症例	埋込時年齢	性別	原因疾患	失聴期間	腫瘍切除	アプローチ	使用機器	使用数/機器の電極数	コード化法	語音聴取成績 (%)*			合併症
										口話のみ	ABIのみ	ABI+口話	
1	25	男	NF2	4M	−	経迷路	NCI 8+1	5/8	SPEAK	10	0	10	デバイス露出・摘出 水頭症
2	26	女	NF2	2Y	+	後頭下	N24	9/24	SPEAK	0	0	0	
3	63	男	NF2	16Y	−	後頭下	Combi40+	11/12	CIS	35	31	78	
4	67	男	髄膜炎による内耳の骨化	17Y	+	後頭下	Combi40+	9/12	CIS	62	21	89	
5	58	女	NF2	6Y	+	後頭下	Pulser100	8/12	CIS	8	0	0	両側反回神経麻痺
6	42	女	NF2	5Y	+	後頭下	Pulser100	8/12	CIS	75	7	96	
7	39	男	NF2	2Y	−	後頭下	Pulser100	9/12	CIS	15	0	26	
8	64	男	NF2	1Y2M	−	後頭下	Pulser100	3/12	CIS	63	0	53	

＊CI-2004 学童用日常会話文

IV. 電極埋め込み手術

CN へのアプローチ方法としては，経迷路法と後頭下法の2つの方法がある。後頭下法アプローチは経迷路法に比べて外側陥凹の確認や挿入角度の点から ABI の埋め込みには不利であると考えられていたが，実際には本法は視野が広く，他のランドマークも見やすいため，最近では全例，脳神経外科と協力して，後頭下法アプローチで行っている[5]。

蝸牛神経核は図3のように延髄外側に位置し，その大きさは幅2mm，長さ12mmである。ただし，通常のアプローチで直接これを見ることはむずかしいので，適切な位置の確認には術中に電気刺激によるモニタリングを要する。電気刺激後，III・IV・V波はそれぞれ1〜1.3，1.5〜2.3，2.5ms の潜時をもって出現する。このうち筋電図を最小とし，より多くのピークが検出され，振幅が最大となり，かつ筋原性反応を最小にする電極位置が最適位置である[6]。

電極を蝸牛神経核上に置き，生体糊で固定する（図4）。刺入するわけではないので安全である。電極周囲のダクロン膜がクモ膜と線維性に結合し，電極の移動率は8%程度である。

V. 術後成績

これまでに当院で施行した8例の ABI 埋め込み症例について，プロフィールと術後の聴取成績を表1に示す。手術時年齢は25〜67歳，男性5例，女性3例，原因疾患の内訳は神経線維腫症第2型に伴う両側聴神経腫瘍7例，髄膜炎に伴う両側内耳の骨化1例であった。

腫瘍切除と同時に埋め込みが行われたものは4例，段階手術として ABI 埋め込みだけが行われた例が4例である。手術は1例が経迷路法で，7例が後頭下法で行われた。

装用閾値は全症例にて30〜50dBHL で得られた。開始当初の2例では ABI のみによる語音弁別は困難であった[7]が，最終的には8例中3例（症例3，4，6）で，ABI のみでの語音弁別能がそれぞれ31，21，7%となった。この3例では ABI による聴覚と口話併用では，78%，89%，96%と良好な聴取能が得られた。

さらに活用状況を図5に示す。環境音の知覚，Ling6音の検出は全例で可能であった。また，8例中7例では ABI 併用によって，日常場面での口話能の増強が認められた。平均の装用時間は，8.5時間/日であったが，ABI のみでの語音聴取が可能であった3症例は終日装用となっており平均16時間/日であり，生活上必要不可欠なものになっていた。その他の症例は必要時またはごく短時間の使用で平均4時間/日であったが，読話併用での効果があり，また環境音の知覚が可能といった理由から，非装用者はいなかった。音楽を楽しめてい
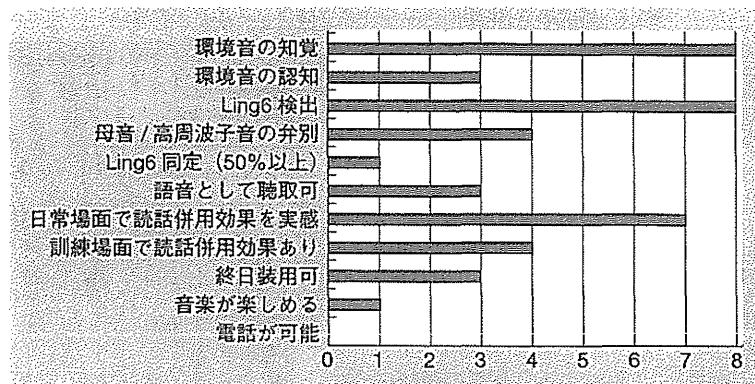
図 5 当院の ABI 埋め込み症例の活用状況

るのは1例のみであった。

術後の聴取成績については個人差が大きく，活用レベルもさまざまであった。大幅な閾値変動を起こす例も多く，聴覚管理だけでも長期に及ぶフォローが必要とされる。さらに聴取レベルに応じた聴能訓練，活用訓練，カウンセリングなどにも十分な時間をかけ，疾患の予後にも対応したりハビリテーションの工夫が必要であると考えられた[8]。

VI. 術後合併症

ABI 術後合併症として，デバイスの皮膚露出による摘出1例，水頭症併発1例，両側反回神経麻痺1例が認められた。デバイス露出例は多数回の聴神経腫瘍切除後で側頭骨皮膚弁の血流不全があったため，と考えられた。2例でマップ上での軽度の電極移動が示唆された。皮膚の知覚刺激や筋の痙攣などを除いて，電気刺激による重篤なバイタルサインの変化などの副作用は認められなかった。

VII. 聴性中脳インプラント（Auditory midbrain implant：AMI）

最近，延髄の蝸牛神経核よりもさらに上位中枢である，中脳の下丘 inferior colliculus（図1）に電極を置く auditory midbrain implant（AMI）が開発されている。延髄の蝸牛神経核よりも聴覚以外の電気刺激の副作用が少ないという利点がある。

これには現在2つの方式があり，Colletti ら[9]は

市販の MED-EL 社の ABI 電極を下丘に置く方式を採用し，Lim と Lenarz ら[10]は新たに 20 チャンネルのプラチナ電極が付いた直径 0.4 mm，長さ 6.2 mm のストレート電極を開発し，これを下丘に刺入している。ただし，聴神経腫瘍の摘出とアプローチ，視野が異なるので，実際には適応症例が問題となろう。

文　献

1) Hitselberger WE, House WF, Edgerton BJ, et al：Cochlear nucleus implant. Otolaryngol Head Neck Surg 92：52-54, 1984.

2) Kumakawa K, Takeda H, Seki Y, et al：A Nucleus CI8+1 channel auditory brainstem implant in a steged operation. Cochlear Implants；An update. pp553-557, Kugler Publications, 2002.

3) Colletti V, Carner M, Fiorino F, et al：Hearing restoration with auditory brainstem implant in three children with cochlear nerve aplasia. Otol Neurotol 23：682-693, 2002.

4) Temple RH, Axon PR, Ramsden RT, et al：Auditory rehabilitation in neurofibromatosis type 2；A case for cochlear implantation. J Laryngol Otol 113：161-163, 1999.

5) 熊川孝三：聴性脳幹インプラントの手術手技と微小解剖顕微鏡下手術のための脳神経外科解剖．ＸＸサイメッド・パブリケーションズ，120-125頁，2008.

