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Changes in cell proliferation in rat and guinea pig cochlea after aminoglycoside-induced damage

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Abstract

Cell proliferation in the cochleae of guinea pigs and rats was investigated after systemic application of kanamycin sulfate (KM) and ethacrynic acid (EA). Bromodeoxyuridine (BrdU) was injected daily for 10 days, after which the number of BrdU-positive cells was counted in paraffin sections of the cochlea. Only a few BrdU-positive cells were present in the spiral ligament and among the acoustic nerve fibers in the non-deafened control animals. Animals treated with KM and EA had profound hearing loss and significant increases in the number of BrdU-positive cells in the spiral ligament and among the acoustic nerve fibers. No BrdU-positive cells were found in the auditory sensory epithelium of any animal. These findings suggest that in the mature mammalian cochlea cell proliferation increases in nonsensory regions after ototoxic damage but may not occur in the auditory sensory epithelium.

Keywords: Mitosis; Spiral ligament; Hair cell; Supporting cell; Regeneration; Cochlear nerve; Kanamycin; Ethacrynic acid

Mammalian auditory hair cells have been believed to be produced only during the embryonic stage of life [16] and not replaced when they die [3,15]. Studies done in the last decade, however, have shown that sensory hair cells in chicks and in lower vertebrates lost owing to injury can be replaced by the production of new ones [3,6]. It has been shown that supporting cell proliferation is an early event and that the supporting cells are progenitors of a new generation of hair cells after injury [5,9]. The possibility of a nonproliferative regeneration process also has been reported [1]. Hair cell replacement in mammals also occurs in the vestibular sensory epithelium. Immature-appearing stereocilliary bundles appear in situ in the vestibular epithelia of adult guinea pigs (GPs) several weeks after aminoglycoside-induced hair cell loss [7]; moreover, sensory epithelial cells proliferate in organotypic explants of mature GP utricular macula after aminoglycoside-induced damage [18]. Experiments on adult GPs that involved continuous infusion of a cell proliferation marker to the inner ear after drug damage confirmed that a small amount of sensory epithelium cell proliferation is induced in vivo [11].

These findings suggest that sensory epithelium cell proliferation or even hair cell regeneration might occur in the mature mammalian cochlea, but no in vivo evidence has been reported. Lefebvre and colleagues showed that in vitro retinoic acid stimulated the regeneration of rat auditory hair cells in the ototoxic-poisoned organ of Corti explants [10], but other researchers failed to confirm this [4]. Using [^{3}H]thymidine labeling to detect mitotic activity, Roberson and Rubel found no cell division within the cochlear sensory epithelia of gerbils after 14 h exposure to two-octave band noise at 130 dB SPL [15]. As this severe acoustic overstimulation caused significant trauma to the entire acoustic epithelium, the insult may have damaged any potential hair cell precursors and prevented sensory epithelial cell proliferation. Sensory epithelial cell proliferation also may occur in mature cochleae of other mammalian species. We therefore investigated changes in cell proliferation in mature cochleae of GPs and rats after damage induced by kanamycin sulfate (KM) and ethacrynic acid (EA). KM and EA were used because their co-administration reliably destroys almost all the outer hair cells and some inner hair cells, whereas the supporting cells are well preserved and form a scar [2,14].

Sixteen pigmented GPs and ten albino rats, each weighing 250–350 g, were used. These animals had auditory brainstem response (ABR) thresholds at 2, 4, 8,

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and 20 kHz within the normal laboratory range. The ABR measurement technique has been described elsewhere [13]. Ten GPs and six rats were deafened by the systemic application of KM (400 mg/kg, s.c.) followed 2 h later by EA (50 mg/kg, i.v.). The other six GPs and four rats received 5 ml saline s.c. and served as the controls. Mitotic tracer bromodeoxyuridine (BrdU, 150 mg/kg per day, i.p.) was then injected to all the animals daily for 10 days. After the last BrdU injection, ABR thresholds were measured, after which the animals were decapitated under anesthesia with xylazine (10 mg/kg, i.m.) and ketamin (40 mg/kg, i.m.). The cochleae were perfused with 10% buffered neutral formalin through the oval and round windows and immersed in the same fixative for 2 days. Specimens were rinsed in 0.1 M phosphate buffer saline (PBS), decalcified in 10% EDTA for 7 days, dehydrated through a graded alcohol series, and embedded in paraffin. The embedded tissues were cut in 6 μm thick sections parallel to the modiolus and mounted on glass slides. The sections were deparaffinized and hydrated, rinsed with PBS, and incubated with 3% hydrogen peroxide in methanol for 30 min. After another PBS rinse, the sections were immersed in 2 N HCl for 30 min, then incubated overnight in a solution containing mouse monoclonal antibody to BrdU (Clone Bu20a; DAKO A/S, Glostrup, Denmark) at 4 °C. After six PBS rinses, these sections were incubated for 30 min with a peroxidase-conjugated secondary antibody (Histofine, simple stain PO Max (Multi), Nichirei, Tokyo, Japan). After another six rinses, they were allowed to react with diaminobenzidine (Histofine, simple stain DAB; Nichirei).

The presence of BrdU-positive cells was checked in 50 sections taken from around the modiolus of both ears. The numbers of BrdU-positive cells in the spiral ligament and among the acoustic nerve fibers were counted in the basal, second, and third turns of the GPs and in the basal and apical turns of the rats. Numbers for the controls and deafened animals were compared to determine if there was a difference in cell proliferation among the turns. Numbers also were compared between the controls and deafened animals to investigate changes in cell proliferation after KM and EA treatment. A one-way analysis of variance (ANOVA) and the Student–Newman–Keuls test were used for the statistical analysis.

ABR thresholds essentially were unchanged in the controls, whereas no ABR waves were elicited at any frequency, even at 110 dB SPL, in animals treated with KM and EA, indicative of marked increases in hearing thresholds. None of the 2600 sections from the study population showed evidence of BrdU labeling of hair cells, supporting cells, or any other cells within the sensory epithelium proper. BrdU-positive cells, however, were present in many other cochlear structures, most commonly in the spiral ligament and among the acoustic nerve fibers coursing toward the modiolus, but much less frequently in the stroma of the spiral limbus, tympanic border cells, strial cells, and on Reissner's membrane (Fig. 1). Only a small number of BrdU-positive cells were present in the spiral ligament and among the acoustic nerve fibers of the controls. After the systemic application of KM and EA, there were significant increases in the number of BrdU-positive cells in the spiral ligament ($P < 0.01$) and among the acoustic nerve fibers ($P < 0.01$) in both the GPs and rats (Fig. 2). No significant difference was found in the number of BrdU-positive cells among the turns in either the controls or deafened animals.

The spiral ligament of 4- to 5-week-old mice treated with BrdU showed significant increases in BrdU-positive cells after intraperitoneal injection of dihydrostreptomycin sulfate (SM) [19]; the number of BrdU-positive cells in the spiral ligament in the 2nd and 3rd turns per 5–6 μm thick

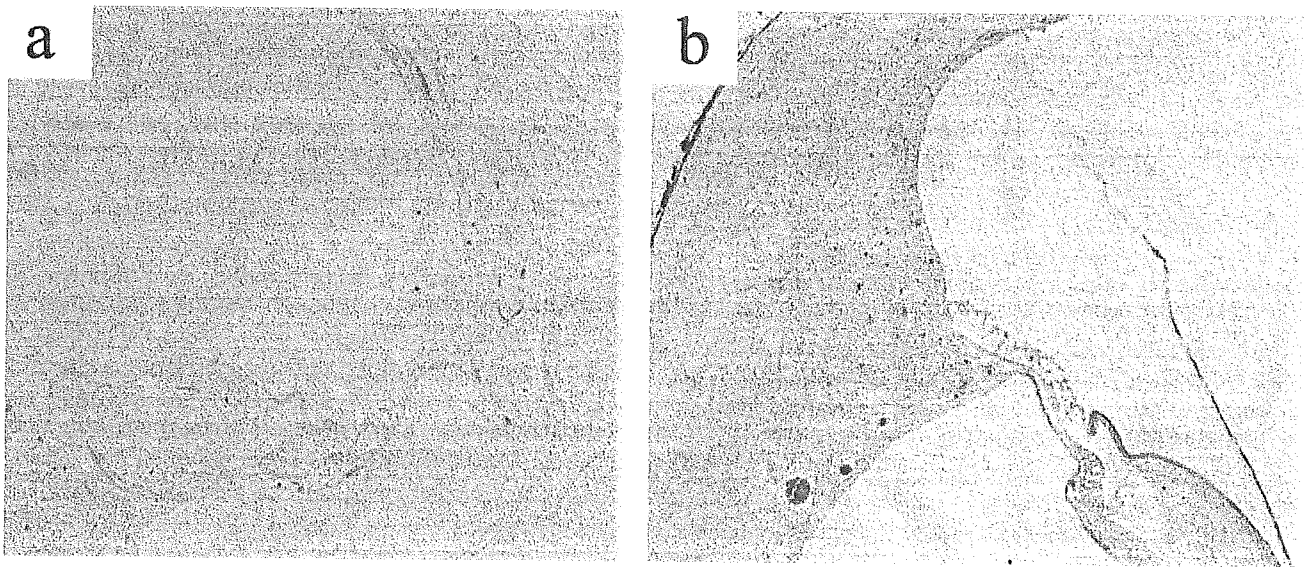

Fig. 1. BrdU-positive cells in the spiral ligament and among the auditory nerve fibers in the second turn in a deafened GP (a) and in the basal turn in a deafened rat (b).

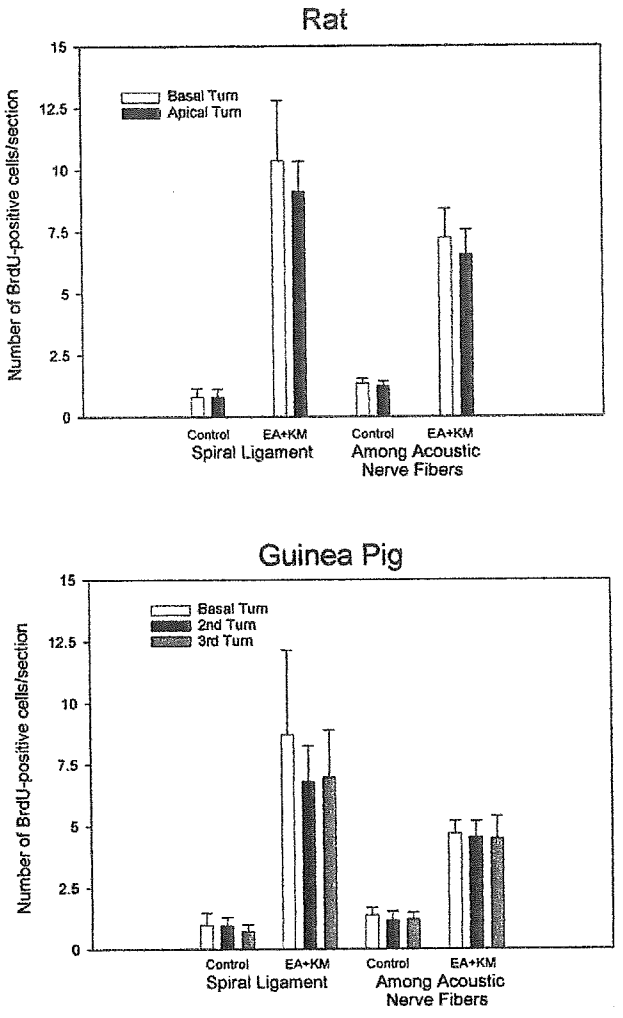

Fig. 2. Numbers of BrdU-positive cells in the spiral ligament and among the acoustic nerve fibers in the controls and deafened rats and GPs. BrdU-positive cells are significantly increased ($P < 0.05$) in both regions in the rats and GPs after deafening by treatment with KM and EA. There is no significant difference among the turns for these species.

section was 6 ± 1 for the controls and 14 ± 5 for the test mice 14 days after SM injection. The number of mitotic cells under normal conditions and the extent of their increase in the spiral ligament after ototoxic insult appear to be similar for GPs, rats, and mice. The BrdU-positive cells in the spiral ligament presumably are fibrocytes undergoing division. Fibroblast growth factor receptor and connexin 43 are reported to be expressed in the spiral ligament where there are BrdU-labeled cells [19]. The gap junction in fibrocytes is believed to have an important function in ion transport in the spiral ligament and to be involved in the regulation of cell proliferation [12].

