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（分担）研究報告書

従来の初期治療法による急性高度難聴の治療成績の検討

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研究要旨

京都大学医学部附属病院で施行中の生体吸収性徐放ゲルによるIGF1内耳局所投与による急性高度難聴治療第Ⅰ－Ⅱ相臨床試験の次の段階にあたる第Ⅱ相臨床試験のプロトコルデザインのための調査として、突発性難聴症例の発症数および現在の治療の有効率を調べる目的で、当院での2年間の突発性難聴の症例数および治療成績について検討した。結果、2年間で入院治療を行った症例は29例であり、11例、40%が軽度回復あるいは不変となり、これらの症例が第Ⅱ相試験の対象となることが推察された。

A. 研究目的

京都大学医学部附属病院で現在、ステロイド全身投与が無効であった急性高度難聴症例を対象として、生体吸収性徐放ゲルによるIGF1内耳局所投与による急性高度難聴治療第Ⅰ－Ⅱ相臨床試験を施行中である。現時点で、17例の登録が行われ、明らかな有害事象は認められておらず、今後有効性を検証するための第Ⅱ相臨床試験へと進むことが予想される。臨床試験プロトコル作成に際しては、倫理面、臨床疫学的な妥当性、統計学に基づいたデザインが重要であるが、リクルート出来る症例数についての適切な予測も確実に臨床試験を完遂するためには不可欠な要素である。本研究では、第Ⅱ相臨床試験における対象症例リクルートの予測を行うことを目的として、従来の初期治療を施行した急性高度難聴症例の当院における過去2年間の臨床統計を行った。

B. 研究方法

2007年1月1日～2008年12月31日までの2年間に突発性難聴で虎の門病院耳鼻咽喉科に入院し，安静・点滴などの初期治療を施行した重症例について、治療法、有効性を解析した。有効性は、投与開始後12週時点での治療効果を厚生省特定疾患突発性難聴研究班が策定の突発性難聴・聴力回復の判定基準にて判定した。また、初期治療方法の違いによる有効性への影響について調べた。

C. 研究結果

対象となる症例は、29 例であり、Hydrocortisone soudium succinate（ソルコーテフ）500mg からの 2 週間での漸減投与例が 24 例であり、Predonisolone sodium succinate（プレドニン）40mg からの 2 週間での漸減投与例を行った症例が 5 例であった。投与開始後 12 週時点での治療効果を厚生省特定疾患突発性難聴研究班が策定の突発性難聴・聴力回復の判定基準による判定では、治癒 10 例 34.5%、著明回復 8 例 27.6%、回復 3 例 10.3%、不変 8 例 27.6%であった。施行したステロイド治療の違いによる有効性の違いは認められなかった。

D. 考察

従来の入院,安静, 点滴を行う治療方法では 18 例 60%が治癒あるいは著明回復例となることが判明した. しかし, 11 例 40%は軽度回復, あるいは不変例であり, これらは今後, 内耳薬物投与システムを応用した感音難聴治療技術の適応となるであろうことが推測された。ただし、本検討は入院治療例のみを対象としていることから、外来加療を含めた突発性難聴症例数はかなり多いことが推察できるが、本院では重症例では基本的に入院治療を行っていることから、臨床試験の対象となるであろう症例は、ほぼ今回検討した重症例に含まれるのではないかと推察する。

E. 結論

本院における生体吸収性徐放ゲルによる IGF1 内耳局所投与による急性高度難聴治療第 II 相臨床試験の対象となりうる症例数を予測するために、過去 2 年間の入院治療を行った突発性難聴症例の検討を行った。結果、年間 5〜6 症例が対象となることが予測された。

F. 研究発表

なし

G. 知的所有権の取得状況

1) 特許取得
　なし
2) 実用新案登録
　なし
3) その他
　なし

研究成果の刊行に関する一覧表

著書

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OTOLOGY

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

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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

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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

