70% corresponded to a mechanical impedance of 0.14, 0.56, and 1.7 ns/m, respectively, when the device was calibrated using weight. For our measurements in the cochlear implantees, there was a great difference between the RORs of the stapes (15–20% in Case 1 and 35–45% in Case 2), whereas the RORs of the malleus and incus were within the same range. This was thought to correspond to the partial calcification of the cochlea noted in Case 2. In otosclerosis, with the stapes fixed at the oval window, the ROR of the stapes ranged from 70 to 80%. When mobility was measured in the malleus-incus fixation anomaly (Case 4), the ROR of the malleus and incus was in the range of 60 to 70%. Although the study examined too few patients to make general conclusions, our results suggest that the ROR of the stapes is normally around 20% and increases with the degree of stapes fixation. The ROR of the malleus or incus was normally around 30% and also increased with fixation. This study also showed that the test-retest reliability of the device was reasonable based on the results of repeated measurements.

This clinical trial revealed some drawbacks of the device: 1) it is affected by the surgeon's hand tremor; 2) it requires pushing against the target; 3) there is a risk for inner ear damage; and 4) the probe shaft moves in a direction different from that of the target ossicle.

The effects of hand tremor during measurement are inevitable because the device is designed for handheld use. One must keep the device as stable as possible during measurements. This was actually not very difficult because we could obtain repeatable results. The dispersion of the data was only 5 to 10%. The effects of pushing against the target are also critical for accurate assessment of the ossicular mobility: low pressure causes a lag in the articulation between the probe tip and the target ossicle, resulting in underestimation of the ossicular resistance, whereas high pressure dislocates the ossicle, resulting in overestimation of the ossicular resistance. Careful control of the pressure is essential for correct measurement, and this is actually somewhat difficult to achieve. We think that the risks for our measuring device are similar to those of the standard surgical procedure in which the ossicles are manipulated using an ear pick. Nevertheless, the potential for causing inner ear trauma exists. In this study, the loading amplitude of the probe was set to 0.15 mA, which caused reciprocal vibration of the shaft corresponding to the vibration of the stapes at 100 dB SPL at 1,600 Hz. This is thought to be the limit of the permanent threshold shift that causes inner ear damage via transmission of the vibration (14). The direction of the probe shaft movement differs from that of the ossicle, and the measurement can be performed only in the direction of probe vibration. Therefore, the results differ from the natural ossicular resistance. To obtain a precise value of the stapes impedance, we need to expose the ossicles as widely as possible and place the probe tip perpendicular to the stapes footplate. We think that a rough estimate of the ossicular resistance using this procedure is sufficient for the clinical evaluation of ossicular mobility.

Further clinical studies are necessary, especially to analyze the normal value of the ossicular mobility using this procedure. Nevertheless, we think that our device is useful for diagnosing ossicular mobility during middle ear surgery.

CONCLUSION

The ossicular vibration tester developed for use during middle ear surgery is small and handy. The device was applied in four patients without any side effects. The measurements were performed safely, and the ossicular mobility gave us important information concerning the surgical procedures. Further clinical studies are necessary to fully evaluate the usefulness of this device.

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Cochlear Protection by Local Insulin-Like Growth Factor-1 Application Using Biodegradable Hydrogel

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Objective: **The aim of this experimental study was to examine the potential of local recombinant human insulin-like growth factor-1 (rhIGF-1) application through a biodegradable hydrogel for the treatment of cochleae.** ***Methods:*** **A hydrogel immersed with rhIGF-1 was placed on the round window membrane of Sprague-Dawley rats while a hydrogel immersed with physiological saline was applied to control animals. On day 3 after drug application, the animals were exposed to white noise at 120 dB sound pressure level (SPL) for 2 hours. Cochlear function was monitored using measurements of auditory brain stem responses (ABRs) at frequencies of 8, 16, and 32 kHz. The temporal bones were collected 7 or 30 days after noise exposure and the loss of hair cells was quantitatively analyzed.** ***Results:*** **Local rhIGF-1 treatment significantly reduced the elevation of ABR thresholds on days 7 and 30 after noise exposure. Histologic analysis revealed that local rhIGF-1 treatment significantly prohibited the loss of outer hair cells.** ***Conclusions:*** **These findings demonstrate that local IGF-1 application through the biodegradable hydrogel has the potential for protection of cochleae from noise trauma.** ***Key Words:*** **Drug delivery, cochlea, hair cell, protection, growth factor, acoustic trauma, rat.**

