Table 1
E-prostanoid receptors; signaling, second messenger, effect on vasculature, effect on neurons and possible actions in cochleae. EP1: E-prostanoid receptor 1, EP2: E-prostanoid receptor 2, EP3: E-prostanoid receptor 3, EP4: E-prostanoid receptor 4, PLC: phospholipase C, IP3: inositol-1,4,5-trisphosphate, AC: adenylyl cyclase, cAMP: cyclic adenosine monophosphate.

	Signaling	Second messenger	Vasculature	Neuron	Possible actions in cochleae
EP1	PLC/IP3	Increase IP3, Ca^{2+}	Vasoconstriction	Toxic	Decrease blood flow Ototoxic
EP2	AC/cAMP	Increase cAMP	Vasodilation	Protective in acute injury Toxic in chronic inflammation	Increase blood flow Protective
EP3	AC/cAMP	Decrease cAMP	Vasoconstriction	Toxic	Decrease blood flow Ototoxic
EP4	AC/cAMP	Increase cAMP	Vasodilation	Protective	Increase blood flow Protective

Interestingly, biological actions of some EPs are contradictory to those of other EPs. In hemodynamics, EP1 induces vasoconstriction in the peripheral vasculature, while EP2 and EP4 signaling induces vasodilation. The activation of EP2 and EP4 increases intracellular cAMP levels, while EP3, which is regarded as an inhibitory receptor, decreases cAMP formation. Recent studies with gene targeted mice, in which single EP was deleted, gave new insights on the various actions of PGE2 (Sugimoto and Narumiya, 2007), although their auditory function has not been examined in detail. Therefore, the development of subtype-specific EP agonists/antagonists will boost pharmacological actions of PGE1 or PGE2.

4. EP signaling in the central nervous system

In the central nervous system, some subtype-specific EP signaling events mediate toxic effects but others appear to mediate paradoxically protective effects. In addition, protective or toxic effects of a particular EP signaling pathway can differ depending on the context of cerebral injury, for example, in excitotoxicity/hypoxia paradigms versus inflammatory-mediated secondary neurotoxicity (Andreasson, 2010).

Regarding pathological roles, in general, EP1 signaling plays neurotoxic roles. In N-methyl-D-asparate (NMDA) excitotoxicity, increased calcium ion influx through NMDA receptors is a primary mechanism for neurotoxicity. The activation of EP1 causes an increase of intracellular calcium ion levels, which enhances NMDA neurotoxicity. Actually, the normalization of intracellular calcium ion levels with EP1 blockade or genetic deletion attenuates NMDA neurotoxicity (Ahmad et al., 2006; Kawano et al., 2006). EP1 is also known to induce vasoconstriction in the peripheral vasculature, indicating that EP1 signaling is involved in ischemia-induced brain injury (Saleem et al., 2007). In addition, direct effects of EP1-mediated neurotoxicity have been demonstrated in models of neurodegenerative disease including Parkinson's diseases (Carrasco et al., 2007).

In contrast to EP1, EP2 signaling plays a role in neuroprotection against acute neurotoxic events. Direct actions of the EP2 in neurons mediate pro-survival and anti-apoptotic functions (Jiang et al., 2010). Activation of the EP2 is neuroprotective in paradigms of NMDA toxicity, which is mediated by protein kinase A (PKA) activation via cAMP signaling (Liu et al., 2005; McCullough et al., 2004; Mori et al., 2009). However, stimulation with PGE2 has no effect on NMDA toxicity, which is consistent with the idea of toxic EP1 activation and protective EP2 activities. In setting of cerebral ischemia, EP1 activation decreases blood flow, whereas EP2 activation increases blood flow by vasodilation. Further complexity is emerging in EP2 effects in the central nervous system. Effects of the EP2 activation differ depending on the context of brain injury. In acute toxicity, EP2 signaling is neuroprotective, while in chronic inflammation EP2 signaling shows pro-inflammatory neurotoxic effects. In lipopolysaccharide (LPS)-mediated toxicity, microglial EP2 activation induces generation of reactive oxygen species (ROS) (Montine et al., 2002). In short, neuronal EP2 signaling mediates neuroprotection in acute injury models via cAMP-PKA signaling, while microglial EP2 signaling leads to secondary neurotoxicity via ROS generation.

The EP4 receptor is positively coupled to cAMP production similarly to the EP2 receptor. EP4 signaling functions to confer neuroprotection in excitotoxic (Ahmad et al., 2005) and ischemia (Li et al., 2008) models, likely EP2 signaling. The EP4 receptor has also been demonstrated to modulate the inflammatory response. As described above, EP2 signaling is neurotoxic in models of chronic inflammation, while EP4 signaling mediates an anti-inflammatory effect in some contexts. EP4 signaling suppresses brain inflammation in models of LPS-induced inflammation (Shi et al., 2010) and multiple sclerosis (Esaki et al., 2010). Pharmacological inhibition of EP4 signaling reverses attenuation of spinal cord injury by PGE1 analog (Umemura et al., 2010).

The EP3 receptor is regarded as an inhibitory receptor, because of its effect decreasing cAMP production. Hence, EP3 signaling may be neurotoxic by its inhibitory effects against EP2 and/or EP4 neuroprotective effects. In fact, an EP3 antagonist attenuates and an EP3 agonist augments glutamate-induced excitotoxicity in hypocampal neurons (Ikeda-Matsuo et al., 2010). In addition, local application of EP3 agonist worsens infarct volume in a model of transient focal ischemia (Ahmad et al., 2007). However, in models of neurological diseases, the function of the EP3 receptor so far is not firmly defined. The murine EP3 receptor consists of three distinct isoforms derived by alternative splicing of the carboxy terminus; these isoforms differ in downstream signaling pathways, desensitization, and constitutive activity (Bilson et al., 2004; Hasegawa et al., 2000). Thus, genetic deletion of EP3 results in total ablation of all three isoforms whereas administration of EP3 agonist may activate one or more isoforms depending on the cellular expression patterns of the EP3 isoforms.

These findings indicate that the development of subtype-specific EP agonists or antagonists is necessary for establishment of novel therapeutic approaches for neuronal degeneration associated with COX-PGE2 signaling, and emphasize importance of selective stimulation or inhibition of specific EP signaling depending on contexts of neuronal diseases.

5. COX-PGE2 signaling in cochleae

PGs are generated by oxidation of AAs by cyclooxygenase-1 (COX-1) and/or -2 (COX-2) and act in an autocrine and paracrine fashion. The presence of COX-1 and COX-2 in the cochlea was demonstrated using immunohistochemistry. The constitutive expression of COX-1 was found in various types of cochlear cells, while COX-2 expression in normal cochleae is still controversial (Stjernschantz et al., 2004; Ziegler et al., 2004). Immunoreaction for COX-1 was found in the stria vascularis, spiral ligament, spiral limbus, spiral ganglion and organ of Corti. These findings indicate the constitutive formation of PGs in the cochlea. In vitro radioimmunoassay using cochlear tissue supports this hypothesis of constitutive PG formation in cochleae, which showed the synthesis of PGD2, PGF2α and most abundantly PGE2 in cochleae (Kawata

et al., 1988). Altogether, PGs, most abundantly PGE2, is constitutively generated in the cochlea.

