Inner ear drug delivery

Chapter 12

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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. Here we review recent progress in basic and clinical studies for cochlear drug delivery.

KEY WORDS: Drug delivery · Cochlea · Biodegradable polymer · Sensorineural hearing loss

Therapeutic targets for treatment of SNHL

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. In addition, the cochlear lateral wall is also included in important components for auditory function, because of its critical role in the maintenance of the endocochlear potential.[2,3] Therefore, cochlear cells in the cochlear lateral wall including the stria vascularis(SV) and the spiral ligament(SL). All together, the aim of pharmacological treatments using inner ear drug delivery systems is to protect or regenerate HCs, SGNs and cells in the SV and SL.

Cochlear drug delivery

Intratympanic injections have been used for local application of aminoglycosides or steroids during therapy for Ménière's disease and acute SNHL. There are a number of clinical reports showing the efficacy of intratympanic injections of these drugs.[4] 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.[5-7] 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.[4] 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 distributed throughout the cochlear fluids. 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[8] and computer simulation.[9]

To achieve sustained delivery of drugs into the cochlea, implantable mini-pumps have been used for local drug delivery to the cochlea in animal experiments. Several clinical reports have described the efficacy of local glucocorticoid application when using a semi-implantable mini-pump.[10-12] In addition, a new device for cochlear drug delivery through the RWM has been reported.[13] 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. More recently, the use of cochlear implant electrodes for drug delivery has been investigated.[14] However, this method was limitedly applied for patients with cochlear implants.

The use of biomaterials for local drug delivery has recently gained attention as an alternative to the implantable mini-pumps. 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.[4, 15] Usually, biomaterilas are disappeared via degradation by enzymes. Therefore, there is no need of surgery to remove the materials after drug application differed from implantable mini-pumps.

In considering clinical application, the surgical approach to the RWM is critical issue for local drug delivery to the cochlea, if biomaterials are used as a carrier of drugs. In rodents, it is easy to visualize the RWM by opening the otic bullae. Therefore, in animal experiments there are few obstcles for approach to the RWM. On the other hand, in clinical application, the trans-tynmapic approach will be used for approach to the RWM for its low invasiveness. In humans, 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. We then examined the potential of a specially modified microendoscope for a transtympanic approach to the RWM using ten human temporal bones.[16] The results revealed that the transtympanic microendoscopic approach enabled visualization of the RWM in all specimens, whereas the transtympanic microscopic approach only permitted visualization in three specimens.[16] These findings indicated that the transtympanic microendoscopic approach can be utilized for cochlear drug delivery through the RWM in the clinic.

Gelatin hydrogel

Previously, we have developed cochlear drug delivery systems using two kinds of biomaterials, gelatin polymers[17-21] and poly lactic/glycolic acid(PLGA) polymers.[22] 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.[23] 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. 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).[17] BDNF plays a crucial role in the development of the inner ears and in the maintenance of the auditory function. In addition, previous studies have demonstrated the effects of local BDNF application when using an osmotic mini pump[24] or adenovirus.[25] We measured BDNF concentrations in the cochlear fluid after placing a gelatin hydrogel that contained this agent onto the RWM.[17] 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.[17] 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.

Next, we examined the efficacy of gelatin hydrogels for cochlear delivery of clinically available growth factor, insulin-like growth factor 1(IGF1), as a pre-clinical examination. First we confirmed sustained delivery of recombinant human IGF-1(rhIGF1) into the cochlear fluid in guinea pigs.[19] Then cochlear protection by local application of rhIGF1 against noise trauma[18, 19] or ischemic injury[20] was examined. Local application of rhIGF1 via gelatine hydrogels exhibited significant protection of cochlear hair cells against noise and ischemia. 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 inner ears

Based on the findings in animal experiments, we have started phase I-IIa clinical trial to test the efficacy and the safety of local rhIGF1 therapy using gelatin hydrogels for acute SNHL that was resistant for systemic glucocorticoid application. Primary outcomes were set to evaluate hearing levels in pure tone audiometory 12 weeks after the treatment. Secondary outcomes were rates for the occurrence of adverse effects including middle ear inflammation and vertigo. The target sample size was 25 cases, and case registration has been already finished. In January 2010, the observation period will be ended. In the near future, we will present the outcomes of this clinical trial.

Recently, we also examined the potential of another candidate that were applied locally using gelatin hydrogels. Hepatocyte growth factor(HGF) was originally identified as the protein that is responsible for stimulating hepatocyte proliferation.[26] It is present in various cells and is a paracrine cellular growth and morphogenetic factor.[27, 28] Previous reports have documented the potential use of gelatin hydrogel for a sustained release of HGF.[23, 29] Firstly, we assessed protective effects of HGF on mouse cochlear hair cells against neomycin toxicity using explant culture systems.[30] The application of HGF to cochlear explant cultures significantly reduced the hair cell loss induced by neomycin.[30] Immunohistochemistry showed c-Met, HGF receptor, expression in normal auditory hair cells and its

increase in response to neomycin-induced damage.[30] These findings demonstrate that a functional HGF/c-Met coupling is present in the cochlea and HGF application exerts protective effects on hair cells, indicating the potential of HGF as a therapeutic agent for sensorineural hearing loss. We then examined the efficacy of the local application of gelatin hydrogels containing HGF in protecting cochlear hair cells from noise-induced damage.[21] Local HGF treatment significantly reduced the noise exposure-caused ABR threshold shifts and the loss of hair cells.[21] HGF could be used for inhibition of cochlear fibrosis following cochlear implantation, because HGF has a potential to inhibit fibrosis in connective tissues.[31,32]

