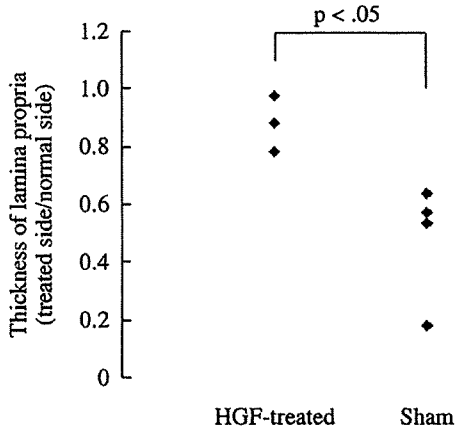

Fig 5. Lamina propria was significantly thinner in sham group than in HGF-treated group ($p < .05$).

Hepatocyte growth factor is thought to regulate excessive collagen deposition, prevent tissue contraction, and stimulate HA production. Histologic evaluation of the HGF-treated group in the present study indicated a remarkable reduction of collagen deposition and tissue contraction, with favorable restoration of HA in the lamina propria. The current HGF DDS also restored elastin deposition, whereas elastin was not restored by local injection of HGF solution in previous studies.[10,11]

The current results imply that the effects of HGF on vocal fold regeneration may be improved by the present DDS instead of injection of solution. However, further studies are needed to determine the ideal dose and administration frequency of HGF hydrogels to achieve complete regeneration of scarred vocal folds. It is also important to confirm the actual controlled release of HGF from the hydrogels in the vocal folds in the future, although such release has been clarified in other areas of the body.

CONCLUSIONS

The present study demonstrated that the HGF DDS significantly improved the vibratory properties of scarred vocal folds in a canine model. It reduced excessive collagen deposition and tissue contraction, with favorable restoration of HA and elastin. The results also suggest that the HGF DDS may produce more consistent effects on restoration of scarred vocal folds than does local administration of HGF solution.

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Drug delivery systems for the treatment of sensorineural hearing loss

TAKAYUKI NAKAGAWA & JUICHI ITO

Department of Otolaryngology-Head and Neck Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan

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

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

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in industrial countries. Excessive noise, ototoxic drugs, genetic disorders and aging can all initiate SNHL. Endolymphatic hydrops-associated diseases including Meniere's disease also cause SNHL. Severe to profound SNHL affects 1 in 1000 newborns, and another 1 in 2000 children before they reach adulthood. About 60% of individuals older than 70 years will manifest SNHL. Despite the high prevalence of SNHL in our society, therapeutic strategies for the treatment of SNHL today are limited to hearing aids and cochlear implants. These therapeutic tools do not provide complete restoration of hearing ability, although they have significant clinical benefits. Based on such backgrounds, many attempts have been made to provide alternative means of biological therapy, which have identified a number of candidates for therapeutic molecules. Experimentally, protective effects of neurotrophins have been demonstrated [1,2], and inhibitors of apoptosis and glutamate antagonists have also shown the ability to promote hair cell survival [3–5]. Recently, local application of genes by virus vectors was shown to induce hair cell regeneration in the mammalian auditory epithelium [6,7], and silencing the mutant gene by RNA interference restored hearing loss in a genetic mouse model [8].

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

Previous methods for local application

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

Correspondence: Takayuki Nakagawa, MD PhD, Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, 606-8507 Kyoto, Japan. Tel: +81 75 751 3346. Fax: +81 75 751 7225. E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

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

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

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

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

Controlled-release systems

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

Siloxane-based polymers

Siloxane-based polymers have been used for years in medical applications in contact with the human body. Silicone-transdermal patches have been widely used in clinic. The drug release in this system is controlled by its diffusion through the silicone network [30]. The releasing rate in this system is determined by the composition of the polymer. This system is particularly suitable for application of lipophilic and low-molecular weight molecules. Arnold et al. [26] have utilized this system for local application of beclomethasone into cochlear fluids. A silicone-microimplant was placed onto the RWM