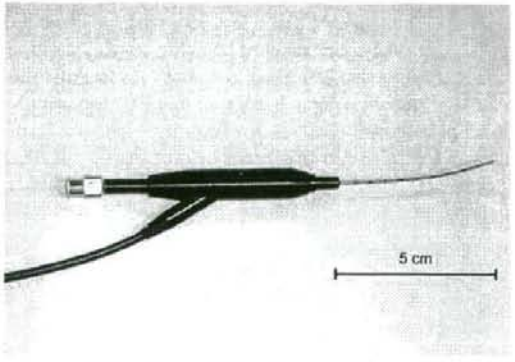

Fig. 1 A microendoscope specially modified for better visualization of the RWM. The outer diameter is 0.9 mm and the length is 50 mm. The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter)

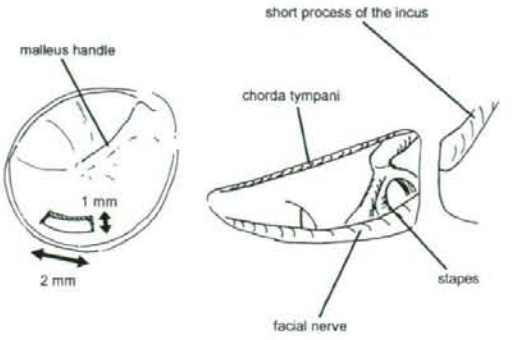

Fig. 2 A small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife. Posterior hypotympanotomy was made as large as possible. In all specimens, the facial nerve and chorda tympani were skeletonized

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).

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.

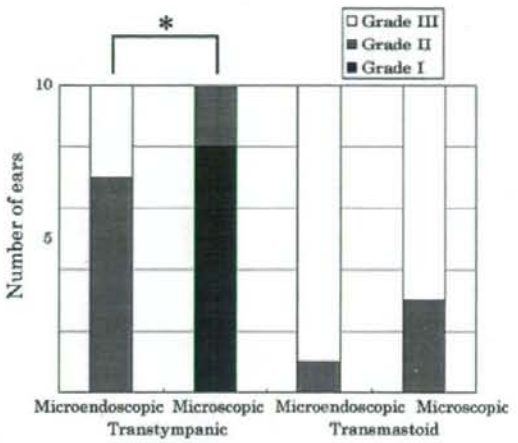

Fig. 3 The visibility of the RWM for four approaches. Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. The visibility through the transtympanic microendoscopic approach was better than that with transtympanic microscopic approach

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

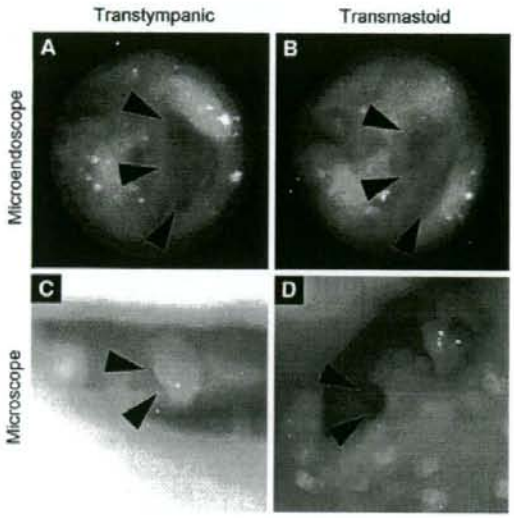

Fig. 4 The RWM of bone three observed through four approaches (*arrow heads*). The transtympanic microendoscopic approach (**a**), transmastoid microendoscopic approach (**b**), and transmastoid microscopic approach (**d**) provided good views. In the transtympanic microscopic approach (**c**), only 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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Conflict of interest We do not have a financial relationship with the organization that sponsored the research.

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Local Drug Delivery to Inner Ear for Treatment of Hearing Loss

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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-

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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.

