefits of these nanoparticles for enhancing the delivery of BP in acute sensorineural hearing loss.

KEYWORDS: cochlea ▪ drug delivery ▪ glucocorticoid ▪ hair cell ▪ hearing impairment ▪ noise trauma

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Sensorineural hearing loss (SNHL) is one of the commonest disabilities, for which we currently have limited therapeutic options. Many investigations have examined novel therapeutic strategies for SNHL, and have identified several agents with therapeutic activity in experimental SNHL. However, for chronic or slowly progressive SNHL, therapeutic options are limited to hearing aids and cochlear implants. Acute or progressive SNHL has a considerable impact upon an individual's quality of life. For acute SNHL, the use of systemic steroids has been a primary therapeutic choice; however, no significant hearing recovery is achieved in approximately half of the patients treated [1,2]. In addition, there is no evidence that explains the mechanism for the efficacy of systemic steroids in acute SNHL [3–5]. Recently, several experimental studies have demonstrated the presence of glucocorticoid receptors in the cochlea [6,7], and some clinical trials have shown the efficacy of local steroid application in treating acute SNHL [8,9]. These results showed that therapeutic targets for steroids are present in the cochlea, suggesting the potential importance of targeted, sustained delivery of steroids in the ear.

Recent advances in drug-delivery systems have produced several techniques that can be applied to the treatment of the inner ear. Encapsulating bioactive molecules in nanoparticles made from biodegradable polymers, such as polylactic acid (PLA) and polylactic/glycolic acid (PLGA), allows the sustained release of bioactive molecules in a controlled manner [10]. Previously, we examined the potential of systemically applied PLGA/PLA nanoparticles for delivering drugs to the cochlea, but found that these nanoparticles did not significantly target the drugs, and that most of the PLGA/PLA nanoparticles were metabolized in the liver [11]. Recently, stealth nanoparticles, in which PLA is coated with polyethylene glycol (PEG), have been developed. PEG-coated PLA nanoparticles overcome the problems associated with conventional nanoparticles by reducing opsonization and preventing interactions with the reticuloendothelial system in the liver or spleen [12–14]. This study aimed to investigate the efficacy of PEG-coated PLA nanoparticles, stealth nanoparticles, for delivering steroids to the cochlea and their therapeutic potential for the treatment of noise-induced hearing loss. We systemically administered PEG-coated PLA nanoparticles containing betamethasone phosphate (BP) or the red fluorescent dye, rhodamine B, to mice that had been exposed to intense noise, and examined the distribution of rhodamine fluorescence, the BP concentrations in tissues, and the function and histology of the mouse cochleae.

Materials & methods

Experimental animals

Male CBA/N mice, 4–6 weeks old and weighing 18–22 g, were purchased from Japan SLC Inc. (Hamamatsu, Japan). The Animal Research

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Committee at the Graduate School of Medicine, Kyoto University, Japan, approved all experimental protocols, and animal care was supervised by the Institute of Laboratory Animals at the Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with NIH guidelines for the care and use of laboratory animals.

PEG-coated PLA nanoparticles

Poly-D,L-lactic acid was purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). PEG-block-PLA was synthesized by a ring-opening polymerization of D,L-lactide, which had been purified by recrystallization in ethyl acetate, in the presence of monomethoxy-PEG (MW: 5580; NOF Co., Tokyo, Japan).

Polyethylene glycol-coated PLA nanoparticles were prepared by the oil-in-water solvent diffusion method as described elsewhere [14,15]. In brief, a mixture of 7.8 mg PEG–PLA and 42.2 mg PLA was dissolved in 1 ml of acetone. To this solution, 500 µl of an acetone solution of DEA (15 mg/ml), followed by 68 µl of an aqueous solution of zinc chloride (1 M; pH 1.9), and then 28 µl of an aqueous solution of BP (350 mg/ml) or 50 µl ethanol containing rhodamine B (20 mg/ml, Sigma-Aldrich, MO, USA) were added; the mixture was then allowed to stand for 30 min at room temperature. A 26-gauge needle was used to add the mixture drop-wise to distilled water stirred at 1000 rpm, and 0.5 M citrate (Wako Pure Chemicals) and Tween 80 (Wako Pure Chemicals) were added immediately to chelate BP–zinc complexes. Finally, the PEG-coated PLA nanoparticles were purified and concentrated by ultrafiltration (Centriprep YM-50; Millipore Corporation, Bedford, MA, USA). We prepared PEG-coated PLA nanoparticles encapsulating rhodamine B (stealth-nano–rhodamine) with an average diameter of 120 nm and a loading efficiency of rhodamine in the nanoparticles of 0.1% (wt/wt) and nanoparticles encapsulating BP (stealth-nano–BP) with an average diameter of 116 nm and a loading efficiency of BP in the nanoparticles of 10.7% (wt/wt).