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INTRODUCTION

In recent years, there has been increasing interest in the treatment of inner ear disorders using the local, rather than systemic, application of therapeutic agents, because the former has fewer side effects and is more target-specific. The establishment of clinically applicable strategies for the local application of therapeutic agents should therefore open a new window for the treatment of inner ear disorders. For methods of drug delivery to be viable in clinical settings, it is crucial for the procedure to be technically undemanding and as minimally invasive as possible. Based on such a background, the use of biodegradable polymers for cochlear drug delivery has been investigated.[1–3] Biodegradable polymers, which enable the sustained release of drugs to the cochlear fluid space, can be applied through an intratympanic injection. Among biodegradable polymers, we have reported the efficacy of the biodegradable hydrogel, which is made from porcine type-I collagen, for delivery of brain-derived neurotrophic factor (BDNF) into the cochlear fluid and successful protection of spiral ganglion neurons (SGNs) from degeneration as a result of the loss of cochlear hair cells.[3]

Insulin-like growth factor-1 (IGF-1) is a mitogenic peptide that plays essential roles in the regulation of growth and development in various parts of the body, including the inner ear.[4] IGF-1 is also known to be a neuroprotective agent.[5] In addition, previous studies on the inner ear have suggested the possibility of inner ear protection by IGF-1.[6,7] Moreover, recombinant human IGF-1 (rhIGF-1) has already been approved for clinical use. Our ultimate goal is for local neurotrophin application to be clinically approved for the treatment of inner ears. In the present study, we then selected rhIGF-1 as a suitable neurotrophin for local application to the cochlea using a biodegradable hydrogel as a vehicle for drug delivery. We evaluated whether the application of rhIGF-1 in this manner was effective in protecting against noise-induced hearing loss.

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MATERIALS AND METHODS

Experimental Animals

Sprague-Dawley rats (Japan SLC Inc., Hamamatsu, Japan) at 10 weeks of age were used as experimental animals. The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all experimental protocols. 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 U.S. National Institutes of Health guidelines for the care and use of laboratory animals.

Preparation of Hydrogels

The biodegradable hydrogels were prepared as described previously.[8] Briefly, the gels were generated by the glutaraldehyde crosslinking of porcine type-I collagen (Gunze, Ayabe, Japan). The rates of degradation were determined according to the concentration of glutaraldehyde. The present study used a hydrogel that was made with 60 mol/L glutaraldehyde, which could release basic fibroblast growth factor[8] and BDNF[3] for 7 days in vivo.

Local Application of Insulin-Like Growth Factor-1

RhIGF-1 was provided by Astellas Pharma Inc., Tokyo, Japan. After measuring the auditory brain stem responses (ABRs), the otic bulla of the left temporal bone was exposed using a retroauricular approach under general anesthesia with ketamine (100 mg/kg intramuscularly [IM]; Sankyo Co., Tokyo, Japan) and xylazine (9 mg/kg IM; Bayer, Tokyo, Japan). A small hole was made on the left bulla to expose the round window niche. A hydrogel in dry condition was cut into the size of 2 mm^3 under the microscope and immersed with rhIGF-1 (400 μg dissolved in 40 μL physiological saline) 30 minutes before application. The hydrogel was then placed on the round window membrane (RWM) of the IGF group animals (n = 10). The animals applied a hydrogel-immersed physiological saline were used as controls (n = 10).

Noise Exposure and Measurement of Hearing

On day 3 after the IGF-1 application, we measured the ABRs to eliminate animals that showed threshold shifts of more than 10 dB at any frequencies from the experiments. In consequence, no animals showed threshold shifts over 10 dB after local drug application in the present study. The animals were then exposed to white noise at 120 dB sound pressure level (SPL) for 2 hours in a ventilated sound exposure chamber. The sound levels were monitored and calibrated at multiple locations within the sound chamber to ensure uniformity of the stimulus.

Auditory function was assessed by recording ABRs. The measurements of ABR thresholds were performed at frequencies of 8, 16, and 32 kHz before noise exposure and days 7 and 30 after noise exposure. Animals were anesthetized with ketamine (100 mg/kg) and xylazine (9 mg/kg) and kept warm with a heating pad. Generation of acoustic stimuli and subsequent recording of 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 and 1-ms plateau), were delivered monaurally through a speaker (ES1spc; Bioresearch Center, Nagoya, Japan) connected to a funnel fitted into 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, Inc., Belmont, CA) 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 mastoid subcutaneous electrodes were amplified with a digital amplifier (MA2; Tucker-Davis Technologies, Alachua, FL). Thresholds were determined from a set of responses at varying intensities with 5-dB SPL intervals and electrical signals were averaged for 1024 repetitions. Thresholds at each frequency were verified at least twice.