PLGA microparticle

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.[33,34] PLGA and PLA are familiar substances to surgeons, as they are the materials that make up absorbable sutures. We applied this technique for cochlear lidocaine delivery aimed to attenuate tinnitus. Lidocaine is a local anesthetic that is known to suppress tinnitus via systemic or local application; however, this effect has only limited duration. We then examined the potential of PLGA microparticles for sustained delivery of lidocaine into the cochlea.[35] Lidocaine-loaded PLGA microparticles were produced and their in vitro-release profiles were examined. The results demonstrated that the microparticles were capable of the sustained delivery of lidocaine for 30 days.[35] The lidocaine concentrations in the cochlear fluid were measured at different time points following the application of the lidocaine-loaded PLGA microparticles to the RWM of guinea pigs. The possible adverse effects of the local application of lidocaine-loaded PLGA microparticles were also examined. The in vivo experiments demonstrated the sustained delivery of lidocaine into the cochlear fluid for 14 days, and the maintenance of high lidocaine concentrations in the perilymph for up to 3 days after application.[35] Nystagmus and inflammation in the middle-ear mucosa were not detected after the local application of lidocaine-loaded PLGA microparticles.[35] These findings demonstrated that lidocaine-loaded PLGA microparticles are capable of the sustained delivery of lidocaine into

the cochlea, suggesting that they could be used for the attenuation of peripheral tinnitus.

Nanoparticle

Nanoparticles are capable to be applied either locally or systemically, differed from microparticles. In addition, nanoparticles can be targetable to selected cell populations in the inner ear, not only equipped with controlled drug release. The first documentation on the potential of nanoparticles for cochlear drug delivery was reported using PLGA nanoparticles loaded rhodamine.[22] After systemic application of PLGA nanoparticles loaded rhodamine, pharmacokinetic analyses demonstrated that PLGA nanoparticles have no capacity for sustained or targeted drug delivery to the cochlea.[22] However, after local application onto the RWM, rhodamine fluorescence was identified in cochlear specimens, indicating that PLGA nanoparticles can penetrate through the RWM.[22] These findings indicate that PLGA nanoparticles have no capacity for targeted delivery to the cochlea after systemic application, and that PLGA nanoparticles are capable to penetrate the RWM suggesting the potential as a tool for intracochlea drug release.

Recently, extensive research of nanoparticles for inner ear treatment has been performed. In EU, the NANOEAR project, of which goal is to develop multifunctional nanoparticles for inner ear treatment, has been launched in 2007. Several types of nanoparticles have been made and examined their potential for drug delivery to specific inner ear cells, in particular following local drug delivery.[36, 37] However, therapeutic potential of these nanoparticles has not been elucidated. In the near future, therapeutic potentials of various types of nanoparticles for SNHL will be evaluated. In addition, their potential for targeted delivery to the cochlea after systemic application should be examined.

Conclusions

In the last decade, several methods for cochlear drug delivery have been developed, and clinical studies for a part of cochlear drug delivery systems have already been launched. In this paper, recent advance in local drug application to the cochlea are reviewed. But, technologies reviewed here may be utilized for gene or peptide delivery into the cochlea. Recent clinical

trials using cochlear drug delivery systems are aimed to examine the efficacy for acute SNHL. Further progress in experimental studies on cochlear drug delivery systems might make possible its application for cochlear regeneration leading to development of novel biological therapies for chronic SNHL.

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1．細胞増殖因子
14）内耳

中川隆之・伊藤壽一

内耳の発達や機能維持に，神経栄養因子や細胞増殖因子は重要な役割を果たしている。また，障害された内耳の治療にも有効であることが基礎的に示されており，臨床応用が期待されている。しかし，臨床応用に際しては，内耳にこれらの因子を適切に送達する方法を開発する必要があった。われわれは，ゼラチンハイドロゲルによる薬物徐放を内耳への神経栄養因子や細胞増殖因子の徐放に応用し，基礎的にその有効性を確認し，インスリン様細胞増殖因子１の内耳徐放による急性高度難聴治療の臨床試験を行っている。