Noise exposure & drug administration

Baseline auditory brain stem response (ABR) thresholds were measured within 7 days of the mice being exposed to the initial noise, which was 8 kHz octave band noise at a sound pressure level (SPL) of 120 dB for 2 h. This was carried out in a ventilated sound exposure chamber with the mice under general anesthesia, using 10 mg/kg midazolam, 37.5 mg/kg medetominide and 0.5 mg/kg butorphanol tartrate. Sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure stimulus uniformity.

After exposure to traumatic noise, the animals received intravenous injections into the tail vein of 200 µl of a 3 µg/ml solution of rhodamine B (n = 6), 200 µl stealth-nano–rhodamine solution, containing the same total mass of rhodamine B as administered to the mice given rhodamine B alone (n = 6), 200 µl of a 250 µg/ml BP solution (n = 12), 200 µl stealth-nano–BP solution, containing the same total mass of BP as administered to the mice given BP alone (n = 12), or physiological saline (n = 4).

Rhodamine distribution

The animals that had been treated with rhodamine B or stealth-nano–rhodamine were sacrificed 2 or 24 h after drug administration by asphyxiation in CO_2 for 15 min. Cochleae were harvested from the mice (n = 4 for each group), immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 4 h, and then decalcified in 0.1 M ethylenediaminetetraacetic acid (EDTA) in PBS for 4 days at 4°C. The liver was dissected from each animal and processed in the same way as cochlear specimens. Tissue specimens were cut into 10-µm thick sections. Two mid-modiolus sections from the cochlea of each animal and two randomly selected sections from the liver were used for histological analysis. Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen, Carlsbad, CA, USA). The specimens were mounted in Vector Shield (Vector Laboratories Inc., Burlingame, CA, USA), and viewed with a fluorescence microscope (BX50, Olympus, Tokyo, Japan).

Betamethasone concentration

Animals that had been given BP or stealth-nano–BP were assessed for BP concentrations in the cochlea and liver. The cochleae and livers were collected under general anesthesia 1, 12 or 24 h after drug administration (n = 4 for each time point). Immediately after dissection, specimens were homogenized in PBS, and then stored at -80°C. The tissue concentration of BP was quantified using a time-resolved fluoroimmunoassay kit for betamethasone (Shionogi & Co., Ltd., Osaka, Japan) as described elsewhere [10]. The detection limit for BP in this assay was 0.01 µg/ml.

Immunohistochemistry for glucocorticoid receptor

Animals received an intravenous injection of saline, BP or stealth-nano–BP immediately after noise exposure (n = 4 for each condition). Cochleae were harvested from the mice 24 h after noise exposure, fixed with 4% PFA for 1 h and prepared as frozen sections in 10-μm thickness. Three mid-modiolar sections separated by 30 μm from each cochlea were provided for immunohistochemistry for glucocorticoid receptor (GR). A polyclonal rabbit anti-GR antibody (5 mg/ml; Affinity Bioreagents, Carlsbad, CA, USA) was used as the primary antibody, and Alexa-594-conjugated antirabbit goat IgG (1:500; Invitrogen, Carlsbad, CA, USA) was used as the secondary antibody. Nuclear staining with DAPI was performed. For quantitative analyses, to quantify the nuclear translocation of GR from the cytoplasm in hair cells, the numbers of inner hair cells (IHCs) and outer hair cells (OHCs) with immunoreactivity for GR in the nuclei were counted using the mid–basal portion of cochleae, respectively. We defined GR translocation rate (%) as:

$$GR\,translocation\,rate\,(\%) = \frac{Number\,of\,GR\,translocated\,hair\,cells}{Number\,of\,counted\,hair\,cells} \times 100$$

The average value in three sections from each cochlea was used as the data for the animal.