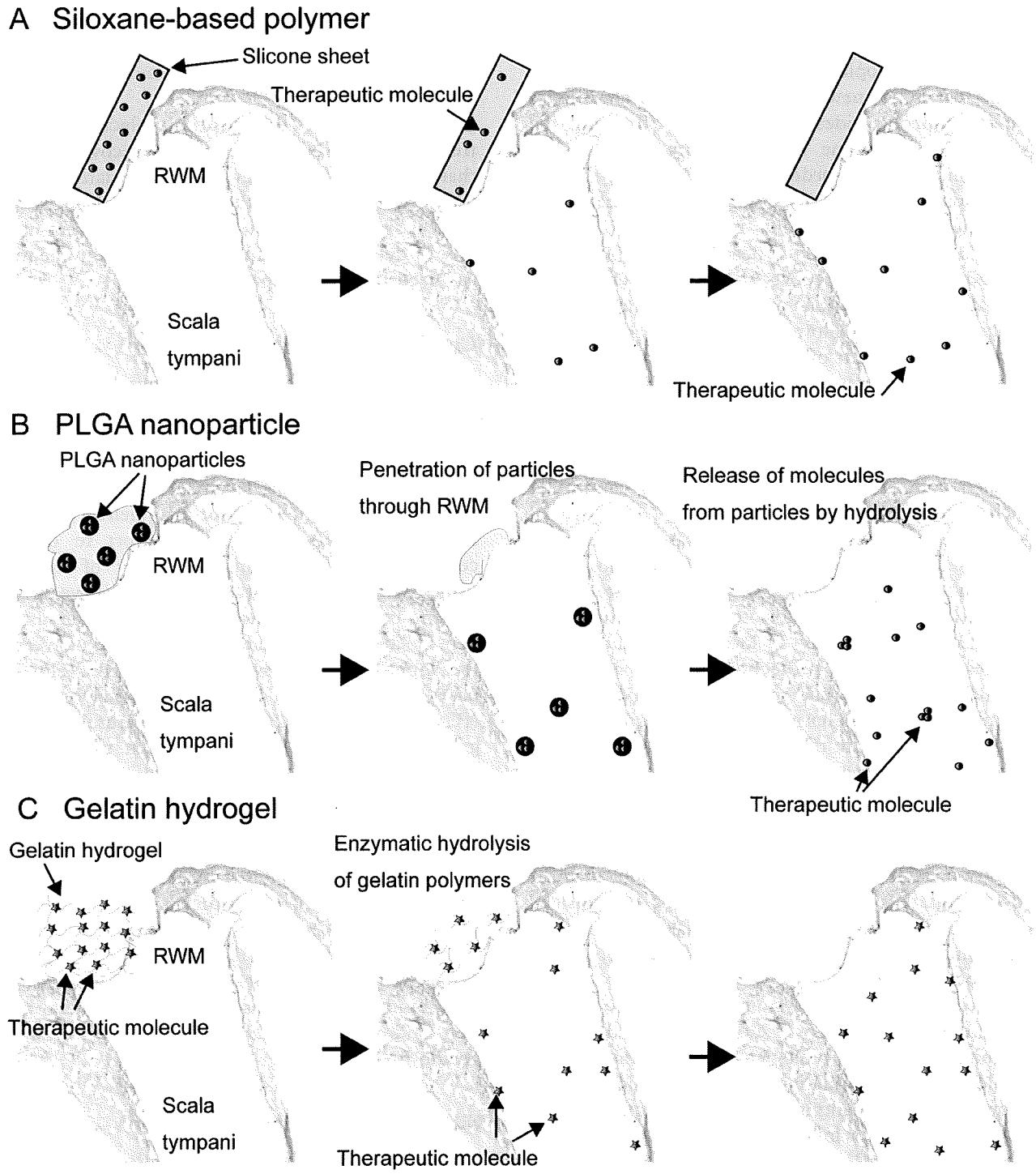

Figure 1. Mechanisms for controlled release of therapeutic molecules from biopolymers. (A) Therapeutic molecules dissolved in siloxane-based polymers move into the scala tympani by diffusion. A silicone sheet remains on the round window membrane (RWM). (B) Poly lactic/glycolic acid (PLGA) nanoparticles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

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

PLGA nanoparticles

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

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

Gelatin hydrogel

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

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

Conclusions for clinical application

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

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

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ORIGINAL ARTICLE

Local application of hepatocyte growth factor using gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs

TAKATOSHI INAOKA[1], TAKAYUKI NAKAGAWA[1], YAYOI S. KIKKAWA[1], YASUHIKO TABATA[2], KAZUYA ONO[1], MITSUHIRO YOSHIDA[1], HIROHITO TSUBOUCHI[3], AKIO IDO[3] & JUICHI ITO[1]

[1]*Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine,* [2]*Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Science, Kyoto University, Kyoto and* [3]*Department of Digestive and Life-style Related Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan*

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Abstract

Conclusion: Local application of hepatocyte growth factor using biodegradable gelatin hydrogels attenuates noise-induced hearing loss in guinea pigs. *Objectives:* To develop an inner ear drug delivery system using gelatin hydrogels that is capable of a sustained delivery of growth factors to the cochlea. We examined the efficacy of the local application of gelatin hydrogels containing hepatocyte growth factor (HGF) in protecting cochlear hair cells from noise-induced damage. *Materials and methods:* A piece of gelatin hydrogel previously immersed in either HGF or saline was placed on the round window membrane of a guinea pig 1 h after noise exposure (4 kHz octave band noise at 120 dB sound pressure level for 3 h). Auditory function was monitored using auditory brainstem responses (ABRs), and the loss of hair cells was evaluated quantitatively. *Results:* Local HGF treatment significantly reduced the noise exposure-caused ABR threshold shifts and the loss of outer hair cells in the basal portion of the cochleae.

Keywords: *Cochlea, drug delivery, growth factor, protection, hair cell*

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities. However, available therapeutic options are limited to hearing aids and cochlear implants. Therefore, many investigations have concentrated on finding novel therapeutic molecules that could possibly be used in the treatment of SNHL. These studies have discovered several agents that exhibit therapeutic activity against SNHL. Despite such basic research progress, the translation of these basic findings into useful therapeutic clinical agents has yet to be achieved. One considerable obstacle to the development of such clinical applications revolves around the current lack of a safe and effective method for drug delivery to the cochlea. As a way of resolving this, we have developed a new method for local inner ear treatment that uses gelatin hydrogel as the inner ear drug delivery system [1]. Biodegradable gelatin hydrogel has been used previously for the sustained release of proteins or peptides, including growth and trophic factors [2]. We have previously demonstrated the efficacy of gelatin hydrogels in the sustained delivery of brain-derived neurotrophic factor [3] and insulin-like growth factor 1 (IGF-1) [4,5] in animal experiments. In addition, we are currently performing a clinical trial designed to examine local IGF-1 therapy that uses gelatin hydrogels for treating acute SNHL (http://www.kuhp.kyoto-u.ac.jp/~ent/ClinicalTrial/Gel_Eng.html).

Hepatocyte growth factor (HGF) was originally identified as the protein that is responsible for stimulating hepatocyte proliferation [6]. It is present in various cells and is a paracrine cellular growth and morphogenetic factor [7,8]. Hearing impairment caused by aminoglycosides is ameliorated after the transfer of the HGF gene to the inner ear via an

Correspondence: Takayuki Nakagawa MD PhD, Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, 606-8507 Kyoto, Japan. Tel: +81 75 751 3346. Fax: +81 75 751 7225. E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

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intrathecal injection of the viral vector [9]. The HGF gene transfer for the treatment of SNHL has been published and patented (US Patent 7390482). Thus, local, sustained application of rhHGF might be effective for the treatment of SNHL and could potentially be approved for clinical applications in the near future.

Previous reports have documented the potential use of gelatin hydrogel for a sustained release of HGF [2,10]. Therefore, based on the previous reported data, we designed the current study to examine the efficacy of using gelatin hydrogels for local rhHGF application to treat noise-induced hearing loss (NIHL) in guinea pigs.

Materials and methods

Experimental animals

A total of 18 male 4-week-old adult Hartley guinea pigs weighing 300–350 g (Japan SLC, Hamamatsu, Japan) served as the experimental animals. Animal care was conducted under the supervision of the Institute of Laboratory Animals at the Kyoto University Graduate School of Medicine. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Biodegradable gelatin hydrogels

The biodegradable hydrogels were prepared as described previously [3–5]. Since other studies have analyzed the in vitro HGF release profiles from hydrogels and demonstrated that a hydrogel made with 10 mM glutaraldehyde allows for optimal HGF delivery [2,10], we designed the present study to use the same type of hydrogel.

Noise exposure and drug application

Baseline auditory brainstem response (ABR) thresholds were measured just before the noise exposure. Animals were then exposed to a 4 kHz octave band noise at 120 dB sound pressure level for 3 h in a ventilated sound exposure chamber. Sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure stimulus uniformity.

A 2 mm^3 piece of hydrogel was immersed in 20 µl physiological saline that contained either 1.0 µg/µl rhHGF or physiologic saline alone (control). Under general anesthesia using midazolam (2 mg/kg, intramuscular; Astellas, Tokyo, Japan) and xylazine (2 mg/kg, intramuscular; Bayer, Tokyo, Japan), the piece of hydrogel was then placed on the round window membrane in the left ear of the animals 1 h after the noise exposure ($n = 6$ for each group).

