持細胞が再び分裂，増殖する可能性がある．$p27^{Kip1}$の発現制御メカニズムとしては，$p27^{Kip1}$遺伝子からの転写，翻訳レベルと$p27^{Kip1}$タンパク質の細胞内での分解レベルの2つがある．後者のメカニズムにかかわるのが，Fボックスタンパク質の1つであるskp2である．skp2は$p27^{Kip1}$のユビキチン化に関与し，skp2などの働きでユビキチンを付加された$p27^{Kip1}$はプロテアソームにて分解される．

発達段階に応じてマウス内耳感覚上皮でのskp2の発現変化を組織学的に解析することで，内耳感覚上皮で細胞増殖が活発な時期には，skp2により$p27^{Kip1}$の発現が抑制されており，内耳感覚上皮で有毛細胞，支持細胞への分化運命が決定されるタイミングにおいては，$p27^{Kip1}$の発現は支持細胞のみに限定され，有毛細胞では消失することが明らかになった．この有毛細胞でのp27の発現の消失にskp2は関与せず[8]，遺伝子からの転写レベルよりも上流で$p27^{Kip1}$の発現が制御されている．したがって，成熟した内耳感覚上皮支持細胞でのp27発現抑制には，skp2過剰発現によるタンパク質分解の促進もしくはRNA干渉による翻訳の抑制が有効な手段と推察される．蓑田らは，ウイルスベクターを用いたskp2の内耳感覚上皮での過剰発現が細胞増殖を誘導すると報告している[9]．しかし，細胞周期にリエントリーすることは，細胞死の誘導につながる可能性もあり[10]，今後の検討が注目される．

内耳再生への試み②：細胞移植

哺乳類内耳の再生能力が限られているのならば，再生能力のある細胞を内耳に送り込んでやればよいのではないか，という発想で内耳への細胞移植実験は開始された．最初の内耳への細胞移植実験は，内耳と同じ外胚葉系の幹細胞である神経幹細胞を用いたものである．有毛細胞障害を惹起した内耳に神経幹細胞を移植すると，内耳組織内に移植細胞の侵入を示唆する所見が認められた．また，ごく限られてはいるが，前庭感覚上皮内に侵入した移植細胞の一部が内耳有毛細胞のマーカーであるミオシン7aを発現している所見が認められた[11]．この結果は，神経幹細胞は障害を受けた内耳感覚上皮には侵入できるということを示しており，有毛細胞に分化する可能性があることを呈示するものとして内外の注目を集めた．内耳組織由来の細胞や胚性幹細胞由来の細胞移植でも，感覚上皮内に移植細胞が侵入し，有毛細胞様の細胞に分化することが報告されているが，機能再生に関連する報告はなされていない．いかに，有毛細胞や支持細胞といった感覚上皮特有の細胞に効率よく分化する細胞を開発するか，また，移植した細胞をいかにして感覚上皮内へと誘導するかが，解決すべき問題点として残されている．

細胞移植による内耳再生研究では，ラセン神経節細胞が主な研究対象とされている．現在，高度難聴に対する唯一の治療法は人工内耳であるが，人工内耳を埋め込んでもラセン神経節細胞に障害がある場合，良好な聞き取りは得られない．細胞移植によって，ラセン神経節細胞が再生すれば，人工内耳での聞き取りも向上すると期待される．このような臨床的背景に加え，神経細胞は種々の細胞から比較的分化誘導しやすいことにより，細胞移植によるラセン神経節細胞再生に関する研究が活発に行われている．種々の細胞が移植細胞のソースとして用いられているが，最も神経細胞の再生能力が高い細胞が胚性幹細胞（ES細胞）といえる．ES細胞では，高率に神経細胞へと分化誘導する方法が確立されており，*in vitro*，*in vivo*での移植実験がいくつか行われている．最も注目すべき点としては，*in vivo*の移植実験で機能回復を示唆する所見が認められているという点である．

神経細胞へ分化誘導したマウスES細胞を，ラセン神経節変性をあらかじめ誘導したモルモットに移植し，4週間後に，電気刺激聴性脳幹反応にて機能評価したところ，コントロールとしたシャムオペ（偽手術）群よりも有意に機能が回復していることが認められた[12]．組織学的にも，移植細胞由来神経細胞の蝸牛軸での局在が確認されている．今後，移植細胞由来神経細胞が直接的に機能回復に寄与しているのか，栄養因子の供給などの間接的な効果なのかを検討する必要がある．

臨床応用への展望

第一に内耳に内在する細胞を活性化し，内耳の再生を誘導し，内耳性難聴の治療に応用する試みについては，内耳再生を妨げている因子を抑制し，再生を誘導する方法が最も臨床に近い方法といえる．しかしながら，この方法による機能回復に関する知見は，報告されておらず，今後の課題といえる．

細胞移植による内耳再生では，人工内耳とも深いかかわりをもつラセン神経節細胞の再生が最も臨床応用に近い位置にある．すでに霊長類での実験が開始されている．最大の問題は，移植細胞ソースにかかわる倫理的な面といえる．ES細胞は他の幹細胞に比べて，均一な性質をもつ細胞を大量に準備することが可能であり，ラセン神経節細胞再生に関しても現在のところ最も能力の高い細胞といえる．しかし，わが国では，ヒトES細胞を使用した研究承認へのハードルはきわめて高い．このような背景から，ES細胞をソースとした研究で得られた成果を他の自己由来細胞で実現しようとする方向性が模索されている．最近，線維芽細胞からES細胞と同様の性質をもった細胞をつくることが可能であることも報告されており[13]，自己由来細胞からES細胞様の細胞をつくることが可能となれば，大きく展開は変わる可能性がある．

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『中枢神経の機能再生―細胞治療と人工臓器―』，Medical Bio，オーム社，2007年7月号

■筆頭著者プロフィール

中川隆之：1989年大阪市立大学医学部卒業，'95年同大学院医学研究科修了，内耳のアポトーシス研究を行い，医学博士取得．2001年より京都大学大学院医学研究科耳鼻咽喉科頭頸部外科助教．現在，内耳再生，特に新しい感音難聴治療方法の開発を目的として研究を行っている．

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Novel Therapy for Hearing Loss: Delivery of Insulin-Like Growth Factor 1 to the Cochlea Using Gelatin Hydrogel

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Hypothesis: Local application of recombinant human insulin-like growth factor 1 (rhIGF-1) via a biodegradable hydrogel after onset of noise-induced hearing loss (NIHL) can attenuate functional and histologic damage.
Background: The biodegradable gelatin hydrogel makes a complex with drugs by static electric charges and releases drugs by degradation of gelatin polymers. We previously demonstrated the efficacy of local rhIGF-1 application via hydrogels before noise exposure for prevention of NIHL.
Methods: First, we used an enzyme-linked immunosorbent assay to measure human IGF-1 concentrations in the cochlear fluid after placing a hydrogel containing rhIGF-1 onto the round window membrane of guinea pigs. Second, the functionality and the histology of guinea pig cochleae treated with local rhIGF-1 application at different concentrations after noise exposure were examined. Control animals were treated with a hydrogel immersed in physiologic saline alone.
Results: The results revealed sustained delivery of rhIGF-1 into the cochlear fluid via the hydrogel. The measurement of auditory brainstem responses demonstrated that local rhIGF-1 treatment significantly reduced the threshold elevation from noise. Histologic analysis exhibited increased survival of outer hair cells by local rhIGF-1 application through the hydrogel.
Conclusion: These findings indicate that local rhIGF-1 treatment via gelatin hydrogels is effective for treatment of NIHL.
Key Words: Biomaterial—Drug delivery—Growth factor—Noise trauma—Round window.
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Sensorineural hearing loss (SNHL) is one of the most common disabilities, but therapeutic options are limited to hearing aids and cochlear implants. Many investigations have therefore examined novel therapeutic molecules for SNHL and have discovered several agents exerting therapeutic activity against SNHL. Despite such progress in basic research, translation of these basic findings into clinical use is very rare. The lack of safe and effective methods for drug delivery to the cochlea has formed a considerable obstacle to clinical applications. Systemically applied drugs have great difficulty reaching cochlear cells because of 1) the blood-labyrinth barrier (1) acting as an obstacle to the transfer of drugs from serum to cochlear cells and 2) the limited blood flow to the cochlea (2). The development of local drug delivery systems to the cochlea thus remains crucial for the clinical application of basic findings in this field.