Auditory function

Auditory function was assessed using ABR recordings. ABR thresholds were measured at frequencies of 8, 16 and 32 kHz 5 min after drug administration and again after 4, 7 and 14 days. Animals that had been given BP (n = 4), stealth-nano–BP (n = 4) or saline (n = 4) were anesthetized using midazolam, medetominide and butorphanol tartrate, and kept warm with a heating pad. Acoustic stimuli were generated and the evoked potentials were recorded using a PowerLab/4sp (AD Instruments, Castle Hill, Australia). Acoustic stimuli, consisting of tone-burst stimuli (for 0.1 ms with a cos 2 rise/fall and 1 ms plateau), were delivered to one ear through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) connected to a funnel fitted into the external auditory meatus. To record bioelectrical potentials, stainless steel needle electrodes were inserted subdermally at the vertex (ground), and ventrolateral (active) and contralateral (reference) to the ear being monitored. Stimuli were calibrated against a quarter-inch free-field microphone (ACO-7016; ACO Pacific, Belmont, CA, USA) connected to an oscilloscope (DS-8812 DS-538; Iwatsu Electric, Tokyo, Japan) or a sound level meter (LA-5111; Ono Sokki, Yokohama, Japan). Responses between the vertex and mastoid subdermal electrodes were amplified using a digital amplifier (MA2; Tucker-Davis Technologies, Alachua, FL, USA). Thresholds were determined from a set of responses at varying intensities at 5 dB SPL intervals and electrical signals were averaged over 1024 repetitions. Threshold measurements at each frequency were repeated at least twice.

Hair cell protection

After ABR measurements had been made 14 days after drug administration, the animals were anesthetized with lethal doses of anesthetics and given intracochlear perfusions of 4% paraformaldehyde in PBS. The temporal bones were excised and decalcified by immersion in the same fixative at 4°C for 4 h, and then in 0.1 M EDTA in PBS. Hair cell damage was evaluated in whole mounts from groups of mice (n = 4) treated with BP, stealth-nano–BP and saline. F-actin was stained with 3 μg/ml fluorescein isothiocyanate-conjugated phalloidin (Sigma-Aldrich) to identify cochlear hair cells in the whole mounts. These whole mounts were inspected using a Leica TCS SPE confocal microscope (Leica Microsystems Inc., Wetzlar, Germany) and the number of remaining IHCs and OHCs were counted. We defined apical as the region 20–40% from the apex and basal as the region 60–80% from the apex. The numbers of IHCs and OHCs were counted in a 0.2-mm-long strip in the apical and the basal portions of each cochlea.

Statistical analyses

All statistical analyses used GraphPad Prisms® (GraphPad Software, La Jolla, CA, USA). The statistical assessment of BP concentrations and ABR thresholds used two-way analysis of variance (ANOVA) followed by multiple t-tests with Bonferroni corrections. The numbers of IHCs and OHCs, and GR translocation rates were assessed using one-way ANOVA followed by Tukey's multiple comparison tests. Any p-values below 0.05 were considered significant. Values were expressed as the means ± standard deviation.

Results

Rhodamine distribution

After administering free rhodamine B to mice, a few red dots of rhodamine fluorescence were observed in the liver after 15 min, numerous red fluorescence dots were found after 2 h,

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.