In models of noise-induced hearing loss as a pathophysiological condition of cochleae, alterations in expression of COX-1 and COX-2 and effects of several COX inhibitors were investigated. Alterations in COX-1 or COX-2 expression were examined using guinea pig exposed to 8 kHz tone bursts at 70 or 90 dB sound pressure level (SPL) for 60 min, which is moderate intensity and induces limited irreversible degeneration in cochleae (Heinrich et al., 2006, 2010). Both COX-1 and COX-2 expression in the organ of Corti decreased after noise exposure. A slight increase of COX-1 expression was found only in the nerve fibers of the osseous spiral lamina. No remarkable alteration in COX-2 expression was found in other cochlear constructions. These findings indicate that noise-induced damage causes a decrease of COX enzymes in the organ of Corti, and no obvious up-regulation of COX enzymes in cochleae. Effects of several COX inhibitors were tested in mice exposed to an intense noise, a 4 kHz pure tone of 128 dB SPL for 240 min (Hoshino et al., 2008). Indomethacine, a COX-1 inhibitor, showed significant hair cell protection in the apical portion of cochleae and attenuation of noise-induced hearing loss at low frequencies. Three COX-2 inhibitors exhibited no protective effects against noise trauma. Altogether, excessive formation of PGs including PGE2 may not occur in cochleae after noise exposure.

6. Expression of EPs in cochleae

Biological effects of PGE2 on cochleae may largely depend on the distributions and amounts of EPs in cochleae. Previously, the expression of EP1 and EP3 was investigated in normal guinea pig cochleae using immunohistochemistry (Stjernschantz et al., 2004). EP1 expression was found in the stria vascularis, spiral ligament, spiral ganglion, and organ of Corti. The expression of EP3 exhibited a similar distribution to that of EP1. Recently, the expression of EP2 and EP4 in mouse cochleae was examined by immunohistochemistry and reverse transcription polymerase chain reaction (Hori et al., 2009, 2010). The expression of EP2 and EP4 was found in the stria vascularis, spiral ligament, spiral ganglion and organ of Corti similarly to that of EP1 or EP3. These findings showed the presence of four subtypes of PGE2 receptors, EP1–4, in cochlear structures that play important roles in auditory function. Hence, activation or inhibition of specific EP signaling is important to boost therapeutic potential of PGE2 signaling in cochleae, probably depending on contexts of cochlear diseases similarly to the central nervous system.

7. Possible role of EP signaling in the cochlear blood flow

PGE1 has long been used for the treatment of SSHL aiming to increase cochlear blood flow (Nakashima et al., 2003). Expression of EP1–4 in the stria vascularis of cochleae suggests an involvement of EP signaling in the regulation of the cochlear blood flow. In general, EP2 and EP4 mediate vasodilatation, and EP1 and EP3 mediate vasoconstriction in many tissues (Legler et al., 2010). Animal experiments demonstrated an increase of cochlear blood by local application of PGE1 or PGE2 using a laser Doppler flowmeter (Umemura and Nakashima, 1997; Rhee et al., 1999; Tominaga et al., 2006), which indicates that EP2 and/or EP4 signaling dominantly works in the cochlear vasculatures rather than EP1 or EP3. In addition to direct actions of EP2 and/or EP4 activation, vasodilatation mediated by vascular endothelial growth factor (VEGF) could be involved in mechanisms for vasodilatation in cochleae by PGE2 signaling. Activation of EP2 and EP4 signaling in cochleae induces the formation of VEGF, which has effects of vasodilatation (Huang et al., 2010), in cochleae (Hori et al., 2010).

On the other hand, noise trauma is known to reduce cochlear blood flow (Miller et al., 2003; Yamane et al., 1991). Studies on expression of COX enzymes in cochleae indicated down-regulation of COX enzymes following noise exposure (Heinrich et al., 2006, 2010), which can lead to a decrease of endogenous PGE2 levels in cochleae. Therefore, down-regulation of PGE2 signaling, particularly EP2 and EP4 signaling, could be involved in mechanisms for reduction of cochlear blood flow due to noise exposure.

However, the localization of EPs in the stria vascularis was demonstrated in a light microscopic level. It is, therefore, unclear which type of cells in the stria vascularis, marginal, intermediate or basal cells, express which subtype of EPs. In addition, alteration in expression of EPs in the stria vascularis following noise exposure has not been investigated. Further studies are required to determine distinct roles of EP signaling in the regulation of cochlear blood flow.

8. Possible role of EP signaling in the cochlear sensory system

Previous studies have demonstrated an involvement of excitotoxicity in cochlear degeneration due to noise trauma (Duan et al., 2000; Hakuba et al., 2000; Puel et al., 1998). Excessive efflux of glutamate from inner hair cells causes degeneration in afferent nerve endings of spiral ganglion neurons following noise exposure. In the central nervous system, activation of EP2 or EP4 functions to confer neuroprotection (Ahmad et al., 2005; McCullough et al., 2004; Liu et al., 2005), while EP1 or EP3 signaling plays neurotoxic roles in models for excitotoxicity (Candelario-Jalil et al., 2005; Ahmad et al., 2006; Ikeda-Matsuo et al., 2010). Therefore, activation of EP2 or EP4 or inhibition of EP1 or EP3 was expected to protect cochlear sensory cells against noise trauma. Cochlear protection by local application of an EP4 agonist, ONO-AE1-329 (Ono Pharmaceutical, Co., Ltd., Osaka, Japan), was investigated using a guinea pig model of noise-induced hearing loss (Hori et al., 2009). In histology, we focused on cochlear outer hair cells, because they are susceptible for noise trauma. Local application of an EP4 agonist before or after noise exposure successfully attenuates noise-induced hearing loss and protects outer hair cells from noise trauma. Therefore, specific activation of EP4 signaling in cochleae may be an effective strategy for attenuation of cochlear damage associated with excitotoxicity. Since ischemic injury of cochleae also involves excitotoxicity (Hakuba et al., 1997; Pujol et al., 1992), specific activation of EP4 signaling might have protective effects on cochlear sensory cells against ischemia-induced damage similarly to the central nervous system (Li et al., 2008).