Roberson and Rubel found that in gerbils after noise exposure cells undergoing mitosis were common in the stria vascularis and among the acoustic nerve fibers but less frequent in the spiral ligament [15]. Acoustic overstimulation alters strial blood flow or capillary permeability [17] and causes degeneration of strial cells [8]. No apparent degenerative changes were seen in the stria vascularis after aminoglycoside treatment in our study and in a previous study [19]. This may explain why mitosis commonly occurs in the stria vascularis after acoustic trauma but not after aminoglycoside-induced damage. The BrdU-positive cells among the acoustic nerve fibers presumably are glia undergoing division in response to nerve fiber degeneration that occurs secondary to hair cell loss, regardless of its cause.

In conclusion, after test animals were deafened with KM and EA, cell proliferation increased in nonsensory regions, most commonly in the spiral ligament and among the acoustic nerve fibers in mature cochleae of GPs and rats. There was no evidence of cell proliferation in the sensory epithelia of the controls or animals deafened by KM and EA treatment, although supporting cells were well preserved. Potential hair cell precursors, presumably the supporting cells, may lose the ability to proliferate in the mature mammalian cochlea.

Acknowledgements

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References

[1] H.J. Adler, M. Komeda, Y. Raphael, Further evidence for supporting cell conversion in the damaged avian basilar papilla, Int. J. Dev. Neurosci. 15 (1997) 375–385.
[2] M.E. Beaugard, S. Asakuma, J.B. Snow Jr., Comparative ototoxicity of chloramphenicol and kanamycin with ethacrynic acid, Arch. Otolaryngol. 107 (1981) 104–109.
[3] O. Bermingham-McDonogh, E.W. Rubel, Hair cell regeneration: winging our way towards a sound future, Curr. Opin. Neurobiol. 13 (2003) 119–126.
[4] S. Chardin, R. Romand, Regeneration and mammalian auditory hair cells, Science 267 (1995) 707–711.
[5] J.T. Corwin, D.A. Cotanche, Regeneration of sensory hair cells after acoustic trauma, Science 240 (1988) 1772–1774.
[6] D.A. Cotanche, K.H. Lee, Regeneration of hair cells in the vestibulocochlear system of birds and mammals, Curr. Opin. Neurobiol. 4 (1994) 509–514.
[7] A. Forge, L. Li, J.T. Corwin, G. Nevill, Ultrastructural evidence for hair cell regeneration in the mammalian inner ear, Science 259 (1993) 1616–1619.
[8] K. Hirose, M. Liberman, Lateral wall histopathology and endocochlear potential in the noise-damaged mouse cochlea, J. Assoc. Res. Otolaryngol. (2003) in press.
[9] J.E. Jones, J.T. Corwin, Regeneration of sensory cells after laser ablation in the lateral line system: hair cell lineage and macrophage behavior revealed by time-lapse video microscopy, J. Neurosci. 16 (1996) 649–662.
[10] P.P. Lefebvre, B. Malgrange, H. Staecker, G. Moonen, T.R. Van de Water, Retinoic acid stimulates regeneration of mammalian auditory hair cells, Science 260 (1993) 692–695.
[11] L. Li, A. Forge, Morphological evidence for supporting cell to hair

cell conversion in the mammalian utricular macula, Int. J. Dev. Neurosci. 15 (1997) 433–446.
[12] F. Miragall, P. Albiez, H. Bartels, U. de Vries, R. Dermietzel, Expression of the gap junction protein connexin43 in the subependymal layer and the rostral migratory stream of the mouse: evidence for an inverse correlation between intensity of connexin43 expression and cell proliferation activity, Cell Tissue Res. 287 (1997) 243–253.
[13] A. Pourbakht, T. Yamasoba, Cochlear damage caused by a continuous and intermittent noise exposure, Hear. Res. 178 (2003) 70–78.
[14] Y. Raphael, R.A. Altschuler, Scar formation after drug-induced cochlear insult, Hear. Res. 51 (1991) 173–183.
[15] D.W. Roberson, E.W. Rubel, Cell division in the gerbil cochlea after acoustic trauma, Am. J. Otol. 15 (1994) 28–34.
[16] R.J. Ruben, Development of the inner ear of the mouse: a radioautographic study of terminal mitoses, Acta Otolaryngol. Suppl. 220 (1967) 1–44.
[17] M. Suzuki, T. Yamasoba, T. Ishibashi, J.M. Miller, K. Kaga, Effect of noise exposure on the blood-labyrinth barrier in the guinea pig, Hear. Res. 164 (2002) 12–18.
[18] M.E. Warchol, P.R. Lambert, B.J. Goldstein, A. Forge, J.T. Corwin, Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans, Science 259 (1993) 1619–1622.
[19] H. Yamashita, H. Shimogori, K. Sugahara, M. Takahashi, Cell proliferation in spiral ligament of mouse cochlea damaged by dihydrostreptomycin sulfate, Acta Otolaryngol. 119 (1999) 322–325.

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Ebselen attenuates cochlear damage caused by acoustic trauma

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Abstract

Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one), a seleno-organic compound, mimics glutathione peroxidase and reacts with peroxynitrite. It is reported to protect against gentamicin- and cisplatin-induced ototoxicity. We investigated whether it protects the cochlea from acoustic trauma. Male pigmented guinea pigs (250–300 g) with normal auditory brainstem response (ABR) thresholds were exposed for 5 h to 125 dB sound pressure level octave band noise centered at 4 kHz. One hour before and 18 h after exposure, they received orally 0.25 ml chloroform solution containing 0, 10, or 30 mg/kg ebselen ($n = 6$, 5 and 5, respectively). The protective effect of ebselen was evaluated by ABR measurement and quantitative hair cell assessment. Treatment significantly ($P < 0.01$) reduced the extent of permanent threshold shifts and outer hair cell loss. Interestingly, the protective effect of a 30 mg/kg dose was less than that of a 10 mg/kg dose. There were no adverse systemic or auditory function effects in three unexposed control subjects given 30 mg/kg ebselen. These findings indicate that ebselen attenuates noise-induced cochlear damage. The concentration that provides optimal protection against such damage has now to be determined.

Key words: Noise-induced hearing loss; Cochlea; Ebselen; Hair cell

1. Introduction

Noise-induced hearing loss (NIHL) is the primary cause of acquired hearing loss in industrialized countries. It is particularly important because it may affect the individual's safety and work performance. A variety of mechanisms have been proposed to account for hearing loss after high intensity sound exposure. There are two main ones: (1) direct mechanical trauma to the organ of Corti and (2) overuse of metabolically dependent processes in the inner ear. The generation of reactive oxygen species (ROS) in the cochlea constitutes a mechanism for metabolic damage to the organ of Corti. The importance of ROS in NIHL is supported by previous findings: (1) ROS are significantly increased in the cochlea after noise exposure (Ohlemiller et al., 1999; Yamane et al., 1995); (2) noise trauma is also associated with significant increases in the activity level of endogenous antioxidant enzymes such as glutathione (GSH) (Yamasoba et al., 1998b); and (3) enhancement of ROS scavengers by various antioxidant interventions attenuates NIHL (Seidman et al., 1993; Quirk et al., 1994; Hu et al., 1997; Yamasoba et al., 1999).

Another suggested mechanism in NIHL is NO-induced tissue damage. NO is present in several cochlear mechanisms, including neurotransmission and blood flow regulation, and may be cytotoxic under pathological conditions (Takumida et al., 1997; Takumida and Anniko, 1988; Amaee et al., 1997; Franz et al., 1996). The NO generated under pathological conditions reacts with superoxide anion (O_2^-), forming peroxynitrite ($ONOO^-$) which may mediate oxidation, nitration, or nitrosation reactions leading to impaired function, toxicity and alteration of signaling pathways (Arteel et al., 1999). Oxidative stress after anoxia and noise trauma increases the release of excitatory amino acid (EAA) (Coyle and Puttfarcken, 1993) and produces excess release of EAA, causing excitotoxic damage to dendrites beneath the inner hair cells (IHCs) (Pujol and Puel, 1999). Excessive release of EAA contributes to ROS formation and the excessive synthesis of NO (Dawson et al., 1991; Yamauchi et al., 1998).

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Neuronal-type NO synthase (NOS) I and endothelial-type NOS III are present in guinea pig cochlea (Fessenden and Schacht, 1998). Popa et al. (2001) reported NOS I staining in the spiral ganglion, cochlear nerve fibers, outer hair cells (OHCs), supporting cells and stria vascularis. Compared to NOS I, there was less intense staining by NOS III in the OHCs, supporting cells and spiral ganglion cells. Weak NOS II staining was present only in a few cochlear nerve fibers. The fluorescence intensity of NO in guinea pig cochlea is enhanced by L-arginine or glutamate stimulation and increases significantly after inoculation with lipopolysaccharide (LPS) (Takumida and Anniko, 2001). Carboplatin increases NO levels significantly, as well as the activities of xanthine oxidase and manganese-superoxide dismutase, in rat cochlea (Husain et al., 2001). Shi and associates (Shi et al., 2002) showed that broadband noise exposure (3 h/day at 120 dB sound pressure level (SPL) on three consecutive days) increases the NO concentration in the perilymph of guinea pig cochlea and that specific dyes for NO and ROS show greater fluorescence in the IHCs and OHCs of noise-exposed animals than in unexposed controls. Moreover, a neuronal NOS inhibitor reduces the threshold shift of the compound action potential in guinea pigs subjected to 30 min of ischemia (Tabuchi et al., 2001). These findings indicate that NO has importance in noise-induced cochlear damage.

Ebselen, 2-phenyl-1,2-benzisoselenazol-3(2H)-one, mimics GSH peroxidase (GSH Px), reacts with $ONOO^-$ and inhibits the enzymes lipoxygenase, NO synthase, NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase, protein kinase C and H^+/K^+-ATPase (Schewe et al., 1994). Although not a strong scavenger of ROS, it effectively scavenges organic hydroperoxides. Because organic hydroperoxides promote lipid peroxidation, their removal is analogous to scavenging ROS. Ebselen is reported to protect against the cochlear damage induced by gentamicin (Takumida et al., 1999), cisplatin (Kopke et al., 1997; Rybak et al., 1999; Rybak et al., 1999, 2000) and LPS-induced labyrinthitis (Takumida et al., 1999), probably by scavenging NO and inhibiting lipid peroxidation. Because NO generation is also thought to be involved in noise-induced cochlear damage, we investigated whether ebselen prevents or attenuates NIHL in the guinea pig.

2. Materials and methods

2.1. Animals

Nineteen healthy, otomicroscopically normal, pigmented guinea pigs weighing 250–300 g were used. Three days after arrival, their hearing was confirmed to be within the normal range (within one standard deviation of the normative lab baseline) by auditory brainstem response (ABR) measurements.

Animals were randomly allocated to the treatment and control groups. Treatment guinea pigs were randomly assigned to one of three groups, then exposed to noise for 5 h. Treatment and control solutions were administered orally by gavage 1 h before and 18 h after noise exposure (24 h after the first treatment). Ebselen is soluble in dimethyl sulfoxide (DMSO) or chloroform. Because DMSO has antioxidant properties, chloroform was the solvent used. The control solution (vehicle) consisted of 0.25 ml of 99% chloroform (group I, $n = 6$). The treatment solutions were 10 mg/kg ebselen (group II, $n = 5$) and 30 mg/kg ebselen (group III, $n = 5$) dissolved in 0.25 ml of the vehicle. A 30 mg/kg dose of ebselen dissolved in 0.25 ml of vehicle was administered to three animals not exposed to noise (drug controls, group IV) to determine whether ebselen had any adverse effects on the subjects and their auditory functions.

This study was reviewed by the Committee for Ethics in Animal Experiments of the University of Tokyo and carried out under Japanese law and the Guidelines for Animal Experiments of the University of Tokyo.

2.2. Noise exposure

Two days after the first baseline ABR measurement, the animals were subjected to a 5-h noise exposure (125 dB SPL, 4 kHz octave band noise) generated within a single-walled, sound-deadened chamber. Two separately caged animals were tested at one time and allowed to move freely during exposure. The sound chamber was fitted with speakers driven by a noise generator and power amplifier. A 0.5-inch Bruel and Kjaer condenser microphone and a Fast Fourier Transform analyzer were used to measure and calibrate the sound level at various locations within the chamber to ensure stimulus uniformity within ± 1 dB.