Several methods of local drug delivery to the cochlea have been reported (3). Implantable minipumps has frequently been used for local drug delivery to the cochlea in animal experiments (4). In addition, several clinical reports have described the efficacy of local steroid application using a semi-implantable minipump (5,6). However, the use of implantable minipumps has not been widely adopted because of the need for surgical procedures similar to tympanoplasty. Local drug delivery using biodegradable polymers has thus gained attention as an alternative to implantable minipumps. In general, biodegradable polymers containing therapeutic molecules are

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placed on the round window membrane (RWM), and therapeutic molecules released from the polymers transfer into the perilymph of cochleae through the RWM (3,7). We have reported the efficacy of gelatin hydrogel for sustained delivery of brain-derived neurotrophic factor (BDNF) to the cochlear perilymph in guinea pigs (8). Brain-derived neurotrophic factor delivered by gelatin hydrogels efficiently protect spiral ganglion neurons from secondary degeneration caused by hair cell loss. However, BDNF is not currently clinically applicable. Insulin-like growth factor 1 (IGF-1) is a mitogenic peptide that plays essential roles in the regulation of growth and development in the inner ear. The gelatin hydrogel system was examined as a vehicle to deliver recombinant human IGF-1 (rhIGF-1) to the cochlea because this drug has already been approved for clinical use. Local rhIGF-1 application through the gelatin hydrogel before noise exposure significantly protects cochleae from functional and histologic losses induced by noise trauma (9).

Our goal is the clinical use of local rhIGF-1 application via the gelatin hydrogel as a therapeutic option for the treatment of SNHL. The current study examined whether posttraumatic application of rhIGF-1 to the cochlea through gelatin hydrogel attenuates noise-induced hearing loss (NIHL). In addition, we examined IGF-1 concentrations in cochlear perilymph after placing rhIGF-1–containing hydrogel onto the RWM of guinea pigs to determine the efficiency of the gelatin hydrogel system for cochlear application of rhIGF-1.

MATERIALS AND METHODS

Experimental Animals

Twenty-six adult female Hartley guinea pigs weighing 250 to 300 g (Japan SLC, Hamamatsu, Japan) served as experimental animals. Animal care was conducted under the supervision of the Institute of Laboratory Animals at the Graduate School of Medicine, Kyoto University. All experimental procedures were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Biodegradable Gelatin Hydrogel

A biodegradable hydrogel has been developed for sustained delivery of peptides, including growth and trophic factors (10). In this approach, a positively charged protein is electrostatically complexed with negatively charged polymer chains, which form the components of the biodegradable hydrogel. The biodegradation of the polymer chains leads to the release of peptide. Biodegradable hydrogels are generated by glutaraldehyde cross-linking of gelatin. The rates of degradation are determined according to the concentration of glutaraldehyde. A previous analysis of in vitro IGF-1 release profiles from hydrogels has demonstrated that a hydrogel made with 10-mmol/L glutaraldehyde allows for optimal IGF-1 delivery (11). We therefore used this type of hydrogel in the present study.

In Vivo IGF-1 Release Profile

Guinea pigs were anesthetized using ketamine (dose, 80 mg/kg intramuscularly; Sankyo, Tokyo, Japan) and xylazine (dose, 9 mg/kg intramuscularly; Bayer, Tokyo, Japan). A sheet of hydrogels in dried condition was cut to a size of 1.5 to 2 mm^3 under microscopy. A piece of hydrogel immersed in rhIGF-1 (amount, 400 μg dissolved in 40-μL physiologic saline; Astellas, Tokyo, Japan) was positioned on the left RWM of each animal in the treated group. Perilymph was collected on Days 1, 3, or 7 (n = 4 each) after drug application in treated groups and from nontreated animals (n = 4). For each animal, a small hole was made in the basal turn of the cochlea 2 mm from the RWM, under general anesthesia, and 3 μL of perilymph was collected through the hole using a micropipette. The same amounts of cerebrospinal fluid (CSF) and serum were obtained from each animal. Insulin-like growth factor 1 proteins were quantified using enzyme-linked immunosorbent assay (ELISA), performed using a Quantikine human IGF-1 immunoassay kit according to standard protocols (R&D Systems, Minneapolis, MN, USA). Triplicate measurements were averaged.

Noise Exposure and Drug Application

Baseline auditory brainstem response (ABR) thresholds were measured within 7 days before initial noise exposure. Animals were then exposed to 4-kHz octave band noise at 120-dB sound pressure level for 5 hours in a ventilated sound exposure chamber. Sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure stimulus uniformity. Under general anesthesia with ketamine and xylazine, a piece of hydrogel immersed in rhIGF-1 at a concentration of 1 or 10 μg/μL in 40 μL physiologic saline (n = 5 for each concentration) was placed on the RWM in the left ear of animals 5 hours after noise exposure; then, the hydrogel immersed in physiologic saline was placed on the RWM of the right ear.

Functional Analysis

Auditory function was assessed by using ABR recordings. Measurements of ABR thresholds were performed at frequencies of 4, 8, and 16 kHz before noise exposure and on Days 3, 7, 14, and 21 after drug application. The animals were anesthetized using ketamine and xylazine and were kept warm with a heating pad. Generation of acoustic stimuli and subsequent recording of evoked potentials were performed using a PowerLab/4SP data acquisition system (ADInstruments, Castle Hill, Australia). Acoustic stimuli, consisting of tone-burst stimuli (0.1-ms cos 2 rise/fall and 1-ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) connected

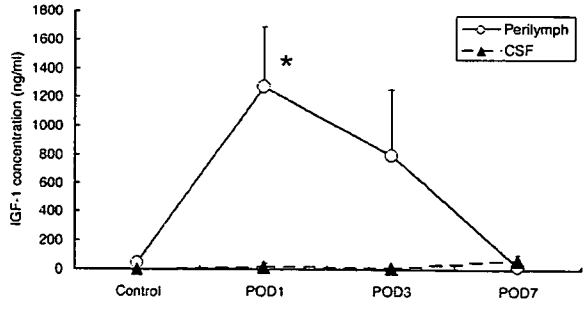

FIG. 1. Graph showing concentrations of human IGF-1 in cochlear perilymph and CSF. A significant increase in concentration of IGF-1 is found on postoperative Day 1 for perilymph ($^*p < 0.05$), whereas no significant changes are observed in concentrations of IGF-1 in CSF. Bars represent SEM. POD indicates postoperative day.

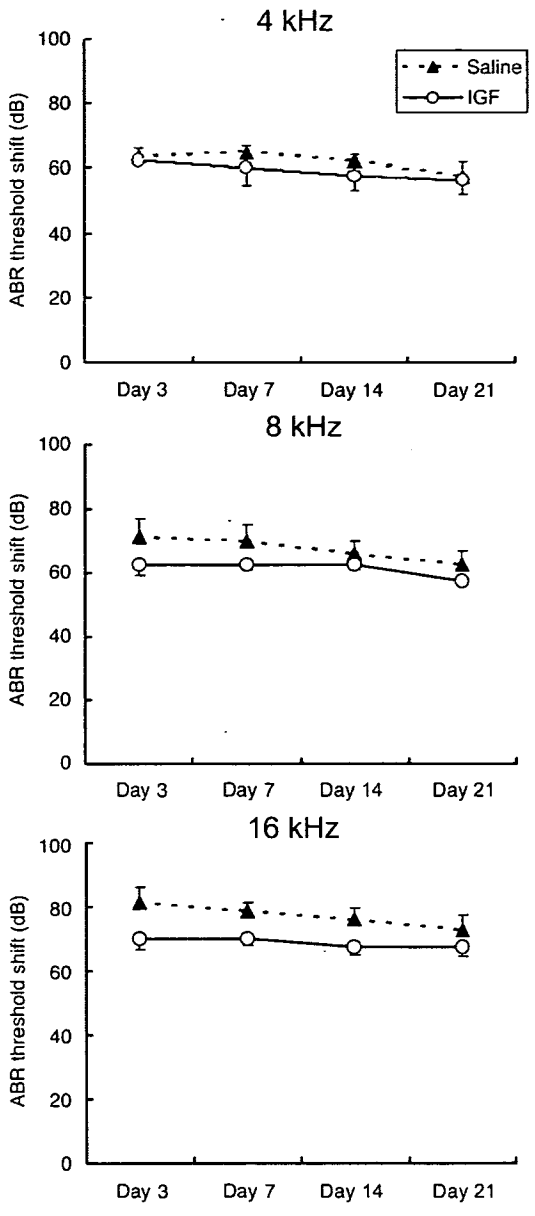

FIG. 2. Graphs showing time courses of alterations in threshold shifts of ABRs in cochleae treated with 1-μg/μl rhIGF-1 and cochleae treated with saline at frequencies of 4, 8, and 16 kHz. No significant differences are present in ABR threshold shifts between rhIGF-1– and saline-treated cochleae at any frequencies. Bars represent SEM.

to a funnel fitted into the external auditory meatus. To record bioelectric 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). The responses between the vertex and the 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 sound pressure level intervals; then, electric signals were averaged for 1,024 repetitions. The thresholds at each frequency were verified at least twice.