Activation of EP2 or EP4 can directly promote the survival of cochlear sensory cells via cAMP-mediated signaling pathways. EP2 and EP4 signaling is also known to act as neuroprotective via induction of growth factor formation (Chang et al., 2005; Takahashi et al., 1996). Since numerous studies demonstrated formation of VEGF by activation of EP2 or EP4 signaling in other systems (Chang et al., 2005; Inoue et al., 2002; Weiss et al., 2004; Bradbury et al., 2005; Jain et al., 2008), we examined VEGF formation in inner ears following local application of an EP2, ONO-AE1-259-01 or EP4 agonist, ONO-AE1-329 (both from Ono Pharmaceutical, Co., Ltd.), and the localization of VEGF and VEGF receptors in mouse cochleae (Hori et al., 2010). The levels of VEGF mRNA and protein increased after local EP2 or EP4 agonist application, showing VEGF formation in inner ears in response to EP2 or EP4 activation. Immunohistochemistry for VEGF indicates that VEGF is predominantly generated in the spiral ganglion neurons (Hori et al., 2010). The expression of VEGF receptors was found in the stria vascularis, spiral ligament, spiral ganglion and organ of

Corti. Previous studies have indicated that VEGF has protective effects on cochlear hair cells (Picciotti et al., 2006; Selivanova et al., 2007). Therefore, VEGF formation via EP4 activation may contribute to protection of outer hair cells against noise trauma by local EP4 agonist treatment.

9. Possible role of EP signaling in the cochlear immunity

PGE2 has been referred to as a classical pro-inflammatory mediator. The relevance of PGs during the promotion of inflammation is emphasized by the effectiveness of non-steroidal anti-inflammatory drugs (NSAIDs) acting as COX inhibitors (Simmons et al., 2004). However, the role of PGE2 in the regulation of immune responses is even more complex. Studies on knock-out mice deficient for individual EPs clearly revealed that PGE2 not only acts as a pro-inflammatory mediator, but also exerts anti-inflammatory responses (Sugimoto and Narumiya, 2007). In addition, recent investigations have demonstrated significant roles of PGE2 signaling, in particular EP2 and EP4 signaling, in innate and adaptive immune systems (Divangahi et al., 2010; Gagliardi et al., 2010; Mandapathil et al., 2010; Zhu et al., 2010). PGE2 is classically known as an immunosuppressant, because it inhibits helper T cell differentiation (Harris et al., 2002; Sugimoto and Narumiya, 2007). However, recent studies demonstrate that PGE2 can function as an immunoactivator via EP2 and EP4 under some conditions (Krause et al., 2009; Yao et al., 2009).

The innate immune system of the central nervous system is principally composed of microglia and astrocytes (Rothwell and Luheshi, 2000). Microglia play a key role in EP2-mediated neurotoxic effects in models of chronic inflammation (Jin et al., 2007; Liang et al., 2008; Montine et al., 2002). Activation of EP2 signaling in microglia induces ROS generation, which results in degeneration of neurons. Expression of the EP2 receptor is highly inducible in microglia of the cerebral cortex and hippocampus in the LPS model of innate immunity (Zhang and Rivest, 1999). Interestingly, EP4 signaling mediates opposite actions of EP2 signaling in the context of chronic inflammation in the central nervous system, although the EP4 receptor is positively coupled to cAMP production similarly to the EP2 receptor. EP4 signaling mediates an anti-inflammatory effect in models of chronic inflammation. EP4 signaling suppresses brain inflammation in models of LPS-induced inflammation (Shi et al., 2010) and multiple sclerosis (Esaki et al., 2010).

The immune system of the inner ear has not been fully understood. However, recent studies indicate importance of the spiral ligament in the immune reaction in the cochlea. Macrophages and/or microglia are constitutively present in the spiral ligament and accumulate in response to injury (Hirose et al., 2005; Okano et al., 2008; Sato et al., 2008). The spiral ligament fibrocytes produce cytokines in response to exposure to bacterial toxins (Moon et al., 2006, 2007) and noise exposure (Fujioka et al., 2006). EP signaling could mediate immune reactions in the spiral ligament, because of the presence of EPs in the spiral ligament (Hori et al., 2009, 2010; Stjernschantz et al., 2004).

However, roles of EP signaling in the spiral ligament and in models of chronic inflammation in cochleae have not been investigated. In future, responses of spiral ligament fibrocytes and cochlear microglia/macrophages for individual EP signalings should be examined to reveal roles of EP signaling in the regulation of immune responses in cochleae. Roles of EP2 signaling in models of cochlear chronic inflammation are particularly interesting, because EP2 activation confers neuroprotection in acute injury models, but mediates neurotoxicity in chronic inflammation models in the central nervous system.

10. Conclusions

Accumulating evidence for EPs indicate significant roles of individual EP signaling in neurodegenerative diseases. In the inner ear, PGs were paid particular attention as regulators of cochlear blood flow. In addition, PGE1 has long been used as a vasodilator for the treatment of SSHL in clinic. However, little is known about roles of EP signaling in the auditory function. Previous studies have demonstrated that PGE2 is constitutively produced in the cochlea and all EP subtypes are expressed in various types of cochlear cells, suggesting that EP signaling may play roles in physiology and pathophysiology of the cochlea. It is necessary to examine the auditory function in individual EP-deficient mice in normal and pathological conditions, which may bring new insights on roles of PGE2 signaling in cochleae. In addition, various antagonists or agonists for individual EPs are available, which may be useful tools for elucidation of roles of EP signaling in the auditory function. We believe that future investigations on EP signaling in cochleae must lead to the development of novel therapeutic options for SNHL.

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The Laryngoscope

Adipose Tissue-Derived Stromal Cells Protect Hair Cells From Aminoglycoside

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Background: Previous studies have demonstrated the therapeutic paracrine activity of adipose tissue-derived stromal cells (ADSCs). This study aimed to examine the ADSC potential for protecting auditory hair cells from aminoglycoside toxicity via paracrine of multiple growth factors and cytokines.

Study Design: Experimental study.

Methods: We assessed hair cell protection from neomycin toxicity by ADSC-derived factors using an explant culture system, in which cochlear explants and ADSCs were separated by a culture mesh insert to avoid direct contact. We measured the levels of growth factors and cytokines in ADSC culture media using an enzyme-linked immunosorbent assay (ELISA).

Results: Neomycin induced severe degeneration of auditory hair cells in cochlear explants, but co-culture with ADSCs significantly increased the number of surviving hair cells in explants. ELISA analysis revealed that ADSCs secreted insulin-like growth factor-1, nerve growth factor, vascular epithelial growth factor, transforming growth factor β1, monocyte chemotactic protein-1, and most prominently hepatocyte growth factor.

Conclusions: These findings demonstrate that ADSCs have the capacity to protect auditory hair cells, and can be a useful strategy to develop therapy for deafness in the clinic. The multiple paracrine growth factors and cytokines secreted by ADSCs might be involved in this effect.

Key Words: Adipose tissue-derived stromal cell, protection, cochlea, cell therapy.