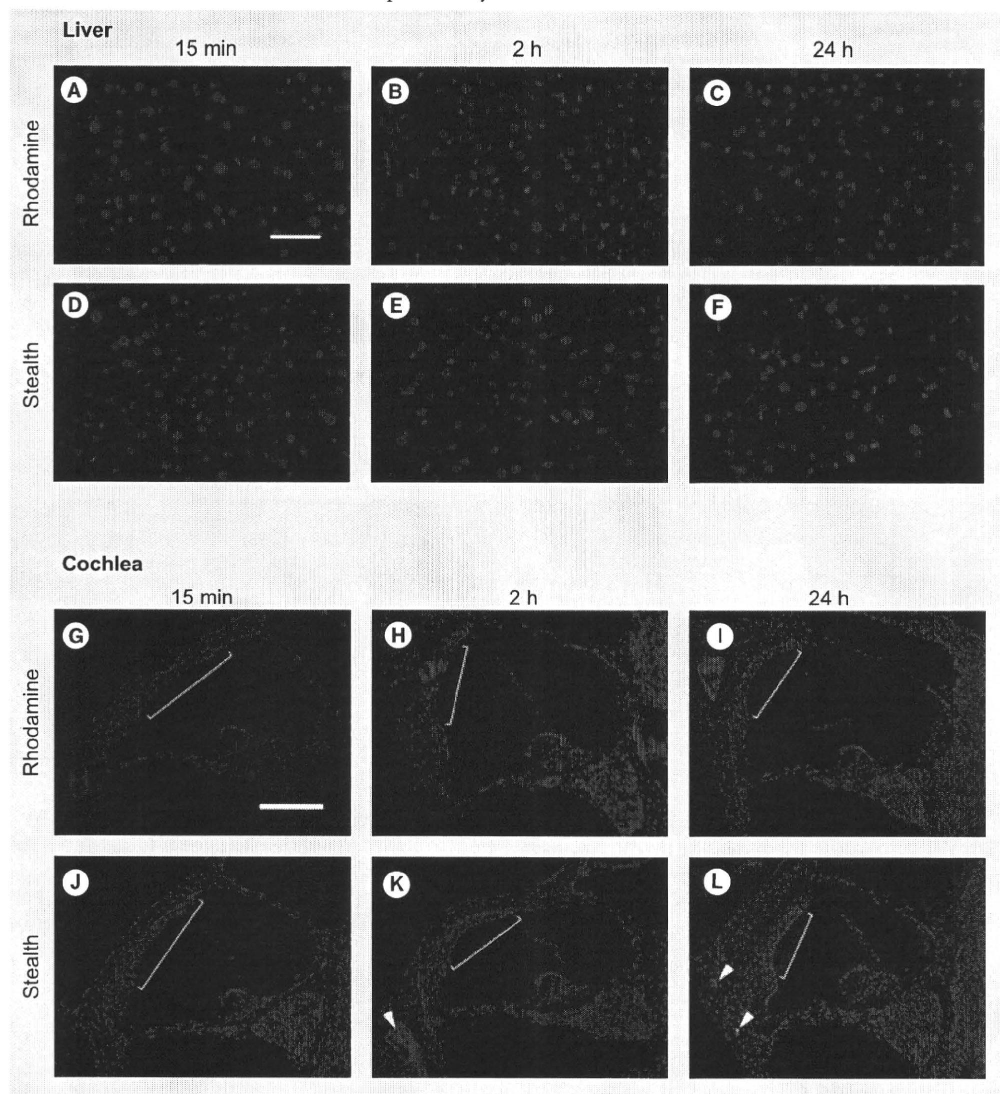

Figure 1. Rhodamine distribution in the liver and cochlea. In the liver in mice after application of free rhodamine **(A–C)**, numerous rhodamine fluorescence dots (red) were observed 2 h after application. In mice treated with stealth-nano–rhodamine **(D–F)**, rhodamine fluorescence was visible at each 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.

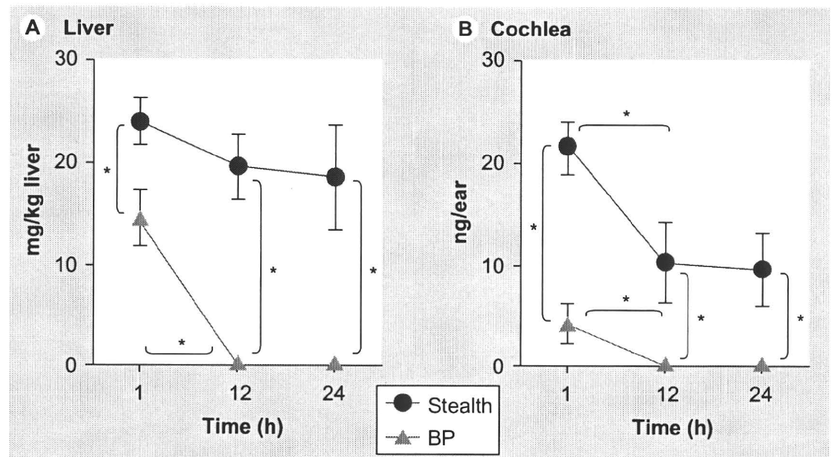

Figure 2. Betamethasone concentrations in the liver and cochlea. Tissue concentrations of betamethasone in the liver **(A)** and the cochlea **(B)** were determined at 1, 12 and 24 h after systemic application of free BP or stealth-nano–BP. Betamethasone concentrations after stealth-nano–BP application 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