Functional analysis

ABRs were measured to assess the auditory function, with the ABR threshold measurements performed at the 4, 8, and 16 kHz frequencies. ABRs were obtained before and after exposure to the noise, and on days 3, 7, 14, and 21 after the drug application. Animals were anesthetized using midazolam and xylazine and kept warm using a heating pad. Generation of acoustic stimuli and the recordings of the evoked potentials were performed using a PowerLab/4sp (AD Instruments, Castle Hill, Australia). Acoustic stimuli, consisting of tone-burst stimuli (0.1 ms $\cos^2$ rise/fall with a 1 ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) that was connected to a funnel fitted to the external auditory meatus. To record bioelectrical potentials, subdermal stainless steel needle electrodes were inserted at the vertex (ground), ventrolateral to the measured ear (active) and contralateral to the measured ear (reference). Stimuli were calibrated against a ¼-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 subcutaneous 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 with 5 dB SPL intervals. Electrical signals were averaged for 1024 repetitions. Thresholds at each frequency were verified at least twice.

Histological analysis

On day 21 after the drug application, animals were deeply anesthetized with midazolam and xylazine and the cochleae were exposed. After removal of otic vesicles, 4% paraformaldehyde in 0.01 mol/l phosphate-buffered saline (PBS) at pH 7.4 was gently introduced into the perilymphatic space of the cochleae. Temporal bones were then excised and immersed in the same fixative at 4°C for 4 h. After rinsing with PBS, cochleae were dissected from temporal bones and subjected to histological analysis in whole mounts. To quantitatively assess the hair cell loss, we examined three regions of the cochlear sensory epithelia that were at a distance of 40–60%, 60–80% or 80–100% from the apex.

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Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin were performed to label the surviving inner hair cells (IHCs) and outer hair cells (OHCs). Anti-myosin VIIa rabbit polyclonal antibody (1:500; Proteus Bioscience, Ramona, CA, USA) was used as the primary antibody, and Alexa-546-conjugated anti-rabbit goat IgG (1:500; Molecular Probe, Eugene, OR, USA) was used as the secondary antibody. Following immunostaining for myosin VIIa, specimens were then stained with FITC-conjugated phalloidin (1:300; Molecular Probe). Specimens were viewed under a confocal microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany). To test the non-specific labeling, the primary antibody was omitted from the staining procedures. Three authors (T.I., T.N., and Y.S.K.) counted the numbers of IHCs and OHCs in 0.2 mm long regions of the apical, middle or basal portions of the cochleae. The average of the values was used as the data for each animal.

Statistical analysis

Overall effects of rhHGF application on ABR threshold shifts were examined using a two-way factorial analysis of variance. When interactions were significant, multiple comparisons with Fisher's protected least significant difference (PLSD) were used for pairwise comparisons. Differences in the IHC and OHC numbers for each region of the cochlea between the rhHGF- and saline-treated cochleae groups were examined using a Student's t test. Values of $p < 0.05$ were considered statistically significant. Values are expressed as the mean ± the standard error.

Results

Auditory function

Time courses of the alterations in the ABR threshold shifts at 4, 8, and 16 kHz after the application of rhHGF or saline are shown in Figure 1. Local application of rhHGF showed a significant effect on the reduction of the ABR threshold shifts at the 16 kHz frequency ($p = 0.030$). There was also a significant difference in threshold shifts on day 21 between the rhHGF- and saline-treated animals, as shown by the Fisher's PLSD test ($p = 0.045$). No significant differences were found for the threshold shifts between the two groups at 4 or 8 kHz.

Histological protection

Immunostaining for myosin VIIa and phalloidin staining demonstrated partial degeneration of the OHCs in the 60–80% distance regions from the apex

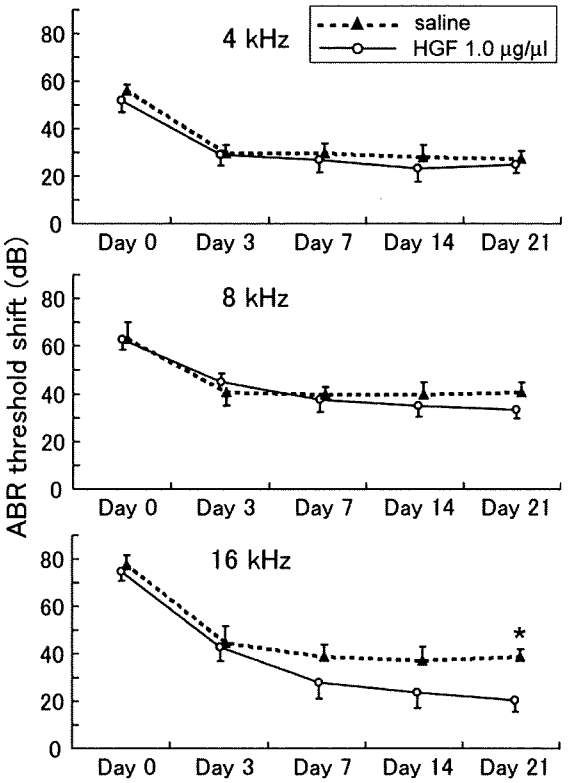

Figure 1. ABR threshold shifts after noise exposure in saline- and HGF-treated animals. An overall effect of HGF application is significant at 16 kHz (two factorial ANOVA, $p = 0.030$), not at 4 or 8 kHz. The difference in threshold shifts between saline- and HGF-treated animals is significant on day 21 at 16 kHz. *$p = 0.045$, Fisher's PLSD.

in the saline-treated cochleae (Figure 2A). The same region for the 1.0 μg/μl rhHGF-treated cochleae exhibited almost normal morphology (Figure 2B). In both experimental groups, OHC loss was not apparent in the 40–60% or 80–100% distance regions from the apex. IHCs were well maintained in every region of the cochleae in both groups. Quantitative assessments revealed a significant difference in OHC numbers in the 60–80% distance region from the apex between the saline- and rhHGF-treated cochleae (Figure 3, $p = 0.003$). No significant differences in OHC numbers were observed in the 40–60% or 80–100% distance regions. There were also no significant differences in the IHC numbers noted in any of the cochleae regions between the two experimental groups.