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INTRODUCTION

Sensorineural hearing loss (SNHL) is one of the most common disabilities, occurring in 60% of individuals aged over 65 years[1] and in 1/1,000-2,000 newborns.[2] However, therapeutic options for SNHL are limited to hearing aids and cochlear implants for chronic SNHL. For acute SNHL, the most acceptable therapeutic option is systemic glucocorticoid application, but approximately 30% of patients exhibit no response following treatment.[3] In addition, there are currently no secondary choices of therapeutic options that have demonstrated clinical efficacy. A major obstacle for the development of novel therapeutic options for SNHL is the inability to regenerate sensory hair cells in the mammalian cochleae.[4] Therefore, practical strategies have focused on the protection of cochlear hair cells.

Previous experimental studies have demonstrated the efficacy of several growth or neurotrophic factors in the protection of cochlear hair cells against various ototoxic insults. A combination of glial cell line-derived neurotrophic factor and transforming growth factor β1 (TGF-β1) reduces the loss of hair cells due to aminoglycoside ototoxicity.[5] Local application of insulin-like growth factor-1 (IGF-1) into cochleae provides functional and histological protection of cochlear hair cells against noise trauma[6,7] and ischemic injury.[8] More recently, the protective effects of hepatocyte growth factor (HGF) on cochlear hair cells against aminoglycoside toxicity[9] and noise trauma[10] have also been reported.

Transplantation of stem or stem cell-like cells is an alternative strategy for the local, sustained delivery of growth or trophic factors and cytokines into tissues, because they have the capacity to secrete several growth factors and cytokines. For this purpose, stromal cells derived from adipose tissue have been frequently used. Adipose tissue-derived stromal cells (ADSCs) are known to secrete multiple growth factors and cytokines,[11] and ADSC transplantation has contributed to angiogenesis[12,13] or functional restoration of the liver[11] via paracrine of solvable factors. We therefore highlighted the potential of ADSCs for paracrine of multiple growth factors and cytokines, and investigated the effects of ADSC on the protection of cochlear hair cells in an explant culture system where cochlea explants and ADSCs were co-cultured without their direct contact. Here, we report on the significant attenuation of aminoglycoside-induced damage in cochlear hair cells co-cultured with ADSCs.

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MATERIALS AND METHODS

Animals

Imprinting control region (ICR) mice (Japan SLC, Hamamatsu, Japan) used in this study were cared for in the Institute of Laboratory Animals of the Kyoto University Graduate School of Medicine, Japan. The Animal Research Committee of the Kyoto University Graduate School of Medicine approved all experimental protocols, which were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Isolation and Expansion of ADSCs

Mouse ADSCs were isolated and expanded according to methods described previously.[14] Briefly, femoral adipose tissue samples were obtained from 20 male ICR mice at 8 weeks of age under general anesthesia with midazolam (10 mg/kg; Astellas, Tokyo, Japan) and xylazine (10 mg/kg; Bayer, Tokyo, Japan). Samples obtained from five animals were transferred into one conical tube. A total of four conical tubes from 20 mice were used to isolate ADSCs.

Adipose tissues were digested in a lacto-Ringer solution containing 0.035 mg/mL Liberase Blendzyme 3 (Roche Applied Science, Mannheim, Germany) under agitation for 40 minutes at 37°C, then rested for 5 minutes to separate the stromal cell fraction from adipocytes. The stromal cell fraction was filtered through a 100 μm cell strainer (BD Biosciences, San Jose, CA) and centrifuged at 500 $\times$ g for 5 minutes. The cell pellet was resuspended in a lacto-Ringer solution and filtered through a 40 μm cell strainer (BD Biosciences). Isolated cells were seeded at 6×10^4 cells/cm^2 in 10-cm plastic plates and allowed to adhere in basic medium (DMEM/F12 with 10% fetal bovine serum, 50 μg/mL gentamycin, 250 ng/mL amphotericin B) for 24 hours. The medium was changed once daily until the cells were 80% confluent. Nonadherent cells were removed during the medium-change procedure. After the fourth passage, the cells were seeded at 1.6×10^4 cells/mL in 6-well plastic plates and cultured until they were 80% confluent. Culture media were then replaced with DMEM/F12 without supplements. ADSCs were cultured for a further 7 days without medium changes.

Cochlear Explants

Postnatal day 3, ICR mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected, and the cochleae freed from the surrounding tissue and placed in 0.01-M phosphate buffered saline (pH 7.4). After removing the cochlear lateral wall, the cochlear epithelia were dissected from the cochlear modiolus. The tissue samples were then placed on culture mesh inserts (Falcon, Billerica, MA) and cultured initially in serum-free minimum essential medium (MEM) (Invitrogen, Carlsbad, CA), supplemented with 3 g/L glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/L penicillin G (Wako Pure Chemicals), for 24 hours at 37°C in humidified (95%) air at 5% atmospheric CO_2. In total, 26 cochlear explants were used. As the hair cells in the apex are resistant to aminoglycosides, the basal (60%–80% from the apex) and upper-basal portions (40%–60% from the apex) of the cochlea were used in this study. We used five cochlea explants without any toxic drugs to confirm that this explant culture system did not damage the hair cells.

Neomycin Application and Co-Culture of Cochlear Explants With ADSCs

Neomycin (Wako Pure Chemicals) was added to the culture wells of 6-well plastic plates containing ADSCs at a final concentration of 1 mM. To avoid attachment between ADSCs and cochlear explants, the latter (n = 9) were transferred with culture-mesh inserts to culture wells containing ADSCs and 1 mM neomycin. In this co-culture study, we used ADSCs from the same origin. Other cochlear explants (n = 12) were transferred with culture-mesh inserts to culture wells containing fresh DMEM/F12 supplemented with 1 mM neomycin, and served as controls. Cultures were maintained for 24 hours, then fixed for 15 minutes in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Hair Cell Protection Assay

Cochlear explants were provided for the immunostaining of myosin VIIa and F-actin staining with phalloidin. Specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1:500; Proteus Bioscience Inc., Ramona, CA). Alexa-Fluor 568 goat anti-rabbit IgG (1:500; Invitrogen) was used as the secondary antibody. Specimens were then incubated in fluorescein isothiocyanate-conjugated phalloidin (1:80; Invitrogen) and viewed with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany).

To quantify cochlear hair cell damage, inner hair cells (IHCs) and outer hair cells (OHCs) with organized stereocilia were counted over 200-μm-longitudinal distances from two separate regions in the basal and upper-basal portions, respectively. The average value was used as the data for each culture. All values are expressed as the mean $\pm$ 1 standard error (SE). Differences in the number of hair cells with organized stereocilia between specimens co-cultured with ADSCs and control specimens cultured without ADSCs were analyzed by unpaired t tests. P values $<.05$ were considered to be statistically significant.

Measurement of Growth Factors and Cytokines in Culture Media

We expanded ADSCs from the four conical tubes into four separate culture wells. Following a 7-day culture of ADSCs, 500 μL of culture media were collected from each culture well, and used for an enzyme-linked immunosorbent assay (ELISA) of growth factors and cytokines using the assay kits shown in Table I. ELISA analyses were performed according to the manufacturers' instructions. Triplicate measurements were performed for each sample, and an average taken. All values are expressed as the mean $\pm$ 1 SE.