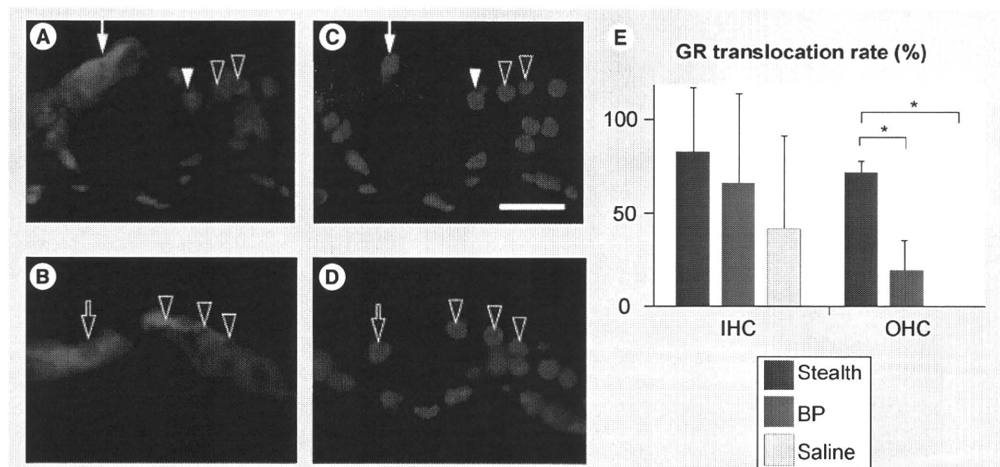

Figure 3. Nuclear translocation of glucocorticoid receptors in cochlear hair cells. Immunostaining for GRs demonstrates the cellular location of GRs in hair cells **(A & B)** and the nuclear locations are demonstrated by DAPI staining **(C & D)**. Arrows indicate IHCs and arrowheads indicate OHCs **(A & 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