The potential use of the gelatin hydrogel system was initially investigated for cochlear delivery of brain-derived neurotrophic factor (BDNF) [32]. BDNF plays a crucial role in the development of the inner ears [38] and in the maintenance of the auditory function [41]. In addition, previous studies have demonstrated the effects of local BDNF application when using an osmotic mini pump [3] or adenovirus [26]. We measured BDNF concentrations in the cochlear fluid after placing a gelatin hydrogel that contained this agent onto the RWM [32]. The results revealed a sustained delivery of BDNF into the cochlear fluid *via* the hydrogel over a seven-day period. The functional and histological protection of the SGNs by BDNF that was applied through the gelatin hydrogel was then examined using a guinea pig model of SGN degeneration. The measurement of electrically evoked auditory-brainstem responses, which reflect SGN function, demonstrated that BDNF delivered *via* gelatin hydrogels was able to significantly reduce the threshold elevation [32]. Histological analysis demonstrated an increased survival of SGNs due to BDNF application through gelatin hydrogels. These findings indicate that gelatin hydrogel can be utilized for drug delivery to the cochlea.

Subsequently, we examined the efficacy of cochlear delivery of insulin-like growth factor-1 (IGF1) for the protection of auditory HCs against acoustic trauma [33]. IGF1 is a mitogenic peptide that plays essential roles in the regulation of growth and development in the inner ear [42]. In addition, previous studies on the inner ear have suggested the possibility of inner ear protection by IGF1 [43,44]. Moreover, recombinant human IGF-1 (rhIGF1) has already been approved for clinical use. Therefore, we selected rhIGF1 as a suitable trophic factor for local cochlear application using a gelatin hydrogel. Local rhIGF1 application through the gelatin hydrogel prior to noise exposure has been shown to efficiently protect the hearing from noise trauma. Additionally, histological analysis also revealed that local rhIGF-1 treatment ameliorated the loss of HCs [33].

Our ultimate goal is the clinical use of a local rhIGF1 application using gelatin hydrogel as a therapeutic option for the treatment of SNHL. Therefore, we examined whether post-traumatic application of rhIGF1 to the cochlea *via* gelatin hydrogels could attenuate noise-induced hearing loss. The results demonstrated that functional and histological efficacy of local rhIGF1 treatment on the attenuation of noise-induced hearing loss occurred in a dose-dependent manner [34]. We also measured IGF1 concentrations in the cochlear fluid, cerebrospinal fluid (CSF) and serum after placing a gelatin hydrogel containing rhIGF1 onto the RWM of guinea pigs. The results demonstrated that there was sustained delivery of rhIGF1 into the cochlear fluid, in addition to no alterations of the IGF1 levels in CSF and serum [34]. There were also no adverse effects due to local rhIGF1 treatment found in any of the experimental animals. These findings document both the effectiveness and the safety of local rhIGF1 treatment using gelatin hydrogels for noise-induced hearing loss.

CELL TRANSPLANTATION

Chronic SNHL is usually incurable because of the loss of HCs and SGNs, and which at the present time is irreversible. Therefore, an alternative means of biological therapy, including cell therapy is required. Indeed, recent studies have indicated that cell therapy could be utilized to regenerate HCs [45] and SGNs [46]. In contrast, cell transplantation is an alternative that can be used as a method for drug delivery where the transplanted cells for this purpose have the ability to survive and generate therapeutic agents. Several stem cells have been reported to have the ability to secrete trophic factors [47-49]. Cell transplantation has been used as a means of delivering peptides or proteins into the central nervous system, demonstrating its viable use as a delivery vehicle for therapeutic molecules [50,51].

Iguchi *et al.* have reported on the ability of neural stem cell-derived cells being used for the production of BDNF and glial cell line-derived neurotrophic factor (GDNF) after engraftment into the cochlea [47]. In addition, transplantation of neural stem cells into the cochlea has the potential of being able to attenuate HC damages due to transient ischemia of the cochlea [48]. Bone marrow derived cells also have the potential for secreting trophic factors. Implantation of bone marrow stromal cells has been reported to contribute to functional recovery of the brain [52] and spinal cord [53] by means of producing trophic factors. Furthermore, previous studies have revealed the potential of bone marrow derived cells surviving in the cochlea [54,55]. Yoshida *et al.* have demonstrated a significant increase in the protein level of GDNF in cochlear specimens and the prevention of HC death due to transient cochlear ischemia by engraftment of hematopoietic stem cells [49]. These findings indicate that cell transplantation into the cochlea may be a novel strategy for treatment of SNHL by providing a means for local application of trophic factors within the cochlea.