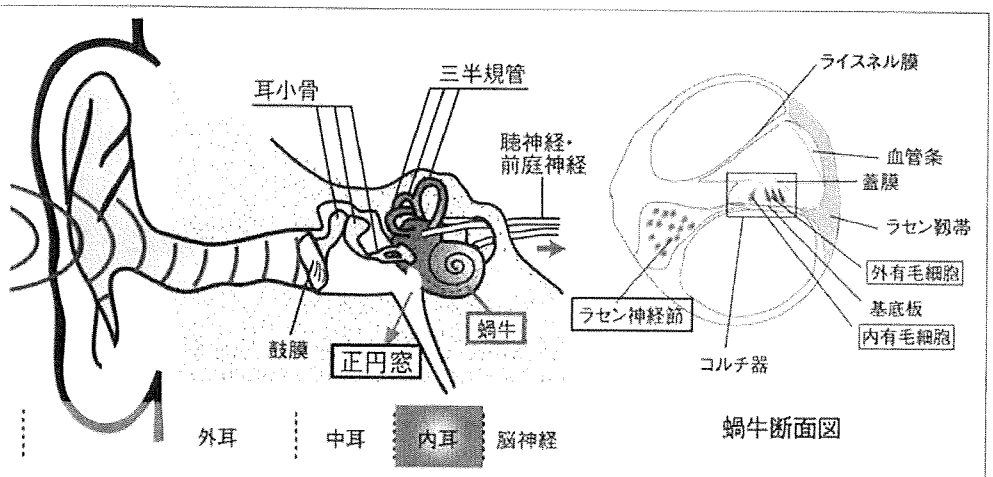
はじめに

感音難聴は，最も頻度の高い身体障害の１つである。身体障害者レベルの高度難聴者は約36万人あり，65歳以上の高齢者の60%には何らかの感音難聴が存在するとされている。しかしながら，いったん喪失した聴力を元に戻す方法は現在のところ存在しない。感音難聴の多くは内耳にある蝸牛の障害が原因であり，特に音刺激を受容する有毛細胞[用語1]と有毛細胞が受容した音刺激を脳に伝えるラセン神経節[用語2]細胞の障害が主因と考えられている（図❶）。現在，高度難聴者に対しては人工内耳[用語3]が広く用いられるようになり，対費用効果の高い治療法として評価されている。人工内耳では，音響刺激を電気刺激に変換し，蝸牛内に挿入された電極が直接蝸牛の神経細胞を刺激することにより聴覚を獲得する。人工内耳で得られる聴覚は，自然な聴覚とはかなり異なるものであるが，その有益性が高く評価されているという事実は，逆に聴覚障害が生活の質に与える影響の大きさを物語っている。このような背景から，聴覚再生を目的とした基礎的研究が活発に行われており，一般市民の新規治療法開発に対する期待も高い。

一方，近年の聴覚保護・再生を目的とした基礎的研究により，いくつかの新しい治療法の可能性が呈示されている。薬物治療に関する研究においても，いくつかの治療的効果が期待できる薬物が動物実験レベルで明らかにされており，神経栄養因子や細胞増殖因子もこれらに含まれる。多くの動物実験では，埋め込み型ポンプやウイルスベクターを用いた遺伝子導入が内耳への神経栄養因子や細胞増殖因子投与の方法として用いられている[1,2]。つまり，十分な効果を発揮するためには，一定期間以上の期間，内耳に神経栄養因子や細胞増殖因子が徐放されることが不可欠であるといえる。したがって，神経栄養因子や細胞増殖因子を用いた感音難聴治療を実現するためには，臨床応用が可能な安全かつ簡便な方法で，神経栄養因子や細胞増殖因子を内耳に一定期間徐放できるシス

key words
感音難聴，脳由来神経栄養因子，インスリン様細胞増殖因子１，ゼラチンハイドロゲル，薬物徐放，局所投与，有毛細胞，蝸牛，突発性難聴

図❶ 中耳，内耳解剖



音響刺激は外耳を通り，鼓膜を振動させる。鼓膜の振動は耳小骨を介して内耳蝸牛に伝達される。蝸牛内では，基底板が振動し，コルチ器にある内有毛細胞が刺激され，脱分極し，ラセン神経節細胞に刺激が伝達される。外有毛細胞は基底板振動をモディファイすることにより，より選択的に内有毛細胞が刺激される働きを担っている。

テムが必要となる。われわれは臨床応用への観点から，バイオマテリアルを用いた神経栄養因子や細胞増殖因子の内耳への徐放を考え，研究開発を行ってきた。この方法では，神経栄養因子や細胞増殖因子を含浸させたバイオマテリアルを中耳に留置するという簡単で安全な方法で薬物投与ができる。現在この方法を用いて，一部の研究成果は臨床応用に至っている。本稿では，バイオマテリアルとして生体吸収性ゼラチンハイドロゲルを用い，内耳に神経栄養因子や細胞増殖因子を投与する新しい感音難聴治療について紹介する。

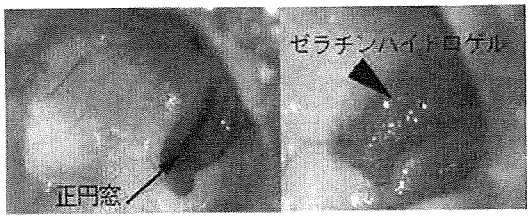
Ⅰ．内耳と神経栄養因子，細胞増殖因子

神経栄養因子や細胞増殖因子は，他の臓器と同様に内耳の発生段階において重要な役割を果たしていることが数多くのノックアウトマウスを用いた研究から明らかにされている。さらに，成熟後においても神経栄養因子や細胞増殖因子が内耳の細胞の生存維持や機能に不可欠であることが知られている。例えば，有毛細胞が受容した音響刺激を中枢に伝える役割を担っているラセン神経節細胞は，脳由来神経栄養因子（BDNF）やNeurotrophin3が欠如すると細胞死に陥る[1]。BDNFやNeurotrophin3は有毛細胞が産生し，パラクラインによりラセン神経節細胞の生存が維持されている。さらに，障害を受けた内耳に神経栄養因子や細胞増殖因子を体外から投与することにより，有毛細胞やラセン神経節細胞を細胞死から回避させることができることが報告されている[1][2][4]。このような背景から，神経栄養因子や細胞増殖因子は感音難聴を含めた内耳障害の治療薬として期待されている。