Discussion

Our findings indicate that local application of rhHGF using biodegradable gelatin hydrogels is effective in the attenuation of OHC damage due to noise trauma, resulting in the reduction of ABR

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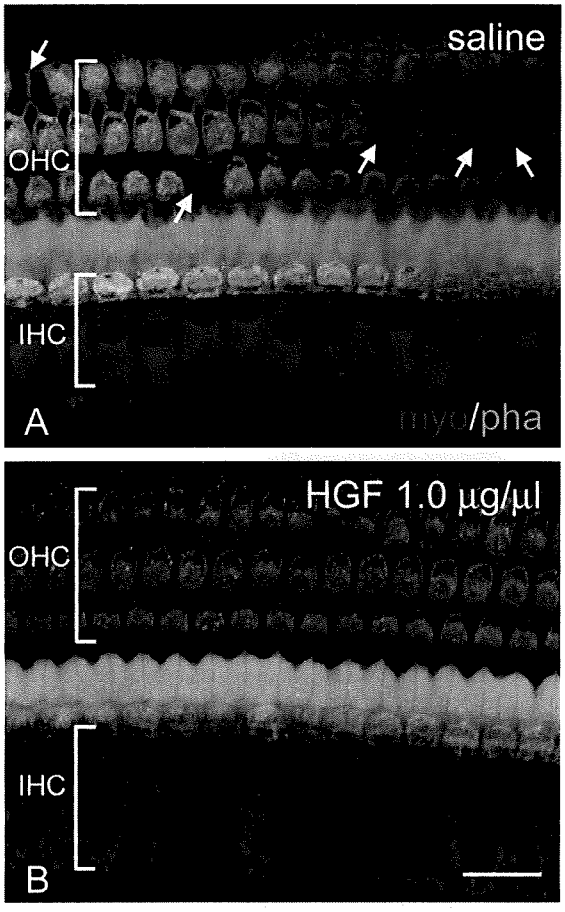

Figure 2. Immunostaining for myosin VIIa (myo) and phalloidin staining (pha) demonstrated loss of outer hair cells (OHC) in the upper basal portion of the saline-treated cochlea (A) and preservation of OHC in that of the HGF-treated cochlea (B). Arrows indicate loss of OHC. IHC, inner hair cells. Scale bar represents 20 μm.

thresholds. ABR measurements demonstrated that post-traumatic local application of rhHGF via gelatin hydrogels had a significant effect on the attenuation of threshold shifts at 16 kHz. Histological analyses demonstrated significant protection of the OHCs in the 60–80% distance from the apex, which is the region responsible for the 10–20 kHz hearing range [11].

Our previous study using IGF-1 indicated that there was a significant reduction of ABR threshold shifts at 4 or 8 kHz [9]. The present findings demonstrated that local HGF treatment caused significant effects at 16 kHz. The spread of the growth factors from the base to the apex of the cochlea occurred by diffusion. Thus, the molecular weights of growth factors could influence the distribution of these factors within the cochlea. The molecular weight of HGF is 69 kDa for the α-subunit and 34 kDa for the β-subunit, while that for

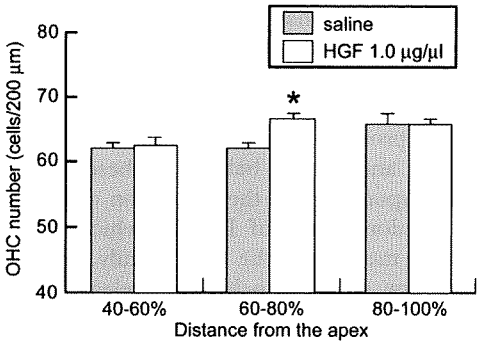

Figure 3. Means of numbers of surviving outer hair cells (OHCs) in saline- and HGF-treated cochleae. In the 60–80% distance region from the apex, the value of HGF-treated cochleae is significantly higher than that of saline-treated cochleae. $*p = 0.003$, t test. Bars represent standard errors.

IGF-1 is 7.6 kDa. Therefore, HGF may be abundantly distributed in the more basal portions of the cochlea as compared with that seen for the IGF-1 distribution.

Previous studies have demonstrated that several agents ameliorate NIHL when they are applied before noise exposure; however, only limited agents including IGF-1 [5] show protective effects by post-exposure administration. Local application of $_{D}$-Jun N-terminal kinase-1 ($_{D}$-JNK-1) peptide, an inhibitor of c-Jun N-terminal kinase, 12 h after noise exposure attenuates NIHL [12]. The efficacy of $_{D}$-JNK-1 peptide has been demonstrated by application via an osmotic mini-pump or a hyaluronic acid gel. In the current study, we used the gelatin hydrogel for sustained delivery of rhHGF into the cochlea. This system may also be utilized for local delivery of $_{D}$-JNK-1 peptide, because the gelatin hydrogel is suitable for sustained delivery of peptides [1,2]. The efficacy of local $_{D}$-JNK-1 peptide application via gelatin hydrogels will be evaluated in the near future. Post-exposure administration of edaravone, a free radical scavenger, also rescues cochleae from NIHL [13]. Locally applied edaravone via an osmotic mini-pump can rescue OHCs even when it is applied 21 h after noise exposure. Edaravone is clinically available; however, how to deliver edaravone into the cochlea continuously is an obstacle for clinical use. Gelatin hydrogels are not suitable for sustained delivery of edaravone, because edaravone is not soluble in water [1,2]. Therefore, drug delivery systems that fit for edaravone should be developed before clinical application of local edaravone treatment.

The mechanisms of cochlear hair cell protection by HGF are not well understood. The cochlear hair cells are degraded through the process of apoptosis after exposure to intense noise [14]. Exposure to intense sound causes production of hydroxyl radicals

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in the cochlear hair cells [15], which leads to peroxidation of the mitochondrial membrane and the release of cytochrome *c* from the mitochondria to the cytosol. The Bcl-2 family proteins, Bcl-xL and Bak, are produced in the hair cells following noise exposure, and it is the balance of these two proteins that is responsible for the regulation of this process [16]. Predominance of Bcl-xL, which is an anti-apoptotic member of the Bcl-2 family, results in the suppression of the cytochrome *c* release, whereas a predominance of the pro-apoptotic member, Bak, leads to the promotion of the cytochrome *c* release. HGF is known to up-regulate Bcl-xL, which is mediated by the phosphorylation of STAT3 [17]. Therefore, OHCs might be protected against noise through the same pathway. HGF also has anti-oxidant activity [18], which contributes to the protection of cells from apoptosis. This mechanism could possibly involve the same mechanism of protection provided by HGF for the OHCs. In the mechanisms of NIHL, disruption of afferent dendrites attached to IHCs is also involved [19]. Therefore, a regrowth of the nerve fibers and a re-afferentiation of the IHC is important for recovery of hearing after noise trauma. After spinal cord injury, HGF promotes axonal regrowth resulting in functional recovery [18]. This mechanism could also be involved in the significant reduction of ABR threshold shifts observed in the present study. In order to be able to elucidate the HGF distinct mechanism for the protection of auditory systems, further investigations are required.