Transplantation of cells that have been genetically manipulated ex vivo has been used as a means of delivering peptides or proteins into the central nervous system [56-58]. In comparison with the stem cell transplantation that has been described above, this strategy has an advantage in that aimed gene-encoded products are applicable. In addition, use of non-viral vectors for ex vivo gene transfer potentially could resolve the problem of viral vector toxicity in cochlear gene therapy. Therefore, we conducted an examination of the efficacy of cell-gene delivery in the application of therapeutic molecules into the cochlea [59]. NIH3T3 cells were chosen as a delivery vehicle for the gene. NIH3T3 cells are a well-established fibroblast cell line, thus, it is easy to optimize conditions for gene transfer and to select gene-expressing cells for use *in vitro*. In addition, such fibroblasts are available from various human sources, which may be advantageous for extending future clinical investigations. NIH3T3 cells were transfected with the BDNF gene using lipofection, with the cells expressing the BDNF gene being selected for use. We examined the potential for transplanting transfected NIH3T3 cells into the inner ear of the mouse. Immunohistochemistry and Western blotting demonstrated the survival of the grafted cells within the cochlea, and a BDNF-specific enzyme-linked immunosorbent assay revealed a significant increase in BDNF production in the inner ear following cell transplantations [59]. These findings indicate that cell-gene delivery with non-viral vectors may be applicable for the local, sustained delivery of therapeutic

molecules into the cochlea. Cell-gene delivery of therapeutic molecules into the inner ear is suitable for protection of inner ear cells against gradually progressive degeneration. Presbycusis, which is an age-related hearing loss, may also need to be included as one of the targets for cell-gene therapy. BDNF application *via* cell-gene delivery could be an effective strategy for survival promotion of SGNs in cases involving cochlear implants, which require the opening of the cochlea for the purpose of inserting an electrode.

CONCLUSIONS

The lack of effective methods for drug delivery to the cochlea has been a considerable obstacle with regard to developing novel therapeutic strategies for SNHL. However, recent findings in studies examining drug delivery systems using biomaterials and cell therapy demonstrate the efficacy of these strategies for cochlear drug delivery, which in the future may contribute to the establishment of novel therapeutic strategies for SNHL.

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Insulin-like growth factor I treatment via hydrogels rescues cochlear hair cells from ischemic injury

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This study was designed to investigate the protective effects of recombinant human insulin-like growth factor I (rhIGFI), applied locally via a hydrogel, against ischemic damage of the cochleae in gerbils. A hydrogel was immersed in rhIGFI or saline and was applied on the round window membrane 30 min after the ischemia. Local rhIGFI treatment significantly reduced the elevation of auditory brain responses thresholds at a frequency of 8 kHz on days 1, 4, and 7 after ischemia. A histological analysis revealed increased survival of inner hair cells in the animals treated with rhIGFI via the hydrogel 7 days after ischemia. These findings showed that local rhIGFI application using a hydrogel has the potential to protect the cochleae from ischemic injury. *NeuroReport* 19:1585–1588 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: cochlear, drug delivery, gerbil, growth factor, hair cell, ischemia, protection

Introduction

Sensorineural hearing loss (SNHL) is one of the most common disabilities in Japan, but therapeutic options for SNHL are currently limited. Studies are being conducted to provide alternative means of biological therapy for the inner ear, and several agents have been shown to exert therapeutic activity against SNHL. The delivery of therapeutic molecules to the inner ear is, however, currently a significant obstacle to their clinical application. Systemically applied drugs have great difficulty reaching inner ear cells because of the blood–labyrinth barrier [1], which acts as an obstacle to the transfer of drugs from the serum to cochlear cells, and the limited blood flow to the cochlea [2]. Therefore, the development of a local drug delivery system for the cochlea is crucial for the clinical application of therapeutic agents. On the basis of this need, drug delivery systems using biodegradable materials such as gelatin hydrogels have been investigated for use in treatment of the inner ear. Recent studies have demonstrated the efficacy of gelatin hydrogels for this purpose [3–5]. After gelatin hydrogels containing therapeutic molecules are placed on the round window membrane (RWM), the therapeutic molecules are released from the gelatin polymers and move through the RWM into the perilymph of the cochlea.