Ⅱ．ゼラチンハイドロゲルを用いた内耳薬物投与

ドラッグデリバリーは，再生医学や組織工学の分野で注目されている研究テーマであり，世界で活発な研究が行われており，広義での再生医療の臨床応用の鍵を握る技術といえる。われわれは，ドラッグデリバリー分野の研究成果を内耳に応用することにより，内耳への薬物徐放という問題は解決できるのではないかと考え，神経栄養因子や細胞増殖因子の徐放に適したゼラチンハイドロゲルを用いた研究を展開した。田畑らは，化学的架橋によりゼラチンを重合させたポリマーを作製し，静電結合により薬物をゼラチンポリマーに結合させ，ゼラチンポリマーの生体内での分解により薬物を徐放するシステムを開発し，種々の神経

図❷　ゼラチンハイドロゲルの正円窓留置



モルモット中耳の正円窓および正円窓窩にゼラチンハイドロゲルを留置したところ。

栄養因子や細胞増殖因子の徐放が可能であることを示している[5]。静電結合の強さやゼラチンポリマーの重合の強さによって徐放のスピードは調整可能とされている。

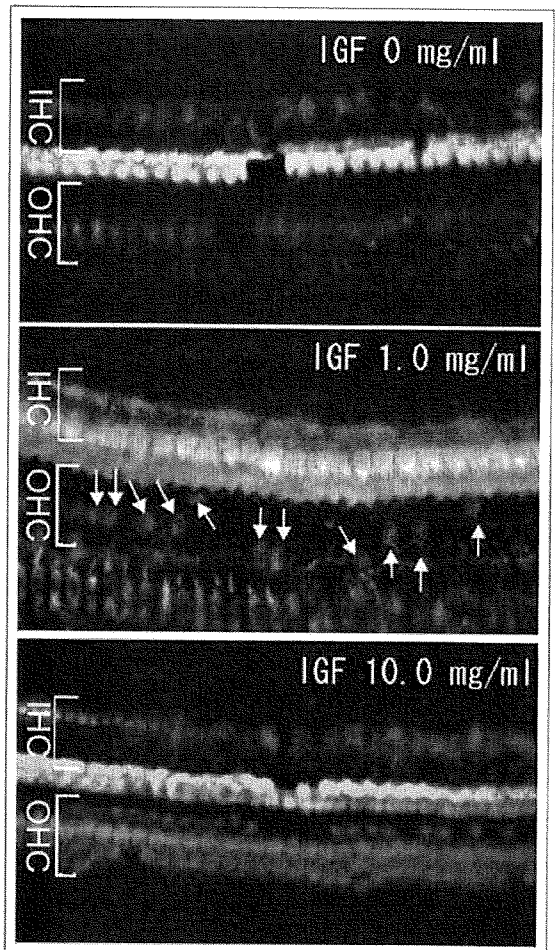
われわれは，まずBDNFを投与薬物として選択し，蝸牛のラセン神経節細胞に対する保護効果を検討した。BDNFは，ラセン神経節細胞の発生・生存に深く関与していることが知られており[7]，最近では聴覚刺激の中枢への伝達調整にも関与していることが示されている[6]。さらに，埋め込み型ポンプや遺伝子導入を用いた実験などで，ラセン神経節細胞に対する細胞死からの保護効果が示されていた[11,12]。したがって，BDNFによるラセン神経節細胞保護実験は，ゼラチンハイドロゲルの神経栄養因子や細胞増殖因子を内耳徐放する方法としての有効性を検証するよいモデルと考えられた。まず，BDNFを含浸させたハイドロゲルを中耳正円窓[用語A]膜上に留置し（図❷），蝸牛外リンパ中のBDNF濃度を経時的にELISA法にて計測したところ，1週間以上の徐放効果が期待できることがわかった[7]。次に，耳毒性薬物全身投与により蝸牛有毛細胞を喪失させ，二次的なラセン神経節細胞変性が誘導されるモデルを用い，ハイドロゲルによるBDNF局所投与によるラセン神経節細胞の組織学的・機能的な保護効果について調べた。ゼラチンハイドロゲルによるBDNF投与により，組織学的にラセン神経節細胞の減少が抑制され，機能が保持されることが電気刺激聴性脳幹反応にて示された[7]。ゼラチンハイドロゲルを用いた場合の組織学的・電気生理学的な保護効果は，埋め込み型ポンプを用いた場合[1]と同等であり，ゼラチンハイドロゲルを用いることにより埋め込み型ポンプと同等の薬物投与効果が得られることが示唆された。つまり，ゼラチンハイドロゲルは内耳への神経栄養因子や細胞増殖因子の投与に有用な方法であることが示されたといえる。