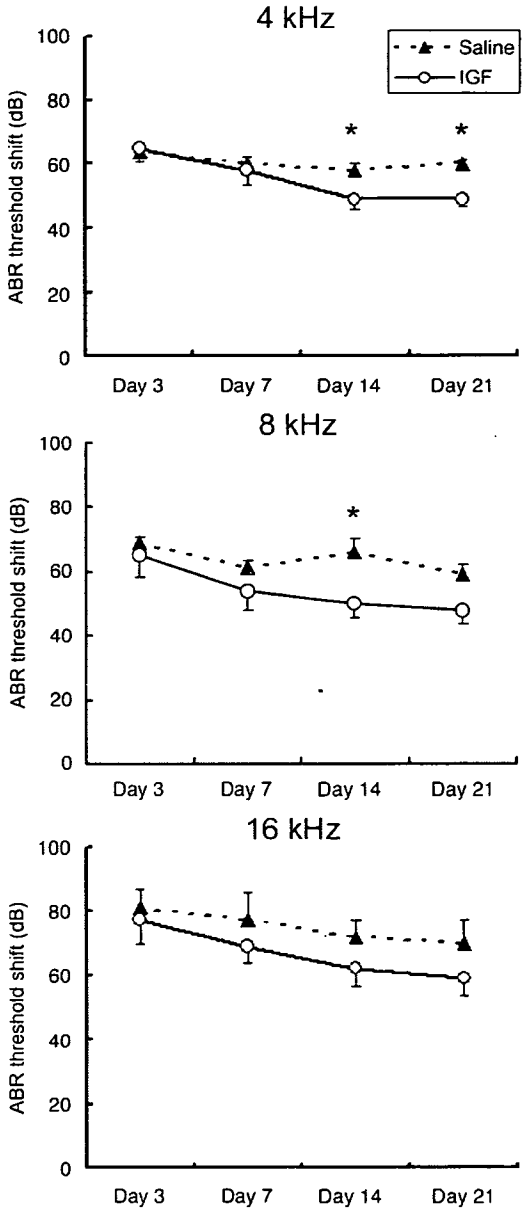

FIG. 3. Graph showing time courses of alterations in threshold shifts of ABRs in cochleae treated with 10-μg/μL rhIGF-1 and cochleae treated with saline at frequencies of 4, 8, and 16 kHz. The differences in ABR threshold shifts at 4 kHz between rhIGF-1– and saline-treated cochleae on Days 14 and 21 are significant, and the difference in ABR threshold shifts at 8 kHz on Day 14 is significant ($^*p < 0.05$). Bars represent SEM.

Histologic Analysis

On Day 21 after drug application, the animals were anesthetized with ketamine and xylazine, and the cochleae were exposed. After removal of the stapes, 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 a temperature of 4°C for 4 hours. After rinsing with PBS, cochleae were dissected from temporal bones and were subjected to histologic analysis in whole mounts. Three regions of cochlear sensory epithelia were used at a distance of 30 to 50% (apical), 50 to 70% (middle), or 70 to 90% (basal) from the apex for quantitative assessments of hair cell loss.

Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin were used to label the surviving inner hair cells (IHCs) and the outer hair cells (OHCs). Anti–myosin VIIa rabbit polyclonal antibody (dilution, 1:500; Proteus BioSciences, Ramona, CA, USA) was used as the primary antibody, and Alexa 546–conjugated goat anti–rabbit immunoglobulin G (dilution, 1:400; Molecular Probe, Eugene, OR, USA) was the secondary antibody. After immunostaining for myosin VIIa, the specimens were stained with fluorescein isothiocyanate–conjugated phalloidin (dilution, 1:300; Molecular Probe). Specimens were viewed under confocal microscopy (TCS SP2; Leica Microsystems, Wetzlar, Germany). Nonspecific labeling was tested by omitting the primary antibody from the staining procedures. The numbers of IHCs and OHCs in 0.2-mm-long regions of the apical, middle, or basal portion of cochleae were counted by the 3 authors (K.Y.L., T.N., and T.O.). The average of the values was used as the data for the animal.

Adverse Effects

We examined the incidence of loss of body weight at times of sample collection compared with weight at the beginning of the experiments. As a local adverse effect, incidence of otitis media was examined at times of sample collection. Otitis media was considered present if effusion was identified in the middle ear cavity.

Statistics

The overall effects of rhIGF-1 application on ABR threshold shifts were examined using two-way factorial analysis of variance. When interactions were significant, multiple comparisons using the Fisher protected least significant difference test were performed for pairwise comparisons. Differences in IHC and OHC numbers in each region of the cochlea between rhIGF-1– and saline-treated cochleae were examined using Student t test. Values of p less than 0.05 were considered statistically significant. Values are expressed as mean ± standard error of the mean (SEM).

RESULTS

In Vivo IGF-1 Release Profile

Enzyme-linked immunosorbent assay analysis of human IGF-1 proteins of the perilymph was performed to examine in vivo IGF-1 release profiles of hydrogels (Fig. 1). Very limited levels (mean ± SEM, 45.4 ± 31.0 ng/mL) of IGF-1 proteins were detected in samples obtained from nontreated cochleae. A marked increase in IGF-1 protein levels was observed on postoperative Days 1 (mean ± SEM, 1278 ± 413 ng/mL) and 3 (mean ± SEM, 801.6 ± 456 ng/mL). The IGF-1 protein levels then decreased to the control level by postoperative Day 7 (mean ± SEM, 23.7 ± 17 ng/mL). Differences in IGF-1 protein level between control and postoperative Day 1 and between postoperative Days 1 and 7 were significant (control versus postoperative Day 1, p = 0.019; postoperative Days 1 versus 7, p = 0.017). Level of IGF-1 protein in the CSF of each experimental group was almost the same as that in the perilymph of control animals (Fig. 1), and no significant differences in IGF-1 protein level were observed among experimental groups. The IGF-1 protein levels in the serum were undetectable in each experimental group.

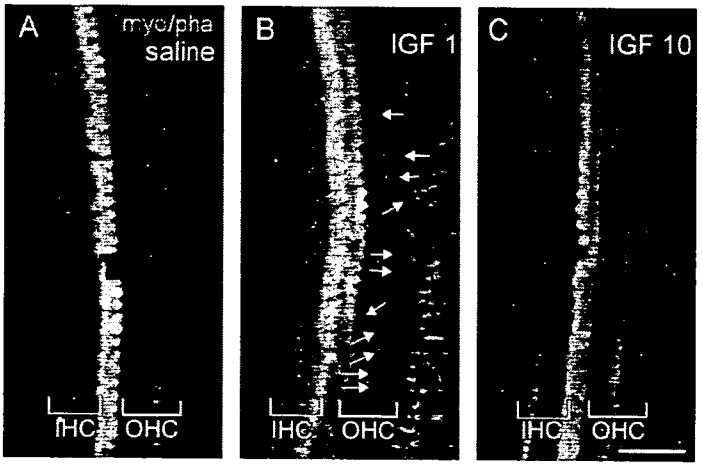

FIG. 4. Image showing immunostaining for myosin VIIa (*myo* [*red*]) and F-actin labeling with phalloidin (*pha* [*green*]) of cochlear sensory epithelia in the basal portion of cochleae. Severe loss of OHCs is observed in the saline-treated cochlea (*A*). Surviving OHCs in the specimen treated with 1-μg/μl recombinant human insulin-like growth factor 1 (rhIGF-1) exhibit scattered distribution (*B*; *arrows*). In cochleae treated with 10-μg/μl rhIGF-1, OHCs are comparatively preserved (*C*). Bar represents 50 μm.