2.3. Auditory brainstem measurement

For groups I, II and III, left-ear ABRs were recorded 2 days before and 14 days after noise exposure. ABRs for the same ear were recorded for group IV 2 days before and 14 days after treatment with 30 mg/kg ebselen. Prior to ABR measurement, the animals were anesthetized intramuscularly with a mixture of xylazine (10 mg/kg) and ketamine (40 mg/kg). An active needle electrode was placed subcutaneously below the left (test) ear, a reference electrode at the vertex and a ground electrode below the right ear. The sound stimulus consisted of a 15 ms tone burst with a rise–fall time of 1 ms

at 2, 4, 8 and 16 kHz. To obtain waveforms, 1024 tone presentations at 12.5 s^{-1} were averaged in a microcomputer with custom software. Near the threshold, the sound intensity was decreased in 5-dB steps. The ABR threshold was defined as the lowest stimulus intensity that produced a reliable (definite and repeatable) ABR waveform peak 3 or 4. Thresholds obtained 2 days before noise exposure were used as the baseline thresholds for estimating noise-induced threshold shifts.

2.4. Histological examination

Animals in groups I, II and III under deep anesthesia produced by a mixture of xylazine and ketamine were decapitated 15 days after noise exposure. Animals in group IV were decapitated 15 days after receiving an oral dose of 30 mg/kg ebselen. The left temporal bones were immediately excised. Under a dissecting microscope, the perilymphatic spaces were perfused for 1 h with 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4, then washed in buffer. For specific F-actin staining whole mounts of the organ of Corti were stained with rhodamine phalloidin for 60 min after being made permeable by 0.3% Triton X-100 treatment for 5 min. The tectorial membrane, Reissner's membrane, osseous spiral lamina and cochlear nerve were removed under a dissecting microscope, and the individual turns of the organ of Corti mounted on glass slides.

Specimens were observed under a fluorescence microscope, and reticules whose length (bin width) at 40× was 0.45 mm were used to count the numbers of total and missing hair cells. The length of the organ of Corti showed only small variation across the animals, and the organ itself was observable from the area next to the apex to the base in all the animals. Because specimens were not always well preserved in the most apical and basal areas (the so-called hook portion, 18 mm or more from the apex), counting was begun at 2.25 mm and completed at 18 mm from the apex. Hair cells that showed an identifiable cell body and cuticular plate were considered to be present. Distinctive scar formation produced by convergence of adjacent phalangeal processes was regarded to show a missing hair cell. The percentages of hair cell loss for the IHCs and first, second and third rows of OHCs were calculated for each segment from each animal. The average for each segment was then determined for each group and plotted from the apex to the base to produce an average cytocochleogram.

2.5. Statistical analysis

Sigma Stat™ statistical software was used. Baseline ABR thresholds before noise exposure and threshold shifts at each frequency thereafter, as well as the percentages of missing IHCs and OHCs, were compared for the ebselen-treated and -untreated groups by a one-way analysis of variance. Significant differences found were compared with each other by the Student–Newman–Keuls test.

3. Results

3.1. Baseline ABR thresholds

ABR thresholds 2 days before noise exposure were essentially equivalent in all the ears. No significant differences in the baseline ABR thresholds among the groups were found at any frequency.

3.2. Protective effects on permanent threshold shifts

Fig. 1 shows the ABR threshold shifts (average change in the threshold level, relative to the pre-noise ABR threshold) across the frequencies tested 14 days after noise exposure. Means of threshold shifts are plotted for the left ears as a function of the treatment group. The vehicle-only animals (group I) had substantial ABR threshold shifts across the frequencies. Given the exposure frequency centered at 4 kHz, in group I the greatest threshold shifts were at 4 and 8 kHz. There were also significant threshold shifts at 2 and 16 kHz.

In comparison, those given 10 or 30 mg/kg ebselen (groups II and III) had much smaller threshold shifts at all the frequencies tested. A statistically significant ($P < 0.01$) difference in ABR threshold shifts was found between groups I and II and I and III. At all test fre-

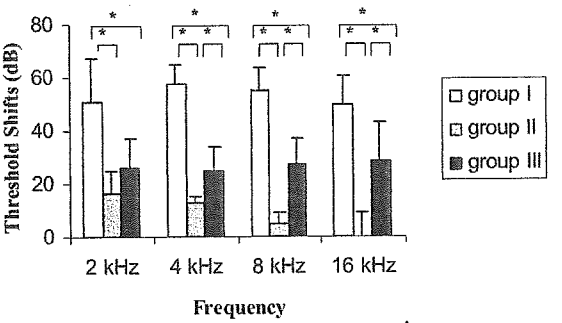

Fig. 1. ABR threshold shifts (mean ± S.D.) at each test frequency 14 days after noise exposure (125 dB SPL, 4 kHz octave band noise, 5 h). Group I (white bar, $n = 6$) vehicle only, group II (hatched bar, $n = 5$) 10 mg/kg ebselen, and group III (black bar, $n = 5$) 30 mg/kg ebselen. Groups II and III had significantly smaller threshold shifts than group I at all frequencies ($P < 0.01$). Compared to group II, group III had greater shifts at all frequencies. This difference was statistically significant at 4, 8 and 16 kHz ($P < 0.01$).

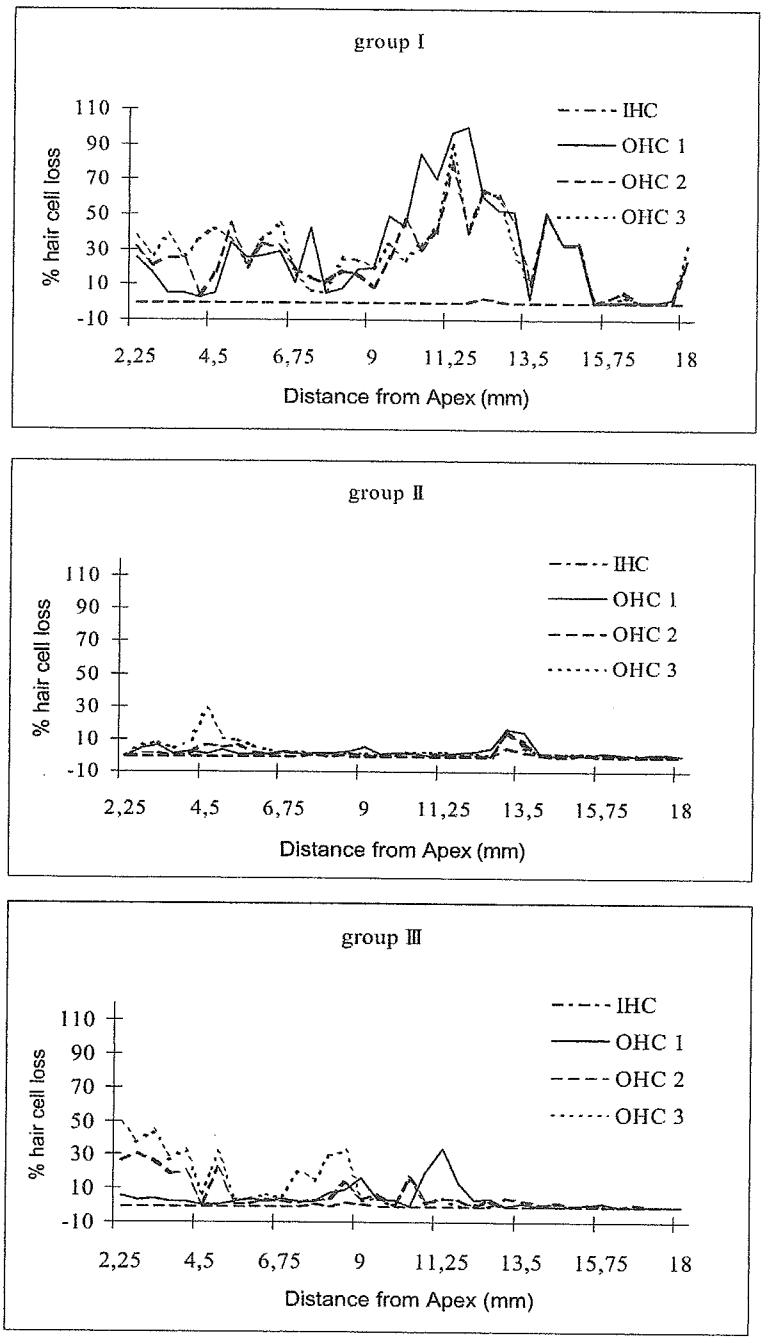

Fig. 2. Average cytocochleograms for the three groups. In group I, damage to the OHCs was greatest in the lower second and upper basal turns. There was less hair cell loss in the same region in groups II and III treated, respectively, with 10 and 30 mg/kg ebselen.

quencies, the threshold shifts were reduced by ebselen pre-treatment.

When groups II and III were compared, group III had consistently greater threshold shifts at all frequencies. These differences were statistically significant ($P < 0.01$) at 4, 8 and 16 kHz. Interestingly, the threshold shift was essentially stable in group III, whereas in group II the shift decreased with the increase in frequency.

3.3. Hair cell protection

Fig. 2 presents the average cytocochleograms for groups I, II and III, showing the average percentages of IHC and OHC loss in rows 1, 2 and 3. In group I, OHC damage is greatest in the region 9.0–15.5 mm from the apex (i.e. the lower second and upper basal turns). In this region, row 1 OHCs are the most severely damaged, followed by rows 2 and 3, whereas IHCs are

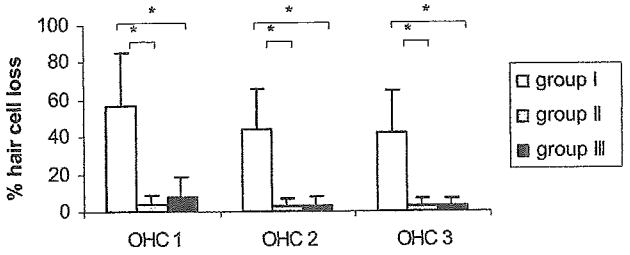

Fig. 3. Means and standard deviation of OHC loss in the region 9.0–15.5 mm from the apex. Asterisks indicate significant differences ($P < 0.01$) in group I ($n = 6$) versus groups II ($n = 5$) and III ($n = 5$). There was no significant difference between groups II and III at any test frequency.

well preserved. Such severe damage is not seen in groups II and III. In those groups, the average cytocochleograms for the ebselen-treated ears show reduced hair cell loss and lesion width.

Fig. 3 shows the extent of hair cell loss in OHC rows 1, 2 and 3 in groups I, II and III in the area 9.0–15.5 mm from the apex. Differences were statistically significant ($P < 0.01$) between groups I and II and I and III for the extent of OHC loss, whereas there was no significant difference between groups II and III.

3.4. Adverse effects of ebselen at high doses

Although ebselen was protective against acoustic trauma at both doses (10 and 30 mg/kg), threshold shifts were greater in animals treated with the higher dose. Additionally, in the apical area less than 9 mm from the apex there was greater hair cell loss for OHC rows 2 and 3 in group III than in group II. It was therefore necessary to determine whether 30 mg/kg of ebselen had adverse systemic effects or affected auditory function. ABRs measured 14 days after 30 mg/kg ebselen treatment without noise exposure showed virtually no threshold change at any frequency. Nor was there significant hair cell loss 15 days after ebselen treatment. These findings for group IV indicate that the higher ebselen dose had no adverse effects on guinea pig ears unexposed to noise.

4. Discussion

We found that ebselen (10 and 30 mg/kg) administered orally by gavage to guinea pigs attenuated noise-induced permanent threshold shifts and cochlear damage. Its protective effects on hearing function and hair cell survival were statistically significant for both concentrations. The higher dose, however, produced less attenuation of ABR threshold shifts and greater hair cell damage in the noise-exposed animals; however, it was not harmful to cochleas of control animals unexposed to noise. These findings suggest that in the guinea pig optimal protection by ebselen against noise-induced cochlear damage is obtained at about 10 mg/kg. In the presence of noise, high ebselen doses may harm the guinea pig cochlea.

The protection against oxidative challenge provided by ebselen is mainly due to the compound's ability to mimic GSH Px, which reacts with $ONOO^-$. A trio of endogenous enzymes (superoxide dismutase (SOD), catalase and GSH Px) cooperatively limits free radical toxicity (Siesjo et al., 1989). SOD catalyzes the dismutation of superoxide to hydrogen peroxide and then catalase and GSH Px subsequently degrades hydrogen peroxide to water. GSH Px also degrades organic hydroperoxides, such as the lipid hydroperoxides formed by free radical attack on membrane lipids, thereby further limiting free radical-mediated injury (Dawson et al., 1995). The therapeutic efficacy of these enzymes, however, is limited because of their rapid metabolization (Lunec, 1990) and high molecular weights.