In conclusion, the present findings suggest that HGF potentially has a role as a protector of OHCs from noise trauma. We are currently in the process of developing a clinical treatment for SNHL that administers local IGF-1 via gelatin hydrogels. Present results strongly suggest that HGF is the next therapeutic candidate that can be used as a local treatment agent via gelatin hydrogels in SNHL clinical trials.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Eur Arch Otorhinolaryngol (2009) 266:367–371
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OTOLOGY

Efficiency of a transtympanic approach to the round window membrane using a microendoscope

Harukazu Hiraumi · Takayuki Nakagawa · Juichi Ito

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Abstract There has been increasing interest in cochlear drug delivery through the round window membrane (RWM). However, placing drugs on the RWM is difficult because of anatomical barriers. We examined the efficacy of a microendoscope for a transtympanic approach to the RWM. We evaluated the visibility of the RWM using four approaches: transtympanic microendoscopic, transtympanic microscopic, transmastoid microendoscopic, and transmastoid microscopic in ten human temporal bones. For the transtympanic approach, we made a fenestration (2 × 1 mm) in the postero-inferior quadrant of the tympanic membrane. For the transmastoid approach, conventional posterior hypotympanotomy was performed. The transtympanic microendoscopic approach enabled visualization of the RWM in all specimens, whereas the transtympanic microscopic approach only permitted visualization in three specimens. Through the transmastoid approach, the RWM was visible in all specimens using either a microendoscope or a microscope. The transtympanic microendoscopic approach can be utilized for cochlear drug delivery through the RWM.

Keywords Microendoscope · Round window membrane · Cochlea · Drug delivery

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in industrial countries. Systemic administration of steroids has been widely used for the treatment of acute profound hearing loss [1]; however there are limitations in their clinical efficacy [2]. At present, therapeutic strategies are limited to hearing aids and cochlear implants for patients with chronic SNHL. Based on this background, basic investigations have elucidated several agents that are effective for the treatment of SNHL. However, the problem of how to deliver drugs to the inner ear has been a considerable obstacle to the development of treatments for SNHL. The blood-inner ear barrier prevents the transportation of serum drugs to the inner ear, and the blood flow to the inner ear is very limited.

Drug transduction through the round window membrane (RWM) is one option for delivering drugs into the inner ear. Continuous infusion of RWM with an osmotic pump and microcatheter has been reported as an effective and safe approach [3]. However, it requires surgery and the invasion cannot be overlooked. Recently, new local drug application procedures using biodegradable substances are gaining interest [4, 5]. The inner ear is one of the targets for local drug administration using biodegradable gelatin hydrogels [6, 7]. In this drug delivery system, positively charged proteins or peptides are electrostatically trapped in negatively charged gelatin polymer chains. As the gelatin polymer chains degrade, proteins or peptides are released from the hydrogel. The released protein is conveyed through the RWM into the inner ear via a concentration gradient. Therefore, close contact of biodegradable hydrogels with the RWM is critical for efficient drug delivery to inner ear fluids.

The RWM is situated perpendicular to the tympanic membrane and deep in the round window niche. In some cases, a false membrane covers the RWM. For safe and certain drug administration, hydrogels containing drugs should be placed on the RWM under direct visualization. Use of a

H. Hiraumi (✉) · T. Nakagawa · J. Ito
Department of Otolaryngology, Head and Neck Surgery,
Graduate School of Medicine, Kyoto University,
Kawaharacho 54, Shogoin, Sakyo-ku, 606-8507 Kyoto, Japan
e-mail: hhiraumi@ent.kuhp.kyoto-u.ac.jp

microendoscope is an effective method for visualization of the RWM [8]. It is equipped with a working channel, which can be used in drug administration. However, the potential of microendoscopes for placing substrates on the RWM has not been evaluated, and it is important to clarify the prevalence of subjects in whom the RWM is microendoscopically visible. In the present study, we examined the potential of a specially modified microendoscope for a transtympanic approach to the RWM using human temporal bones.

Materials and methods

Ten formalin-fixed temporal bones with no middle or inner ear diseases were obtained from six individuals (aged from 68 to 76 years at death, five male, and one female). A microendoscope (0.9 mm in outer diameter, 50 mm in length; FiberTech, Tokyo, Japan) was specially modified in the fit angle for observation of the RWM through the tympanic membrane. The tip is curved 15° (Fig. 1). The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter).

We used four different approaches to observe the RWM as follows: (1) transtympanic microendoscopic, (2) transtympanic microscopic, (3) transmastoid microendoscopic, and (4) transmastoid microscopic. For the transtympanic approach, a small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife (Fig. 2). The microendoscope was inserted into the middle ear through this fenestration and set to provide the best view of the RWM. For observation with a microscope, the fenestration edge in the tympanic membrane was gently pushed with a curved needle to obtain the best access to the RWM. For transmastoid approaches, canal-wall up complete mastoidectomy and posterior hypotympanotomy were performed under conventional microscopy (Leica M300, Leica Microsystems, Wetzlar, Germany). The bones covering the middle cranial fossa dura, the posterior fossa dura, and the sigmoid sinus were drilled to be as thin as possible. The bony wall of the external auditory canal was preserved. The facial nerve and chorda tympani nerve were skeletonized and the facial recess was opened as large as possible (Fig. 2).

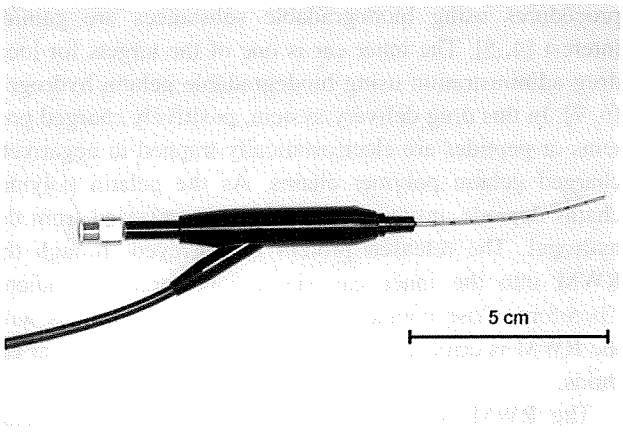

Fig. 1 A microendoscope specially modified for better visualization of the RWM. The outer diameter is 0.9 mm and the length is 50 mm. The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter)