6) 熊川孝三，武田英彦，射場　恵，他：聴性脳幹インプラントに必要な聴覚検査．JOHNS 24（5）：807-812, 2008.

7) 熊川孝三, 望月羲也, 高橋直一, 他：8チャンネル聴性脳幹インプラント埋め込み症例の語音聴取能. 日耳鼻 104：510-513, 2001.

8) 熊谷文愛, 射場　恵, 河村さやか, 他：聴性脳幹インプラント装用者の長期経過. Audiology Japan 52：353-354, 2009.

9) Colletti V, Shannon R, Carner M, et al：The first successful case of hearing produced by electrical stimulation of the human midbrain. Otology and Neurotology 28：39-43, 2007.

10) Lim H, Lenarz T, Joseph G, et al：Effects of phase duration and pulse rate on loudness and pitch percepts in the first auditory midbrain implant patients；Comparison to cochlear implant and auditory brainstem implant results. Nueroscience 15：370-380, 2008.

* * *

第7回　頭頸部表在癌研究会のお知らせ

第7回頭頸部表在癌研究会を下記のごとく開催いたします。奮ってご参加をお願い申し上げます。

記

会　期：2010年6月12日（金）9：30〜16：00（第34回日本頭頸部癌学会最終日の翌日です）

会　場：東京医科歯科大学5号館4階講堂（東京都文京区湯島 1-5-45, 03-3813-6111）

プログラム：1）主題－Ⅰ：下咽頭癌の経口腔的手術の合併症とその対応

　　　　　　2）主題－Ⅱ：中下咽頭癌の 0-Ⅱ c/0-Ⅲ型表在癌はあるか

　　　　　　3）主題－Ⅲ：喉頭蓋・舌根部表在癌の取り扱い

　　　　　　4）一般演題：頭頸部表在癌に関する演題（症例報告を含む）

代表世話人：海老原 敏（杏雲堂病院 院長）

＊当研究会は『日本耳鼻咽喉科学会専門医制度』の認定学術集会であり, 5単位を取得できます。

お問い合わせ先：

　　　東京医科歯科大学頭頸部外科（〒 113-8519 東京都文京区湯島 1-5-45）

　　　Tel：03-5803-5911, Fax：03-5803-0215, E-mail アドレス：kishi.hns@tmd.ac.jp

　　　＊なお, 参加人数把握とプログラム等の詳細送付のため, 演題を申し込まず研究会に出席される先生方も, 上記アドレス宛にその旨ご連絡いただけると幸いです。

　　　第7回頭頸部表在癌研究会当番世話人　岸本誠司

informa
healthcare

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*

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

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.

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 $\pm$ 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
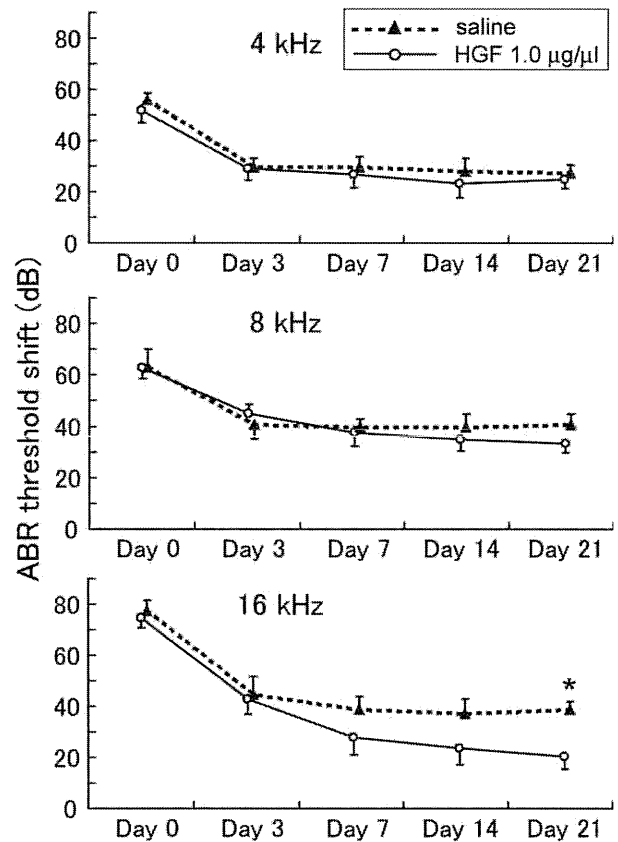

Figure 1. ABR threshold shifts after noise exposure in saline- and HGF-treated animals. An overall effect of HGF application is significant at 16 kHz (two factorial ANOVA, $p = 0.030$), not at 4 or 8 kHz. The difference in threshold shifts between saline- and HGF-treated animals is significant on day 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
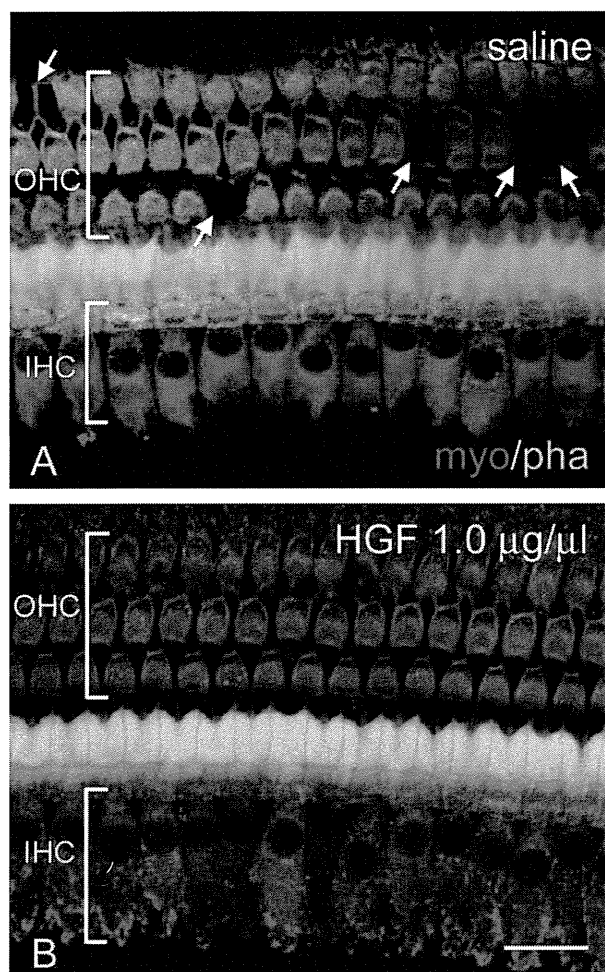
Figure 2. Immunostaining for myosin VIIa (myo) and phalloidin staining (pha) demonstrated loss of outer hair cells (OHC) in the upper basal portion of the saline-treated cochlea (A) and preservation of OHC in that of the HGF-treated cochlea (B). Arrows indicate loss of OHC. IHC, inner hair cells. Scale bar represents 20 μm.
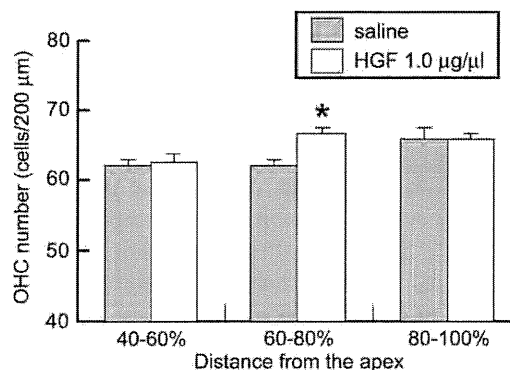

Figure 3. Means of numbers of surviving outer hair cells (OHCs) in saline- and HGF-treated cochleae. In the 60–80% distance region from the apex, the value of HGF-treated cochleae is significantly higher than that of saline-treated cochleae. $*p = 0.003$, t test. Bars represent standard errors.