RESULTS

Hair Cell Protection by ADSCs

We estimated the protective effects of ADSC on cochlear hair cells against neomycin toxicity using an explant culture system. Previously, we established an explant culture model to analyze aminoglycoside toxicity on cochlear hair cells, in which we confirmed that any damage was not seen in the hair cells without toxic drug treatments, and a 24-hour culture with 1 mM neomycin destroyed approximately 70% of IHCs and OHCs in the explants.[9] These were deemed suitable conditions to evaluate the protective effects on cochlear hair cells. Morphology of the hair cell surface in cochlear explants is shown in Figure 1. In control specimens that were cultured with 1 mM neomycin alone, both IHCs and OHCs were severely degenerated in the basal (Fig. 1d–1f) and upper-basal portions (Fig. 1j–1l), as observed in our previous investigation.[9] In the basal portion of cochlear

TABLE I.
Measured Growth Factors, Cytokines, and Enzyme-Linked Immunosorbent Assay Kits.

Protein	Kit Name	Supplier
BDNF	Human BDNF Quantikine kit	R&D systems, Minneapolis, MN
BMP2	Human BMP2 Quantikine kit	R&D systems, Minneapolis, MN
EGF	EGF Mouse, ELISA Kit, Quantikine	R&D systems, Minneapolis, MN
bFGF	Human FGFb Quantikine kit	R&D systems, Minneapolis, MN
HGF	HGF, DuoSet(R)ELISA Development kit	R&D systems, Minneapolis, MN
IGF-1	Mouse IGF-1 Quantikine ELISA Kit	R&D systems, Minneapolis, MN
NGF	NGF Emax (R) ImmunoAssay System	Promega, Madison, WI
PDGF	Mouse/Rat PDGF-BB Quantikine ELISA Kit	R&D systems, Minneapolis, MN
TGF-b1	Canine, Mouse, Rat, Porcins TGF-b1 Quantikine kit	BenderMedSystems, Vienna, Austria
VEGF	VEGF, Mouse, ELISA Kit, RayBio	BenderMedSystems, Vienna, Austria
G-CSF	Mouse G-CSF Single Analyte ELISArray Kit	SABiosciences, Frederick, MD
GM-CSF	Mouse GM-CSF Platinum ELISA	BenderMedSystems, Vienna, Austria
M-CSF	M-CSF ELISA	Peprotech, Rocky Hill, NJ
MCP-1	Mouse MCP-1 kit	BenderMedSystems, Vienna, Austria
MIP-1a.	MIP-1α(Ccl3)ELISA Kit, Single Analyte ELISArray	Peprotech, Rocky Hill, NJ
MIP-1b	MIP-1β(Ccl4) ELISA Kit, Single Analyte ELISArray	Peprotech, Rocky Hill, NJ
SDF-1	Mouse SDF-1, Quantikine kit	R&D systems, Minneapolis, MN

BDNF: Brain-derived nuerotrophich factor, BMP2: Bone morphogenetic protein-2, EGF: Epidermal growth factor, bFGF: basic fibroblast growth factor, HGF: Hepatocyto growth factor, IGF-1: Insulin-like growth factor-1, NGF: Nerve growth factor, PDGF: Platelet-derived growth factor, TGF-b1: Transforming growth factor-beta1, VEGF: Vascular endothelial growth factor, G-CSF: Granulocyte-colony stimulating factor. GM-CSF: Granulocyte-macrophage colony-stimulating factor, M-CSF: Macrophage colony-stimulating factor, MCP-1: Monocyte chemotactic protein-1MIP-1a: Macrophage inflammatory protein-1 alpha, MIP-1b: Macrophage inflammatory protein-1beta, SDF-1a Stromal cell-derived factor-1alpha.

explants, 4.7 ± 0.5 IHCs and 12.4 ± 1.5 OHCs were present in a 200-μm length region, and the numbers of surviving IHCs and OHCs were 4.8 ± 0.9 and 12.1 ±1.9 in a 200-μm length region of the upper-basal portion of cochlear explants, respectively.

In contrast to control specimens, IHCs and OHCs were well maintained in both the basal (Fig. 1a–1c) and upper-basal portions (Fig. 1g–1i) of cochlear explants following co-culture with ADSCs. The numbers of surviving IHCs and OHCs in the basal portion were 20.3 ± 1.1 and 61.1 ± 4.6 in a 200-μm length region, and those in the upper-basal portion were 19.0 ± 0.7 and 64.4 ± 4.2. Differences in numbers of IHCs and OHCs between co-cultured and control specimens were

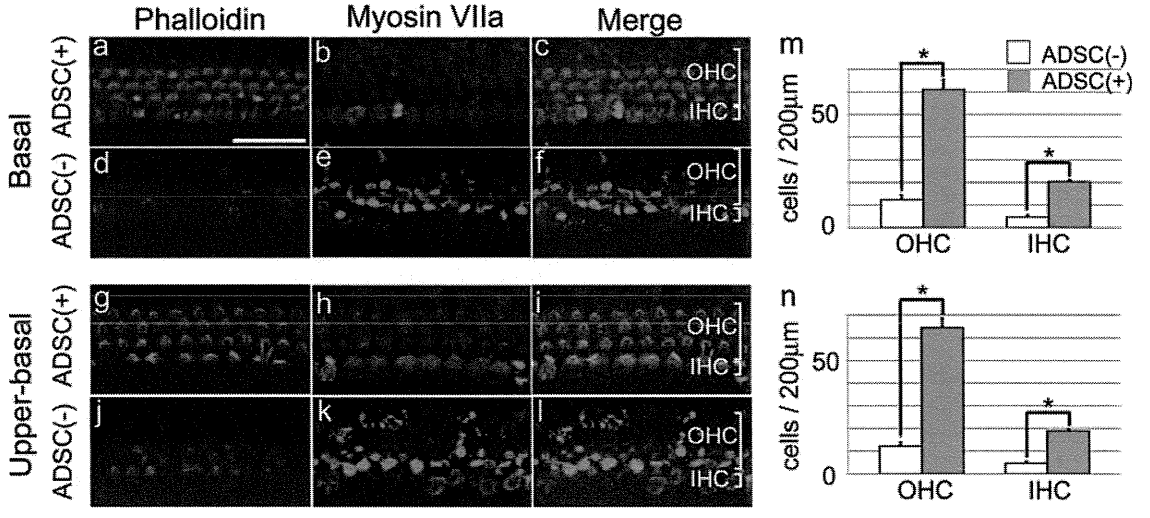

Fig. 1. Hair cell protection by paracrine effects of adipose tissue-derived stromal cells (ADSCs) against aminoglycoside toxicity. Basal (a–f) and upper-basal portions (g–l) of cochlear epithelia cultured with neomycin are shown. Phalloidin staining and immunostaining for myosin VIIa demonstrate that inner hair cells (IHCs) and outer hair cells (OHCs) are well maintained in cochleae cultured with ADSCs (a–c, g–i), whereas severe degeneration is found in both IHCs and OHCs in cochleae cultured without ADSCs (d–f, j–l). Quantitative analyses reveal significant differences in surviving IHC and OHC numbers between the two groups in the basal (m) and upper-basal (n) portions, respectively (*). Scale bar in (a) represents 50 μm (a–l).