1．ゼラチンハイドロゲルを用いたIGF1投与による内耳保護

臨床応用への次のステップとして，すでに臨床での使用が認可されている薬物である細胞増殖因子を用いた実験を行った。いくつかの細胞増殖因子が臨床に供されているが，インスリン様細胞増殖因子1（IGF1）を選択した。IGF1を選択した理由は，第一に日本および米国ですでに市販されている薬物であったこと，第二に内耳の発生に関与し，内耳保護効果を示唆する基礎的な研究結果[8,9]があったという2点である。また，臨床的にも重篤な有害事象が報告されておらず，安全性が高いことも理由に含まれる。しかしながら，BDNFと比較して，IGF1の内耳保護効果は十分に検討されているとはいえなかったので，まず効果が期待しやすい条件，すなわち障害を与える前に薬物投与を行う条件での実験を行った。BDNF実験と同様の方法，すなわち，IGF1を含浸させたゼラチンハイドロゲルを正円窓膜上に留置し，その後，強大音響曝露を行った。音響外傷による感音難聴は，主として有毛細胞の障害・消失によって引き起こされるが，ラセン神経節細胞や血管条など他の蝸牛組織も障害される，いわば蝸牛の総合的な障害モデルととらえることができ，急性の感音難聴に対する治療効果を調べるモデルとして妥当と考えた。結果，ゼラチンハイドロゲルによるIGF1投与により，恒久的な聴覚閾値上昇をほぼ完全に抑制することができ，組織学的な有毛細胞の保護も確認された[10]。つまり，IGF1は有毛細胞保護効果を有し，ゼラチンハイドロゲルによるIGF1局所投与は音響外傷による急性高度難聴に対して有効である可能性が示された。

次の段階として，治療的効果を調べるために，薬物投与を障害後，すなわち難聴発症後に投与する実験を行った。急性高度難聴を対象とした臨床試験を想定し，音響外傷モデルに加え，内耳虚

図❸ IGF1による蝸牛感覚上皮保護効果



投与したIGF1の濃度が高くなるにつれて，残存している外有毛細胞（OHC）が増加している。IHCは内有毛細胞を示す。

血障害モデル[11]での有効性も検討した。内耳虚血は，突発性難聴[用語5]の病態の1つと考えられている。音響外傷後にIGF1を投与した場合，音響曝露前投与に比べると効果は劣るものの，有意の聴覚閾値上昇抑制が確認され，有毛細胞の保護効果も確認された（図❸）[12]。内耳虚血モデルでも聴覚閾値の上昇が抑制され，組織学的にも蝸牛有毛細胞生存促進効果が確認された[13]。したがって，音響外傷および内耳虚血という異なった病態による急性感音難聴に対して，ゼラチンハイドロゲルによるIGF1投与が治療的効果を有すると考えられた。さらに，中耳炎などの有害事象も認められず，安全性が高い治療法であることも示唆された[12]。これらの結果に基づき，ゼラチンハイドロゲルを用いたIGF1内耳投与による急性高度難聴治療の臨床試験の準備に着手した。

2. ゼラチンハイドロゲルによるIGF1内耳投与臨床応用

上記の研究成果をふまえ，ゼラチンハイドロゲルを用いたIGF1内耳投与による急性高度難聴治療について，安全性と少数例での治療効果を調べる第Ⅰ～Ⅱ相臨床試験のデザインを京都大学医学部附属病院探索医療センターの協力のもとに行った。対象は，突発性難聴を含める急性高度難聴とし，厚労省班研究の突発性難聴診断基準での確実例および疑い例とした。確実なエビデンスはないが，ステロイドの全身投与は急性高度難聴に対する一般的な治療法として世界で広く認知されている。今回の臨床試験では，倫理的な配慮から，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た。ただし，治療の有効性という面から考えると，発症後聴力低下が固定した症例では効果が期待しにくいことから，発症後30日未満という条件を設けた。症例数はヒストリカルコントロールとし，過去に京都大学耳鼻咽喉科頭頸部外科で突発性難聴ステロイド無効例に施行した高気圧酸素療法の有効性の後ろ向き研究の結果に基づいて行い，目標登録症例数を25例とした。エンドポイントは，①ゼラチンハイドロゲルによるIGF1局所投与がステロイド無効急性高度難聴例（発症後30日未満）に対してどの程度の有効性が期待できるのか，②有害事象はどの程度発生するのかを明らかにすることとした。2007年12月より登録を開始，順調に症例登録は進んでおり，いくつかの症例で有効性が確認されている。

おわりに

これまでに神経栄養因子や細胞増殖因子が内耳障害に有効であることは，多くの基礎的研究成果から明らかであった。しかしながら，有効性を発揮するためには，一定の期間標的となる細胞が神経栄養因子や細胞増殖因子に曝露されている必要があり，いかにして内耳に神経栄養因子や細胞増

殖因子を徐放するかは重要な課題であった。われわれが開発したゼラチンハイドロゲルによる内耳への神経栄養因子や細胞増殖因子の徐放システムは，この問題に対する1つの回答といえる。現在の臨床試験では1回投与での有効性を調べているが，複数回投与を行うことによりかなり長期の徐放も可能となり，更なる有効性が期待できる。また，複数の神経栄養因子や細胞増殖因子を同時に徐放することも技術的には可能であり，今後複数の神経栄養因子や細胞増殖因子の投与による相乗効果も調べていく必要がある。神経栄養因子や細胞増殖因子は，他の神経疾患にも応用することが可能であり，今後さらに多くの神経栄養因子や細胞増殖因子が臨床的に使用可能となることが期待される。特に耳鼻咽喉科や眼科領域では局所投与が有効であり，適切な徐放システムを使用すれば高い有効性と副作用の軽減が期待できる。今後さらに基礎的研究開発を進め，使用できる薬物の選択肢を増やすとともに，積極的に臨床応用の可能性を探っていきたい。本稿で紹介した治療法は，内耳の細胞を細胞死から保護することにより，いったん障害された聴力を回復させるものであるが，今後消失した細胞を再生させる方向性での研究にも，今回開発した内耳への神経栄養因子や細胞増殖因子の徐放システムを応用していきたい。