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

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

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.

Acknowledgements

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

References

[1] Nakagawa T, Ito J. Drug delivery systems for the treatment of sensorineural hearing loss. Acta Otolaryngol Suppl 2007; 557:30–5.

[2] Young S, Wong M, Tabata Y, Mikos A. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. J Control Release 2005;109:256–74.

[3] Endo T, Nakagawa T, Kita T, Iguchi F, Kim T, Tamura T, et al. Novel strategy for treatment of inner ears using a biodegradable gel. Laryngoscope 2005;115:2016–20.

[4] Iwai K, Nakagawa T, Endo T, Matsuoka Y, Kita T, Kim T, et al. Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. Laryngoscope 2006;116:529–33.

[5] Lee K, Nakagawa T, Okano T, Hori R, Ono K, Tabata Y, et al. Novel therapy for hearing loss: delivery of insulin-like growth factor 1 to the cochlea using gelatin hydrogel. Otol Neurotol 2007;28:976–81.

[6] Gohda E, Tsubouchi H, Nakayama H, Hirono S, Sakiyama O, Takahashi K, et al. Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J Clin Invest 1988;81:414–9.

[7] Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor. Nature 1989;342:440–3.

[8] Funakoshi H, Nakamura T. Hepatocyte growth factor: from diagnosis to clinical applications. Clin Chim Acta 2003;327: 1–23.

[9] Oshima K, Shimamura M, Mizuno S, Tamai K, Doi K, Morishita R, et al. Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats. FASEB J 2004;18:212–4.

[10] Ozeki M, Ishii T, Hirano Y, Tabata Y. Controlled release of hepatocyte growth factor from gelatin hydrogels based on hydrogel degradation. J Drug Target 2001;9:461–71.

[11] Viberg A, Canlon B. The guide to plotting a cochleogram. Hear Res 2004;197:1–10.

[12] Wang J, Ruel J, Ladrech S, Bonny C, van de Water TR, Puel JL. Inhibition of the c-Jun N-terminal kinase-mediated mitochondrial cell death pathway restores auditory function in sound-exposed animals. Mol Pharmacol 2007;71:654–66.

[13] Tanaka K, Takemoto T, Sugahara K, Okuda T, Mikuriya T, Takeno K, et al. Post-exposure administration of edaravone attenuates noise-induced hearing loss. Eur J Pharmacol 2005;522:116–21.

[14] Hu B, Guo W, Wang P, Henderson D, Jiang S. Intense noise-induced apoptosis in hair cells of guinea pig cochleae. Acta Otolaryngol 2000;120:19–24.

[15] Ohlemiller K, Wright J, Dugan L. Early elevation of cochlear reactive oxygen species following noise exposure. Audiol Neurootol 1999;4:229–36.

[16] Yamashita D, Minami S, Kanzaki S, Ogawa K, Miller J. Bcl-2 genes regulate noise-induced hearing loss. J Neurosci Res 2008;86:920–8.

[17] Nakagami H, Morishita R, Yamamoto K, Taniyama Y, Aoki M, Matsumoto K, et al. Mitogenic and antiapoptotic actions of hepatocyte growth factor through ERK, STAT3, and AKT in endothelial cells. Hypertension ;37(2 Part 2001;2): 581–6.

[18] Kitamura K, Iwanami A, Nakamura M, Yamane J, Watanabe K, Suzuki Y, et al. Hepatocyte growth factor promotes endogenous repair and functional recovery after spinal cord injury. J Neurosci Res 2007;85:2332–42.

[19] Ruel J, Wang J, Rebillard G, Eybalin M, Lloyd R, Pujol R, et al. Physiology, pharmacology and plasticity at the inner hair cell synaptic complex. Hear Res 2007;227:19–27.

OTOLOGY

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

Harukazu Hiraumi · Takayuki Nakagawa · Juichi Ito

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 adminis-

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

tration 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

🎴 Springer

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
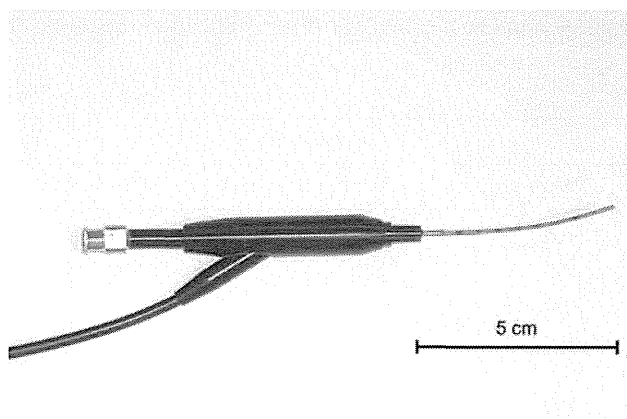
Fig. 1 A microendoscope specially modified for better visualization of the RWM. The outer diameter is 0.9 mm and the length is 50 mm. The view angle is 70°. It is equipped with a working channel (0.3 mm in diameter)
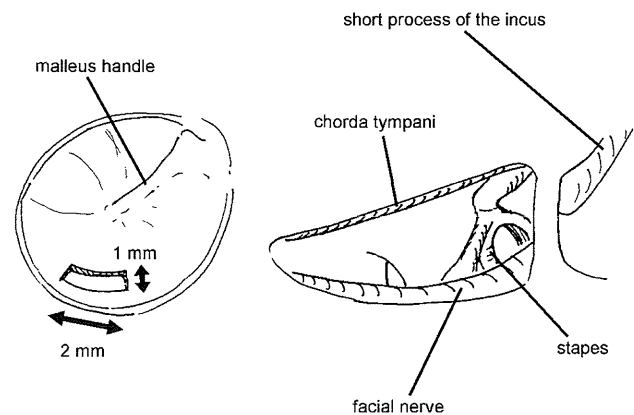
Fig. 2 A small fenestration (2 × 1 mm) was made in the posterior inferior quadrant of the tympanic membrane using a knife. Posterior hypotympanotomy was made as large as possible. In all specimens, the facial nerve and chorda tympani were skeletonized

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.