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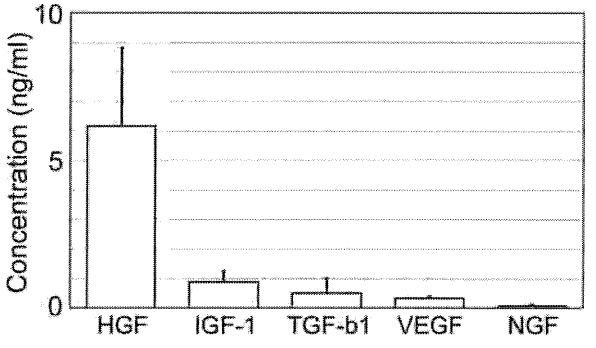

Fig. 2. Paracrine factors in ADSC-conditioned media. Hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF) were detected by enzyme-linked immunosorbent assay (ELISA). Bars represent 1 standard error.

statistically significant ($P < 0.01$ for each condition) (Fig. 1m–1n), demonstrating that co-culture with ADSCs significantly attenuates neomycin-induced damage of cochlear hair cells. This suggests that ADSCs may secrete protective factors for cochlear hair cells into culture media.

Growth Factors and Cytokines Secreted by ADSCs

To examine the ability of ADSCs to secrete growth or trophic factors and cytokines, we measured the protein levels of several factors in culture media collected after a 7-day culture of ADSCs using ELISA. Analyses revealed the presence of five growth factors: HGF, IGF-1, vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and TGF-β1 at detectable levels for each assay kit (Fig. 2). The protein levels of brain-derived neurotrophic factor, bone morphogenic protein-2, epidermal growth factor, basic fibroblast growth factor, and platelet-derived growth factor were undetectable by ELISA, whereas the protein level of HGF was 6154.62 $\pm$ 2673.18 pg/mL. The levels of IGF-1, TGF-β1, VEGF, and NGF were 889.69 $\pm$ 355.21, 502.00 $\pm$ 502.00, 327.53 $\pm$ 63.90, and 76.53 $\pm$ 33.89 pg/mL, respectively. For cytokines, ELISA analyses revealed the presence of one cytokine: monocyte chemotactic protein-1 (MCP-1), whereas the protein levels of other cytokines, granulocyte-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, macrophage inflammatory protein-1α, β (MIP-1α, β), and stromal cell-derived factor-1α (SDF-1α), were not detectable. The level of MCP-1 was 6310.67 $\pm$ 2101.91 pg/mL. These findings demonstrate that HGF, IGF-1, VEGF, NGF, TGF-β1 and MCP-1 are secreted from ADSCs.

DISCUSSION

Cell transplantation can contribute to the functional restoration of various organs through two different mechanisms. One is the differentiation of transplants into functional cells and the formation of functional connections with host cells. The second is paracrine of therapeutic factors from transplanted cells that promote self-repair systems in host organs. Several cell types have had their potential tested for use as transplants in cell-based therapy. Of these, mesenchymal stromal cells have often been used because of their ready and easy availability from bone marrow or adipose tissue, particularly in the case of autologous cells.[15] Recent studies have also demonstrated the high potential of human ADSCs for the secretion of various growth factors.[11] Furthermore, ADSCs have some advantages over bone marrow-derived stromal cells (BMSCs). First, collecting ADSCs is easier and less invasive than collecting BMSCs. Complications of bone marrow aspiration include osteomyelitis and accidental puncture of adjacent vital organs. Second, we can collect larger amount of cells at one time from adipose tissue than from bone marrow. It is possible that multiple passages of ADSCs are not needed before there are enough cells to be transplanted. Third, autologous fat transplantation have been performed for improving facial and body contour depressions and scars since 1898,[16] and the safety of adipose tissue transplantation is well established. For such reasons, we investigated the paracrine effects of ADSCs in the protection of mammalian cochlear hair cells.

In this study, we tested the potential of ADSCs in the rescue of cochlear hair cells from aminoglycoside toxicity ex vivo. To exclude the possibility of ADSCs' penetration into cochlea explants, the cochlear explants were separated from ADSCs by culture mesh inserts to avoid direct contact. We demonstrated that co-culture with ADSCs significantly promotes the survival of cochlear hair cells in explants against neomycin toxicity. We then measured the protein levels of several growth and trophic factors in the culture media of ADSCs using ELISA, revealing the presence of IGF-1, VEGF, TGF-β1, NGF, and robust amounts of HGF, which has protective effects on cochlear hair cells against neomycin toxicity[9] and noise-induced damage.[10] IGF-1 also demonstrates important protective effects on cochlear hair cells against noise trauma[6,7] and ischemic injury.[8] VEGF is associated with attenuation of noise-induced damage on cochlear hair cells,[17] whereas TGF-β1 exhibited hair cell protection against aminoglycosides in combination with glial cell line-derived neurotrophic factor.[5] In short, most ADSC-secreted growth factors presented in the current study have the potential to protect cochlear hair cells, which strongly supports our hypothesis about the paracrine effects of ADSCs.

In comparison with previous studies showing the direct effects of growth factors on hair cell protection, the levels of growth factors demonstrated in the present study were considerably low. HGF levels (6 ng/mL) were the highest of all growth factors measured in this study, but were still less than HGF levels reported to demonstrate protective effects on cochlear hair cells in explant cultures against neomycin toxicity.[9] Although we used the same explant culture system in both studies, our previous work showed significant protective effects of as much as 20 ng/mL HGF on cochlear hair cells against 1 mM neomycin, with no protection provided by 4 ng/mL

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HGF.[9] Based on these findings, we speculate that the protection observed in the present study was achieved by the synergistic effects of multiple growth factors secreted from ADSCs, although we cannot exclude the possibility that the recombinant HGF we used in the previous report had weaker activity than HGF ADSCs secreted in this study because of the difference in the post-transcriptional regulation. Indeed, TGF-β1 enhanced the protective effects of glial cell line-derived neurotrophic factor on cochleae against aminoglycosides,[5] which supports our synergistic hypothesis.