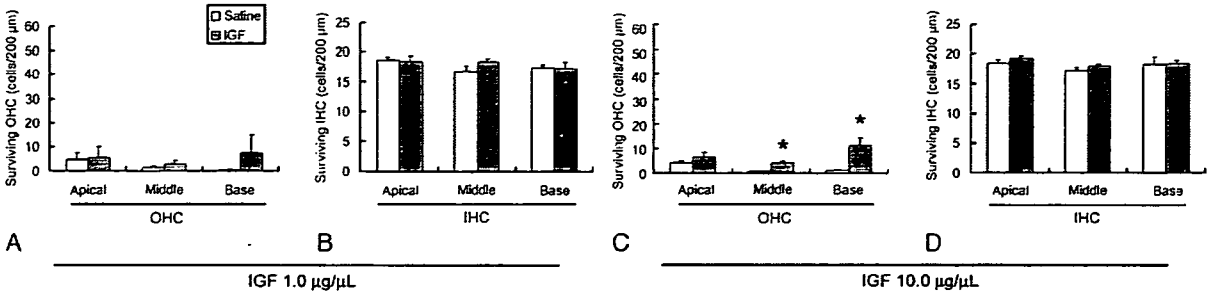

FIG. 5. Graphs showing numbers of surviving inner and outer hair cells in apical, middle, and basal portions of cochleae. No significant differences in numbers of OHCs (*A*) or IHCs (*B*) are found between saline- and 1-μg/μl rhIGF-1–treated cochleae. Differences in numbers of surviving OHCs between saline- and 10-μg/μl rhIGF-1–treated cochleae are significant in basal and middle portions of cochleae (*C*; $^*p < 0.05$), whereas significant differences in numbers of IHCs are not found between the 2 groups (*D*). Bars represent SEM.

Auditory Function

Time courses of alterations in ABR threshold shifts after drug application at 4, 8 and 16 kHz are shown in Figures 2 and 3. Local rhIGF-1 treatment at the concentration of 1 μg/μL demonstrated no significant effects on ABR threshold shifts at each frequency, although a trend toward lower threshold shifts was observed in comparison with controls (Fig. 2). Conversely, the overall effect of rhIGF-1 application on ABR threshold shifts at a concentration of 10 μg/μL was significant at 4 and 8 kHz, but not at 16 kHz (Fig. 3; $p = 0.014$ for 4 kHz, 0.005 for 8 kHz, and 0.074 for 16 kHz). Differences in threshold shifts at 4 kHz between 10-μg/μL rhIGF-1– and saline-treated cochleae on Days 14 and 21 were significant at multiple comparisons using the Fisher protected least significant difference test (Day 14, $p = 0.01$; Day 28, $p = 0.01$), and the difference in threshold shifts at 8 kHz on Day 14 was significant ($p = 0.016$).

Histologic Damage

Immunostaining for myosin VIIa and phalloidin staining demonstrated severe degeneration of OHCs in the apical, middle, and basal portions of saline-treated cochleae. Surviving OHCs were rarely found in middle and basal portions of saline-treated specimens (Fig. 4A). Outer hair cells degeneration in 1-μg/μL rhIGF-1–treated specimens was also severe. Surviving OHCs exhibited scattered distribution (Fig. 4B). In specimens treated with 10-μg/μL rhIGF-1, loss of OHCs was still observed; however, the degree of OHC degeneration was moderate (Fig. 4C). Inner hair cells were well maintained in all the experimental groups (Fig. 4, A–C). Quantitative assessments revealed no significant differences in numbers of surviving OHCs in any cochlear portion between saline- and 1-μg/μL rhIGF-1–treated cochleae (Fig. 5A). Differences in numbers of surviving OHCs between saline- and 10-μg/μL rhIGF-1–treated cochleae were significant in basal and middle portions of cochleae but not in the apical portion (Fig. 5C; basal and middle turns, $p = 0.009$; apical turn, $p = 0.387$). No significant differences were observed in numbers of surviving IHCs between saline- and 1- or 10-μg/μL rhIGF-1–treated cochleae (Fig. 5, B and D).

Incidence of Adverse Effects

No experimental animals exhibited loss of body weight. Scar formation was identified at the surgical site of the bulla, but no cochleae exhibited collection of effusion in the middle ear cavity indicative of otitis media.

DISCUSSION

These findings demonstrate that local rhIGF-1 treatment using gelatin hydrogel is effective for the treatment of NIHL in guinea pigs. Enzyme-linked immunosorbent assay in the present study revealed sustained delivery of rhIGF-1 to the cochlear fluid for 3 days, which is reasonable given the previous findings regarding the in vitro release profile of the hydrogel (11). The hydrogel used in the present study releases approximately 80% of IGF-1 into PBS for 3 days in the presence of collagenase, which is also present in the middle ear (12,13). The present findings from ELISA measurements also demonstrated no influences of cochlea rhIGF-1 application on levels of IGF-1 in CSF or serum. In addition, no systemic or local adverse effects were found in experimental animals. These findings indicate that rhIGF-1 application using the hydrogel offers safe and efficient delivery to the cochlea.

In the present study, ABR measurements exhibited significant effects of local rhIGF-1 treatment through gelatin hydrogels on attenuation of threshold shifts due to noise exposure. Although a tendency toward reduced threshold shifts was observed, local rhIGF-1 application at 1-μg/μL concentration showed no significant reduction in threshold shifts, whereas local rhIGF-1 application at 10-μg/μL concentration (the concentration recommended by the supplier for clinical use) exhibited significant attenuation of threshold shifts at frequencies of 4 and 8 kHz. Local rhIGF-1 application via hydrogels also exhibited significant histologic protection. Similar to functional protection, local rhIGF-1 treatment at a

concentration of 10 μg/μL significantly increased the numbers of surviving OHCs, whereas treatment at a concentration of 1 μg/μL had no significant effect on the numbers of surviving OHCs. These findings indicate dose dependency for the effects of local rhIGF-1 treatment on the attenuation of NIHL.

Previous studies have demonstrated that several agents ameliorate NIHL when they are applied before noise exposure; however, only limited agents show protective effects by postexposure administration. Local application of D-JNK-1 peptide, an inhibitor of c-Jun N-terminal kinase, 12 hours after noise exposure attenuates NIHL (14). The efficacy of D-JNK-1 peptide has been demonstrated by application via an osmotic minipump or a hyaluronic acid gel. Postexposure administration of edaravone, a free-radical scavenger, also rescues cochleae from NIHL (15). Locally applied edaravone via an osmotic minipump can rescue OHCs even when it is applied 21 hours after noise exposure. In addition, these agents offer stronger protection of cochleae than does rhIGF-1. The drug delivery system via a gelatin hydrogel may be used for cochlear delivery of D-JNK-1 peptide because the gelatin hydrogel is suitable for sustained delivery of peptides (7–11). However, D-JNK-1 peptide is not clinically applicable. On the other hand, 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 (7). Therefore, other drug delivery systems that are fit for delivery of water-insoluble agents, including encapsulating in polylactic/glycolic acid particles (16), may be required for sustained delivery of edaravone.

The present findings indicate the effectiveness and safety of local rhIGF-1 treatment using gelatin hydrogels for NIHL. Clinical use of gelatin hydrogel as a drug delivery system has already started for angiogenesis of the inferior limb in Japan (17). In addition, rhIGF-1 is clinically applicable. The present findings may help advance the clinical application of local rhIGF-1 treatment using gelatin hydrogel for the treatment of SNHL.