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
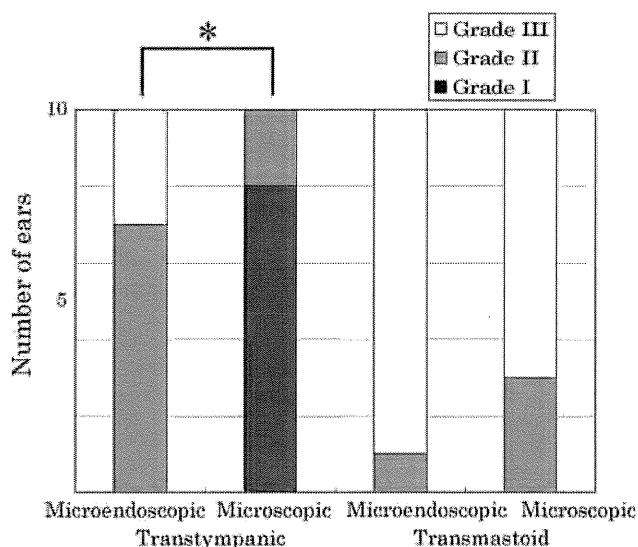

Fig. 3 The visibility of the RWM for four approaches. Grade I as no or little visualization of the RWM (<20%), Grade II as defined by >20%, and Grade III as defined by >70%. The visibility through the transtympanic microendoscopic approach was better than that with transtympanic microscopic approach

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

Transtympanic Transmastoid

Microendoscope
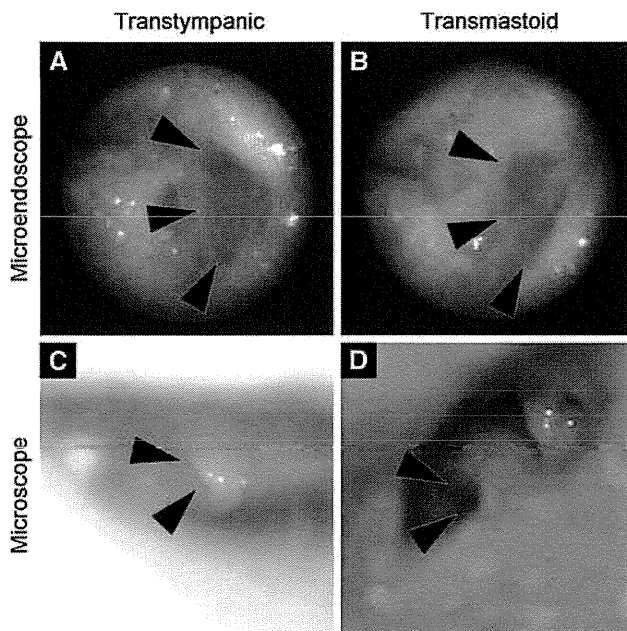
Microscope

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.

References

1. Wilson WR, Byl FM, Laird N (1980) The efficacy of steroids in the treatment of idiopathic sudden hearing loss. A double-blind clinical study. Arch Otolaryngol 106:772–776
2. Conlin AE, Parnes LS (2007) Treatment of sudden sensorineural hearing loss: I. A systematic review. Arch Otolaryngol Head Neck Surg 133:573–581. doi:10.1001/archotol.133.6.573
3. Plontke SK, Zimmermann R, Zenner HP et al (2006) Technical note on microcatheter implantation for local inner ear drug delivery: surgical technique and safety aspects. Otol Neurotol 27:912–917. doi:10.1097/01.mao.0000235310.72442.4e
4. Jeong B, Bae YH, Lee DS et al (1997) Biodegradable block copolymers as injectable drug-delivery systems. Nature 388:860–862. doi:10.1038/42218
5. Tabata Y, Yamada K, Miyamoto S et al (1998) Bone regeneration by basic fibroblast growth factor complexed with biodegradable hydrogels. Biomaterials 19:807–815. doi:10.1016/S0142-9612(98)00233-6
6. Endo T, Nakagawa T, Kita T et al (2005) Novel strategy for treatment of inner ears using a biodegradable gel. Laryngoscope 115:2016–2020. doi:10.1097/01.mlg.0000183020.32435.59

7. Iwai K, Nakagawa T, Endo T et al (2006) Cochlear protection by local insulin-like growth factor-1 application using biodegradable hydrogel. Laryngoscope 116:529–533. doi:10.1097/01.mlg.0000200791.77819.eb

8. Plontke SK, Plinkert PK, Plinkert B et al (2002) Transtympanic endoscopy for drug delivery to the inner ear using a new microendoscope. Adv Otorhinolaryngol 59:149–155

9. Karhuketo TS, Puhakka HJ, Laippala PJ (1997) Endoscopy of the middle ear structures. Acta Otolaryngol Suppl 529:34–39. doi:10.3109/00016489709124074

10. Silverstein H, Rowan PT, Olds MJ et al (1997) Inner ear perfusion and the role of round window patency. Am J Otol 18:586–589

11. Kakehata S, Futai K, Kuroda R et al (2004) Office-based endoscopic procedure for diagnosis in conductive hearing loss cases using OtoScan Laser-Assisted Myringotomy. Laryngoscope 114:1285–1289. doi:10.1097/00005537-200407000-00027

12. Remley KB, Swartz JD, Harnsberger HR (1998) The external auditory canal. In: Swartz JD, Harnsberger HR (eds) Imaging of the temporal bone, 3rd edn. Thieme, New York, pp 16–20

13. Lee KY, Nakagawa T, Okano T et al (2007) Novel therapy for hearing loss: delivery of insulin-like growth factor 1 to the cochlea using gelatin hydrogel. Otol Neurotol 28:976–981

14. Schicker S (1957) Das runde Fenster. Laryngologie 36:149–153

Hydrogen protects auditory hair cells from free radicals
Yayoi S. Kikkawa, Takayuki Nakagawa, Rie T. Horie and Juichi Ito

Reactive oxygen species (ROS) play a role in the degeneration of auditory hair cells because of aging, noise trauma, or ototoxic drugs. Hydrogenation is a fundamental reduction/deoxidation reaction in living organisms. This study thus examined the potential of hydrogen to protect auditory hair cells from ROS-induced damage. To generate ROS, we applied antimycin A to explant cultures of auditory epithelia, and examined the effect of hydrogen on the protection of hair cells against ROS. Incubation with a hydrogen-saturated medium significantly reduced ROS generation and subsequent lipid peroxidation in the auditory epithelia, leading to increased survival of the hair cells. These findings show the potential of hydrogen to protect auditory hair cells from ROS-induced

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

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

Introduction
Hearing disorders affect nearly 10% of the world population. The common causes of sensorineural hearing loss because of cochlear injury are aging, hereditary disorders, noise trauma, and ototoxic drugs. The mechanisms underlying cochlear injury are still not completely known. However, numerous studies have suggested that they involve the production of reactive oxygen species (ROS), which cause cellular injury in the cochlea resulting in sensorineural hearing loss [1–4]. Although they are probably intended to fight against invasive pathogens, ROS seem to produce substantial collateral damage through DNA strand breaks, lipid and protein oxidation [5–7].

Hydrogenation is a fundamental reduction/deoxidation reaction in living organisms. Many reduction processes in the body involve electron transfer from molecular hydrogen. This molecule was recently established as an antioxidant that selectively reduces the hydroxyl radical, and was shown to decrease the cerebral infarction volume after ischemia in rats [8]. Subsequently, protective effects of hydrogen gas have been demonstrated in a mouse model for hepatic injury [9] and in a rat model for myocardial infarction [10]. In the nervous system, hydrogen-rich water was shown to prevent superoxide formation in mouse brain slices [11], and to prevent stress-induced impairments in learning tasks during chronic physical restraint in mice [12]. Moreover, a clinical study showed that consuming hydrogen-rich pure water improves lipid and glucose metabolism in type 2 diabetes patients [13].

The ex-vivo study reported here tested the hypothesis that molecular hydrogen, hydrogen gas, protects against cochlear impairment by scavenging free radicals. We initially generated in situ ROS in the cochlea using an inhibitor of mitochondrial respiratory chain complex III, antimycin A, and showed that they caused direct damage to the hair cells. Then, using a hydrogen (hydrogen gas)-saturated culture media, we demonstrated that hydrogen gas alleviated ROS-induced ototoxicity, suggesting that hydrogen gas has the potential to act as an antioxidant for the treatment of cochlear damage. We also evaluated the generated hydroxyl radicals by fluorescence emission of 2-[6-(4′-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF) and lipid peroxidation by immunohistochemistry for 4-hydroxynonenal (HNE).