Beckman et al. (1990) were the first to propose that when overproduced, NO tends to react with O_2^-, forming peroxynitrite. At the whole-organism level, the reactive chemistry of $ONOO^-$ is considered beneficial because of the radical's cytotoxicity to bacteria. Excessive production, however, mediates oxidation, nitration, or nitrosation reactions, leading to impaired function, toxicity and alterations in signaling pathways. The $ONOO^-$ oxidation reaction causes DNA damage, producing base modification and mutation as well as single- and double-strand breaks (Roussyn et al., 1996). Protein tyrosine nitration by peroxynitrite may interfere with phosphorylation/dephosphorylation signaling pathways (Kong et al., 1996) or alter protein function. For homogeneous systems, multiplication of the concentration of a given compound by the corresponding rate constant for its reaction with $ONOO^-$ gives the rate of disappearance of $ONOO^-$. Ebselen reacts very rapidly with $ONOO^-$, the second-order rate constant being $2 \times 10^6\ M^{-1}\ s^{-1}$ (Masumoto and Sies, 1996).

The reduced, but not the oxidized, form of ebselen effectively protects against peroxynitrite oxidation and nitration reactions. In fact, ebselen oxidation produces one or more covalent bindings of selenium with oxygen and decreases its protective action, apparently because of the decreasing nucleophilicity of the selenium atom (Sies et al., 1997). Prolonged hypoxia after noise exposure is therefore of physiological interest.

Multiple defense mechanisms against $ONOO^-$ exist in animals in vivo: prevention of $ONOO^-$ formation by control of NOS and NO levels by oxyhemoglobin, and control of O_2^- levels by SOD (Masumoto and Sies, 1996). Other compounds, such as ascorbate, cysteine, GSH and methionine, when present in sufficiently high concentrations, protect against damage by biomol-

ecules formed in reactions with $ONOO^-$ (Briviba et al., 1996). In previous studies, prior treatment with GSH-promoting drugs dramatically reduced the effects of noise. Hu et al. (1997) administered R-PIA topologically to the round window of the chinchilla and reported less permanent threshold shifts in R-PIA-treated ears, probably because of up-regulation of the endogenous antioxidant. GSH ester applied to the round window membrane (Hight et al., 2003) and L-NAC (Kopke et al., 2000) by intraperitoneal injection substantially prevented noise-induced hair cell loss by increasing intracellular GSH. It is difficult to determine whether ebselen is more or less effective in attenuating NIHL than other agents by a comparison of our findings with those of previous studies because experimental conditions, e.g. the animal species and paradigms of noise exposure, differ across the studies. In other systems, however, ebselen is reported to give approximately 100- to 1000-fold more protection against the oxidation and nitration reactions caused by $ONOO^-$ than do cysteine, GSH and methionine (Briviba et al., 1996).

A common reason for the many diverse effects reported is that ebselen, due to its being a GPx mimic, can lower the tissue peroxide tone (Helmer et al., 1979). This is important in the control of lipoxygenases and cyclooxygenases and leads to the diminution of oxidative injury (Parnham and Sies, 2000). Because peroxynitrite formation requires peroxide, its removal might be sufficient to attenuate noise trauma.

Takumida and Anniko (1998b) reported that 100 mg/kg ebselen administered by gavage to guinea pigs inhibited LPS-induced labyrinthitis. Interestingly, in our study the optimal dose of ebselen (10 mg/kg) required for protection against noise-induced cochlear damage was 10 times less than that cited in their study. NOS II is crucial to the development of LPS-induced vestibular disorders (Takumida and Anniko, 1998b). Enhanced NO production by NOS II and of O_2^-, resulting in subsequent $ONOO^-$ formation, may be an important cause of pathological damage to the cochlear end organs. The high dose of ebselen required to protect against LPS-induced cochlear damage may reflect high levels of free radicals generated by endotoxin rather than noise exposure.

Why 30 mg/kg ebselen provided less protection against noise-induced cochlear damage than 10 mg/kg in our study is not known. Dawson and co-workers (Dawson et al., 1995) assessed the neuroprotective efficacy of ebselen (10 and 30 mg/kg) in a rat model of transient focal ischemia. They found that the lower dose of ebselen (10 mg/kg) did not significantly reduce the extent of ischemic injury, whereas the higher one (30 mg/kg) did. In contrast, in a permanent middle cerebral artery occlusion study (Matsui et al., 1990), treatment with 10 mg/kg ebselen significantly reduced the infarct area, whereas 30 mg/kg did not alter it. Lynch et al. (2003) found that 16 mg/kg ebselen provided significant protection for rats developing PTS after both single and repeated noise exposures, but they also found that a larger dose gave less protection (Kil et al., personal communication). This may be because ebselen itself could have adverse effects on auditory function and general conditions. We therefore investigated whether there were adverse effects when 30 mg/kg of ebselen was administered to guinea pigs unexposed to noise, but found none. At 30 mg/kg, ebselen is probably not harmful to the cochlea.

The protective effect of ebselen depends on the presence of thiols such as GSH. GSH depletion is known to switch neurotrophic nitric oxide effects to cell death. Barker et al. (1996) reported that experimental depletion of GSH potentiates the toxicity of $ONOO^-$ in astrocytes. Reduction of the endogenous antioxidant GSH increases NIHL (Yamasoba et al., 1998a; Ohinata et al., 2000). The lesser protection provided by the higher dose of ebselen may be related to changes in GSH metabolism brought about by ebselen pre-treatment. Further study is needed to determine why the higher dose of ebselen gives less protection.

In group I, the extent of missing hair cells was very limited to the 4 and 8 kHz range, and the greatest threshold shifts were at 4 and 8 kHz. There were also significant threshold shifts at 2 and 16 kHz, where hair cell damage was very mild. A similar discrepancy between threshold shifts and the extent of hair cell loss was found in other experiments on guinea pigs under the same conditions of noise exposure (Yamasoba et al., 1999, 2001). Moreover, Liberman and Gao (1995) reported that guinea pigs with an approximately 40 dB hearing loss caused by 10 kHz narrow-band noise had minimal hair cell death. As for other species, Hunter-Duval and Elliot (1973) found no observable hair cell loss in squirrel monkey cochleas that showed threshold shifts greater than 40 dB, whereas Hamernik et al. (1989) reported that in chinchillas the absence of OHCs was a major contributor to the first 30–40 dB threshold shifts and that the loss of the OHCs was almost complete when permanent threshold shifts were more than 40 dB. These results suggest there is varied hair cell susceptibility to noise among animal species. In the current study, we frequently found stereocilia disarray, especially in the first-row OHCs following noise, as did Liberman and Gao (1995), but it was difficult to evaluate the extent of lesions quantitatively using surface preparations. In guinea pigs, this lesion may mainly account for the greater hearing threshold shifts versus the extent of missing hair cells. More subtle changes in the hair cells and other cochlear tissues, which we did not evaluate, may also have contributed to the noise-induced threshold shifts.

Hearing loss and pathological features of the inner ear exposed to 4 kHz OBN showed a significant difference in threshold shift between groups II and III, but no difference in the extent of hair cell loss. The degree of hair cell loss after noise trauma is sometimes difficult to correlate with the auditory threshold shift (Lataye and Campo, 1996; Liberman and Beil, 1979; Ades et al., 1974). In a previous study that used the same noise paradigm, there were significant noise-induced threshold shifts with no significant hair cell loss (Pourbakht and Yamasoba, 2003). Many of these threshold shifts are presumed to reflect damage to the stereocilia of sensory cells, ranging from floppy, disarrayed, broken tip links and broken roots to collapsed, fused and elongated stereocilia (Liberman and Kiang, 1978; Robertson et al., 1980; Slepecky and Chamberlain, 1982; Engstrom et al., 1983; Saunders and Flock, 1986; Tilney et al., 1982).

Fig. 1 shows the significant difference between the ebselen-treated and control ears at the higher frequency. Why ebselen was more effective at 16 kHz than at lower frequencies is not clear. Ebselen was administered orally by gavage; therefore we think it was distributed equally in all parts of the cochlea. One possible explanation may be found in the study by Sha et al. (2001), who reported different concentrations of GSH at the base and apex of the cochlea. There is a significantly less antioxidant GSH in basal OHCs than in apical ones. This suggests that basal OHCs are more sensitive to oxidative damage; therefore antioxidants should protect against hair cell loss. This is consistent with previous reports of the significantly higher survival of basal hair cells produced by L-NAC, GSH, mannitol or cysteine, and R-PIA.

Various studies have shown that NIHL is attenuated by ROS scavengers, but the majority of those scavengers have adverse systemic effects, making human clinical application impractical. For example, α-tocopherol has limited beneficial effects, owing to its co-oxidative destructive action (Schewe, 1995), but this adverse effect does not occur with ebselen. Randomized, placebo-controlled, double-blind clinical studies have shown that ebselen significantly enhances chances of a positive outcome for patients who experience occlusive cerebral ischemia of limited duration (Parnham and Sies, 2000). For human use, ebselen is the most promising of the known agents with antioxidative properties.

In conclusion, ebselen prevents cochlear damage produced by intense noise exposure. Its efficacy probably reflects ebselen's scavenging of $ONOO^-$. It is already used clinically to treat ischemic stroke in humans and has few or no side effects. Further studies of its action are needed, but ebselen appears to be an excellent candidate for the prevention and treatment of NIHL in humans.

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References

Ades, H.W., Trahiotis, C., Kokko-Cunningham, A., Averbuch, A., 1974. Comparison of hearing thresholds and morphological changes in the chinchilla after exposure to 4 kHz tones. Acta Otolaryngol. 78, 192–206.

Amaee, F.R., Comis, S., Osborne, M., Drew, S., Tarlow, M.J., 1997. Possible involvement of nitric oxide in the sensorineural hearing loss of bacterial meningitis. Acta Otolaryngol. (Stockh.) 117, 329–336.

Arteel, G.E., Briviba, K., Sies, H., 1999. Protection against peroxynitrite. FEBS Lett. 44, 226–230.

Barker, J.E., Bolanos, J.P., Land, J.M., Clark, J.B., Heales, S.J., 1996. Glutathione protects astrocytes from peroxynitrite-mediated mitochondrial damage: implications for neuronal/astrocytic trafficking and neurodegeneration. Dev. Neurosci. 18, 391–396.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. USA 87, 1620–1624.

Briviba, K., Roussyn, I., Sharov, V.S., Sies, H., 1996. Attenuation of oxidation and nitration reactions of peroxynitrite by selenomethionine, selenocystine and ebselen. Biochem. J. 319, 13–15.

Coyle, J.T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative diseases. Science 262, 689–695.

Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S., Snyder, S.H., 1991. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc. Natl. Acad. Sci. USA 88, 6368–6371.

Dawson, D.A., Masayasu, H., Graham, D.I., Macrae, I.M., 1995. The neuroprotective efficacy of ebselen (a glutathione peroxidase mimic) on brain damage induced by transient focal cerebral ischemia in the rat. Neurosci. Lett. 185, 65–69.

Engstrom, B., Flock, A., Borg, E., 1983. Ultrastructural studies of stereocilia in noise-exposed rabbits. Hear. Res. 12, 251–264.

Fessenden, J.D., Schacht, J., 1998. The nitric oxide/cyclic GMP pathway: a potential major regulator of cochlear physiology. Hear. Res. 118, 168–176.

Franz, P., Hauser-Kronberger, C., Böck, P., Quint, C., Baumgartner, W.D., 1996. Localization of nitric oxide synthase I and III in the cochlea. Acta Otolaryngol. (Stockh.) 116, 726–731.

Hamernik, R.P., Patterson, J.H., Turrentine, G.A., Ahroon, W.A., 1989. The quantitative relationship between sensory cell loss and hearing thresholds. Hear. Res. 38, 199–212.

Helmer, M.E., Cook, H.W., Lands, W.E., 1979. Prostaglandin biosynthesis can be triggered by lipid peroxides. Arch. Biochem. Biophys. 193, 340–345.

Hight, N.G., McFadden, S.L., Henderson, D., Burkard, R.F., Nicotera, T., 2003. Noise-induced hearing loss in chinchillas pre-treated with glutathione monoethylester and R-PIA. Hear. Res. 179, 21–32.

Hu, B.H., Zheng, X.Y., McFadden, S.L., Kopke, R.D., Henderson, D., 1997. R-phenylisopropyladenosine attenuates noise-induced hearing loss in the chinchilla. Hear. Res. 113, 198–206.