Recombinant human insulin-like growth factor 1 (rhIGF1), which is a mitogenic peptide with essential roles in the regulation of growth and development of the inner ear [6], has been approved for clinical use. Earlier studies have demonstrated that local rhIGF1 treatment via gelatin hydrogels is effective for attenuating noise-induced hearing loss [4,5]; however, its potential for SNHL has not been examined owing to the additional pathogenesis of SNHL. Cochlear ischemia and reperfusion injury has been considered one cause of acute profound SNHL. We thus established a gerbil model for cochlear ischemia and reperfusion that represents hearing loss similar to sudden deafness in humans [7,8]. Sudden deafness can be defined as greater than 30 dB of hearing loss over at least three contiguous audiometric frequencies occurring within 3 days or less. Though several treatment methods can be used to treat sudden deafness, either in combination or alone, there is no universally accepted treatment for sudden deafness.

In this study, we examined the efficacy of local rhIGF1 treatment via gelatin hydrogels in protection against cochlear damage because of ischemia and reperfusion, to investigate the potential clinical use of this treatment for sudden deafness. We applied gelatin hydrogels containing rhIGF1 to the RWM of gerbils after the onset of SNHL produced by cochlear ischemia and evaluated auditory function by measurements of auditory brain stem responses (ABRs) and cochlear histology.

Materials and methods

Animals

Adult male Mongolian gerbils weighing 60–80 g (n=12) were used in this study. The study was conducted in accordance with the Guidelines for Animal Experimentation

of Ehime University School of Medicine, Japan. All experimental procedures were performed in accordance with the US National Institutes of Health guidelines for the care and use of laboratory animals.

Biodegradable gelatin hydrogels

A biodegradable hydrogel has been developed for sustained delivery of peptides, including growth and trophic factors [9]. In this approach, a positively charged protein is electrostatically complexed with negatively charged polymer chains, which form components of the biodegradable hydrogel. Biodegradation of the polymer chains leads to the release of the peptide. Biodegradable hydrogels are generated by glutaraldehyde cross-linking of gelatin, and the rates of degradation are determined by the concentration of glutaraldehyde. We extracted the residual glutaraldehyde in 48.5 mg of the gelatin hydrogel measured by the gas chromatography/mass spectrography method. The residual glutaraldehyde ratio was 0.14 ppm. An earlier analysis of in-vitro IGF1 release from hydrogels demonstrated that a hydrogel made with 10 mmol/l glutaraldehyde allows optimal IGF1 delivery *in vitro* [10] and enables the sustained delivery of rhIGF1 to cochlear fluids after placement on the RWM *in vivo* [5]. We therefore used this type of hydrogel in this study.

Transient cochlear ischemia and drug application

Baseline ABR thresholds were measured within 7 days before the induction of cochlear ischemia. Cochlear ischemia in gerbils was induced by temporarily occluding the bilateral vertebral arteries in the neck as described previously [7,8]. In brief, under general anesthesia with 1% halothane, the bilateral vertebral arteries were exposed just before their entry into the transverse foramen of the cervical vertebra. A 4–0 silk suture was loosely looped around each vertebral artery, and ischemia was induced by pulling the ligatures with 5 g weights. After 15 min of ischemia, the sutures were removed to allow recirculation, which was confirmed by observation under an operating microscope.

At 30 min after recirculation began, the otic bulla was opened to expose the RWM. A sheet of dried hydrogel was cut to a size of 1.5–2 mm^3 under a microscope. A piece of the hydrogel was immersed in rhIGF1 (400 μg dissolved in 40 μl physiological saline; Astellas, Tokyo, Japan) for 30 min and then applied onto the left RWM of each animal in the IGF group (n=6). In the control group, the hydrogel pieces were immersed in physiological saline (n=6).