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

Cochlear explant culture
Postnatal day 2 (P2) ICR mice were deeply anesthetized with diethyl ether and decapitated. The temporal bones were dissected, and the cochleae were freed from the surrounding tissue and placed in 0.01 M phosphate-buffered saline (PBS; pH 7.4). After removing the cochlear lateral wall, the auditory epithelia were dissected from the cochlear modiolus. The tissue samples were then placed on glass-mesh inserts (Falcon, Billerica, Massachusetts, USA) and cultured initially in serum-free

modified Eagle's medium (MEM; Invitrogen, Eugene, Oregon, USA), supplemented with 3 g/l glucose (Wako Pure Chemicals, Osaka, Japan) and 0.3 g/l penicillin G (Wako), for 24 h at 37°C in a humidified (95%) air: 5% atmospheric CO_2. In total, 20 cochlear explants were used in a single culture, and at least three independent cultures were performed for each condition. As the hair cells in the apex are resistant to free radicals [14], the basal turns of the cochlea were used in this study.

Antimycin A application

The explants were transferred to medium containing antimycin A (Sigma–Aldrich, St Louis, Missouri, USA) at concentrations of 0.1, 1, or 10 µg/ml, with six to nine cochleae incubated at each concentration. The cultures were maintained for 24 h. At the end of the culture period, the samples were fixed for 15 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and then provided for immunostaining for myosin VIIa to evaluate the number of surviving hair cells. The specimens were incubated with primary rabbit polyclonal antibodies against myosin VIIa (1 : 500; Proteus Bioscience Inc., Ramona, California, USA). Alexa-Fluor 568 goat anti-rabbit IgG (1 : 200; Invitrogen) was used as the secondary antibody. Specimens were then incubated in Alexa-Fluor 488-conjugated phalloidin (1 : 250; Invitrogen) to label F-actin. The specimens were examined with a Leica TCS-SP2 laser-scanning confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). To quantify the hair-cell loss in the cochlea after the different treatments, inner hair cells (IHCs) and outer hair cells (OHCs) were counted over a 100-µm-long stretch of the auditory epithelia, in two separate regions of the basal turn in each culture (totaling 200 µm). For each treatment, six to nine cultures were evaluated.

Hydrogen treatment

To assess the efficacy of hydrogen gas for cochlear protection, explants were cultured initially in an airtight box (Chopla Industries, Inazawa, Japan) with reduced-CO_2-dependence media, MEM and Leivovitz's L-15 media (Invitrogen) mixed in a 1 : 1 ratio [15], supplemented with 3 g/l glucose and 0.3 g/l penicillin G, at 37°C in humidified (100%) atmospheric air. After 24 h, the medium was changed to one containing antimycin A at a concentration of 0.1, 1 or 10 µg/ml, with or without hydrogen gas for another 24 h. Hydrogen gas was dissolved directly into the media, and a high content of dissolved hydrogen (1.3 ± 0.1 mg/l) was confirmed using a hydrogen electrode (Model M-10B2; Able Corporation, Tokyo, Japan). The pH of the culture media without hydrogen gas was 7.18 ± 0.02, and that of the culture media with hydrogen gas was 7.52 ± 0.02. The prepared media were used for culture within 30 min. At the end of the experiments, the explants were fixed and provided for histological analysis to evaluate hair-cell survival. Between six and 12 cochleae were used for each condition.

Detection of reactive oxygen species by fluorescent indicators

The cochlear explants were treated with 30 µM HPF (Daiichi Pure Chemicals Co., Tokyo, Japan) for 20 min to detect cellular hydroxyl radicals. Fluorescent images were captured with a Leica TCS-SP2 confocal microscope using excitation and emission filters of 488 and 510 nm, respectively. All images were taken with the same laser intensity, detector gain, and offset values. Fluorescent signals were quantified from two separate OHC regions of the basal turns, each of which was 1250 µm^2 (50 × 25 µm), using ImageJ software (http://rsb.info.nih.gov/ij/; NIH, Bethesda, MD). Intensity measurements were expressed relative to the levels in the control samples.

Lipid-peroxidation assay

Lipid peroxidation was assessed in cultures treated with antimycin A, in the presence or absence of hydrogen gas, by measuring the expression of HNE immunohistochemically. Explants were labeled with mouse anti-HNE monoclonal antibody (1 : 8; Oxis Research, Portland, Oregon, USA) and fluorescein horse anti-mouse immunoglobulin G (1 : 250; Vector Laboratories, Burlingame, California, USA) as the primary and secondary antibodies, respectively. Specimens were then counterstained with Alexa 568 phalloidin (1 : 250; Invitrogen). All images were taken with the same exposure and shutter speed. The green fluorescence intensity was measured in the same area using ImageJ software. Intensity measurements were expressed relative to the levels in the control samples.

Fig. 1
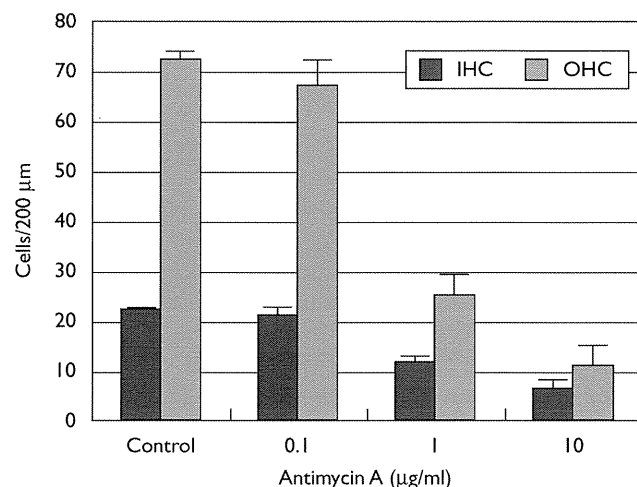
Antimycin A induced dose-dependent auditory hair-cell loss. The graph shows the relationship between the antimycin A concentration and the hair-cell count following 24-h culture. The inner hair cells (IHCs) and outer hair cells (OHCs) were counted in 200-µm-length regions from each cochlea. The hair-cell densities decreased systematically as the antimycin A concentration increased. Bars represent standard errors.

Statistical analysis

The overall effects on the hair-cell number, and the HPF and HNE staining intensities, were analyzed by two-way factorial analysis of variance (ANOVA) using the Statcel2 application (OMS Publishing, Saitama, Japan). P values less than 0.05 were considered to be statistically significant. For interactions that were found to be significant, multiple paired comparisons were analyzed using the Tukey–Kramer test.

Results

### Antimycin A induced dose-dependent hair-cell loss

Initially, we established the dose–response relationship between the antimycin A concentration and its toxic effect on hair cells. The addition of 1 μg/ml antimycin A to cultures for 24 h significantly reduced the hair-cell numbers in both the IHC and OHC regions, with the effect being more severe in the latter (Fig. 1). The

addition of 1 μg/ml antimycin A destroyed $46.2 \pm 4.6\%$ of the IHCs and $65.6 \pm 5.8\%$ of the OHCs. The hair-cell density decreased depending on the concentrations of antimycin A and few could be detected in the auditory epithelia cultured in 10 μg/ml antimycin A (Fig. 2g).