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Drug delivery systems for the treatment of sensorineural hearing loss

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

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

Introduction

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

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

Previous methods for local application

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

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

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

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

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

Controlled-release systems

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

Siloxane-based polymers

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

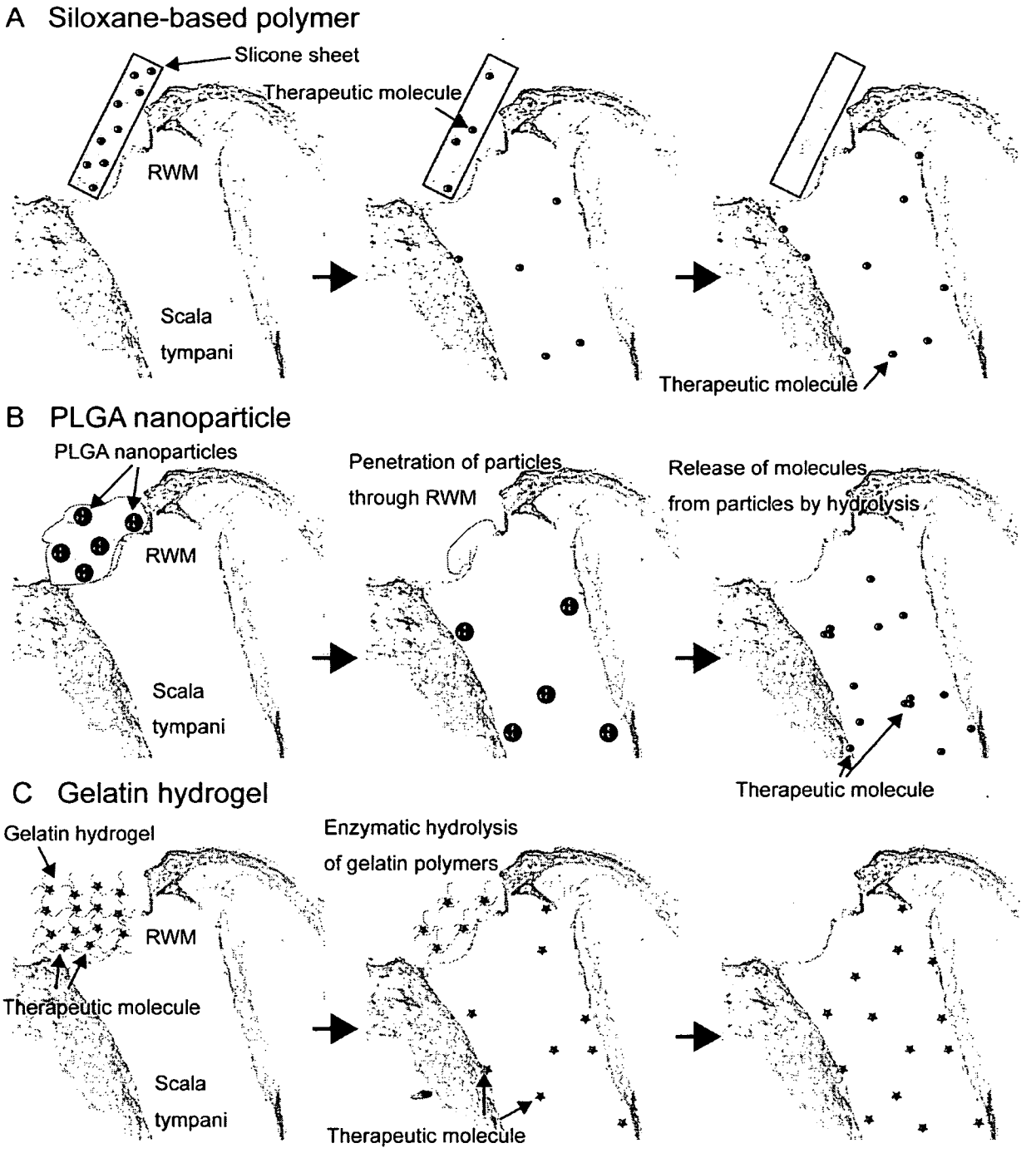

Figure 1. Mechanisms for controlled release of therapeutic molecules from biopolymers. (A) Therapeutic molecules dissolved in siloxane-based polymers move into the scala tympani by diffusion. A silicone sheet remains on the round window membrane (RWM). (B) Poly lactic/glycolic acid (PLGA) nanoparticles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

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

PLGA nanoparticles

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

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

Gelatin hydrogel

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

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

Conclusions for clinical application

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

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

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Pharmacological inhibition of Notch signaling in the mature guinea pig cochlea

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Recent studies using explant cultures have demonstrated that pharmacological inhibition of Notch signaling by γ-secretase inhibitors generates supernumerary hair cells in embryonic or neonatal cochleae. The aim of this study was to examine the effects of such pharmacological inhibition on mature auditory epithelia *in vivo*. Normal adult guinea pig auditory epithelia exhibited weak or no immunoreactivity for Notch1 and Jagged1, whereas ototoxic treatment caused the upregulation of these molecules in damaged auditory epithelia. Local application of a γ-secretase inhibitor in damaged cochleae generated ectopic hair cells in mature auditory epithelia. These findings indicate that pharmacological inhibition of Notch signaling is a possible strategy for hair cell regeneration in adult auditory epithelia. *NeuroReport* 18:1911–1914 © 2007 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: γ-secretase inhibitor, hair cell, hearing loss, inner ear, regeneration

Introduction

Hearing impairment is a common disability in industrialized countries. Hearing depends largely on the presence of hair cells (HCs) in the cochlea, defects of which result in hearing loss or deafness. HC regeneration in mammals occurs naturally over a limited time period during development [1], and many attempts have been made to restore HCs in adult mammals. Recent studies have demonstrated the existence of stem cell-like cells in the mature auditory epithelium that have the potential to differentiate into HCs [2,3]. Notch signaling plays a critical role during auditory epithelium development [4–6]. Even after maturation, viral overexpression of the Notch signaling transcription factor Atoh1 induces transdifferentiation of supporting cells (SCs) into HCs [7–9].

Atoh1 expression is regulated by the expression of Hes1 and Hes5 in Notch signaling [10]. In general, the binding of Notch ligands to their receptors induces the release of Notch intracellular domains from the plasma membrane to the nucleus, resulting in the activation of RBP-J in the nucleus [11]. This induces Hes1 and Hes5 transcription, which, in turn, suppresses Atoh1 expression. Therefore, suppression of Hes1 and Hes5 expression by inhibition of Notch signaling causes an increase in Atoh1 expression [12].

Gamma secretase plays a crucial role in the proteolytic release of Notch intracellular domains from the cytoplasmic membrane. Thus, the pharmacological inhibition of this process results in the inhibition of Notch signaling [12]. Recent studies using explant cultures of auditory epithelia have demonstrated that a γ-secretase inhibitor increases HC numbers in the auditory epithelia of embryonic [13] and newborn mice [14]. These findings prompted us to investigate the potential for pharmacological inhibition of Notch signaling in inducing HC proliferation in mature auditory epithelia.

Our working hypothesis was that pharmacological inhibition of Notch signaling induces the transdifferentiation of SCs into HCs in the damaged cochleae of adult animals. We therefore examined the effects of this pharmacological inhibition in the damaged auditory epithelia of adult guinea pigs *in vivo*.

Materials and methods

Experimental animals

Hartley strain 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 the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All of the experimental procedures were performed in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Ototoxic treatment

The animals were deafened bilaterally with a single intramuscular injection of kanamycin sulfate (KM; 500 mg/kg; Wako Pure Chemical Industries Ltd, Osaka, Japan), followed 2 h later by an intravenous injection of ethacrynic acid (EA; 50 mg/kg; Wako Pure Chemical Industries Ltd). Measurements of auditory function were performed by recording auditory brain-stem responses at

frequencies of 4, 8 and 16 kHz on day 2 or 3 after ototoxic treatment. Animals that exhibited greater than 90 dB sound pressure level of auditory brain-stem response thresholds at all frequencies were used in the following experiments.