謝辞
本稿を終えるにあたり，ゼラチンハイドロゲルを用いた内耳薬物徐放システム開発に多大なご協力をいただいた京都大学再生医学研究所・田畑泰彦教授，内耳虚血モデル実験を中心となって行っていただいた愛媛大学耳鼻咽喉科・暁　清文教授，羽藤直人講師にこの場を借りて深謝いたします。

用語解説

1. **有毛細胞**：聴覚の感覚器である蝸牛および平衡感覚をつかさどる前庭の感覚上皮に存在する。細胞の頂部にある感覚毛の偏位により，脱分極し，細胞底部にある神経終末から神経伝達物質を放出することにより，振動刺激を神経刺激に変換する。
2. **ラセン神経節**：聴覚の感覚器である蝸牛はらせん状の渦巻き構造を有するが，蝸牛の中心にある蝸牛軸に局在し，有毛細胞からの神経刺激を脳幹にある蝸牛神経核に伝達する。
3. **人工内耳**：人工内耳はマイクロホン，音声分析装置，刺激電極，電波の送受信機からなる。刺激電極は蝸牛内に挿入され，ラセン神経節細胞を直接電気刺激することにより，脳へ聴覚刺激を伝達する。2006年で国内での装用者は4150人を超えている。
4. **正円窓**：聴覚の感覚器である蝸牛は骨で囲まれた器官であるが，正円窓でのみ膜で中耳との境界が形成されている。中耳から内耳への薬物や遺伝子の投与経路として用いられる。
5. **突発性難聴**：急激に片側の聴力が低下する原因不明の難聴と定義されている難治性疾患であり，厚労省で班研究が行われている。蝸牛有毛細胞の障害が主因の1つと考えられている。中年に多く，年間35000人に発症する。

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・ゼラチンハイドロゲルによるIGF1内耳投与臨床試験
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RESEARCH ARTICLE Open Access

Role of prostaglandin E receptor subtypes EP2 and EP4 in autocrine and paracrine functions of vascular endothelial growth factor in the inner ear

Ryusuke Hori, Takayuki Nakagawa*, Norio Yamamoto, Kiyomi Hamaguchi and Juichi Ito

Abstract

Background: The physiological effects of prostaglandin E1 (PGE1) and prostaglandin E2 (PGE2) are mediated by the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4, and the respective agonists have been purified. PGE1 and PGE2 can increase the production of vascular endothelial growth factor (VEGF), particularly through EP2 and EP4. The biological effects of VEGF are mediated by the phosphotyrosine kinase receptors fms-related tyrosine kinase-1 (Flt-1) and fetal liver kinase-1 (Flk-1). Here we examined the effects of EP2 and EP4 agonists on the production of VEGF proteins and *VEGF* messenger RNAs (mRNAs) in the inner ear, using an enzyme-linked immunosorbent assay and the real-time quantitative reverse transcription-polymerase chain reaction, respectively. We also examined the localization of EP2, VEGF, Flt-1, and Flk-1 in the cochlea by immunohistochemistry.

Results: The expression of EP2 occurred in the cochlea, and the local application of an EP2 or EP4 agonist increased VEGF protein and *VEGF* mRNA levels in the inner ear. Furthermore, the intensity of the VEGF immunoreactivity in the spiral ganglion appeared to be increased by the local EP2 or EP4 agonist treatment. Immunoreactivity for Flt-1, and Flk-1 was found in the cochlear sensory epithelium, spiral ganglion, spiral ligament, and stria vascularis.

Conclusions: These findings demonstrate that EP2 and EP4 agonists stimulate VEGF production in the inner ear, particularly in the spiral ganglions. Moreover, the Flt-1 and Flk-1 expression observed in the present study suggests that VEGF has autocrine and paracrine actions in the cochlea. Thus, EP2 and EP4 might be involved in the mechanisms underlying the therapeutic effects of PGE1 on acute sensorineural hearing loss via VEGF production.

Background

Sensorineural hearing loss (SNHL) is a common disability. Once hearing has been lost, it is rarely recovered. The systemic application of corticosteroids has been accepted as the treatment of choice for acute SNHL, although its efficacy has not been substantiated [1]. In general, approximately 50% of SNHL cases show no response to the systemic application of corticosteroids [2]. Hence there is an urgent need to develop alternative treatments for acute SNHL. Prostaglandin E1 (PGE1) has often been used as one such treatment; however, its clinical efficacy remains controversial [3-5]. The physiological actions of PGE1 and prostaglandin E2 (PGE2) are mediated by the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4 [6,7]. EP2 and EP4 are coupled to G-protein stimulation, and mediate increases in cyclic adenosine monophosphate (cAMP) that activate protein kinase A (PKA) [7,8]. By contrast, the activation of EP3 decreases cAMP levels, which suggests that the selective activation of EP2 and/or EP4 might increase the clinical efficacy of PGE1 for the treatment of acute SNHL. Based on these findings, we previously examined the expression of EP4 in the cochlea, and the potential of an EP4 agonist to protect the cochlea against noise-induced trauma [9]. Our results demonstrated both EP4 expression in the cochlea, and cochlear protection against noise trauma as a result of the local application of an EP4 agonist. However, the exact mechanisms by which EP4 agonists act in the cochlea are presently unclear and require further research.