Protective effect of hydrogen supplementation

Next, we assessed the potential of hydrogen to protect against antimycin-induced ototoxicity by administering 0, 0.1, 1, or 10 μg/ml antimycin to samples cultured in hydrogen-saturated media in an airtight environment. The addition of hydrogen markedly increased both IHC and OHC survival, with a substantial number of hair cells surviving even at the highest antimycin A dose (Figs 2 and 3). Two-way ANOVA showed that hydrogen gas had a significant effect on the numbers of surviving IHCs and OHCs ($P = 0.00305$ and $P = 0.00016$, respectively). Tukey–Kramer tests for multiple paired comparisons

Fig. 2
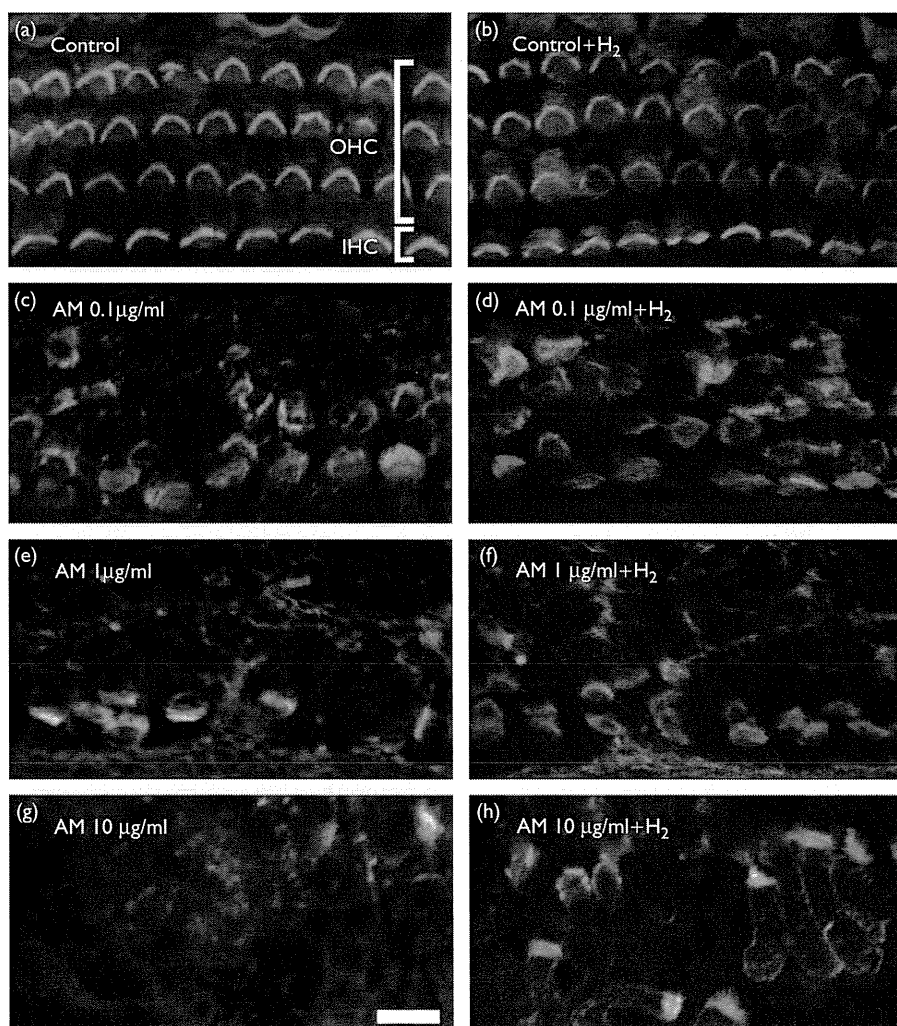
Effect of hydrogen on the survival of cochlear hair cells. (a–h) Photomicrographs of phalloidin (green) and myosin VIIa (red)-labeled cochlear cultures, treated with 0 (a and b), 0.1 (c and d), 1 (e and f), and 10 μg/ml (g and h) antimycin A (AM), with (b, d, f and h) or without (a, c, e and g) hydrogen gas (H₂). Bar, 5 μm. IHCs, inner hair cells; OHCs, outer hair cells.

showed that the loss of OHCs was significantly lower ($P < 0.01$) in the groups treated with 0.1 μg/ml antimycin A and hydrogen than in the groups treated with antimycin A alone (Fig. 3). These data showed that hydrogen protected hair cells against antimycin A-induced toxicity in this model of cochlear damage.

Reactive oxygen species reduction by molecular hydrogen

To investigate the mechanism by which hydrogen alleviated hair-cell damage, we measured the ROS production in the cochlear cultures (Fig. 3). HPF is

a reagent that was developed to detect certain highly ROS directly [16]. In cochlear cultures treated with 0–10 μg/ml antimycin A for 45 min, in the absence of hydrogen gas, the HPF signals increased (Fig. 4a), indicating that antimycin A induced the production of hydroxyl radicals. The intensity of the HPF fluorescence after the treatment with 10 μg/ml antimycin A was 3.61 times greater than that in the absence of antimycin A (Fig. 4a). By contrast, adding hydrogen gas to the cultures resulted in a reduction of the HPF signal intensity.

Fig. 3
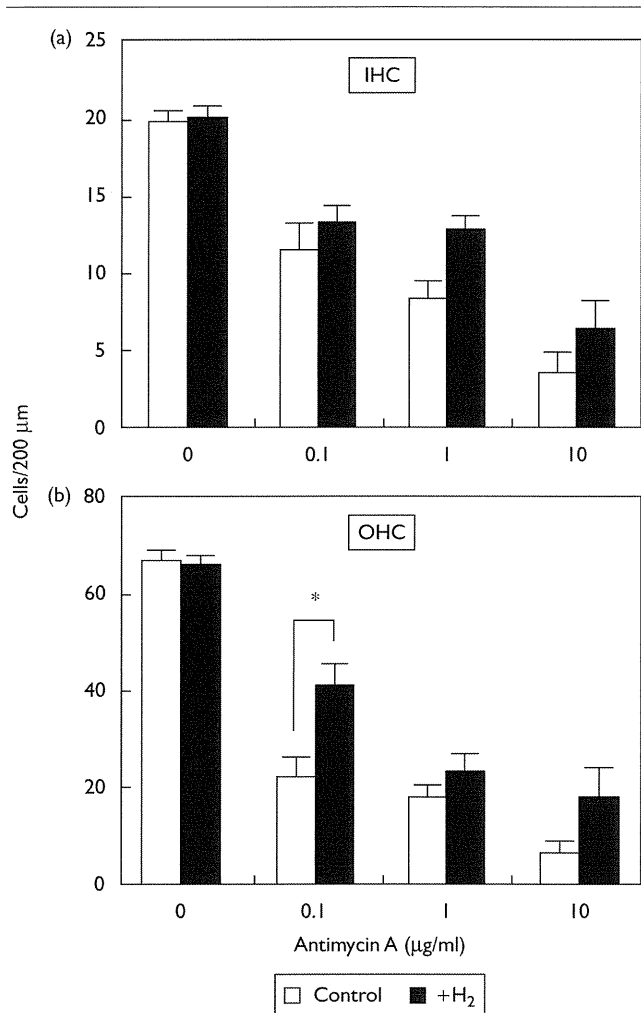
Hair-cell counts in molecular hydrogen-treated cultures. After 24-h culture with antimycin A, inner hair cells (IHCs) (a) and outer hair cells (OHCs) (b) were counted. White box symbols represent the counts from control cultures without molecular hydrogen, and black symbols represent those from cultures with molecular hydrogen. Molecular hydrogen significantly attenuated the loss of IHCs ($P=0.0031$) and OHCs ($P=0.0016$) in antimycin A-damaged cochleae according to a two-way analysis of variance. Post hoc analyses with Tukey–Kramer tests for multiple paired comparisons showed that the OHC loss was significantly lower in cultures treated with antimycin A plus hydrogen gas than in those treated with 0.1 μg/ml antimycin A alone ($*P<0.01$). Bars represent standard errors.