Hunter-Duval, I.M., Elliot, D.N., 1973. Effects of intense auditory

stimulation: hearing losses and inner ear changes in the squirrel monkey II. J. Acoust. Soc. Am. 54, 1179–1183.
Husain, K., Whitworth, C., Somani, S.M., Rybak, L.P., 2001. Carboplatin-induced oxidative stress in rat cochlea. Hear. Res. 159, 14–22.
Kong, S.K., Yim, M.B., Stadtman, E., Chock, P.B., 1996. Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2 (6-20) NH2 peptide. Proc. Natl. Acad. Sci. USA 93, 3377–3382.
Kopke, R.D., Liu, W., Gabaizadeh, R., Jacono, A., Feghali, J., Spray, D., Garcia, P., Steinman, H., Malgrange, B., Ruben, R.J., Rybak, L., Van de Water, T.R., 1997. Use of organotypic cultures of Corti's organ to study the protective effects of antioxidant molecules on cisplatin-induced damage of auditory hair cells. Am. J. Otol. 18, 559–571.
Kopke, R.D., Weisskopf, P.A., Boone, J.L., Jackson, R.L., Wester, D.C., Hoffer, M.E., Lambert, D.C., Charon, C.C., Ding, D.L., McBride, D., 2000. Reduction of noise-induced hearing loss using L-NAC and salicylate in the chinchilla. Hear. Res. 149, 138–146.
Lataye, R., Campo, P., 1996. Applicability of the L(eq) as a damage-risk criterion: an animal experiment. J. Acoust. Soc. Am. 99, 1621–1632.
Liberman, M.C., Gao, W.Y., 1995. Chronic cochlear de-efferentation and susceptibility to permanent acoustic injury. Hear. Res. 90, 158–168.
Liberman, M.C., Kiang, N.Y.S., 1978. Acoustic trauma in cats: cochlear pathology and auditory-nerve activity. Acta Otolaryngol. (Stockh.) Suppl. 358, 1–63.
Liberman, M.C., Beil, D.G., 1979. Hair cell condition and auditory nerve response in normal and noise-damaged cochleas. Acta Otolaryngol. (Stockh.) 88, 161–176.
Lunec, J., 1990. Free radicals: their involvement in disease processes. Ann. Clin. Biochem. 27, 173–182.
Lynch, E.D., Gu, R., Lagasse, J., Fulmer, C., Kil, J., 2003. Ebselen mediated protection from single and repeated noise exposure in rats. Abstr. Assoc. Res. Otolaryngol. Daytona Beach, FL.
Masumoto, H., Sies, H., 1996. The reaction of ebselen with peroxynitrite. Chem. Res. Toxicol. 9, 262–267.
Matsui, T., Johshita, H., Asano, T., Tanaka, J., 1990. The effect of a free radical scavenger, ebselen, on cerebral ischemia. In: Krieglstein, J. (Ed.), Pharmacology of Cerebral Ischemia. Wissenschaftliche Verlagsgesellschaft, Stuttgart, pp. 363–367.
Ohinata, Y., Yamasoba, T., Schacht, J., Miller, J.M., 2000. Glutathione limits noise-induced hearing loss. Hear. Res. 146, 28–34.
Ohlemiller, K.K., Wright, J.S., Dugan, L.L., 1999. Early elevation of cochlear reactive oxygen species following noise exposure. Audiol. Neurootol. 4, 229–236.
Parnham, M.J., Sies, H., 2000. Ebselen: prospective therapy for cerebral ischemia. Exp. Opin. Investig. Drugs. 9, 607–619.
Popa, R., Anniko, M., Takumida, M., Arnold, W., 2001. Localization of nitric oxide synthase isoforms in the human cochlea. Acta Otolaryngol. 121, 454–459.
Pourbakht, A., Yamasoba, T., 2003. Cochlear damage caused by continuous and intermittent noise exposure. Hear. Res. 178, 70–78.
Pujol, R., Puel, J.L., 1999. Excitotoxicity, synaptic repair, and functional recovery in the mammalian cochlea: a review of recent findings. Ann. N.Y. Acad. Sci. 884, 249–254.
Quirk, W.S., Shivapuja, B.G., Achwimmer, C.L., Seidman, M.D., 1994. Lipid peroxidation inhibitor attenuates noise-induced temporary threshold shifts. Hear. Res. 74, 217–220.
Robertson, D., Johnstone, B.M., McGill, T.J., 1980. Effects of loud tones on the inner ear: a combined electrophysiological and ultrastructural study. Hear. Res. 2, 39–53.
Roussyn, I., Briviba, K., Masumoto, H., Sies, H., 1996. Selenium-containing compounds protect DNA from single-strand breaks caused by peroxynitrite. Arch. Biochem. Biophys. 330, 216–218.
Rybak, L.P., Whitworth, C., Somani, S., 1999. Application of antioxidants and other agents to prevent cisplatin ototoxicity. Laryngoscope 109, 1740–1744.
Rybak, L.P., Hussain, K., Morris, C., Whitworth, C., Somani, S., 2000. Effect of protective agents against cisplatin ototoxicity. Am. J. Otol. 21, 513–520.
Saunders, J.C., Flock, A., 1986. Recovery of threshold shift in hair-cell stereocilia following exposure to intense stimulation. Hear. Res. 23, 233–243.
Schewe, C., Schewe, T., Wendel, A., 1994. Strong inhibition of mammalian lipoxygenases by the anti-inflammatory seleno-organic compound ebselen in the absence of glutathione. Biochem. Pharmacol. 48, 65–74.
Schewe, T., 1995. Molecular action of ebselen - an anti-inflammatory antioxidant. Gen. Pharmacol. 26, 1153–1169.
Seidman, M.D., Shivapuja, B.G., Quirk, W.S., 1993. The protective effects of allopurinol and superoxide dismutase on noise-induced cochlear damage. Otolaryngol. Head Neck Surg. 109, 1052–1056.
Sha, S.H., Taylor, R., Forge, A., Schacht, J., 2001. Differential vulnerability of basal and apical hair cells is based on intrinsic susceptibility to free radicals. Hear. Res. 155, 1–8.
Shi, X., Ren, T., Nuttall, A.L., 2002. The electrochemical and fluorescence detection of nitric oxide in the cochlea and its increase following loud sound. Hear. Res. 164, 49–58.
Sies, H., Sharov, V.S., Klotz, L.O., Birviba, K., 1997. Glutathione peroxidase protects against peroxynitrite-mediated oxidations: a new function for selenoproteins as peroxynitrite reductase. J. Biol. Chem. 272, 27812–27817.
Siesjo, B.k., Agardh, C.D., Bengtsson, F., 1989. Free radicals and brain damage. Cerebrovasc. Brain Metab. Rev. 1, 165–211.
Slepecky, N., Chamberlain, S.C., 1982. Distribution and polarity of actin in sensory hair cells of the chinchilla cochlea. Cell Tissue Res. 20, 245–260.
Tabuchi, K., Tsuji, S., Asaka, Y., Hara, A., Kusakari, J., 2001. Ischemia-reperfusion injury of the cochlea: effects of an iron chelator and nitric oxide synthase inhibitors. Hear. Res. 160, 31–36.
Takumida, M., Zhang, D.M., Anniko, M., 1997. Localization of nitric oxide synthase isoforms (I, II and III) in the endolymphatic sac of the guinea pig. ORL 59, 311–316.
Takumida, M., Anniko, M., 1988. Localization of nitric oxide synthase isoforms (I, II and III) in the vestibular end organs of the guinea pig. ORL 60, 67–72.
Takumida, M., Anniko, M., 1998b. Lipopolysaccharide-induced expression of nitric oxide synthase II in the guinea pig vestibular end organs. Eur. Arch. Otorhinolaryngol. 255, 184–188.
Takumida, M., Popa, R., Anniko, M., 1999. Free radicals in the guinea pig inner ear following gentamicin exposure. ORL 61, 63–70.
Takumida, M., Anniko, M., 2001. Direct evidence of nitric oxide production in the guinea pig organ of Corti. Acta Otolaryngol. (Stockh.) 121, 342–345.
Tilney, L.G., Sauunders, J.C., Egelman, E., DeRosier, D.J., 1982. Changes in the organization of actin filaments in the stereocilia of noise-damaged lizard cochlea. Hear. Res. 7, 181–197.
Yamane, H., Nakai, Y., Takayama, M., Iguchi, H., Nakagawa, T., Kojima, A., 1995. Appearance of free radicals in the guinea pig inner ear after noise-induced acoustic trauma. Eur. Arch. Otorhinolaryngol. 252, 504–508.
Yamasoba, T., Nuttall, A.L., Harris, C., Raphael, Y., Miller, J.M., 1998a. Role of glutathione in protection against noise-induced hearing loss. Brain Res. 784, 82–90.

Yamasoba, T., Harris, C., Shoji, F., Lee, R.J., Nuttall, A.L., Miller, J.M., 1998b. Influence of intense sound exposure on glutathione synthesis in the cochlea. Brain Res. 804, 72–78.

Yamasoba, T., Schacht, J., Shoji, F., Miller, J.M., 1999. Attenuation of cochlear damage from noise trauma by an iron chelator, a free radical scavenger and glial cell line-derived neurotrophic factor in vivo. Brain Res. 815, 317–325.

Yamasoba, T., Raphael, Y., Altschuler, R.A., Miller, A.L., Shoji, F., Miller, J.M., 2001. Absence of hair cell protection by exogenous acidic and basic fibroblast growth factors delivered to guinea pig cochlea in vivo. Noise Health 11, 65–78.

Yamauchi, M., Omote, K., Ninomiya, T., 1998. Direct evidence for the role of nitric oxide on glutamate-induced neuronal death in cultured cortical neurons. Brain Res. 780, 253–259.

Brief Communication

Math1 Gene Transfer Generates New Cochlear Hair Cells in Mature Guinea Pigs *In Vivo*

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Hair cell loss in the mammalian cochlea is irreversible and results in permanent hearing loss. *Math1*, the basic helix-loop-helix transcription factor homolog of the *Drosophila atonal* gene, is a positive regulator of hair cell differentiation during cochlear development. Developing hair cells express *Math1*, and nonsensory cells do not. We set out to determine the outcome of overexpression of *Math1* in nonsensory cells of the cochlea on the phenotype of these cells. We demonstrate that *in vivo* inoculation of adenovirus with the *Math1* gene insert into the endolymph of the mature guinea pig cochlea results in *Math1* overexpression in nonsensory cochlear cells, as evident from the presence of Math1 protein in supporting cells of the organ of Corti and in adjacent nonsensory epithelial cells. *Math1* overexpression leads to the appearance of immature hair cells in the organ of Corti and new hair cells adjacent to the organ of Corti in the interdental cell, inner sulcus, and Hensen cell regions. Axons are extended from the bundle of auditory nerve toward some of the new hair cells, suggesting that the new cells attract auditory neurons. We conclude that nonsensory cells in the mature cochlea retain the competence to generate new hair cells after overexpression of *Math1 in vivo* and that *Math1* is necessary and sufficient to direct hair cell differentiation in these mature nonsensory cells.

Key words: hair cell; guinea pig; regeneration; *Math1*; gene therapy; adenovirus; supporting cell

Introduction

The auditory sensory epithelium in the inner ear, the organ of Corti, is an epithelial mosaic made of hair cells and supporting cells. Hair cell loss may result from aging, excessive exposure to loud stimuli, bacterial and viral infections, or ototoxic drugs. Cellular renewal on the basis of stem (basal) cell proliferation is a hallmark of most epithelial tissues. However, the organ of Corti lacks basal cells, and the terminally differentiated auditory hair cells are not replaced once lost (Hawkins, 1973). Thus, cochlear hair cell loss leads to permanent hearing impairment, the most common sensory disorder in humans.

On the basis of data obtained in avian inner ears, differentiated supporting cells are able to change their phenotype and become new hair cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Supporting cells can generate new hair cells by transdifferentiation (Raphael, 1992; Stone and Cotanche, 1994) or by conversion of the phenotype without cell division (Adler and Raphael, 1996; Roberson et al., 1996). Supporting cells are therefore an attractive target for interventions designed to produce new hair cells.