Functional analysis

Auditory function was assessed by ABR recordings. Measurements of ABR thresholds for an 8-kHz tone burst (rise/fall time, 0.5 ms; duration, 10 ms) were performed before and at 1, 4, and 7 days after the ischemic insult. The ABRs were recorded using a signal processor (NEC Synax 1200, NEC Medical Systems, Tokyo, Japan). 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). Cochlear sensory epithelia corresponding to the region of the 8 kHz tone were found to be the most vulnerable to the ischemic injury used in this study [11]. Responses to 300 consecutive stimuli were averaged, and the ABR threshold was determined by measuring the responses in 5-dB steps.

Histological analysis

On day 7 after ischemic injury, the animals were deeply anesthetized with halothane, and the cochleae were harvested. Immediately after removing the otic bulla, the cochlea was perfused intrascalarly with 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 and post-fixed for 2 h with the same fixative at 4°C. After rinsing with phosphate-buffered saline at pH 7.4, sensory epithelia were dissected from the temporal bones and subjected to histological analysis in whole mounts. The specimens were stained with rhodamine–phalloidin (1:250, Molecular Probes, Eugene, Oregon, USA) and Hoechst 33342 (20 μg/ml; Calbiochem-Novabiochem Corporation, La Jolla, California, USA). The specimens were then mounted in carbonate-buffered glycerol and viewed under an Olympus BX60 microscope (Olympus Bx60, Olympus America, Lake Success, New York, USA). The numbers of intact and missing hair cells were counted in the basal turn of each cochlea, and the ratio of missing hair cells was calculated for the inner hair cells (IHCs) and outer hair cells, as described previously [8,11].

Statistical analysis

An overall effect of rhIGF1 application on ABR threshold shifts was examined using two-way factorial analysis of variance. For significant interactions, multiple comparisons with Fisher's protected least-significant difference were used for pairwise comparisons. The differences in the ratios of missing IHCs and outer hair cells between the IGF-treated and control groups were examined using an unpaired t-test. A P value of less than 0.05 was considered statistically significant. Values are expressed as means ± standard deviation.

Results

Functional analysis

In the control animals, which had received gelatin hydrogels immersed in saline, the ABR threshold was elevated to 47.5 ± 6.9 dB on day 1 and gradually decreased to 35.0 ± 7.1 dB on day 7. The degree of the ABR threshold shifts observed in the control group was almost identical to that in earlier studies using temporal occlusion of bilateral vertebral arteries [11,12]. Although elevated ABR thresholds were also found in the IGF group, local rhIGF1 treatment significantly reduced the ABR threshold shifts compared with the control values (Fig. 1). The ABR threshold elevation in the IGF group was significantly lower than that in the control group at each time point. Attenuation of the ABR threshold elevation was especially marked on day 1.

Histological analysis

The loss of hair cells (HCs) was observed in cochlear specimens from the control group (Fig. 2a and b). Degenerative changes that were apparent in the IHC region of control specimens were identical to previous observations [8,11,12]. In contrast to the control specimens, the rhIGF1-treated specimens exhibited limited loss of IHCs (Fig. 2c and d). Quantitative analysis revealed that local rhIGF1 treatment significantly suppressed ischemia-induced IHC loss (Fig. 2e). The number of missing IHCs in the rhIGF1-treated

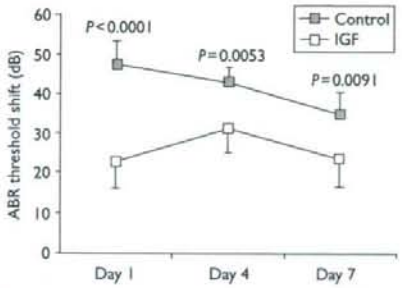

Fig. 1 Changes in the auditory brainstem response (ABR) threshold after transient cochlear ischemia. The ABR was measured in response to an 8000-Hz tone burst. The ABR threshold before ischemia was defined as 0 dB. The average increases in the ABR threshold on days 1, 4, and 7 were significantly less in the insulin-like growth factor (IGF) group than in the control group. All values are means ± SD. (n=6 for each group).