Histologic Analysis

On day 7 or 30 after noise exposure, five cochleae from each experimental group were provided for histologic analysis. The animals were anesthetized with ketamine and xylazine, and the left cochleae were exposed. After removal of otic vesicles, 4% paraformaldehyde in 0.01 mol/L phosphate-buffered saline (PBS)

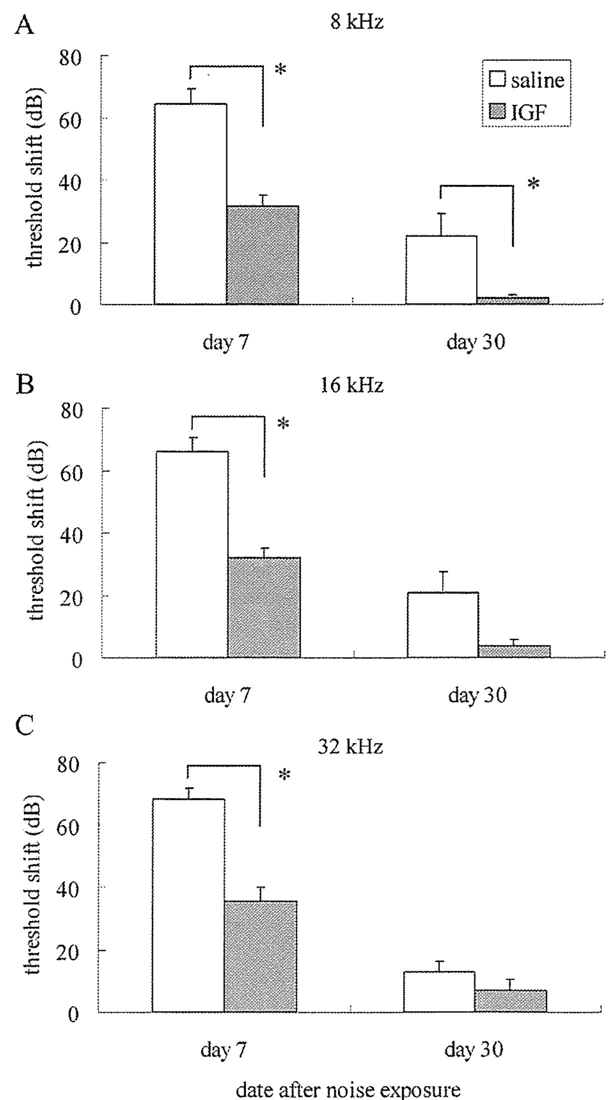

Fig. 1. Auditory brain response threshold shifts for recombinant human insulin-like growth factor-1- (rhIGF-1) and saline-treated cochleae at 8, 16, and 32 kHz on days 7 and 30 after noise exposure. An overall effect of local rhIGF-1 treatment is significant at 8, 16, or 32 kHz (two factorial analysis of variance). Asterisks are indicated significant differences in pairwise comparisons with Fisher's protected least-significant difference. Bars represent standard error (SE).

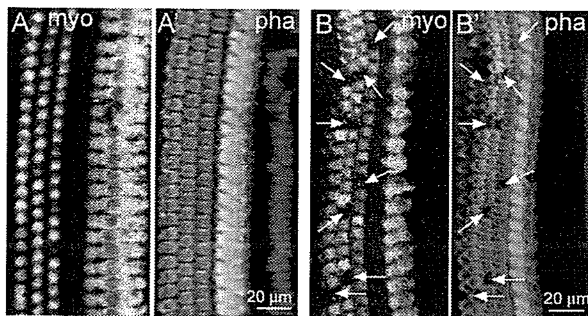

Fig. 2. Photomicrographs of surface preparations stained with myosin VIIa and phalloidin from the second turn of cochleae treated with recombinant human insulin-like growth factor-1 (**A**) or physiological saline (**B**) on day 30. Figures A and B show immunostaining for myosin VIIa (myo), and Figures A' and B' show F-actin labeling by phalloidin. Arrows indicate missing outer hair cells.