The discovery of developmental genes that encode hair cell differentiation facilitates the design of interventions to promote generation of new hair cells in cochleae with hair cell loss. Basic helix-loop-helix (bHLH) transcription factors regulate the development of a variety of systems in vertebrates and invertebrates (Hutcheson and Vetter, 2001; Vetter and Brown, 2001). Mechanoreceptors, including hair cells, depend on bHLH genes for their differentiation (Bermingham et al., 1999; Leonard et al., 2002). Expression of the bHLH transcription factor *Math1*, the mouse homolog of the *Drosophila* gene *atonal*, is essential for generating hair cells (Bermingham et al., 1999; Zine et al., 2001; Chen et al., 2002). After maturation of hair cells, the expression of *Math1* is downregulated (Zheng et al., 2000). Overexpression of *Math1* in cultures of immature rat cochleae results in the production of ectopic hair cells derived from nonsensory epithelial precursors (Zheng and Gao, 2000). The outcome of *Math1* overexpression in the mature inner ear has not been determined. We set out to determine the influence of *Math1* overexpression on the phenotype of supporting cells in the mature cochlea *in vivo*. We demonstrate that, after viral-mediated gene transfer of *Math1*, nonsensory epithelial cells in the mature cochlea express the transgene and retain the competence to generate new hair cells *in vivo*. We also show that some of the new hair cells generated after the *Math1* gene transfer attract auditory neurons.

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Materials and Methods

Adenovirus vectors. The *Math1* cDNA used for the construct was obtained from Huda Zoghbi (Baylor College of Medicine, Houston, TX). The three vectors, Ad.*Math1*.11D, Ad.*LacZ*, and adenovirus with no gene insert, were based on human adenovirus serotype 5 with E1, E3, and E4 regions deleted, as described previously (Brough et al., 1996). Expression of the transgene insert in each of these vectors was driven by the human cytomegalovirus promoter.

Animals and inoculation surgery. We used young adult guinea pigs (4–5 weeks of age) weighing 350–500 gm at the beginning of the experiment. Inoculation surgery and composition of artificial endolymph were essentially as described by Ishimoto et al. (2002), except that the viral vector (or control) solutions were inoculated using an electromechanical infusion pump (Harvard Apparatus, Holliston, MA). The inoculation procedure was performed once (for every animal), serving as a tool for inducing lesion and delivering Ad.*Math1*.11D or control solutions. Animal care and use were in accordance with National Institutes of Health and institutional guidelines. Animals used to detect transgene expression were killed 4 d after inoculation ($n = 6$ for Math1; $n = 5$ for β-galactosidase). Animals used to assess for new hair cells were examined using scanning electron microscopy or myosin VIIa antibody. These animals were killed 30 d ($n = 5$ for scanning electron microscopy; $n = 4$ for myosin VIIa) or 60 d ($n = 9$ for scanning electron microscopy; $n = 5$ for myosin VIIa) after the inoculation. Neurofilament staining was performed on normal animals, as well as those killed 60 d after Ad.*Math1*.11D or control vector inoculation. At least three animals were used for each control group.

Scanning electron microscopy. Guinea pigs were anesthetized and transcardially perfused with saline, followed by 2% glutaraldehyde in cacodylate buffer (0.15 M). Cochleae were processed for scanning electron microscopy using the osmium thiocarbohydrazide method (Osborne and Comis, 1991). Samples were then dehydrated, and the critical point was dried with CO_2 in a SamDri-790 (Tousimis, Rockville, MD), mounted on stubs using silver paste, and photographed digitally using a Philips XL30 Field-Emission Gun scanning electron microscope (FEI, Hillsboro, OR).

Immunocytochemistry. Whole mounts of the auditory sensory epithelium and surrounding tissues were used to localize Math1, myosin VIIa, and neurofilament. We fixed cochleae in 4% paraformaldehyde in phosphate buffer, pH 7.4, removed the spiral ligament, stria vascularis, and tectorial membrane, and then permeabilized the tissue with 0.3% Triton X-100 in PBS for 10 min. Nonspecific binding of secondary antibodies was blocked with 5% BSA in PBS for 20 min. Tissues were reacted with primary antibody, rinsed, and incubated with the secondary antibody. To perform double staining of neurofilaments and myosin VIIa, we used FITC secondary antibody for neurofilaments and tetramethylrhodamine isothiocyanate (TRITC) fluorescence for myosin VIIa. To double stain for F-actin, we used FITC-conjugated phalloidin (1:400; Molecular Probes, Junction City, OR). Specimens were mounted on glass slides using CrystalMount (Biomeda, Foster City, CA). Cryosections of the organ of Corti and surrounding cochlear epithelium were used to localize β-galactosidase and Math1. Cryosections were obtained as described by Ishimoto et al. (2002) and immuno-stained as described above.

Primary antibodies were a rabbit polyclonal anti-myosin VIIa antibody (a gift from Tama Hasson, University of California San Diego, San Diego, CA) diluted 1:200 in PBS with 0.1% BSA for 1 hr, a rabbit polyclonal anti-Math1 (a gift from Jane Johnson, The University of Texas Southwestern Medical Center at Dallas, Dallas, TX) diluted 1:200, a monoclonal antibody against neurofilament 200 kDa (Sigma, St. Louis, MO) diluted 1:200 in PBS for 1 hr, and a rabbit polyclonal against β-galactosidase (Chemicon, Temecula, CA) used as described by Ishimoto et al. (2002). Secondary antibodies were TRITC-conjugated goat anti-rabbit or anti-mouse (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in PBS.

Specimens were examined and recorded using a Leica DMRB epifluorescence microscope (Leica, Eaton, PA) using 40 and 100× oil objectives and a CCD Cooled SPOT-RT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Results

Nonsensory cochlear cells express transgenes

To insert genes into nonsensory cells in the cochlea, we used adenovirus vectors. We constructed an adenoviral vector designated Ad.*Math1*.11D, with the *Math1* cDNA insert, as described previously (Brough et al., 1996). Ad.*Math1*.11D or controls (artificial endolymph, an adenovirus vector with no gene insert or reporter gene vector designated Ad.*LacZ*) were surgically injected into the cochlear endolymph of the left inner ear in mature guinea pigs. Experimental animals had not undergone any treatment before the inoculation. The injected volume (5 μl) was larger than the total volume of endolymph in guinea pigs (Thorne et al., 1999) and therefore resulted in a mechanical trauma, injuring some hair cells and causing degeneration of others (Fig. 1*a–c*). The inoculation procedure was performed once (for every animal), serving as a tool for inducing lesion and delivering Ad.*Math1*.11D or control solutions. The lesion caused by the inoculation was similar in *Math1*-treated animals (Fig. 1*b,c*) and controls (Fig. 2*e*) and appeared milder in areas more remote from the site of inoculation. The lesion was most severe at the site of inoculation, where most hair cells were eliminated and the Hensen cells area appeared hypertrophied (Fig. 3*e,i*).

To detect reporter gene expression, we killed the animals 4 d after Ad.*LacZ* inoculation and analyzed cochlear cryosections. At the site of inoculation, Ad.*LacZ* transgene expression was found in several cell types in the epithelium, including supporting cells of the organ of Corti and adjacent epithelial cells that reside lateral or medial to the organ of Corti (Fig. 1*a*). These epithelial cells included Hensen cells and cells in the inner sulcus and interdental cell regions (Figs. 1*a*, 4).

We assessed the extent of *Math1* transgene expression using a Math1-specific antibody in cochleae processed 4 d after Ad.*Math1*.11D inoculation. Numerous cells in the third turn of Ad.*Math1*.11D-inoculated animals were Math1 positive in the organ of Corti and in adjacent regions, including Hensen cells, inner sulcus areas (Fig. 1*b*), and the interdental cell area (data not shown). Most Math1-positive cells were within the normal boundaries of the organ of Corti (Fig. 1*b,d*). To better localize Math1-positive cells and distinguish hair cells from supporting cells or scars (sites of missing hair cells), cochleae were double stained with FITC phalloidin (Fig. 1*b,c*). We determined that most Math1-positive cells were nonsensory cells (Fig. 1*c*).

We used cryosections to localize Math1-positive cells in the fourth (apical) and second cochlear turns, flanking the site of inoculation. We determined that the extent of lesion decreased in areas distant from the inoculation site, with most hair cells surviving (Fig. 1*d,e*). Many nonsensory epithelial cells were Math1 positive, whereas most hair cells were Math1 negative (Fig. 1*d,e*). Control-inoculated cochleae were Math1 negative (Fig. 1*f,g*), demonstrating the absence of Math1 expression in the mature cochlea. These data demonstrate robust and efficient expression of *Math1* in nonsensory cells of the auditory epithelium after Ad.*Math1*.11D inoculation into the third-turn endolymph.

New and immature hair cells in the cochlea

To assess the surface morphology of the cochlear epithelium, we performed scanning electron microscopy analysis in the inner ears obtained from animals killed 30 or 60 d after the inoculation. After Ad.*Math1*.11D inoculation, we observed hair cells adjacent to the organ of Corti, in which hair cells are typically absent (Fig.

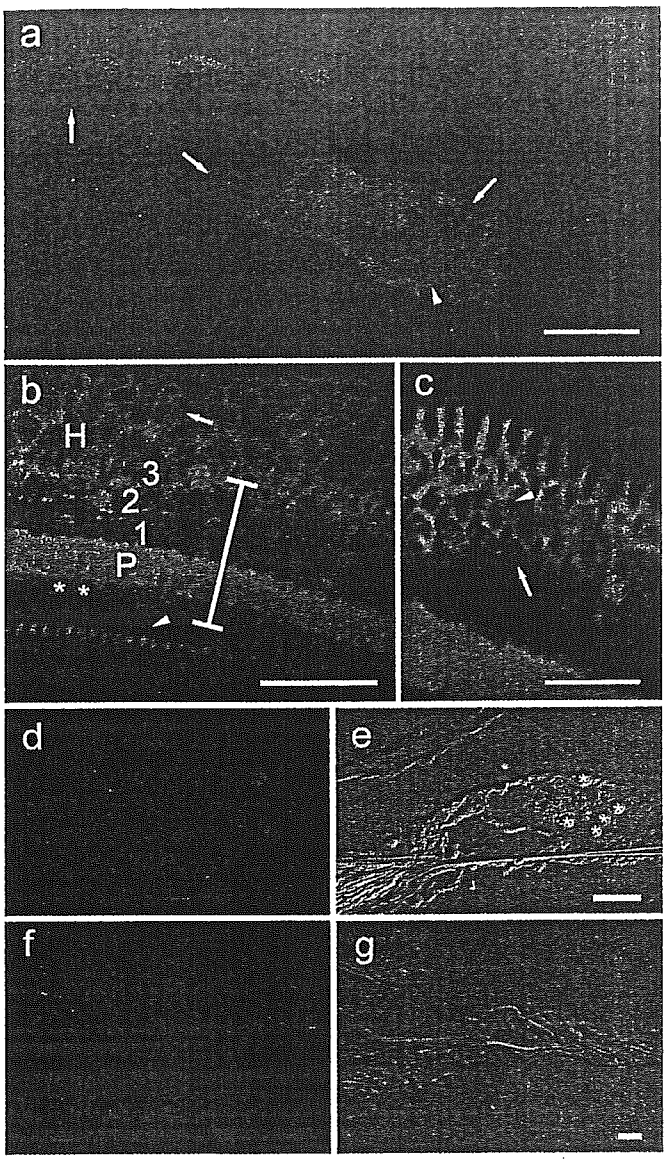

Figure 1. Epifluorescence of β-galactosidase and Math1 in the cochlear epithelium 4 d after Ad.*Math1*.11D inoculation. *a*, A cryosection showing β-galactosidase immunoreactivity in interdental (left arrow), inner sulcus (middle arrow), Hensen (right arrow), and supporting cells of the organ of Corti (arrowhead) in a cryosection of the third cochlear turn. *b*, A whole mount showing that Math1-positive nuclei (red) are in the inner sulcus (arrowhead), organ of Corti (vertical bar spans organ of Corti area; asterisk depicts inner hair cells; P depicts pillar cells; 1, 2, and 3 are first, second, and third row outer hair cells, respectively), and in the Hensen cell area (*H*) in which Math1-positive nuclei (arrow) are observed >30 μm outside the organ of Corti. Phalloidin staining (green) identifies surviving hair cells and sites of hair cell loss. *c*, Remaining hair cells adjacent to the inoculation site (phalloidin stain, green) are Math1 negative (arrowhead points to third row, outer hair cells). Some of the nonsensory cells that replaced lost hair cells (arrow in second row, outer hair cell area) are Math1 positive (red). *d*, *e*, Cryosection (*d*) of second turn of Ad.*Math1*.11D-inoculated organ of Corti showing Math1 immunoreactivity in nuclei of nonsensory cells (*). Outer hair cells (+) and several other cell types are negative. Nomarski optics image of same cryosection (*e*) identified cells shown in *d*. *f*, *g*, Cryosection of second-turn auditory epithelium of Ad.*LacZ*-inoculated cochlea. Math1 immunoreactivity is negative (*f*). Nomarski optics image of same cryosection (*g*) identifies cells. Scale bars: *a*, *b*, 50 μm; *c*, *d*–*g*, 25 μm.