Immunohistochemistry for Notch1 and Jagged1

To evaluate Notch signaling activation, we determined the expression level of Notch1 and Jagged1 in auditory epithelia. Cochlear specimens from guinea pig embryos (embryonic day 50; n=4), normal adult guinea pigs (n=4) and adult guinea pigs on days 2 (n=4), 4 (n=4) and 7 (n=4) following ototoxic treatment were fixed with 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS; pH 7.4) at 4°C for 12 h. After decalcification with 0.1 M ethylenediamine tetra-acetic acid for 14 days at 4°C, 10-μm thick cryostat sections were prepared. Four midmodiolus sections were chosen from each cochlea, and antigen retrieval was accomplished by boiling the slides for 5 min in 0.01 M citrate buffer (pH 6.0). Slides were incubated with antimyosin VIIa rabbit polyclonal antibody (1:500; Proteus Bioscience Inc., Romana, California, USA), anti-Notch1 goat polyclonal antibody (1:200; Santa Cruz Biotechnology Inc., California, USA) and anti-Jagged1 goat polyclonal antibody (1:200; Santa Cruz Biotechnology Inc.) as primary antibodies, followed by Alexa Fluor 555 donkey antirabbit immunoglobulin G (IgG; 1:500; Molecular Probes, Eugen, Oregon, USA) and Alexa Fluor 488 donkey antigoat IgG (1:500, Molecular Probes) secondary antibodies. Nuclei were counterstained with 4′,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in PBS; Molecular Probes). Cochlear specimens obtained from mice at postnatal day 2 (P2) were used as positive controls for Notch1 and Jagged1. Nonspecific labeling was tested by omitting the primary antibody from the staining procedures. The specimens were viewed with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

Pharmacological inhibition of Notch signaling

On day 4 after ototoxic treatment, the γ-secretase inhibitor MDL28170 (MDL; Sigma-Aldrich, St Louis, Michigan, USA) was locally applied to the perilymph. Under ketamine (75 mg/kg) and xylazine (9 mg/kg) general anesthesia, a silicone tube (180 μm in outer diameter) connected to a micro-osmotic mini pump (pumping 0.25 μl/h for 14 days; Alzet, Cupertino, California, USA) was inserted into the scala tympani of the basal turn of the left cochlea of 15 guinea pigs. MDL was dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS to give a final concentration of 1 mM containing 0.3% DMSO. The MDL solution was continuously injected through a micro-osmotic pump into the left cochleae of seven deafened and four normal animals, whereas the corresponding right cochleae received no local drug application (n=7). An additional four deafened animals received PBS containing 0.3% DMSO in the left cochlea instead of MDL solution. On day 14 after local drug application, the temporal bones were collected and immersed in 4% paraformaldehyde in PBS at 4°C for 12 h. After decalcification with 0.1 M ethylenediamine tetra-acetic acid for 14 days at 4°C, the cochleae were subjected to histological analysis of whole mounts.

Cochlear specimens were permeabilized in 0.2% Triton X in PBS for 30 min at room temperature. Immunohistochemistry for myosin VIIa and F-actin labeling by phalloidin was performed to determine the location of the HCs. After immunostaining for myosin VIIa, the specimens were stained with fluorescein-phalloidin (1:400; Molecular Probes), and viewed with a Leica TCS SP2 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). Quantitative analysis for numbers of ectopic myosin VIIa-positive cells and inner HCs (IHCs) was performed. Cochlear specimens treated with systemic KM and EA application followed by local MDL application and those treated with KM and EA alone were used. The numbers of ectopic myosin VIIa-positive cells and IHCs were counted in a 0.2-mm long region of the midbasal portion at 7-mm distance from the hook portion. The difference in numbers of ectopic myosin VIIa-positive cells or IHCs was examined by the unpaired t-test. Values of P less than 0.05 were considered statistically significant. Values are expressed as mean ± standard error of the mean (SEM).

Results

Immunohistochemistry for Notch1 and Jagged1

The expression of Notch1 and Jagged1 was identified in the auditory epithelium of guinea pig embryos, as previously reported in mouse embryos [15]. Strong expression of Notch1 was observed in SCs, and moderate expression was detected in the greater epithelial ridge, which is located between the spiral limbus and IHCs of immature cochleae (Fig. 1a). The expression of Jagged1 was distributed throughout the auditory epithelium of guinea pig embryos, similar to that of Notch1 (Fig. 1e). By contrast, with embryonic cochleae, the auditory epithelium of normal adult guinea pigs exhibited virtually no immunoreactivity for Notch1 or Jagged1 (Fig. 1b, f).

On days 2 and 4 after the systemic application of KM and EA, immunostaining for myosin VIIa demonstrated the loss of outer HCs (OHCs). Immunoreactivity for Notch1 was found in the inner sulcus cells, which are located between the spiral limbus and the IHCs of mature cochleae (Fig. 1c). We observed weak expression of Notch1 in the SCs of other regions of the auditory epithelium. Immunoreactivity for Jagged1 was also identified in the inner sulcus cells and SCs of the organ of Corti (Fig. 1g). Weak or no expression of Notch1 or Jagged1 was observed in the auditory epithelia on day 7 after ototoxic treatment (Fig. 1d, h).

Pharmacological inhibition of Notch signaling

Normal animals treated with local MDL application exhibited no loss of HCs, and no ectopic myosin VIIa-positive cells were seen in their auditory epithelia. Cochlear specimens treated with systemic KM and EA application followed by local DMSO application exhibited total OHC loss in each turn of the cochleae, and partial loss of IHCs in the basal turn similarly to those treated with KM and EA alone (Fig. 2a). Total OHC loss and partial IHC loss were also observed in cochlear specimens treated with systemic KM and EA application followed by local MDL application (Fig. 2b, c). No significant difference in the number of IHCs was found between cochlear specimens treated with KM and EA followed by MDL treatment and those with KM and EA alone (Fig. 2d).

No ectopic myosin VIIa-positive cells were found in the basal portion of cochleae treated with KM and EA alone (Fig. 2a) or followed by DMSO application, whereas in those treated with KM and EA followed by MDL application,

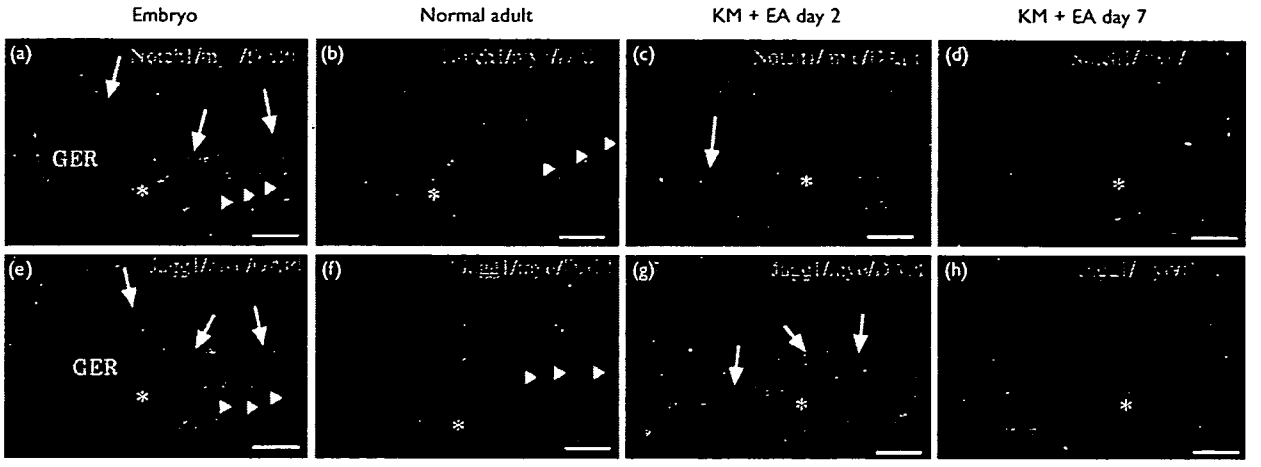

Fig. 1 Expression of Notch1 and Jagged1 in auditory epithelia of embryonic and adult auditory epithelia with or without ototoxic treatment. The embryonic auditory epithelium exhibits immunoreactivity for Notch1 and Jagged1 (Jagg1) in the greater epithelial ridge (GER) and the supporting cells (arrows in a, e), whereas neither expression is found in normal adult epithelia (b, f). The adult auditory epithelium on day 2 after kanamycin and ethacrynic acid application (KM + EA day 2) exhibits immunoreaction for Notch1 in the inner sulcus cells (arrow in c), and that for Jagg1 in the inner sulcus cells and the supporting cells (arrows in g), while neither expression is found in that obtained on day 7 (d, h). Red fluorescence shows immunoreactivity for myosin VIIa (myo), and blue shows DAPI. The locations of inner hair cells are indicated by asterisks and those of outer hair cells are indicated by arrowheads. Bars represent 25 μm. DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

ectopic myosin VIIa-positive cells were identified in the inner sulcus region of the basal turn of cochleae (Fig. 2b, c). Ectopic myosin VIIa-positive cells were found in four of seven experimental animals. The mean and SEM for numbers of ectopic myosin VIIa-positive cells was 0.71 and ±0.29 (cells/200 μm). The difference in numbers of ectopic myosin VIIa-positive cells between specimens with MDL treatment and those KM and EA alone was statistically significant (P=0.028, Fig. 2d).