at pH 7.4 was gently introduced into the perilymphatic space of the cochleae. The temporal bones were then excised and immersed in the same fixative at 4°C for 12 hours. After rinses with PBS, the cochleae were dissected from the temporal bones and subjected to histologic analysis in whole mounts. We used three regions of cochlear sensory epithelia at a distance of 30% to 40% (apical), 50% to 60% (middle) or 80% 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 outer hair cells (OHCs). Anti-myosin VIIa rabbit polyclonal antibody (1:300; a gift from Tama Hasson, San Diego, CA) was used as the primary antibody, and Alexa-594-conjugated antirabbit goat IgG (1:400; Molecular Probe, Eugene, OR) was used as the secondary antibody. After immunostaining for myosin VIIa, specimens were stained with FITC-conjugated phalloidin (1:300; Molecular Probe). Specimens were viewed using a Leica TCS SP2 confocal microscope (Leica Microsystems Inc., Wetzlar, Germany). Nonspecific labeling was tested by omitting the primary antibody from the staining procedures. We counted the numbers of IHCs and OHCs in 0.2-mm long regions of the apical, middle, or basal portion of cochleae, respectively.

Statistical Analyses

An overall effect on ABR threshold shifts of application of rhIGF-1 was examined by the two-way factorial analysis of variance. When the interaction was significant, multiple comparisons with Fisher's protected least-significant difference (PLSD) were used for pairwise comparisons. The differences in OHC numbers in each region of the cochlea between the rhIGF-1- and saline-treated cochleae were examined using the Student t test. A P value less than .05 was considered statistically significant. Values are expressed as the mean ± standard error.

RESULTS

Functional Protection

The time course of alterations in ABR threshold shifts after noise exposure at 8, 16, or 32 kHz is shown in Figure 1. Local rhIGF-1 treatment demonstrated significant effects on ABR threshold shifts at each frequency. An overall effect on data for 8 kHz of rhIGF-1 application was significant ($P < .001$). The differences in threshold shifts between rhIGF-1- and saline-treated cochleae on days 7 and 30 were significant at multiple comparisons with Fisher's PLSD ($P < .001$ for day 7, $P = .039$ for day 30). An overall effect on data for 16 or 32 kHz of rhIGF-1 application was significant ($P < .001$ for 16 kHz, $P = .005$ for 32 kHz). The difference in threshold shifts at 16 kHz between rhIGF-1- and saline-treated cochleae was significant on day 7 ($P < .001$), but not on day 30 ($P = .051$). The difference in threshold shifts at 32 kHz between rhIGF-1- and saline-treated cochleae was significant on day 7 ($P < .001$), but not on day 30 ($P = .48$).

Histologic Protection

Immunostaining for myosin VIIa and phalloidin staining demonstrated degeneration of OHCs in the apical, middle, and basal portions of saline-treated cochleae (Fig. 2B), whereas OHC degeneration was very limited in rhIGF-1-treated cochleae (Fig. 2A). Conversely, IHC loss was not apparent in every region of both saline- and rhIGF-1-treated cochleae. Quantitative assessments revealed the significant differences in the degree of OHC loss between saline- and rhIGF-1-treated cochleae on days 7 and 30 (Fig. 3). The differences in the degree of OHC loss between the saline- and rhIGF-1-treated cochleae were significant in the apical ($P = .0006$), middle ($P < .0001$), and basal portion ($P = <.0001$) of cochleae on day 7, and in the apical ($P = .0006$), middle ($P < .0001$), and basal portion ($P = .002$) of cochleae on day 30. IHC loss was 2.7 ± 1.3% in the basal, 1.0 ± 0.5% in the middle, or 1.1 ± 0.8 in the apical portion of saline-treated cochleae, and 2.0 ±

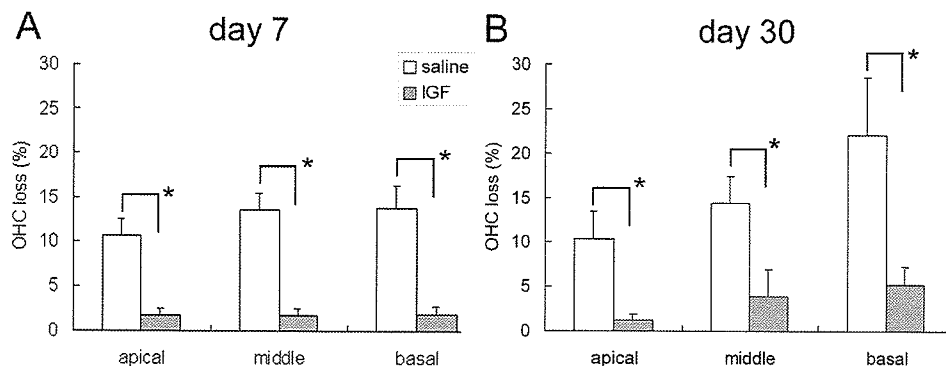

Fig. 3. Means of the percentage outer hair cell loss in the apical, middle, and basal portions of insulin-like growth factor-1- and saline-treated cochleae on days 7 (**A**) and 30 (**B**). Asterisks indicate significant differences with unpaired t test. Bars represent standard error (SE).