Fig. 4
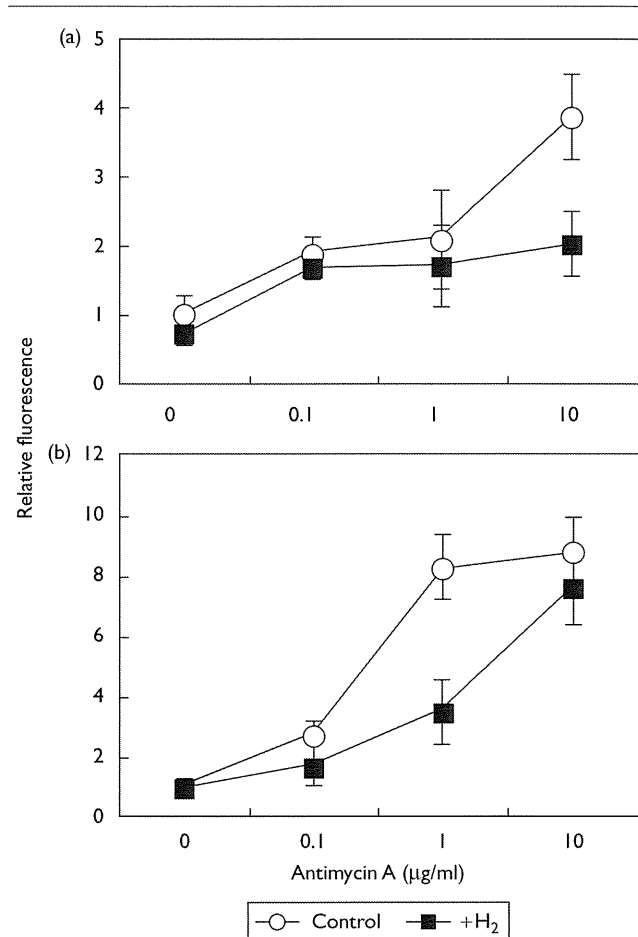
Molecular hydrogen reduced reactive oxygen species (ROS) production and lipid oxygenation. White circle symbols represent the counts from control cultures without molecular hydrogen, and black box symbols represent those from cultures with molecular hydrogen. (a) ROS production was measured in cultures treated with antimycin A with or without hydrogen gas for 40 min, according to the intensity of chemifluorescence of 2-[6-(4'-hydroxy)phenoxy-3H-xanthen-3-on-9-yl] benzoate (HPF). The relative HPF fluorescence intensity in the organ of Corti increased dose dependently in the presence of antimycin A, but was significantly reduced by the addition of hydrogen gas ($P=0.0439$). (b) Lipid oxygenation was measured in cultures treated with antimycin A with or without hydrogen gas for 24 h, according to the intensity of immunohistochemical labeling for 4-hydroxynonenal (HNE). The relative HNE-staining intensity in the organ of Corti increased dose dependently in the presence of antimycin A, but was significantly reduced by the addition of hydrogen gas ($P=0.0447$). Bars represent standard errors.

In the presence of hydrogen gas, the intensity of the HPF fluorescence after the treatment with 10 µg/ml antimycin A was 2.34 times greater than that in the absence of antimycin A (i.e. 52.1% of that without hydrogen). Two-way ANOVA showed that hydrogen had a significant effect on the production of hydroxyl radicals ($P = 0.0439$). No significant differences were identified in multiple paired comparisons with Tukey–Kramer tests.

We also investigated the expression of HNE, which is a lipid-peroxidation marker, in the explant cultures. HNE production increased dose dependently in the presence of antimycin A. The intensity of the HNE fluorescence after the treatment with 10 µg/ml antimycin was 8.68 times greater than that in the absence of antimycin A (Fig. 4b). Adding molecular hydrogen to the cultures resulted in a significant reduction of HNE expression. Two-way ANOVA showed that hydrogen had a significant effect on the production of HNE ($P = 0.0446$), but, no significant differences were identified in multiple paired comparisons with Tukey–Kramer tests. Molecular hydrogen had its greatest effect at an antimycin concentration of 1 µg/ml, when the HNE production was attenuated to 42.4% of the level seen in the absence of molecular hydrogen.

Discussion

To our knowledge, this study was the first to evaluate the therapeutic potential of molecular hydrogen for the auditory system. A quantitative assessment of the hair-cell loss caused by antimycin A showed a dose-dependent effect, indicating that the toxic effects in this explant culture system represented a good model for the cochlea *in vivo*. Treating the cultures with hydrogen gas significantly influenced the dose response for hair-cell loss because of antimycin A, indicating that hydrogen gas has a protective effect on hair cells against ROS toxicity.

We also investigated the mechanisms by which hydrogen gas protected hair cells from damage in the cochlea. Our results showed that the hydrogen gas in fact reduced the production of cellular ROS and subsequent lipid oxygenation. Our antimycin A cochlea culture system, along with chemiluminescence detection, was shown to be useful in screening for antioxidant drugs, because antimycin A directly produces ROS in the cochlea and the direct measurement of ROS was possible when HPF was used.

Hydrogen is one of the most abundant and well-known molecules. Inhalation of hydrogen gas has been used in the prevention of decompression sickness in divers and has shown a good safety profile [8]. Hydrogen has been approved by the US Food and Drug Administration for the treatment of several different diseases. Ohsawa *et al.* [8] demonstrated that hydrogen gas is a potent antioxidant with certain unique properties. First, hydro-

gen gas is permeable to cell membranes and can target organelles, including mitochondria and nuclei. Second, hydrogen gas specifically quenches detrimental ROS, such as the hydroxyl radical and peroxynitrite, while maintaining the metabolic oxidation–reduction reaction and other less-potent ROS, such as hydrogen peroxide and nitric oxide. The first feature is especially favorable in inner-ear medicine, because many therapeutic compounds are blocked by the blood–cochlear barrier and cannot reach cochlear hair cells [17,18]. Therefore, hydrogen therapy could be widely used in medical applications as a safe and effective antioxidant with minimal side effects.

Conclusion

In conclusion, this study showed that hydrogen gas markedly decreased oxidative stress by scavenging ROS, and protected cochlear cells and tissues against oxidative stress. These results have prompted us to perform in-vivo studies to determine whether treatment with hydrogen gas might exert a beneficial effect on damaged cochleae and promote hearing recovery.

Acknowledgments

References

1 Jiang H, Talaska AE, Schacht J, Sha SH. Oxidative imbalance in the aging inner ear. *Neurobiol Aging* 2007; **28**:1605–1612.
2 Yamane H, Nakai Y, Takayama M, Iguchi H, Nakagawa T, Kojima A. Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. *Eur Arch Otorhinolaryngol* 1995; **252**:504–508.
3 Yamashita D, Jiang HY, Schacht J, Miller JM. Delayed production of free radicals following noise exposure. *Brain Res* 2004; **1019**:201–209.
4 Lee JE, Nakagawa T, Kim TS, Endo T, Shiga A, Iguchi F, *et al.* Role of reactive radicals in degeneration of the auditory system of mice following cisplatin treatment. *Acta Otolaryngol* 2004; **124**:1131–1135.
5 Clerici WJ, Hensley K, DiMartino DL, Butterfield DA. Direct detection of ototoxicant-induced reactive oxygen species generation in cochlear explants. *Hear Res* 1996; **98**:116–124.
6 Linseman DA. Targeting oxidative stress for neuroprotection. *Antioxid Redox Signal* 2009; **11**:421–424. Aug 20. [Epub ahead of print]
7 Klein M, Koedel U, Pfister HW. Oxidative stress in pneumococcal meningitis: a future target for adjunctive therapy? *Prog Neurobiol* 2006; **80**:269–280.
8 Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, *et al.* Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007; **13**:688–694.
9 Fukuda K, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. *Biochem Biophys Res Commun* 2007; **361**:670–674.