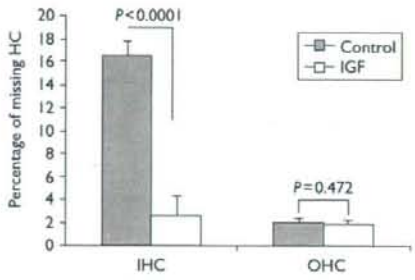

Fig. 3 The rates of inner hair cell (IHC) and outer hair cell (OHC) loss 7 days after transient cochlear ischemia. The rate of IHC loss in the insulin-like growth factor (IGF)-treated group was significantly less than that in the control group. No statistical difference in the rate of OHC loss between the two groups was present. All values are means ± SD. (n=6 for each group).

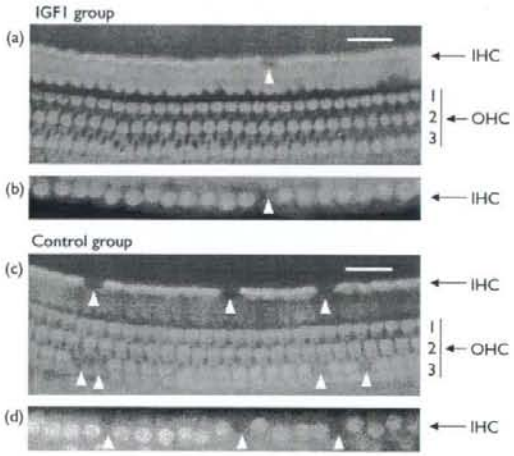

Fig. 2 The surface structure of the organ of Corti 7 days after cochlear ischemia. Representative fluorescence images of the organ of Corti stained with rhodamine-phalloidin (a and c) or Hoechst 33342 (b and d). The organ of Corti samples were obtained from the otic bullae of the (insulin-like growth factor) IGF group (a and b) and control group (c and d). Three rows of outer hair cells (OHCs) and a single row of inner hair cells (IHCs) are present. Fluorescence microscopy revealed fewer IHC deficits in the IGF group compared with the control group. Scale bar=20 μm.

specimens was only 15.8% of that in the control specimens (Fig. 3). In contrast, no significant difference in the percentage of missing OHCs was found between the two experimental groups.

Discussion

These findings showed that the posttraumatic application of rhIGF1 into the cochlea via gelatin hydrogels significantly attenuates hearing impairment and IHC damage attributable to cochlear ischemia, supporting the therapeutic potential of local rhIGF1 treatment for acute SNHL because of cochlear ischemia. Earlier studies on acoustic trauma models have demonstrated the efficacy of local rhIGF1 treatment for acute SNHL resulting from acoustic injury [4,5]. This type of the gelatin hydrogel has been used in a clinical trial for angiogenesis in patients with critical limb ischemia [13], and the safety of the gelatin hydrogel for topical application into the middle ear has been demonstrated in the guinea pig experiments [5]. On the basis of existing evidence, local rhIGF1 treatment should be considered for the treatment of sudden deafness.

In the gerbil model for cochlear ischemia and reperfusion, cochlear damage is divided into two phases, acute and chronic. In the acute phase, afferent dendrites attached to IHCs typically swell [8,14]. Degeneration in the cochlear lateral wall is also found in the acute phase [15]. These histological changes are usually reversible and are thought to cause temporal threshold shifts of the ABRs in this model. IHC loss is characteristic of the chronic phase of ischemia-induced cochlear degeneration and results in permanent threshold shifts of the ABRs [8,14]. These findings showed that local rhIGF1 treatment significantly attenuates both temporal and permanent threshold shifts of the ABRs. In addition, local rhIGF1 treatment has the capacity to protect IHCs against cochlear ischemia. Local rhIGF1 treatment is therefore effective for the attenuation of chronic and irreversible degeneration of cochlear sensory epithelia.