Discussion

In this study, we examined the activation of Notch signaling in adult and embryonic guinea pig auditory epithelia, with or without systemic KM and EA application. Notch1 and Jagged1 immunohistochemistry indicated a quiescent state of Notch signaling in the nonaffected auditory epithelia of adult guinea pigs and the activation of Notch signaling in damaged auditory epithelia. Strong immunoreactivity for Notch1 and Jagged1 was found in the auditory epithelia on days 2 and 4 after systemic KM and EA application. Immunoreactivity for these molecules then returned to the levels seen in normal adult tissues on day 7. These findings indicate that transient activation of Notch signaling might occur in adult auditory epithelia after systemic KM and EA application.

The nonaffected auditory epithelia of adult guinea pigs exhibited no ectopic myosin VIIa-positive cells following local MDL application, which supported the present immunohistochemical findings for Notch1 and Jagged1. By contrast, local MDL application to cochleae on day 4 after systemic KM and EA treatment, which demonstrated Notch1 and Jagged1 expression, induced the appearance of ectopic myosin VIIa-positive cells in the auditory epithelia. Notch1 and Jagged1 expression was found in the inner sulcus region of damaged auditory epithelia, and a limited number of ectopic myosin VIIa-positive cells were identified in this region. These findings indicate that pharmacological inhibition of Notch signaling by a γ-secretase inhibitor generates the appearance of ectopic HCs in adult auditory epithelia.

In this study, we applied an ototoxic treatment that was used previously in a study of Atoh1 overexpression by virus vectors [9]; we also introduced a γ-secretase inhibitor at the time point when the virus inoculation occurred in the previous report [9]. Atoh1 overexpression by virus vectors induced the generation of a number of new HCs in various portions of the auditory epithelia, whereas local MDL application resulted in a limited number of ectopic HCs in the inner sulcus region. In addition, viral overexpression of Atoh1 generated some ectopic HCs in normal adult guinea pig cochleae [8]. Atoh1 might have had a direct effect on the transdifferentiation of SCs into HCs, whereas the pharmacological inhibition of Notch signaling indirectly induced an increase in Atoh1 expression, which might explain the difference in effect on ectopic HCs.Atoh1 overexpression in auditory epithelia, however, requires viral vectors with their associated risk of toxicity, whereas pharmacological inhibition of Notch signaling requires only local drug application to the cochlea. We therefore consider pharmacological inhibition of Notch signaling to be preferable for clinical applications, although its effect on the generation of new HCs is limited.

In this study, the expression of Notch1 and Jagged1 and the generation of ectopic HC-immunophenotypes were observed in the inner sulcus region of the auditory epithelia. The greater epithelial ridge of embryonic and neonatal auditory sensory epithelia corresponds to the inner sulcus region in mature auditory epithelia, and is necessary for the normal development of the auditory epithelium [16]. Several cell-culture studies have demonstrated the existence of progenitor cells, even in adult auditory epithelia, with the potential to differentiate into HCs [2,3,17]. In addition, Malgrange *et al.* [17] reported on the preservation of such progenitor cells in the inner sulcus region of adult animals, suggesting that the inner sulcus cells might be important for the restoration of adult auditory epithelia. Future investigations should elucidate the unique characteristics of these

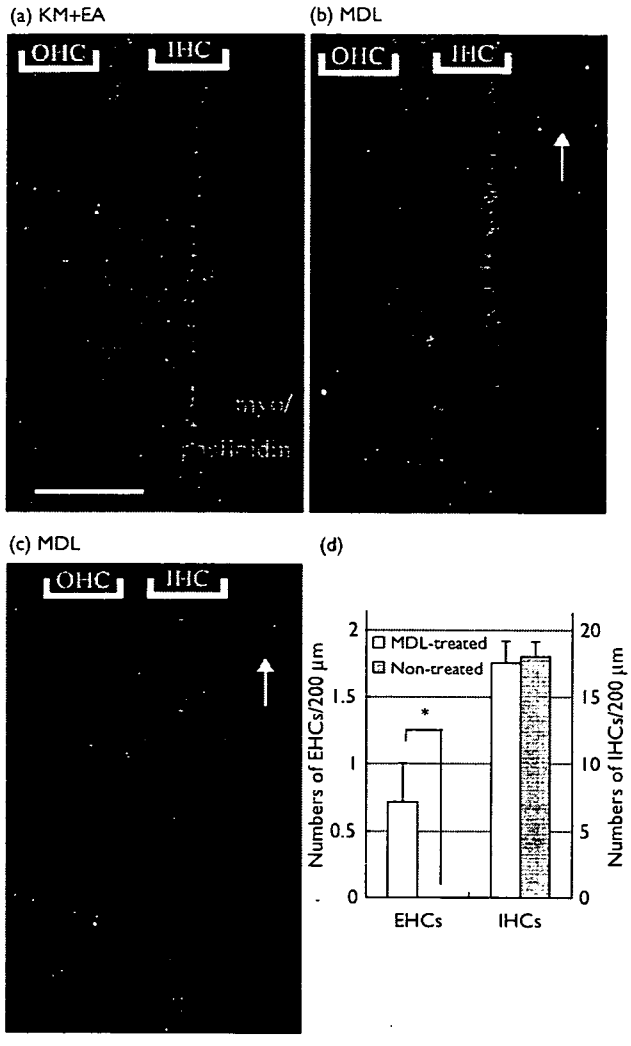

Fig. 2 Ectopic hair cell-immunophenotypes in damaged auditory epithelia. An auditory epithelium affected by kanamycin (KM) and ethacrynic acid (EA) exhibits no ectopic myosin'VIIa (myo)-positive cells (a). In auditory epithelia treated with systemic KM and EA treatment followed by local MDL application, ectopic myo-positive cells are found in the inner sulcus region (arrows in b, c). IHC indicates the location of inner hair cells and OHC indicates that of outer hair cells. Bar represent 50 µm. The numbers of ectopic hair cells (EHCs) and inner hair cells (IHCs) in the midbasal portion of MDL-treated and nontreated cochleae following KM and EA application are shown in d. The difference in EHC numbers between two groups was significant (*P=0.028 in d). No significant difference in IHC numbers was found. Bars represent SEM.

cells in relation to the temporary activation of Notch signaling, which will contribute to the optimization of the pharmacological inhibition of Notch signaling.

Conclusion

The present findings demonstrate that pharmacological inhibition of Notch signaling has the potential to generate ectopic HCs in the damaged auditory epithelia of adult guinea pigs. The functionality of these ectopic HC immunophenotypes, however, remains to be determined. In addition, an increase in the number of new HCs will be necessary for functional recovery. We therefore aim to determine the optimal conditions for the pharmacological inhibition of Notch signaling in our future work.

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The potential use of bone marrow stromal cells for cochlear cell therapy

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This study investigated the potential of bone-marrow stromal cell transplantation for cell replacement therapy in the cochlea. Bone-marrow stromal cells labeled with enhanced green fluorescent protein were injected into the perilymphatic space of normal cochleae in mice. Histological analysis 2 weeks after transplantation demonstrated that transplanted cells settled within the cochlear tissues, especially in the spiral ligament and the spiral limbus, although most transplants were located in the perilymphatic space. Some of the transplanted cells expressed the cochlear gap-junction protein connexin 26. These findings indicate the potential of bone-marrow stromal cells for delivering therapeutic molecules and for the restoration of cochlear cells, particularly in the spiral ligament and the spiral limbus. *NeuroReport* 18:351–354

Keywords: bone-marrow stromal cell, cell therapy, cochlea, migration, transplantation

Introduction

Treatment options for sensorineural hearing loss (SNHL) are currently limited to cochlear implants and hearing aids. Hence, there is a requirement for alternative means of biological therapy, including cell and/or gene therapy. Indeed, recent studies have indicated that cell or gene therapy could be utilized to regenerate hair cells [1,2] and neurons [3] in the inner ear, and to deliver therapeutic molecules to the inner ear [4–6]. More recently, transplantation of gene-transfected cells has been reported as an efficient strategy to deliver genes into the inner ear [7].