3) (for schematic, see Fig. 4). The most remote area that contained ectopic hair cells was the interdental cell region (Fig. 3*a*–*c*). The morphology of some ectopic hair cells appeared similar to normal mature hair cells (Fig. 3*c*). Typically, approximately one-third of the ectopic hair cells exhibited a well differentiated surface morphology. Within the organ of Corti, we detected hair cells with an immature appearance (Fig. 3*d*). No immature-looking hair cells were observed in any of the control-inoculated cochleae (data not shown).

In most Ad.*Math1*.11D-inoculated cochleae, the number of hair cells with an immature appearance was between 25 and 50. However, we could not reliably distinguish between old (preexisting) and new hair cells within the boundaries of the organ of Corti. In the inner sulcus area, which resides immediately medial to the organ of Corti (Fig. 4), we observed some immature hair cells with short stereocilia (Fig. 3*e*,*f*) and some hair cells with longer stereocilia (Fig. 3*g*,*h*). Ectopic hair cells were also found in the Hensen cell area, immediately lateral to the organ of Corti (Figs. 3*i*,*j*, 4).

All Ad.*Math1*.11D-inoculated animals assessed with scanning electron microscopy exhibited new hair cells ($n = 14$). In five animals killed 60 d after the inoculation, the number of ectopic cells varied from 2 to 10 per cochlea. In four animals killed 30 d after Ad.*Math1*.11D inoculation, we observed two to nine ectopic cells per cochlea. Cells observed to have features typical of hair cells in ectopic locations were not counted if there was any doubt as to their phenotypic identification. Cochleae receiving control inoculations did not exhibit any ectopic hair cells (data not shown; $n = 12$). These data demonstrate that nonsensory cells in the mature mammalian cochlea retain the competence to generate hair cells after viral-mediated overexpression of *Math1*.

New hair cells express myosin VIIa

We also characterized the phenotype of new hair cells with antibodies against myosin VIIa, a hair cell-specific marker (Hasson et al., 1995). In normal (noninoculated) ears (Fig. 2*f*), myosin VIIa antibody stains inner and outer hair cells (Hasson et al., 1995). In all of the cochleae that were inoculated with Ad.*Math1*.11D and processed for myosin VIIa immunocytochemistry after 60 d, myosin VIIa-positive cells were found in the organ of Corti and adjacent areas, including the inner sulcus (data not shown), interdental cell (Fig. 2*a*), and Hensen cell areas (Fig. 2*b*). The number of myosin VIIa-positive cells in ectopic sites was similar to that seen in scanning electron microscopy preparations of Ad.*Math1*.11D-inoculated ears ranging from 4 to 10 per cochlea. Expression of *Myo7a* in these cells also identified them as hair cells, consistent with the scanning electron microscopy images.

New hair cells attract neurons

To assess for the presence of axons in the vicinity of new ectopic hair cells, we double stained control-inoculated and Ad.*Math1*.11D-treated cochleae (time point, 60 d) with antibodies to myosin VIIa and neurofilament. Assessment of double-stained cochlear whole mounts revealed that neurofilament staining within the cochlear epithelium was restricted to the organ of Corti in noninoculated cochleae (Fig. 2*f*) and control-inoculated cochleae (Fig. 2*e*), with no axons in the regions of Hensen cells, inner sulcus, and interdental cells. In contrast, in Ad.*Math1*.11D-treated tissues, long and slender neurofilament-stained fibers extended over 50 μm from the organ of Corti toward some myosin VIIa-labeled ectopic hair cells in the interdental cell area (Fig. 2*c*) and toward the Hensen cell area (Fig. 2*d*), suggesting that axons grow toward newly formed ectopic hair cells in the cochlea. Nerve processes have been shown to remain in the area of the traumatized organ of Corti long after hair cells are lost (Strominger et al., 1995). Our data suggest that, when given a new target (a new hair cell), some of these axons will extend and grow toward it.

Discussion

Plasticity and the potential for repair are commonly found in developing tissues. In explants of developing rat cochleae, Math1 was sufficient to produce extra hair cells via phenotypic conversion of nonsensory cells (Zheng and Gao, 2000). Plasticity and the ability to repair injuries during development do not usually persist into adulthood. However, our *in vivo* data indicate that nonsensory cochlear cells maintain their competence to become new hair cells in mature animals, and that *Math1* is a potent transcription factor that induces the nonsensory cochlear cells to generate new hair cells. Thus, Math1 appears sufficient to activate the cellular program, leading mature differentiated cells to recapitulate development.

Using immunocytochemistry with Math1-specific antibodies, we demonstrate that mature hair cells downregulate *Math1* expression. This finding is in agreement with reverse transcription-PCR data showing downregulation of *Math1* in the mature rat cochlea (Zheng et al., 2000) and with the transient developmental expression seen with other bHLH transcription factor genes, such as *Math5* (Brown et al., 1998). These data demonstrate that Math1-positive cells in the inoculated cochleae express the transgene rather than the endogenous *Math1*. Therefore, the results implicate transgenic *Math1* expression in nonsensory cells in signaling the generation of new hair cells. A causative relationship between *Math1* overexpression and new hair cell production is also demonstrated by the findings that all *Math1*-treated ears displayed new hair cells, whereas no new hair cells were found in any of the control-treated ears.

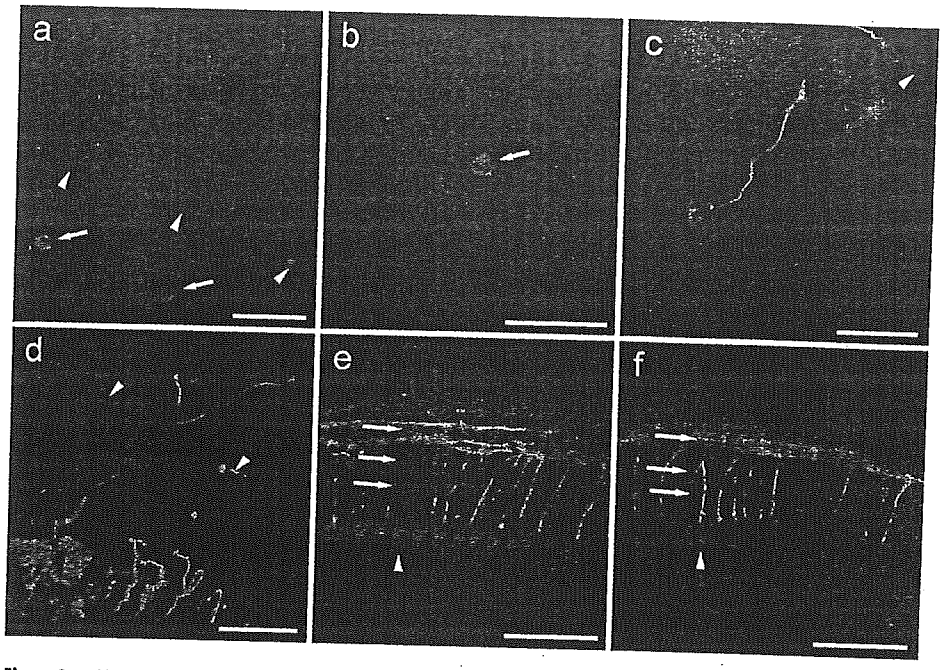

Figure 2. Myosin VIIa and neurofilaments in Ad.*Math1*.11D-treated and normal cochleae. *a*, Myosin VIIa-positive ectopic hair cells (arrows) among interdental cells. Inner hair cells (arrowheads) mark the medial border of the organ of Corti. *b*, A myosin VIIa-positive hair cell (arrow) among Hensen cells. *c*, An ectopic myosin VIIa-positive hair cell (red) in the interdental cell region. An axon (green) extends from the organ of Corti (arrowhead) to ectopic hair cell. *d*, An axon (green) extends laterally into the Hensen cell region (arrowheads, lateral border of organ of Corti). *e*, Myosin VIIa and axons in the organ of Corti (cochlear area similar to that shown in Fig. 1*b*, adjacent to the inoculation site) 60 d after artificial endolymph inoculation. Inner (arrowhead) and outer (arrows) hair cells are myosin VIIa positive. Several outer hair cells are missing. *f*, Neurofilament staining is restricted to the organ of Corti in normal (noninoculated) cochlea. Myosin VIIa (red) is in inner (arrowhead) and outer (arrows) hair cells. Micrographs are oriented with medial (modiolar) side down. Scale bars, 25 μm.

Regenerated hair cells within the organ of Corti are likely to contribute more than ectopic cells toward recovery of hearing. Nevertheless, the potential functional contribution of ectopic hair cells should not be overlooked. Ectopic hair cells in the inner sulcus are adjacent to inner hair cells. Similar to inner hair cells, the neighboring ectopic cells are situated on a part of the basilar membrane that is not free to vibrate (Slepecky, 1996). The luminal fluid movements that generate receptor potentials by deflecting stereocilia of inner hair cells (the primary auditory hair cells) may also stimulate ectopic hair cells. Thus, provided that ectopic cells differentiate and receive innervation, they may contribute to cochlear function.

Most Math1-positive cells that were identified in cochlea 4 d after *Math1* inoculation were within the normal boundaries of the organ of Corti. Surface analysis 2 months later revealed numerous cells with surface morphology resembling immature hair cells within the organ of Corti. Although we cannot unequivocally identify these cells as new hair cells, the absence of such immature cells in control-treated cochleae suggest that they are regenerated hair cells induced by *Math1* overexpression. Future experiments using *Math1* overexpression in cochleae that are completely depleted of their original hair cells may help identify new hair cells within the organ of Corti.

In *Math1* null mice, the auditory sensory primodrium and supporting cells develop normally, but hair cells are not generated (Bermingham et al., 1999; Chen et al., 2002). Similarly, mice with mutations in *Brn-3c*, a likely postranscriptional target of *Math1* (Vetter and Brown, 2001; Leonard et al., 2002), fail to develop cochlear hair cells (Erkman et al., 1996). Mutations in the human homolog *POU4F3* cause hereditary deafness (Vahava et al., 1998). Together with these previous reports, our data suggest that *Math1* is necessary and sufficient to direct hair cell differentiation in the cochlea and may act as a master switch for hair cell differentiation via transcriptional activation of *POU4F3* and potentially other genes.

The bundles of stereocilia on most ectopic hair cells did not reach a level of maturity seen on normal hair cells 2 months after Ad.*Math1*.11D inoculation. It is possible that a longer period of time is required for bundle maturation. However, it is likely that the extracellular environment and cell–cell communication in ectopic locations cannot support the formation of completely normal bundles. As such, ectopic hair cells may be experimentally useful for elucidating the requirements for normal hair cell differentiation.