Interestingly, these findings showed a notable reduction of the ABR threshold elevation on day 1 in rhIGF1-treated animals during the acute phase of cochlear damage. This observation is quite different from the effects of local rhIGF1 treatment on acoustic trauma models [4,5], in which no significant effects were identified in the acute phase of cochlear damage. This difference might have been caused by differences in the mechanisms for acute cochlear dysfunction between ischemic and acoustic injury. The effects of local rhIGF1 treatment on the cochlear lateral wall or afferent dendrites in the ischemia model might have contributed to the different effects in the acute phase. Further investigations are required to elucidate the precise mechanisms for the effects of rhIGF1 on acute damage after cochlear ischemia.

Conclusion

These findings showed the efficacy of local rhIGF1 application using a biodegradable hydrogel for protecting the

cochleae from ischemic injury. Our goal is the clinical use of local rhIGF1 treatment via a gelatin hydrogel as a therapeutic option for sudden deafness. At present, rhIGF1 has been approved for clinical use. These findings may help in advance the clinical application of local rhIGF1 treatment using gelatin hydrogels for the treatment of sudden deafness.

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Bone Marrow-Derived Cells Expressing Iba1 Are Constitutively Present as Resident Tissue Macrophages in the Mouse Cochlea

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Immune-mediated inner ear disorder has been well established as a clinical entity; however, the innate immune system of the inner ear is a poorly understood area of research with high clinical and immunological importance. Although the presence of resident tissue macrophages in the inner ear has been suggested, there has been some controversy. In this study, we analyzed the origin of cochlear resident macrophages and the contribution of hematopoietic bone marrow (BM) to the recruitment of macrophages in the cochlea. To visualize the localization of BM-derived cells, BM chimeric mice were made by transplantation of hematopoietic stem cells, which were genetically labeled with enhanced green fluorescent protein, into lethally irradiated C57BL/6 mice. The distribution and characteristics of BM-derived cells in the mouse cochlea were studied immunohistochemically. We successfully identified the constitutive presence of tissue resident macrophages in the spiral ligament and spiral ganglion that are derived from BM in larger numbers than previously reported. Moreover, cochlear resident macrophages gradually turn over for several months during steady-state replacement by BM-derived cells, and the number of cochlear macrophages immediately increased in response to local surgical stress. The present findings demonstrate the hematopoietic origin of cochlear resident and infiltrating macrophages. Our study provides a novel anatomical and immunological basis for the inner ear and indicates that the cochlear resident macrophages would be a therapeutic target in inner ear disorders.

Key words: hematopoietic stem cell; microglia; innate immunity; inner ear

Macrophages are generally considered to be derived from circulating monocytes and roughly classified into two categories; 1) infiltrating macrophages, which migrate from the circulation into tissues in response to inflammatory signals, and 2) resident tissue macrophages, which are present in tissues during steady-state conditions. Resident tissue macrophages take up residence in virtually every tissue of the body and have a broad role in the innate immune system. Recent studies have demonstrated multiple key functions of resident tissue macrophages not only in phagocytosis of foreign bodies or senescent cells but also in the production and secretion of cytokines and the regulation of specific immune responses (Gordon and Taylor, 2005).

The inner ear was once believed to be an immunoprivileged organ isolated by the blood–labyrinthine barrier similar to the central nervous system (CNS) and the cornea and retina of the eye. Although immune-mediated inner ear disorders have been well established as a clinical entity with progressive and fluctuating bilateral sensorineural hearing loss (SNHL), the innate immune system of the inner ear is a poorly understood area of research with high clinical and immunological importance. Recently, Hirose et al. (2005) reported the existence of mononuclear phagocytes in the spiral ligament of nonnoise-exposed $CX3CR1^{GFP/GFP}$ transgenic mice, and the density of CD45-positive cells in the cochlea was quite different between wild-type and transgenic mice used in the study. Lang et al. (2006) also reported that bone marrow (BM)-derived cells are present in the noninjured inner ear of BM chimeric mice and that 5% of BM-derived cells are macrophages dual-labeled with CD45R and/or F4/80. However, the phenotypes shown by BM-derived cells in the inner ear are as yet only partially understood. Whereas previous studies have reported macrophages infiltrating into the coch-

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