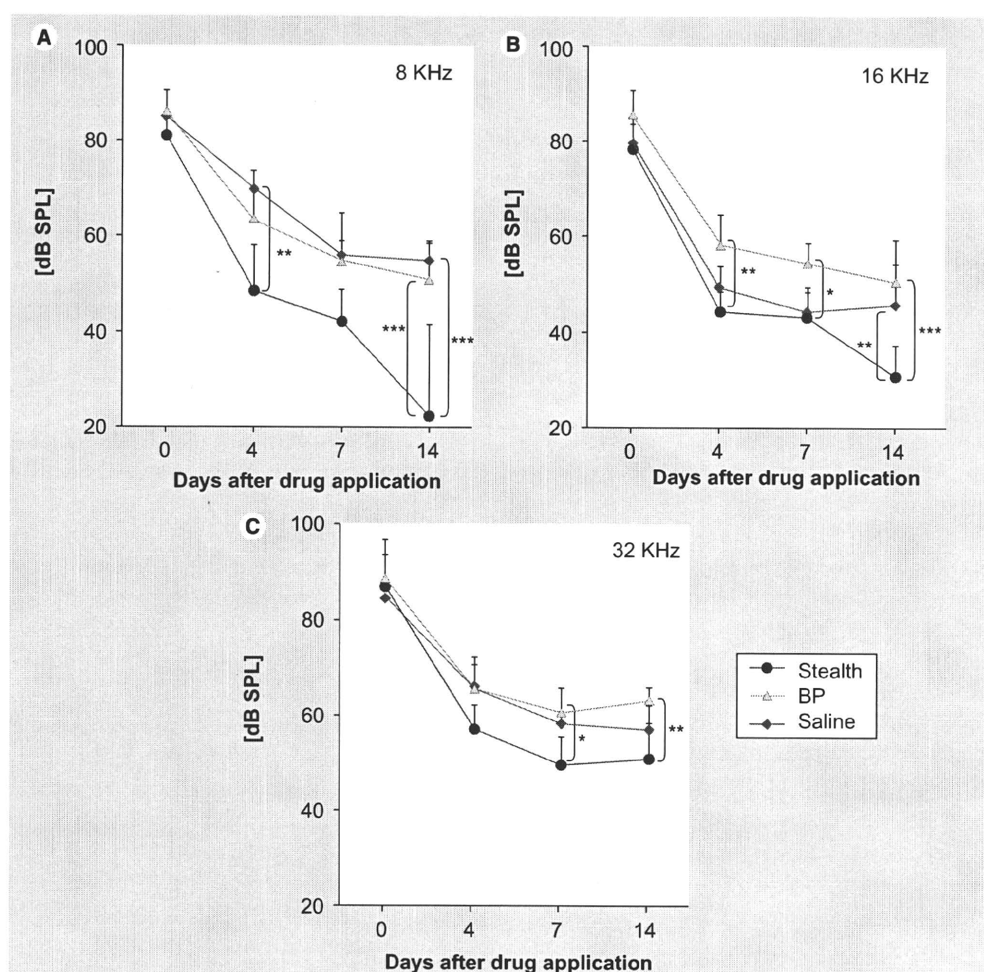

Figure 4. Auditory brain stem response thresholds following saline, free betamethasone phosphate or stealth-nano–betamethasone phosphate. Changes in ABR thresholds at **(A)** 8, **(B)** 16 and **(C)** 32 kHz were determined immediately after the treatment, and 4, 7 and 14 days after treatment. Drug treatment 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.

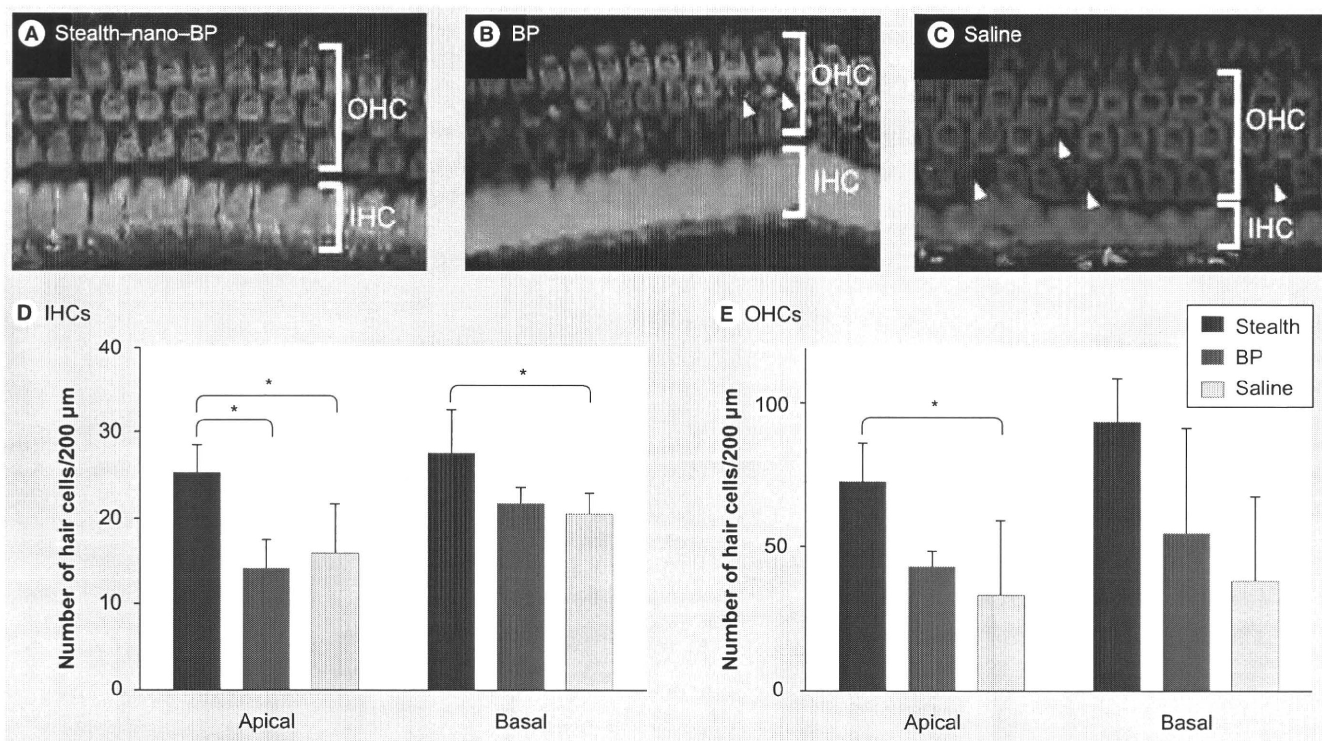

Figure 5. Stealth-nano–betamethasone phosphate attenuates noise-induced damage on cochlear hair cells. Phalloindin staining demonstrated well-maintained IHC and OHC in cochlear specimens of the basal portion treated with stealth-nano–BP **(A)**, while in cochleae treated with free BP **(B)** or saline **(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 fourfold 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

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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.