Among their various physiological and pathophysiological functions, PGE1 and PGE2 are known to increase vascular endothelial growth factor (VEGF) production

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via a cAMP-dependent mechanism, which is mediated by EP2 and EP4 [10-13]. VEGF, which is a 45-kDa heparin-binding homodimeric glycoprotein, plays roles in angiogenesis, vasodilation, differentiation, anti-apoptosis, proliferation, and vascular permeability in endothelial tissues [14-16]. It also has neurotrophic and neuroprotective effects in non-endothelial tissues [17-19]. Recent studies have indicated that VEGF also protects the cochlea against noise trauma [20,21]. Based on these findings, we hypothesized that VEGF is involved in the mechanisms underlying the cochlear protection against noise trauma that results from local EP4 agonist application. We thus examined the expression of EP2 in the cochlea, and the effects of local EP2 and EP4 agonist application on the modulation of VEGF in the inner ear. The current study demonstrated an increase in the levels of VEGF proteins and messenger RNAs (mRNAs) following local EP2 and EP4 agonist application according to an enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, and the real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). We also demonstrated the expression of VEGF receptor-1 (VEGFR-1) or fms-related tyrosine kinase-1 (Flt-1), and VEGF receptor-2 (VEGFR-2) or fetal liver kinase-1 (Flk-1), which mediate the physiological actions of VEGF in the cochlea [22,23].

Results

EP2 expression in cochleae

Our previous study demonstrated the localization of EP4 in the mouse cochlea [9]; however, EP2 expression in the mouse cochlea has not been determined. We thus performed an immunohistochemical analysis of EP2 using normal mouse cochlear specimens in the current study. Immunostaining revealed that the EP2 expression occurred in the stria vascularis, spiral ligament, spiral ganglion neurons, supporting cells, and hair cells (Figure 1). Negative controls using a specific blocking peptide showed no immunoreactivity (data not shown). These findings confirmed that EP2 expression was present in the cochlear cells, similar to EP4 [9].

VEGF increase due to EP2 and EP4 agonists

To examine the changes in the expression of VEGF proteins and *VEGF* mRNAs in the inner ear caused by EP2 and EP4 agonists, we performed ELISA and real-time qRT-PCR analyses of extracts from inner ear specimens following the local application of EP2 and EP4 agonists or control substrates. ELISA analyses revealed significant increases in VEGF protein levels following EP2 agonist application at concentrations of 0.01 and 0.1 mg/ml (Figure 2A; $p < 0.001$ and $p = 0.005$, respectively). No significant increase of VEGF protein levels was found in samples treated with 1 mg/ml EP2 agonist. The maximum VEGF protein increase was found at a concentration of 0.1 mg/ml. Local application of an EP4 agonist also caused a significant increase of VEGF protein levels in the inner ear. Specimens treated with 0.01 or 0.1 mg/ml EP4 exhibited significantly higher VEGF protein levels than those treated with 0.01 or 0.1% dimethyl sulfoxide (DMSO) (Figure 2B; $p = 0.004$ and 0.026, respectively). No significant increase of VEGF protein levels was identified in samples treated with 1 mg/ml EP4 agonist in comparison with those treated with 1% DMSO. The EP4 agonist caused a maximum VEGF protein increase at a concentration of 0.01 mg/ml.

Real-time qRT-PCR analyses demonstrated a significant increase of *VEGF* mRNA levels following the local application of the EP2 and EP4 agonists. The relative *VEGF* expression levels in the samples treated with 0.01 or 0.1 mg/ml EP2 agonist were significantly higher ($p < 0.001$ and $p = 0.007$, respectively) than those in the control samples treated with saline (Figure 3A). There was no significant difference in the relative *VEGF* expression levels between the samples treated with 1 mg/ml EP2 agonist and the control samples, similar to the VEGF protein levels. The relative *VEGF* expression levels in the samples treated with 0.01 and 0.1 mg/ml EP4 agonist were significantly higher ($p = 0.002$ and 0.036, respectively) than those in the control samples treated with DMSO (Figure 3B). The samples treated with 1 mg/ml EP4 agonist exhibited no significant increase of *VEGF* mRNA levels in comparison with the control samples treated with DMSO, similar to the VEGF protein levels. The maximum increases in *VEGF* mRNA levels were observed after treatment with 0.1 mg/ml EP2 and 0.01 mg/ml EP4, similar to the VEGF protein levels. These results demonstrated that the EP2 and EP4 agonists stimulated VEGF production in the inner ear.

VEGF expression in cochleae

Our previous study [9] and immunostaining for EP2 in the present study demonstrated the presence of EP2 and EP4 in various types of cochlear cells. ELISA and real-time qRT-PCR analyses revealed VEGF induction in inner ears by local EP2 or EP4 agonist application. We then examined what type cochlear cells were responsible for VEGF increase in the cochlea in response to local application of EP2 or EP4 agonists. We thus performed immunostaining for VEGF in cochlear specimens treated with EP2 or EP4 agonist and in untreated cochlear specimens. The untreated cochlear specimens showed little or no staining for VEGF in the spiral ganglion neurons (Figure 4A), supporting cells, hair cells, spiral ligament, and stria vascularis (data not shown). In the control specimens treated with saline or DMSO, VEGF immunoreactivity was weakly detectable in some of the spiral ganglion neurons (Figure 4B, D). By contrast, intense immunoreactivity for VEGF was observed in the spiral ganglion