10 Hayashida K, Sano M, Ohsawa I, Shinmura K, Tamaki K, Kimura K, *et al*. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun* 2008; **373**:30–35.

11 Sato Y, Kajiyama S, Amano A, Kondo Y, Sasaki T, Handa S, *et al*. Hydrogen-rich pure water prevents superoxide formation in brain slices of vitamin C-depleted SMP30/GNL knockout mice. *Biochem Biophys Res Commun* 2008; **375**:346–350.

12 Nagata K, Nakashima-Kamimura N, Mikami T, Ohsawa I, Ohta S. Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. *Neuropsychopharmacology* 2008; **34**:501–508.

13 Kajiyama S, Hasegawa G, Asano M, Hosoda H, Fukui M, Nakamura N, *et al*. Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. *Nutr Res* 2008; **28**:137–143.

14 Sha SH, Taylor R, Forge A, Schacht J. Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. *Hear Res* 2001; **155**:1–8.

15 Futai N, Gu W, Song JW, Takayama S. Handheld recirculation system and customized media for microfluidic cell culture. *Lab Chip* 2006; **6**: 149–154.

16 Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. *J Biol Chem* 2003; **278**: 3170–3175.

17 Coimbra RS, Loquet G, Leib SL. Limited efficacy of adjuvant therapy with dexamethasone in preventing hearing loss due to experimental pneumococcal meningitis in the infant rat. *Pediatr Res* 2007; **62**:291–294.

18 Laurell GF, Teixeira M, Duan M, Sterkers O, Ferrary E. Intact blood-perilymph barrier in the rat after impulse noise trauma. *Acta Otolaryngol* 2008; **128**: 608–612.

PROSTAGLANDIN E RECEPTOR SUBTYPE EP4 AGONIST PROTECTS COCHLEAE AGAINST NOISE-INDUCED TRAUMA

R. HORI,[a] T. NAKAGAWA,[a]* Y. SUGIMOTO,[b]
T. SAKAMOTO,[a] N. YAMAMOTO,[a] K. HAMAGUCHI[a] AND
J. ITO[a]

[a]*Department of Otolaryngology–Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku 606-8507, Kyoto, Japan*

[b]*Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan*

Abstract—Prostaglandin E$_1$ is frequently used for the clinical treatment of acute sensorineural hearing loss. However, the mechanisms underlying the effects of prostaglandin E$_1$ on the inner ear have not yet been elucidated. The physiological effects of prostaglandin E$_1$ are mediated by the prostanoid receptors prostaglandin I receptor and the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4, the respective agonists for which have been purified. In the current study, we examined the efficacy of a local EP4 agonist application for the treatment of sensorineural hearing loss. We examined EP4 expression in the mouse cochlea using the reverse transcription–polymerase chain reaction and immunohistochemistry. The protective effects of local EP4 agonist treatment before or after noise exposure were tested in guinea pigs using measurements of auditory brain-stem responses and histological analysis. The results demonstrated EP4 expression in the cochlea, and showed that pre- and post-treatment with an EP4 agonist significantly attenuated threshold shifts of auditory brain stem responses, and significant attenuation in the loss of outer hair cells was found in local EP4 agonist treatment before noise exposure. These findings indicate that EP4 is involved in mechanisms for prostaglandin E$_1$ actions on the cochlea, and local EP4 agonist treatment could attenuate acute sensorineural hearing loss. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: acoustic trauma, hair cell, hearing loss, inner ear, prostanoid.

Sensorineural hearing loss (SNHL) is one of the most frequent disabilities. Once hearing has been lost, it is rarely recovered, because the mammalian auditory system, particularly the sensory hair cells (HCs), has a limited capability for regeneration. Clinically, there are no curative therapeutic options for chronic SNHL, and the curative rate for

acute SNHL is also limited. The systemic application of corticosteroids has been accepted as the primary treatment of choice for acute SNHL, although its efficacy has not been substantiated (Wei et al., 2006). In general, approximately 50% of SNHL cases show no response to the systemic application of corticosteroids (Ogawa et al., 2002). Other options for the treatment of acute SNHL have therefore been required. Prostaglandin E$_1$ (PGE$_1$) has often been used as a secondary choice treatment for acute SNHL. However, its clinical efficacy remains controversial (Ahn et al., 2005; Suzuki et al., 2008; Zhuo et al., 2008). PGE$_1$ is usually applied to improve local blood circulation. An experimental study using guinea pigs demonstrated an increased blood supply in the cochlea following local PGE$_1$ application (Tominaga et al., 2006). However, the actual mechanisms underlying the effects of PGE$_1$ on the inner ear have not yet been elucidated.

The physiological actions of PGE$_1$ are mediated by the prostanoid receptors prostaglandin I receptor (IP) and the prostaglandin E receptor subtypes EP1, EP2, EP3, and EP4 (Coleman et al., 1994; Kiriyama et al., 1997). Recently, these prostanoid receptors have been cloned, and their actions have been elucidated. IP, EP2, and EP4 are coupled to G-protein stimulation and mediate increases in cyclic AMP (cAMP) that activate protein kinase A (PKA) (Coleman et al., 1994; Narumiya et al., 1999). The EP4 receptor in particular has various physiological and pathophysiological actions, including anti-apoptotic (Kataoka et al., 2005), anti-excitotoxicity (Ahmad et al., 2005), and anti-inflammatory (Kabashima et al., 2002; Nitta et al., 2002) effects. The actions of EP4 agonists could protect auditory HCs, and might indicate therapeutic efficacy of PGE$_1$ for acute SNHL. We therefore investigated the potential use of an EP4 agonist in the protection of auditory HCs from noise trauma. In the current study, we demonstrated EP4 expression in the cochlea using the reverse transcription–polymerase chain reaction (RT-PCR) and immunohistochemistry. We also showed the efficacy of local EP4 agonist treatment for protecting auditory HCs against noise-induced damage, by means of auditory brain-stem response (ABR) recordings and histological analyses of cochlear specimens.

EXPERIMENTAL PROCEDURES

Experimental animals

Male C57BL/6 mice at 8 weeks of age and Hartley guinea pigs weighing 350–400 g were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all of the experimental protocols. Animal care was supervised by

*Corresponding author. Tel: +81-75-751-3346; fax: +81-75-751-7225.
E-mail address: tnakagawa@ent.kuhp.kyoto-u.ac.jp (T. Nakagawa).
Abbreviations: ABR, auditory brain-stem response; bp, base pairs; cAMP, cyclic AMP; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetra-acetic acid; EP, prostaglandin E receptor subtype; HC, hair cell; HGF, hepatocyte growth factor; IHC, inner hair cell; IP, prostaglandin I receptor; OHC, outer hair cell; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PGE$_1$, prostaglandin E$_1$; PKA, protein kinase A; RT-PCR, reverse transcription–polymerase chain reaction; RWM, round window membrane; SNHL, sensorineural hearing loss; SPL, sound pressure level; VEGF, vascular endothelial growth factor.