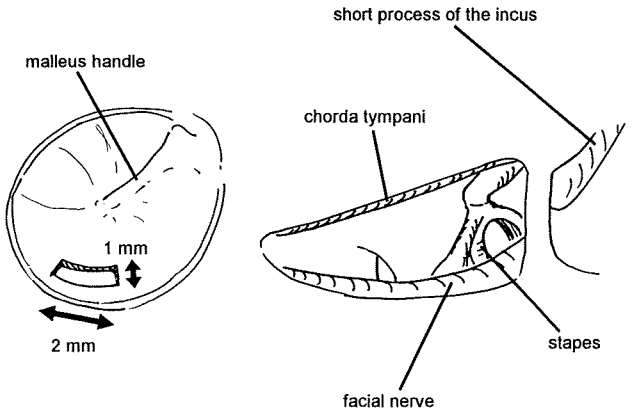

Fig. 2 A small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife. Posterior hypotympanotomy was made as large as possible. In all specimens, the facial nerve and chorda tympani were skeletonized

The RWM was observed through a posterior hypotympanotomy with a microendoscope or a microscope. Surgical procedures were performed by one author (Harukazu Hiraumi). The view of the RWM and surrounding structures using the four approaches was video-captured. Frames showing best view of the RWM were converted into still images, and the area of the RWM was measured using image-processing program, ImageJ. An angled hook (1.0 mm sharp tip) was used as a reference. Total area of the RWM was measured after drilling the round window niche. The visibility of the RWM was calculated and graded into three classes: Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. In three samples, the round window niche was covered with false membranes. In these cases, the false membranes were removed with a curved needle under microscopic view via posterior hypotympanotomy.

Results

A microendoscope was smoothly inserted into the middle ear cavity and the incuidostapedial joint was observed easily in all the specimens. The percentage of the area of the

RWM under direct vision was shown in the Table 1. The transtympanic microendoscopic approach enabled visualization of the RWM in all the specimens (Fig. 3). In three specimens, the RWM was totally observed (Fig. 4a). We used the incuidostapedial joint as a landmark to identify the location of the round window niche and the tip of the microendoscope was safely oriented to the RWM. No hazardous events such as ossicular dislocation or disruption of the tympanic membrane occurred. In contrast to the transtympanic microendoscopic approach, a transtympanic approach using a microscope provided visualization of the RWM in only three specimens (Fig. 3). Even in those three specimens, the view of the RWM was very limited (Fig. 4c). In the other seven specimens, the RWM was not observed, as the overhang of the round window niche was an obstacle for visualization. The visibility of the RWM through the transtympanic microendoscopic approach was significantly superior to that through transtympanic microscopic approach (Fig. 3, $P < 0.01$, Wilcoxon matched-pair signed-rank test).

In all the specimens, the transmastoid approach provided an excellent view of the RWM using either microendoscope (Fig. 4b) or microscope (Fig. 4d). The transmastoid microendoscopic approach provided a wide view of the middle ear cavity; for instance more than 70% of the tympanic membrane was visible in nine (microendoscopic), and seven (microscopic) specimens.

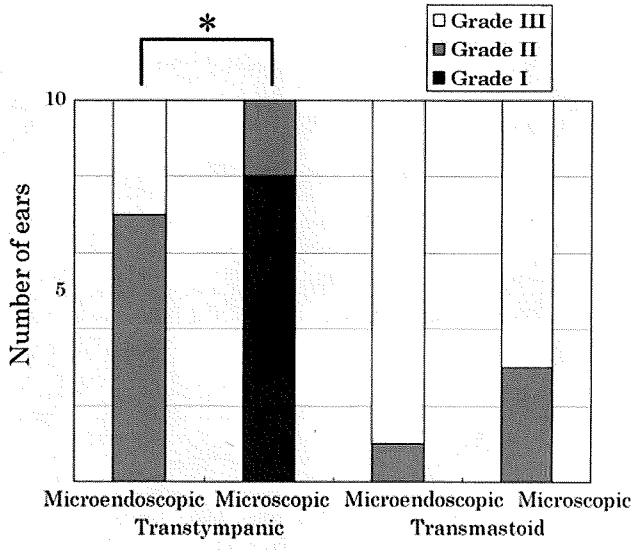

Fig. 3 The visibility of the RWM for four approaches. Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. The visibility through the transtympanic microendoscopic approach was better than that with transtympanic microscopic approach

Discussion

The present results demonstrate that a microendoscope provided a satisfactory view of the RWM through a transtympanic approach with only a 2-mm incision on the tympanic membrane. Although the transmastoid microscopic approach provides an excellent view and favorable access to the RWM, this approach requires mastoidectomy and is not adequate for local drug application for treatment of SNHL. In contrast, the transtympanic microendoscopic approach requires only a small fenestration in the tympanic membrane. Therefore, the transtympanic microendoscopic approach may be applicable for office-based treatment.

Conventional endoscopes with 30° provide good visualization of the RWM [9, 10]. However, endoscopes with attached CCD cameras are not easy to handle. In office-based usage, the endoscope is usually placed just outside of the tympanic membrane [11], and tools used for drug application can hinder the view. The outer diameter is 1.7 mm or larger, requiring larger myringotomy. In addition, use of a conventional endoscope for drug delivery onto the RWM requires another channel for drug application, resulting in

Table 1 The percentage of the visible area of the round window membrane using four approaches

No	Side	Transtympanic		Transmastoid	
		Microendoscope (%)	Microscope (%)	Microendoscope (%)	Microscope (%)
1	Left	80.2	0.0	91.6	70.1
2	Left	54.5	0.0	78.1	72.0
3	Left	78.8	23.0	87.3	79.6
4	Left	59.1	0.0	73.3	84.8
5	Left	48.2	14.6	94.8	71.6
6	Right	49.7	0.0	80.7	61.3
7	Right	79.9	0.0	87.6	75.7
8	Right	39.5	0.0	66.2	42.3
9	Right	62.0	20.1	84.9	83.2
10	Right	56.9	0.0	82.8	65.4