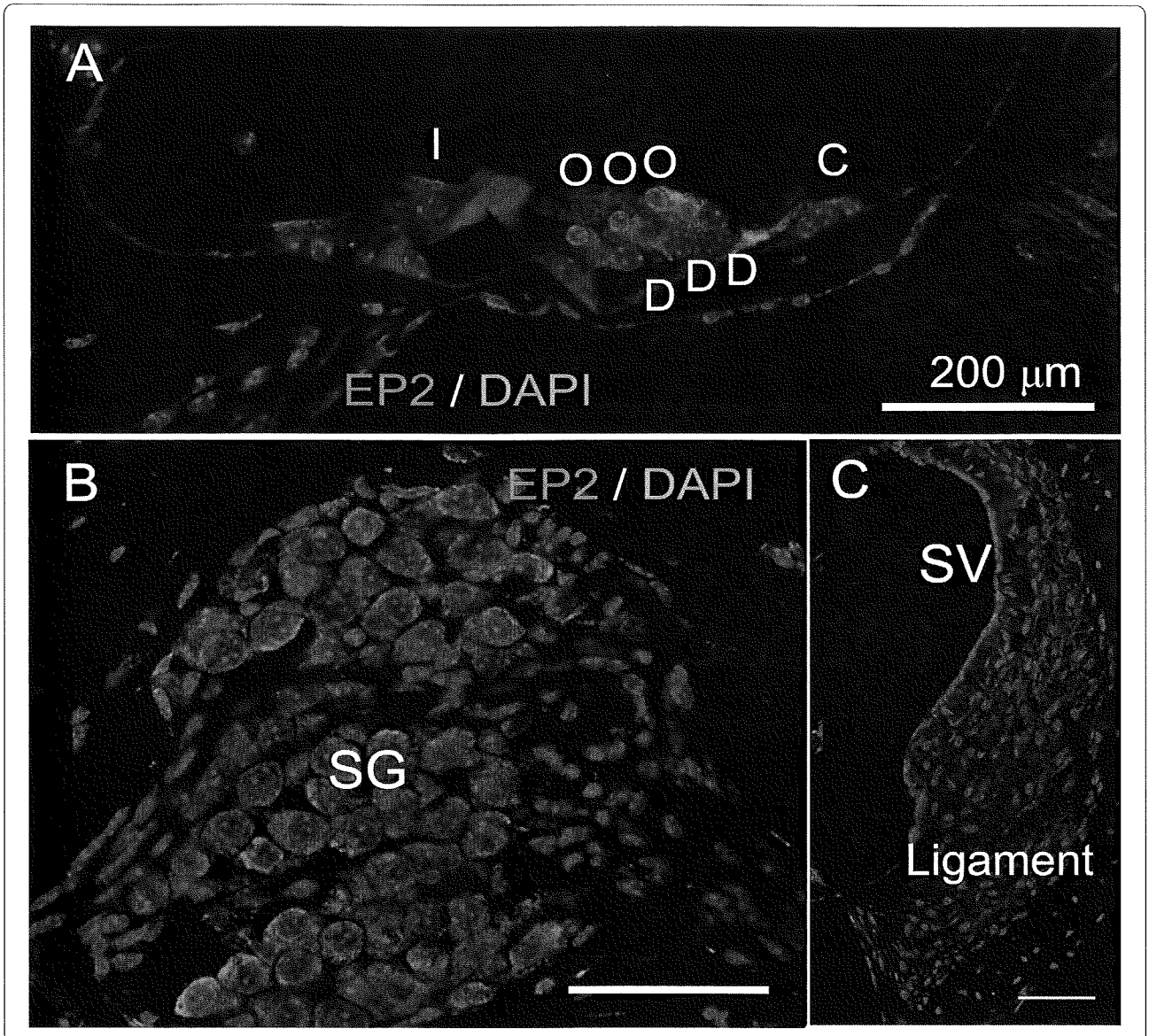

Figure 1 Immunoreactivity for EP2 in the cochlea. EP2 expression (red) was detected n the cochlear sensory epithelium (A; I, inner hair cell; O, outer hair cell; D, Deiter's cell; C, Claudius' cell), spiral ganglion neurons (B; SG), spiral ligament (C; ligament) and stria vascularis (C; SV). Nuclei were labeled with DAPI. The specimens were viewed with 63× oil objective. Scale bar = 200 μm.

neurons of the specimens treated with EP2 and EP4 agonists (Figure 4C, E). The other EP2-positive and EP4-positive regions of the cochlea, including the supporting cells, hair cells, spiral ligament, and stria vascularis, showed little or no immunoreactivity for VEGF under all of the experimental conditions, and no changes in VEGF immunoreactivity were observed. These findings indicate that the spiral ganglion neurons are responsible for the increase in VEGF expression in the cochlea that results from the local application of an EP2 or EP4 agonist.

VEGFR expression in cochleae

To examine the target cells of VEGF in the cochlea, we performed immunostaining for VEGFRs, Flt-1 and Flk-1, using non-treated cochlear specimens. Flt-1 expression was detected in the supporting cells, hair cells, spiral ganglion neurons, and spiral ligament fibrocytes (Figure 5A-C). Flk-1 expression was identified in the supporting cells, hair cells, spiral ganglion neurons, spiral ligament fibrocytes, and stria vascularis marginal cells (Figure 5D-F). These findings suggest that VEGF generated in the spiral