We also measured the levels of several cytokines in ADSC culture media, because cytokines derived from ADSCs could exert protective effects on hair cells. In the present study, ELISA analysis demonstrated the secretion of MCP-1 from ADSCs to the culture media. MCP-1 is a small cytokine belonging to adipocytokines that is also known as chemokine (C-C motif) ligand 2. MCP-1 displays chemotactic activity to monocytes and macrophages. Recently, MCP-1 was also known to play an important role in the regulation of metabolism[18] and in the pathogenesis of neurodegenerative diseases.[19] In the inner ear, the involvement of MCP-1 has been demonstrated in the process of inflammatory responses following exposure to otitis media pathogens[20] and acoustic overstimulation.[21] In addition, previous studies have suggested that migration of macrophages into cochlear tissue following traumatic insults contributes to the protection of cochlear tissues[21,22] However, direct effects of MCP-1 on hair cells have not been elucidated. Therefore, it is unclear whether MCP-1 secreted from ADSCs plays positive or negative roles in hair cell protection against neomycin toxicity. However, the present finding indicates that ADSCs could modulate inflammatory responses in the cochlea under pathological conditions via paracrine of MCP-1.

The cell transplantation approach can be advantageous at some points when we compare it with local application of growth factors. First, transplanted cells can keep secreting growth factors for a long period. Second, the secretion volume can be regulated by cross-talk with surrounding cells including damaged cells. Third, the cells may secrete unknown growth factors that have protective effect on hair cells. Fourth, the growth factors transplanted cells secrete can have higher activity than recombinant growth factors because they were regulated by physiological post-transcriptional modifications. Although these are advantages of the cell transplantation approach, it requires surgical procedure for opening the cochlear fluid space. One possible application of ADSC transplantation is hybrid cochlear implantation,[23] where low frequency auditory stimuli are transmitted to the auditory primary neurons by the remaining hair cells, and high frequency auditory stimuli are transmitted to the auditory primary neurons by direct electrical stimulation via cochlear implant devices. In the case of hybrid cochlear implantation, the preservation of residual hearing at low frequency regions is critical. It is therefore important to protect cochlear hair cells from invasive surgery. During cochlear implant surgery, the cochlear bony wall is opened to insert a cochlear implant electrode; at this stage, therapeutic cells could be transplanted into the cochlea. Because the capacity of ADSCs to protect cochlea hair cells was demonstrated in this study, ADSCs are expected to be applied to clinical use in case of hybrid cochlear implantation in future.

CONCLUSION

The present findings demonstrate that ADSCs have the capacity to protect cochlear hair cells via paracrine effects, suggesting that ADSC transplantation into cochleae may be a useful strategy for the protection of cochlear hair cells in vivo. As a next step, we will examine the effects of ADSC transplantation for cochlear protection using animal models in vivo.

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Insulin-like growth factor 1 protects vestibular hair cells from aminoglycosides

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This study investigated the therapeutic potential of insulin-like growth factor-1 (IGF-1) for vestibular hair cells using explant cultures of mouse utricles. After incubation with the ototoxic drug gentamicin, explants from neonatal mouse utricles were cultured in medium containing IGF-1 at various concentrations. Histological evaluation revealed significant increases in the number of surviving hair cells cultured with IGF-1 at concentrations reflecting a clinical setting. Immunostaining for trio-binding protein and espin showed the maintenance of functional structures in hair bundles at the apex of surviving hair cells. An FM1-43 assay indicated the presence of mechanoelectrical transduction channels in surviving hair cells. These findings indicate that IGF-1 may protect the functionality of vestibular hair cells against drug-induced injury. *NeuroReport* **22:38–43 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Keywords: aminoglycoside, espin, FM1-43, growth factor, inner ear, mechanoelectrical transduction channel, trio-binding protein, utricle

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Introduction

The peripheral vestibular apparatus consists of two otolith organs and three semicircular canals. Vestibular sensory epithelia report the magnitude and direction of angular and linear motion of the head to the central nervous system. Normal sensory processing through the vestibular system is largely unconscious, and we become aware of it only when it malfunctions. Dysfunction of the peripheral vestibular apparatus has an incapacitating influence on every aspect of our lives, and drug-induced damage can cause serious disability as it often leads to bilateral dysfunction.

Hair cells are the primary mechanotransducers of the peripheral vestibular apparatus. A bundle of enlarged microvilli, stereocilia, is present at the apex of each hair cell, and the tilt of these bundles induces the opening of mechanoelectrical transduction (MET) channels, which is the primary step in the sensing of head movement by the vestibular system. The survival of hair cells is therefore crucial for the maintenance of vestibular functions. In mammalian inner ears, cochleae have limited capacity for spontaneous regeneration. Although mammalian vestibular epithelia can undergo hair cell regeneration [1–5], this is not sufficient for functional recovery [1,4–6].

The protection of hair cells is therefore a practical strategy for the treatment of the mammalian vestibular epithelium. Earlier studies have indicated the potential of growth factors, including insulin-like growth factor-1 (IGF-1), for the protection and regeneration of vestibular hair cells [7–11]. This study focused on IGF-1 and examined these effects using explant culture systems with special attention to the functionality of hair cells.

Methods

Animals

ICR mice (Japan SLC Inc., Hamamatsu, Japan) were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Japan. Experimental protocols were approved by the Animal Research Committee of Kyoto University Graduate School of Medicine (MedKyo10119), and complied with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Explant culture

Postnatal day 3 mice were deeply anesthetized with sevoflurane and decapitated. The temporal bones were dissected out and the utricles were removed from the surrounding tissue in 0.01 M phosphate-buffered saline, pH 7.4, which was supplemented with 0.2% glucose. The otoconial membranes were gently removed with a fine needle. Explants of utricle sensory epithelia were placed intact on type I collagen-coated cover glass (Iwaki, Tokyo, Japan) and maintained in 24-well culture plates (Iwaki) in Dulbecco's modified Eagle's medium (Invitrogen, Eugene, Oregon, USA), supplemented with 6 g/l glucose

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(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.