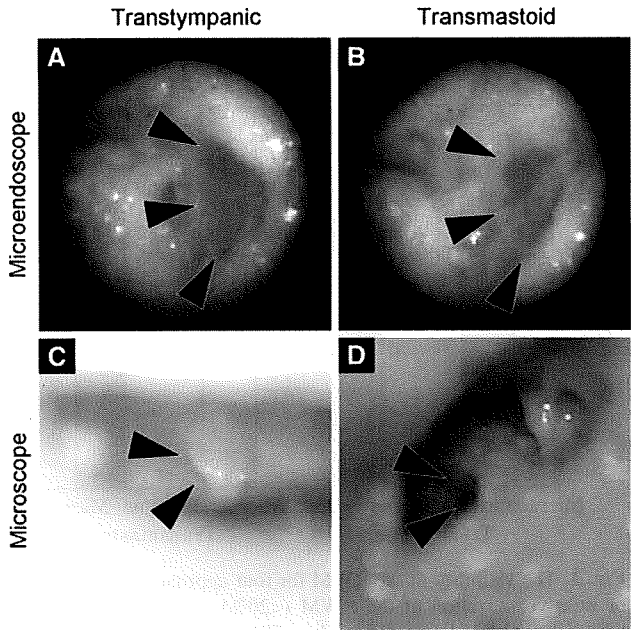

Fig. 4 The RWM of bone three observed through four approaches (*arrow heads*). The transtympanic microendoscopic approach (**a**), transmastoid microendoscopic approach (**b**), and transmastoid microscopic approach (**d**) provided good views. In the transtympanic microscopic approach (**c**), only a small part of the RWM was observed with the aid of a curved needle

increase of surgical invasion on the tympanic membrane. This means that enlargement of the size of tympanotomy or making additional tympanotomy site is necessary. Conventional microendoscopes are made for the inspection of the nasolacrymal ducts, and their tips are straight. The external auditory canal is S-shaped [12], and it is difficult to direct straight microendoscope to the RWM. The modified microendoscope used in the current study is quite smaller than conventional ones, and is connected to a CCD camera system via a cable. The curved tip fitted the external auditory canal. This configuration provides excellent handling of equipment for drug delivery. In addition, the microendoscope used in this study has a working channel that can be utilized for application of substrates onto the RWM.

The aim of the current study was to evaluate the accurate RWM drug application efficacy of a microendoscope with angles modified to ease RWM access. For clinical use of previously developed local drug delivery systems [3, 8], safe and stable visualization of the RWM through the tympanic membrane is necessary. In this manuscript, we compared the transtympanic microendoscopic approach with the transmastoid microscopic approach, since it is the most common procedure to access the RWM. The transmastoid microscopic approach is the most reliable approach for observation of the RWM, and additional removal of the round window niche enabled measurement of the total area of the RWM, which was indispensable for quantitative analysis in the present study. The view provided by a microendoscope is enough to deliver drugs or biomaterials incorporating drugs onto the RWM, although it is not satisfactory for precise surgical procedures. Previous studies have demonstrated the efficacy of biodegradable gelatin hydrogels for local application of brain-derived neurotrophic factor [6] and insulin-like growth factor 1 [7, 13]. The present findings resolve the problem of how to place a hydrogel onto the RWM in the clinic.

This study also found some drawbacks for this instrument. The resolution of the microendoscope is not as high as that of conventional microscopes, which may impede the differentiation of the false membrane from the RWM [14]. Sufficient understanding of the surgical anatomy of the middle ear is necessary for appropriate use of the microendoscope in drug delivery onto the RWM. However, we consider that refinement of the quality of view provided by microendoscopes may resolve this problem.

Conclusion

The transtympanic microendoscopic approach provided satisfactory visualization of the RWM through the tympanic membrane, indicating that the microendoscope is a useful tool for placing drugs or drug-containing materials onto the RWM.

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Local Drug Delivery to Inner Ear for Treatment of Hearing Loss

Takayuki Nakagawa* and Juichi Ito

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Abstract: Sensorineural hearing loss (SNHL) is one of the most common disabilities in our society. Experimentally, many candidates for use as therapeutic molecules have been discovered. However, a considerable obstacle to clinical application is the lack of an effective method for drug delivery to the cochlea. In order to overcome this obstacle, there needs to be development of a local cochlear drug delivery system. Advances in pharmacological technology have provided various drug delivery systems that use biomaterials, and which can be utilized for local drug delivery to the cochlea. Indeed, recent studies have demonstrated the potential of synthetic and natural biomaterials for local drug delivery to the cochlea, indicating that the clinical application of such local drug delivery systems could be used in the near future for therapeutic treatments. Recent progress in cell therapy research also offers a novel drug delivery method for the cochlea. In addition, transplantation of stem cells into the cochlea has been demonstrated to provide protective effects for the auditory function. Transplantation of genetically engineered cells has also resulted in the sustained delivery of aimed therapeutic molecules within the inner ear. Although problems involving clinical application still need to be resolved, these drug delivery systems for the inner ear may hold the future therapeutic options for treatment of SNHL.

Key Words: Drug delivery system, cochlea, biodegradable polymer, cell transplantation, gene therapy.

THERAPEUTIC TARGETS FOR TREATMENT OF HEARING LOSS

Sensorineural hearing loss (SNHL) is one of the most prevalent disabilities in our society. Sound stimuli are received by auditory hair cells (HCs) in the bony, snail-shaped cochlea, followed by transduction of the sound stimuli by the HCs to neural signals. Spiral ganglion neurons (SGNs), which are auditory primary neurons, are located in the central bony axis of the cochlea and responsible for transmitting auditory signals to the central auditory system. Excessive noise, ototoxic drugs, genetic disorders and aging all contribute to the causes of SNHL. Severe to profound SNHL affects 1 in 1000 newborns, and another 1 in 2000 children before they reach adulthood. About 60% of individuals older than 70 years will manifest SNHL. Previous studies on human temporal bones have demonstrated that the loss of HCs and/or SGNs is a major cause of SNHL [1]. Protecting HCs and SGNs from irreversible degeneration is therefore a primary objective due to the limited regeneration capacity of these cells. Acute SNHL sometimes responds to drug treatment; however, there are no therapeutic options for chronic SNHL except for hearing aids and cochlear implants, which are small devices that are surgically implanted into the cochlea in order to stimulate SGNs. However, the success of cochlear implants depends on the remaining SGNs and with their loss, this severely compromises the efficacy of this technique. HCs and SGNs are therefore the major targets for the treatment of SNHL.

WHY IS LOCAL DRUG DELIVERY REQUIRED FOR THE INNER EAR?

Based on the backgrounds described above, studies are being conducted with the hopes of providing an alternative means of biological therapy. Thus far, research has identified a number of candidates for use as therapeutic molecules. Experimentally, protective effects of neurotrophins have been demonstrated [2,3], and inhibitors of apoptosis and glutamate antagonists have also been shown to have the ability to promote HC survival [4-6]. Recently, it has been found that local application of genes by virus vectors induces HC regeneration in the mammalian auditory epithelium [7,8], and additionally, by silencing the mutant gene *via* RNA interference, can restore hearing loss in the genetic mouse model [9]. These therapeutic strategies are attractive and promising for the restoration of SNHL. However, clinical application is still quite limited. The problem of how to deliver such therapeutic molecules to the inner ear has been a considerable obstacle in the development of treatments for SNHL. One of the reasons for the difficulty of drug delivery involves the limited blood flow to the cochlea [10]. In addition, the blood–inner ear barrier, which inhibits the transport of drugs from serum to the inner ear, represents a fundamental obstacle to the use of systemic applications [11]. The inner ear tissues are isolated from the surrounding organs by a bony construction, which allows for the topical introduction of drugs or genes. Based on these considerations, local application has generally been the preferred method for drug administration to the inner ear. The sustained delivery of therapeutic molecules is also critical for the efficient treatment of the cochlea, as bioactive molecules usually require a period of minutes or hours over which they produce their pharmacological actions. Consequently, a number of researchers are currently working to solve these problems and develop methods for the local direct application of these molecules into the cochlea [12].