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1.4% in the basal, 1.2 ± 0.9% in the middle, or 0.6 ± 0.6 in the apical portion of rhIGF-1-treated cochleae on day 30. No significant differences in the loss of IHCs were identified between the two experimental groups.

DISCUSSION

Our findings demonstrate that local rhIGF-1 application using a hydrogel before noise exposure has significant effects on reduction of ABR threshold shifts and of OHC loss. Our previous study has demonstrated the efficacy of local BDNF delivery to the cochlea by the biodegradable hydrogel.[3] The present findings therefore indicate that the biodegradable hydrogel can be used for local rhIGF-1 application to the cochlea. Our previous findings[3] demonstrate that high concentrations of BDNF in the cochlear fluid are maintained during days 3 to 7 after local BDNF application using this system. We then locally applied rhIGF-1 3 days before noise exposure to obtain sufficient concentrations of rhIGF-1 in the cochlear fluid. As expected, the pretreatment with rhIGF-1 demonstrated sufficient protective effects against noise trauma in the present study. However, in clinical settings, drug application after the onset of hearing loss is usually performed. Hence, we should examine the efficacy of local rhIGF-1 treatment after onset of hearing loss in the near future.

In the present study, we focused on degeneration of sensory hair cells in histologic analysis, because the degree of hair cell loss has traditionally been used to evaluate both the extent of noise-induced injury and the efficacy of protective treatments.[9,10] Quantitative assessments in the present study demonstrated significant protection of OHCs from noise trauma by local rhIGF-1 treatment. As for mechanisms of OHC protection by rhIGF-1, several possible explanations are aroused. One possible explanation is the rescue of OHCs from apoptosis resulting from noise by rhIGF-1. IGF-1 is known to inhibit apoptosis by downregulating the expression of proapoptotic genes,[11] and apoptosis is involved in OHC degeneration resulting from noise.[12] Another mechanism is the regulation of glucose transporters in OHCs by rhIGF-1. The expression of glucose transporter-5 (GLUT-5) in OHCs and its importance in their function have been reported.[13,14] GLUTs operate in the first step of glucose utilization by promoting the transport of glucose across the plasma membrane.[15] IGF-1 can regulate the expression of GLUT-5, thereby promoting neuronal cell survival.[16] Such mechanisms might be involved in the rhIGF-1-induced protection of OHCs against noise-induced injury. Further studies are required for elucidation of detailed mechanisms for the rhIGF-1-induced protection of OHCs.

ABR threshold shifts observed on day 7 remarkably recovered on day 30, whereas the damage in the organ of Corti moderately progressed until day 30. This indicates that ABR threshold shifts observed on day 7 may be caused not only by the damage in the organ of Corti, but also by reversible damages in other regions of the cochlea. In addition, the damage in the organ of Corti on day 7, 10% to 14% loss of OHCs and limited loss of IHCs, is not compatible with over 60 dB ABR threshold shifts. Recent studies have indicated involvement of damages in the cochlear lateral wall in noise-induced HL.[17] Local rhIGF-1 treatment significantly reduced ABR threshold shifts on day 7. IGF-1 has also effects on promotion of survival of fibloblasts.[18] Therefore, protective effects of IGF-1 on the fibrocytes in the spiral ligament may be involved in mechanisms for significant reduction of ABR threshold shifts on day 7.

CONCLUSION

This report demonstrates the efficacy of local rhIGF-1 application using a biodegradable hydrogel for the protection of cochleae from noise-induced hearing loss. Because the materials used in the present study are suitable for clinical application, the present findings encourage us to conduct further studies for clinical application of local rhIGF-1 treatment using the biodegradable hydrogel. However, the exact mechanisms by which rhIGF-1 acts in the cochlea are presently unclear and require further research. Furthermore, rhIGF-1 was applied before the onset of noise-induced hearing loss in the present study. The ability of rhIGF-1 to ameliorate cochlear damages when applied locally after the onset of hearing loss should therefore be examined in an experimental model in the near future.

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