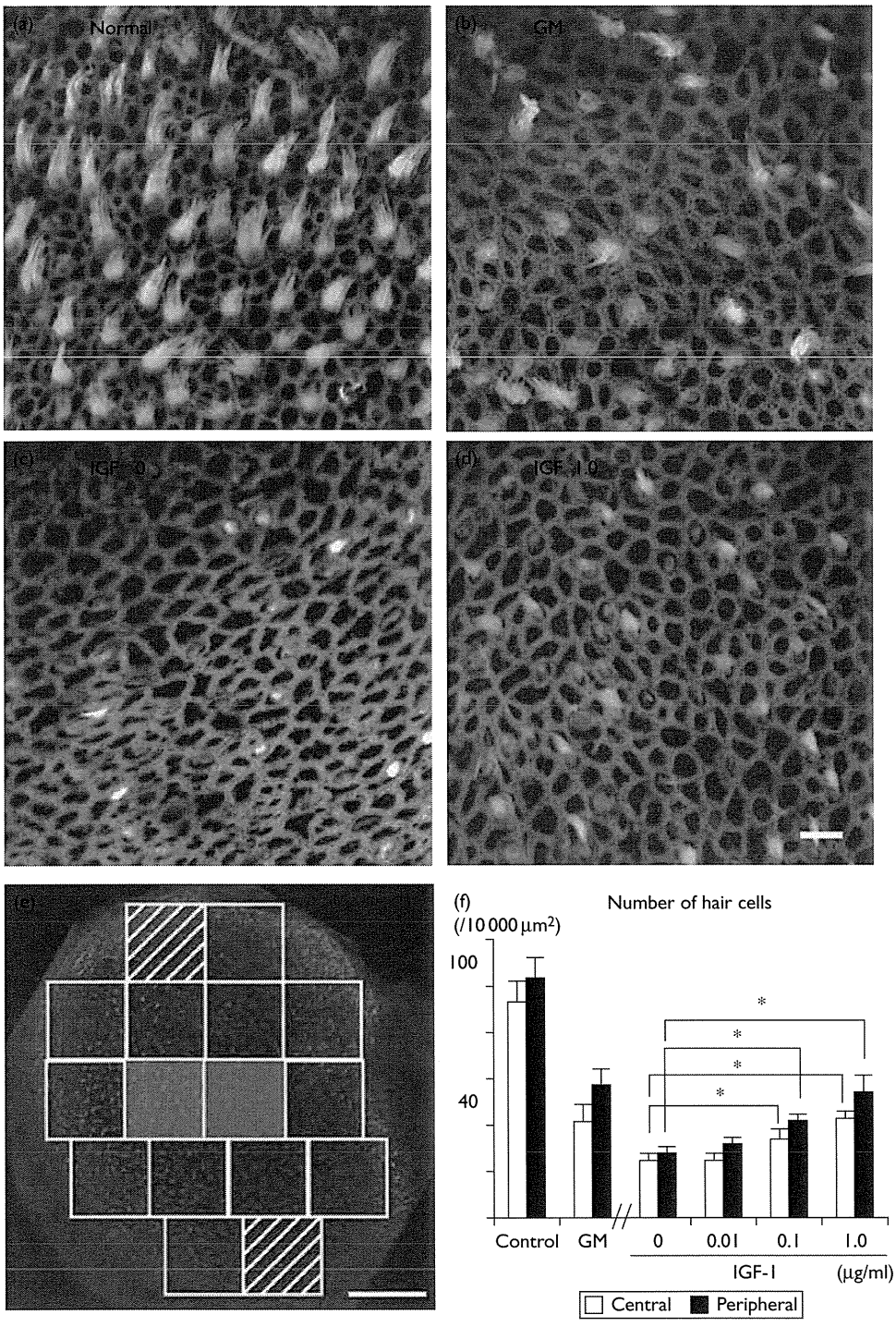
Results

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

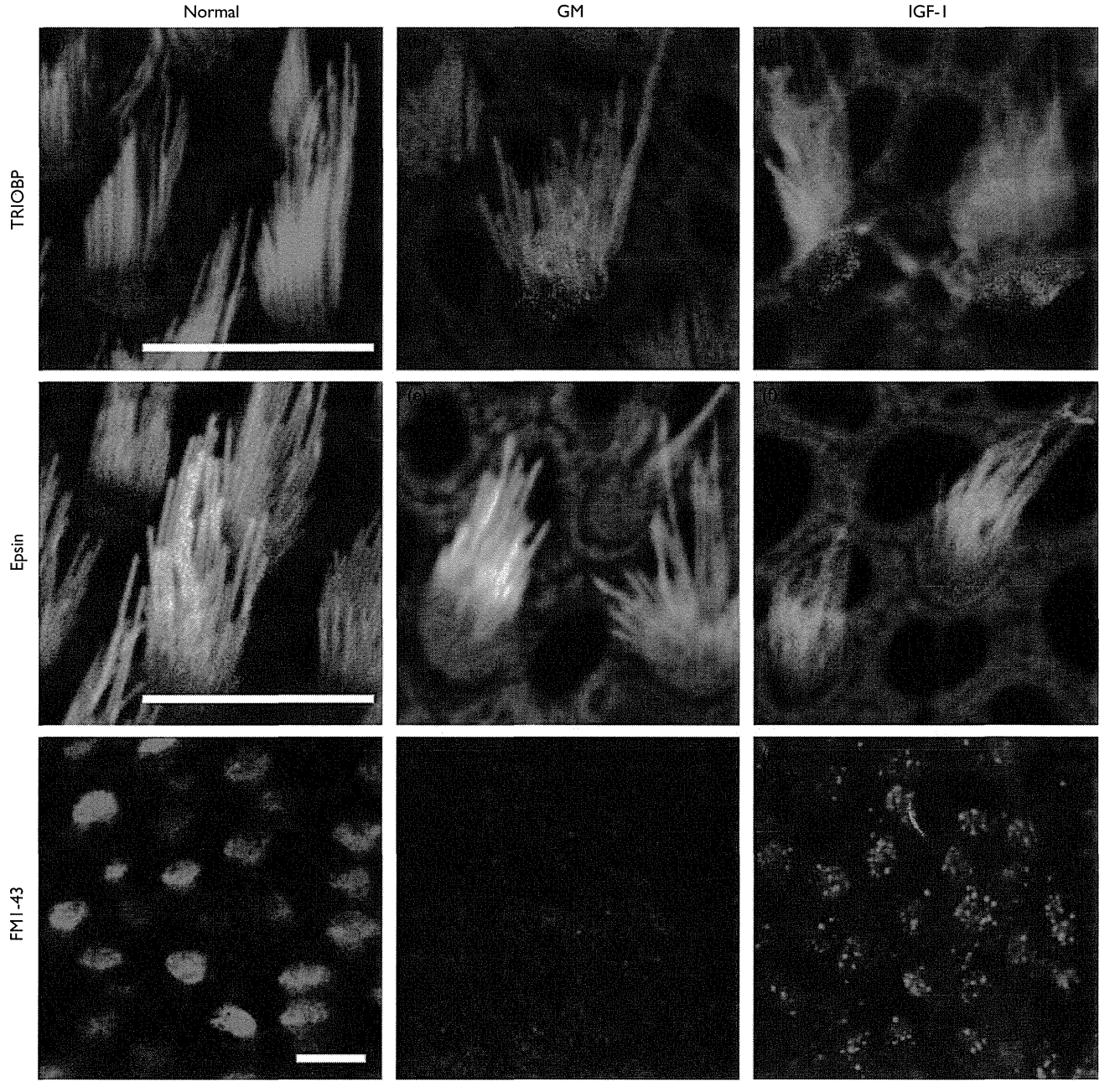


Surface images of the central regions in utricle explants immediately after dissection (a), incubation with 0.5 mM gentamicin (GM) for 24 h (b), following additional 48-h culture with no extra insulin-like growth factor 1 (IGF-1) (c) or with 1.0 μg/ml IGF-1 (d). Scale bar in (d) represents 10 μm for (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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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,