STRATEGIES FOR LOCAL DRUG DELIVERY

The cochlea is connected to the tympanic cavity by the round window membrane (RWM). When substances are applied intratympanically, the assumption is that they will enter the scala tympani through the RWM and then be dis-

*Address correspondence to this author at the Department of Otolaryngology-Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, 606-8507 Kyoto, Japan; Tel: +81-75-751-3346; Fax: +81-75-751-7225;
E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

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tributed throughout the cochlear fluids. The idea of using a topical application of medicine to the inner ear is not new, as local anesthetics and aminoglycosides were applied decades ago, with the compounds passing through the tympanic membrane into the tympanic cavity during the treatment of the inner ear disorders [13,14]. Intratympanic injections have been used for local application of aminoglycosides or steroids during therapy for Ménière's disease and sudden hearing loss. There are a number of clinical reports showing the efficacy of intratympanic injections of these drugs [see review in reference 12]. However, it is very difficult to predict the amounts of drugs that will actually reach the cochlear fluid space. Some reports have indicated that this method can lead to varying results during therapeutic treatment of Ménière's disease [15-17]. While intratympanic injection is a simple and easy method to perform, unfortunately, a controlled and sustained release of drugs cannot be achieved using this method. The pharmacokinetics of drug entry into cochlear fluids is crucial to determine the efficacy of the method for drug delivery into cochlear fluids [12]. Salt and Plontke have indicated importance of sustained delivery of drugs on the RWM by means of perilymph sampling from various regions of the cochlea [18] and computer simulation [19].

Implantable mini-pumps have also frequently been used for local drug delivery to the cochlea in animal experiments [20]. Several clinical reports have described the efficacy of local glucocorticoid application when using a semi-implantable mini-pump [21,22]. However, the use of an implantable mini-pump has not been widely adopted, given the need for surgical procedures similar to tympanoplasty that must be done in order to place the mini-pump. The use of a local viral gene transfer as a sustained treatment of the inner ear can provide sufficient protection from noise, drug toxicity and re-perfusion injury [23-28]. Today, adenoviral vectors or adeno-associated viral vectors are the most widely used for cochlear gene transfer, because of the high efficiency for the transfection, the availability of high titers, and the ease of production. However, their use can potentially initiate an immune response that results in the destruction of the recipient's cochlear cells.

The use of biomaterials for local drug delivery has recently gained attention as an alternative to the implantable mini-pumps or gene transfer using virus vectors. In general, biodegradable polymers containing therapeutic molecules are placed on the RWM, with the therapeutic molecules released into the cochlear fluids from the polymers in a controlled manner *via* the RWM [12,28].

DRUG DELIVERY *VIA* BIOMATERIALS

In the past decade, pharmaceutical technologists have paid increasing attention to controlled or sustained release technology using biomaterials for the delivery of drugs in order to avoid side effects and achieve sufficient drug levels in tissues. In an effort to develop a controlled-release system, a variety of methods using synthetic and natural materials have been undertaken. Recent publications have reported the use of a controlled-release system for local drug delivery to the inner ear. Two synthetic materials, siloxane-based polymers [29] and polylactic/glycolic acid (PLGA) polymers [30], and several natural materials, which include hyaluronic acid [31] and gelatin [32-34], have been used for this purpose.

Siloxane-based polymers have been used for years in medical applications that involve contact with the human body. In the clinic, silicone-transdermal patches have been widely used. In this system, drug release is controlled by its diffusion through the silicone network [35]. The actual release rate is determined by the composition of the polymer. This system is particularly suitable for application of lipophilic and low-molecular weight molecules. Arnold *et al.* [29] have utilized this system for local application of beclomethasone into the cochlear fluids. When using this system, the silicone microimplant remains on the RWM, although it does not induce functional and histological damage in the cochlea. Therefore, repeated treatments require that there is extirpation of the material used during the procedure.

Encapsulating bioactive molecules in PLGA or polylactic acid (PLA) particles has been also used as a method of controlled-release application. Water-insoluble, low-molecular weight agents have been encapsulated in PLGA or PLA microparticles and nanoparticles [36,37]. PLGA and PLA are familiar substances to surgeons, as they are the materials that make up absorbable sutures. Tamura *et al.* [30] examined the potential of PLGA nanoparticles for drug delivery to the cochlea using guinea pigs. To evaluate the use of PLGA nanoparticles (140 to 180 nm in diameter) in the cochlea, rhodamine, which is a red fluorescent dye, was encapsulated and then following local application onto the RWM, its overall distribution was evaluated. PLGA nanoparticles containing rhodamine were observed in the cochlea, indicating that PLGA nanoparticles can penetrate through the RWM. Rhodamine is released from PLGA nanoparticles after penetration of the particles through the RWM. Compared to a silicone microimplant, PLGA nanoparticles have the advantage of being able to be repeatedly applied, as the PLGA is dissolved by hydrolysis. However, there is a limitation with regard to the variation of the drugs that can be applied, since the process of encapsulation in the PLGA particles requires that compounds must be dissolved in acetone. Therefore, this method is not suitable for the delivery of proteins or peptides.

GELATIN HYDROGEL

Gelatin is a commonly used natural polymer that is derived from collagen. In the clinic, gelatin polymers have been widely used as hemostats. Recently, gelatin-based controlled-release systems have been developed [38]. During the fabrication process, the isoelectric point of gelatin can be modified to yield either a negatively charged acidic gelatin or a positively charged basic gelatin. This allows for electrostatic interactions to take place between charged therapeutic molecules and gelatin of the opposite charge, leading to the formation of polyion complexes. The significance of such a system is that it provides the ability for application of water-soluble, comparatively high-molecular weight proteins and peptides. Additionally, this method is also capable of being used for the delivery of plasmid DNA [39]. In this system, therapeutic molecules are released by enzymatic degradation of gelatin polymers, for which the rates can be determined based on the crosslinking density of the gelatin polymers.