Bone-marrow stromal cells (BMSCs) are possible candidates for transplants for cell therapy for the treatment of SNHL. They have the potential for differentiation into various types of cells and are easily obtained from one's own bone marrow. In addition, BMSCs are capable of secretion of several growth factors [8], which are included in cochlear protectants [9–11]. BMSC transplantation, therefore, could be utilized in three different strategies for inner ear treatment, restoration of missing cells, providing growth factors and delivering genes. In this study, we examined the distribution and characteristics of BMSCs after transplantation into cochleae of C57BL/6 mice to evaluate the potential of BMSCs as a source of cells for cell-replacement-therapy for the cochlea.

Materials and methods

Animals

Male C57BL/6 mice (n=6, SLC Japan, Hamamatsu, Japan) aged 10 weeks were used as the recipients. The experimental protocols were approved by the Animal Research Committee of Kyoto University Graduate School of Medicine, and were conducted in accordance with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Bone-marrow stromal cells

The BMSCs were obtained from enhanced green fluorescent protein (GFP)-transgenic mice [strain B6;C3-*Tg(ACtb-EGFP)CX-FM139Osb*] [12]. Under general anesthesia with ketamine (75 mg/kg) and xylazine (9 mg/kg), the tibias and femurs of the animals (n=4) were collected, and the medullary cavity was aspirated to harvest the bone marrow. The BMSCs were cultured in a 25-cm^2 flask with 8 ml of Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, California, USA) supplemented with 20% fetal bovine serum (Thermo Trace, Victoria, Australia), 100 U/ml of penicillin (Nacalai Tesque Inc., Kyoto, Japan) and 100 µg/ml of streptomycin (Nacalai Tesque Inc.). The cells were cultured at 37°C under 5% CO_2. The medium was changed twice weekly until the cells were 80% confluent. Non-adherent cells were removed during the medium-change procedure and the adherent cells were collected. After two passages, the cells were suspended in Iscove's modified Dulbecco's medium at a concentration of 1×10^5 cells/µl.

Transplantation

Cell transplantation was performed under general anesthesia with ketamine and xylazine. A retroauricular incision was made in the left ear of each mouse and the otic bulla

was exposed. The bony wall of the bulla was partially resected to expose the basal turn of each cochlea. A small perforation was then made in the lateral wall at the basal turn of the cochlea corresponding to the location of the scala tympani (ST). Cell suspensions of GFP-labeled BMSCs (2 μl; 10^5 cells/μl) were injected through a fine glass needle using a microinfusion pump. Subsequently, the perforation was plugged with a fat graft and covered with fibrin glue.

Histology

Under general anesthesia, the animals were transcardially perfused with phosphate-buffered saline at pH 7.4, followed by 4% paraformaldehyde in phosphate buffer at pH 7.4 on day 14. The temporal bones were immediately dissected out and immersed in the same fixative for 4 h at 4°C. After decalcification, cryostat sections (8 μm thickness) were cut and immunohistochemical analysis for GFP, CD43, nestin, β-III-tubulin, E-cadherin and Cx26 was performed. BMSCs grown on sterile cover glasses were also subjected to immunocytochemical analysis to determine the characteristics of the BMSCs before transplantation. Anti-GFP mouse monoclonal (1 : 200; Invitrogen, San Diego, California, USA) or rabbit polyclonal (1 : 500, Molecular probes, Eugene, Oregon, USA), anti-CD43 rat monoclonal (1 : 200; Pharmingen, San Diego, California, USA), anti-nestin rat monoclonal (1 : 200; Pharmingen), anti-β-III-tubulin mouse monoclonal (1 : 500, Covance Research Products, Berkeley, California, USA), anti-Cx26 rabbit polyclonal (1 : 500; Zymed, San Francisco, California, USA) and anti-E-cadherin mouse monoclonal antibody (1 : 200; Takara Bio, Otsu, Japan) were used as the primary antibodies. The secondary antibody was Alexa-546 or 488-conjugated anti-mouse, rat or rabbit antibody (1 : 400; Molecular Probes). Counterstaining by 4′,6-diamidino,2-phenylindole dihydrochloride (DAPI; 1 μg/ml in phosphate-buffered saline; Molecular Probes) was performed at the end of the staining procedures. Specimens stained without primary antibodies served as negative controls. Cryostat-sections of mouse cerebellum on embryonic day 12 were used as positive controls for nestin. The specimens were viewed with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) or a Leica TCS-SP2 confocal laser-scanning microscope (Leica Microsystems, Tokyo, Japan).

Four mid-modiolar sections were chosen from each cochlea and subjected to quantitative analysis of the number of transplanted cells in the cochlea. We counted the number of cells expressing both GFP and DAPI as transplant-derived cells. The distribution of the engrafted cells was divided into four compartments: the scala vestibuli (SV), the scala media, the ST and the cochlear tissues. In addition, the cochlear tissues were further subdivided into three compartments: the spiral ligament (SL), the spiral limb (SLB) and the other components of the cochleae. The number of transplant-derived cells expressing CD34, nestin or Cx26 was also counted in four sections from each cochlea. The expression ratio for each marker was then determined by dividing the numbers of transplant-derived cells expressing each marker by those of transplant-derived cells in each section. The average in four sections was defined as the data for the cochlea. The expression ratios for these markers were also calculated using four samples of BMSCs grown on sterile cover glasses. All the data were represented by the means and the standard deviations.

Statistics

Statistical analyses for the location of BMSC-derived cells in the cochlea were performed using one-way analysis of variance followed by the Scheffe's test. The unpaired *t*-test was used in analyses of the expression ratios for CD34, nestin and Cx26. A P value <0.05 was considered statistically significant.

Results

BMSC-derived cells labeled with GFP were found in all the transplanted cochleae (Fig. 1a). The mean number of GFP-positive cells in one mid-modiolar section per cochlea was 180 ± 38 ($n = 6$). The transplanted cells were distributed from the base to the apex of the cochleae. No apparent difference in distribution of transplanted cells was found among the turns of cochleae. BMSC-derived cells were predominantly located in the perilymphatic space of the cochleae: $67.3 \pm 7.3\%$ in the ST and $18.1 \pm 10.8\%$ in the SV (Fig. 1b). The ST was the region in which BMSC-derived cells were most frequently observed. BMSC-derived cells were also observed within the cochlear tissues ($12.8 \pm 9.2\%$), indicating the migration activity of BMSCs into various parts of the cochleae. Of BMSC-derived cells located in cochlear tissues, $57.4 \pm 8.0\%$ were found in the SL (Fig. 1b–d) and $17.3 \pm 3.4\%$ were found in the SLB (Fig. 1b, e, p and s). In addition, cell aggregates of transplants that were located in the perilymphatic space were adjacent to those located in the SL (Fig. 1c and d). Within the SLB, the transplanted cells were located in the medial region, which faced the SV (Fig. 1e, p and s). BMSC-derived cells were also observed in other compartments of the cochleae: the sensory epithelium (Fig. 1f and g), the osseous spiral lamina (Fig. 1e and f) and the acoustic nerve (Fig. 1h).

Before transplantation, $74.2 \pm 17.6\%$ of the BMSCs expressed CD34 (Fig. 1i–k) and $3.5 \pm 2.0\%$ were immunoreactive for nestin (Fig. 1l–n). No immunoreactivity for the other markers used in this study was identified in the BMSCs before transplantation. Two weeks after transplantation into the cochleae, CD34-positive transplants were found (Fig. 1p–r); however, the expression ratio for CD34 in the BMSC-derived cells had significantly decreased to $7.3 \pm 8.3\%$ (Fig. 1o, $P = 0.0003$). Immunoreactivity for nestin was still detected, but was significantly reduced to $0.9 \pm 0.8\%$ (Fig. 1o, $P = 0.04$). Cx26 expression was detected in $4.7 \pm 2.7\%$ of the BMSC-derived cells that had settled in the cochleae (Fig. 1o, s–u). The difference in the ratio of Cx26 expression between before and after transplantation was significant at $P = 0.02$. By contrast, immunoreactivity for E-cadherin and β-III-tubulin was not found in these cells.

Discussion

We hypothesized that BMSC transplantation could be utilized in three different strategies for inner ear treatment, restoration of missing cells, providing growth factors and delivering genes. These findings demonstrate that BMSCs can survive in various parts of the cochleae after injection into the perilymphatic space of cochleae, indicating possible use of BMSC transplantation for providing growth factors in the cochlea. These results, however, demonstrated significant decrease of the rate for CD34 expression in BMSC-derived cells in cochleae in comparison with that in BMSCs before transplantation, indicating maturation