In birds, nonsensory cells of the auditory epithelium spontaneously generate new hair cells after experimentally induced trauma (Corwin and Cotanche, 1988; Ryals and Rubel, 1988). Chick hair cell regeneration can occur via a mitotic (transdifferentiation) or nonmitotic (conversion) mechanism (Adler and Raphael, 1996; Roberson et al., 1996; Stone and Rubel, 2000). It is unclear whether the present results using *Math1* overexpression involve generation of new hair cells via transdifferentiation or conversion. Newly generated hair cells in the avian basilar papilla often appear in pairs (Raphael, 1992). Although we cannot rule

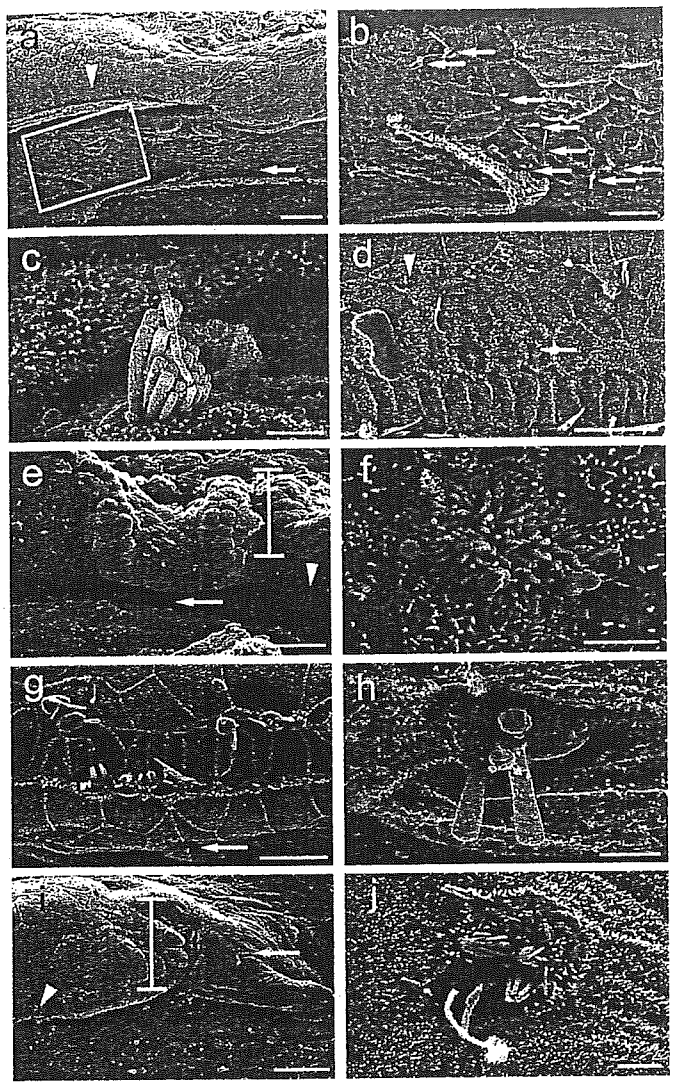

Figure 3. Scanning electron microscopy of cochleae after Ad.*Math1*.11D treatment. *a*, An interdental cell area (boxed) with several ectopic hair cells medial to the organ of Corti (arrowhead). *b*, Box in *a* enlarged to show ectopic hair cells (arrows). *c*, Higher magnification of ectopic hair cell in interdental cell area (*a*, arrow) with a well developed stereocilia bundle. *d*, Inoculation-lesioned organ of Corti exhibits cells with short stereocilia (arrow) and small hair cells (arrowhead). Micrographs are oriented with medial (modiolar) side down. *e*, The site of inoculation showing the injured organ of Corti (arrowhead) and Hensen cells (bar). *f*, An ectopic hair cell with short stereocilia in the inner sulcus (*e*, arrow). *g*, Hair cells in the organ of Corti distant from the inoculation site are missing or injured. *h*, An ectopic hair cell in the inner sulcus (*g*, arrow). *i*, Lateral to the organ of Corti (arrowhead), Hensen cells (bar) exhibit an ectopic hair cell (arrow). *j*, Higher magnification of ectopic hair cell depicted in *i*. Micrographs are oriented with medial (modiolar) side down. Scale bars: *a*, *e*, *i*, 50 μm; *b*, *d*, *g*, 20 μm; *c*, *f*, *h*, *j*, 2 μm.

out a proliferative mechanism, the occurrence of single ectopic hair cells, rather than pairs, in our study lends support to a conversion mechanism. The developmental role of *Math1* as a differentiation factor also supports a conversion mechanism for the regenerative process in the mature animal.

Our data raise several issues regarding the potential for use of *Math1* gene therapy for restoring hearing. First, the inoculation into the endolymph damages the organ of Corti. Candidates for inner ear gene therapy in the future are likely to have preexisting severe hair cell lesions, making the adverse effects of this procedure less troubling. It is also likely that vector inoculation into the larger human cochleae would elicit less mechanical trauma compared with that seen in guinea pigs. Second, in severely traumatized cochleae, supporting cells often become dedifferentiated and appear like cells in the inner sulcus (Leake and Hradek, 1988), making them candidate precursors for efficient generation of new hair cells.

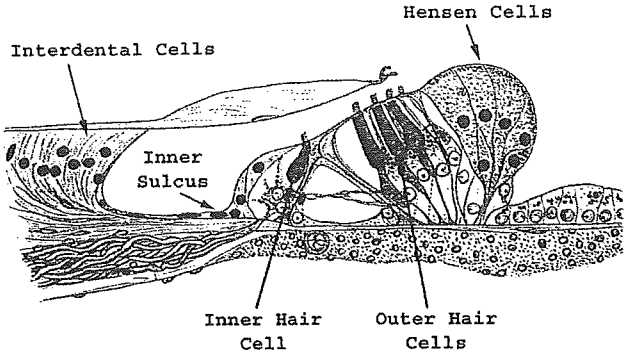

Figure 4. Math1-positive nuclei and ectopic hair cells. The schematic of the organ of Corti is oriented similar to the mid-modiolar cross sections in Figure 1, *a* and *d–g*, in which medial is on left and lateral is on right. The epithelial regions that exhibit Math1-positive nuclei include the organ of Corti (yellow) and ectopic areas adjacent to the organ of Corti (red). Ectopic new hair cells were identified in the interdental cell, inner sulcus, and Hensen cell regions.

Innervation of the new hair cells would be a prerequisite for restoring hearing. The ability of new hair cells to receive new nerve terminals has been demonstrated in the regenerating avian basilar papilla (Ofsie and Cotanche, 1996; Wang and Raphael, 1996). Our data showing axonal extension toward new ectopic hair cells suggest that new hair cells can provide signals to attract axons and that neurons can respond to these signals and extend toward the new hair cells. Because some of the ectopic hair cells did not have a neuron in their vicinity at the time points studied in our experiments, we conclude that new hair cells develop independently of neurons, as shown previously during cochlear development (Fritzsch et al., 1999). Longer survival times after *Math1* overexpression may be needed to allow more new hair cells to receive axonal connections.

In conclusion, we show that new hair cells are generated after *Math1* overexpression via an adenovirus vector in the mature mammalian cochlea. The new hair cells exhibit the typical surface morphology of hair cells and stain for the hair cell-specific protein myosin VIIa. The new hair cells are ectopically positioned and able to attract auditory nerve fibers, raising the possibility that they may be functional. This is the first *in vivo* induction of new hair cell generation in the mammalian cochlea and the first success in inducing regeneration in any tissue in which spontaneous cell replacement does not occur. The ability to generate hair cells in the mammalian organ of Corti may lead to treatments for sensorineural deafness and the development of methods for inducing regeneration and innervation in other organs.

References

Adler HJ, Raphael Y (1996) New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear. Neurosci Lett 205:17–20.

Bermingham NA, Hassan BA, Price SD, Vollrath MA, Ben-Arie N, Eatock RA, Bellen HJ, Lysakowski A, Zoghbi HY (1999) Math1: an essential gene for the generation of inner ear hair cells. Science 284:1837–1841.

Brough DE, Lizonova A, Hsu C, Kulesa VA, Kovesdi I (1996) A gene transfer vector-cell line system for complete functional complementation of adenovirus early regions E1 and E4. J Virol 70:6497–7501.

Brown NL, Kanekar S, Vetter ML, Tucker PK, Gemza DL, Glaser T (1998) Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. Development 125:4821–4833.

Chen P, Johnson JE, Zoghbi HY, Segil N (2002) The role of Math1 in inner ear development: Uncoupling the establishment of the sensory primordium from hair cell fate determination. Development 129:2495–2505.

Corwin JT, Cotanche DA (1988) Regeneration of sensory hair cells after acoustic trauma. Science 240:1772–1774.

Erkman L, McEvilly RJ, Luo L, Ryan AK, Hooshmand F, O'Connell SM, Keithley EM, Rapaport DH, Ryan AF, Rosenfeld MG (1996) Role of transcription factors Brn-3.1 and Brn-3.2 in auditory and visual system development. Nature 381:603–606.

Fritzsch B, Pirvola U, Ylikoski J (1999) Making and breaking the innervation of the ear: neurotrophic support during ear development and its clinical implications. Cell Tissue Res 295:369–382.

Hasson T, Heintzelman MB, Santos-Sacchi J, Corey DP, Mooseker MS (1995) Expression in cochlea and retina of myosin VIIa, the gene product defective in Usher syndrome type 1B. Proc Natl Acad Sci USA 92:9815–9819.

Hawkins Jr JE (1973) Comparative otopathology: aging, noise, and ototoxic drugs. Adv Otorhinolaryngol 20:125–141.

Hutcheson DA, Vetter ML (2001) The bHLH factors Xath5 and XNeuroD can upregulate the expression of XBrn3d, a POU-homeodomain transcription factor. Dev Biol 232:327–338.

Ishimoto S, Kawamoto K, Kanzaki S, Raphael Y (2002) Gene transfer into supporting cells of the organ of Corti. Hear Res 173:187–197.

Leake PA, Hradek GT (1988) Cochlear pathology of long term neomycin induced deafness in cats. Hear Res 33:11–33.

Leonard JH, Cook AL, Van Gele M, Boyle GM, Inglis KJ, Speleman F, Sturm RA (2002) Proneural and proneuroendocrine transcription factor expression in cutaneous mechanoreceptor (Merkel) cells and Merkel cell carcinoma. Int J Cancer 101:103–110.

Ofsie MS, Cotanche DA (1996) Distribution of nerve fibers in the basilar papilla of normal and sound-damaged chick cochleae. J Comp Neurol 370:281–294.

Osborne MP, Comis SD (1991) Preparation of inner ear sensory hair bundles for high resolution scanning electron microscopy. Scanning Microsc 5:555–564.

Raphael Y (1992) Evidence for supporting cell mitosis in response to acoustic trauma in the avian inner ear. J Neurocytol 21:663–671.

Roberson DW, Kreig CS, Rubel EW (1996) Light microscopic evidence that direct transdifferentiation gives rise to new hair cells in regenerating avian auditory epithelium. Aud Neurosci 2:195–205.

Ryals BM, Rubel EW (1988) Hair cell regeneration after acoustic trauma in adult Coturnix quail. Science 240:1774–1776.

Slepecky NB (1996) Cochlear structure. In: The cochlea (Dallos P, Popper AN, Fay R, eds), pp 44–129. New York: Springer.

Stone JS, Cotanche DA (1994) Identification of the timing of S phase and the patterns of cell proliferation during hair cell regeneration in the chick cochlea. J Comp Neurol 341:50–67.

Stone JS, Rubel EW (2000) Cellular studies of auditory hair cell regeneration in birds. Proc Natl Acad Sci USA 97:11714–11721.

Strominger RN, Bohne BA, Harding GW (1995) Regenerated nerve fibers in the noise-damaged chinchilla cochlea are not efferent. Hear Res 92:52–62.

Thorne M, Salt AN, DeMott JE, Henson MM, Henson Jr OW, Gewalt SL (1999) Cochlear fluid space dimensions for six species derived from reconstructions of three-dimensional magnetic resonance images. Laryngoscope 109:1661–1668.

Vahava O, Morell R, Lynch ED, Weiss S, Kagan ME, Ahituv N, Morrow JE, Lee MK, Skvorak AB, Morton CC, Blumenfeld A, Frydman M, Friedman TB, King MC, Avraham KB (1998) Mutation in transcription factor POU4F3 associated with inherited progressive hearing loss in humans. Science 279:1950–1954.

Vetter ML, Brown NL (2001) The role of basic helix-loop-helix genes in vertebrate retinogenesis. Semin Cell Dev Biol 12:491–498.

Wang Y, Raphael Y (1996) Re-innervation patterns of chick auditory sensory epithelium after acoustic overstimulation. Hear Res 97:11–18.

Zheng JL, Gao WQ (2000) Overexpression of Math1 induces robust production of extra hair cells in postnatal rat inner ears. Nat Neurosci 3:580–586.

Zheng JL, Shou J, Guillemot F, Kageyama R, Gao WQ (2000) Hes1 is a negative regulator of inner ear hair cell differentiation. Development 127:4551–4560.

Zine A, Aubert A, Qiu J, Therianos S, Guillemot F, Kageyama R, de Ribaupierre F (2001) Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. J Neurosci 21:4712–4720.

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DIABETES RESEARCH AND CLINICAL PRACTICE

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A case showing an association between type 1 diabetes mellitus and Kabuki syndrome

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Abstract

The case of a 31-year-old female suffering from type 1 diabetes mellitus (DM) and Kabuki syndrome is presented. The patient was diagnosed as having impaired glucose tolerance (IGT) at age 18; secondary amenorrhea occurred at age 20, following acute body weight loss. Extensive examination revealed the patient to have a slowly progressive form of type 1 DM and, based on the physical findings, including her facial features, she was diagnosed as also having congenital Kabuki syndrome. Since then, this patient has experienced several episodes of diabetic ketoacidosis, all of which were brought about by prolonged bronchial infection. Although it is perhaps reasonable at present to consider this case to represent a chance association, further clinical investigations will be carried out to clarify whether or not Kabuki syndrome and type 1 DM have any common pathogenic features.

Keywords: Type 1 diabetes; Kabuki syndrome; Immunodeficiency; Autoimmune diseases; Congenital diseases

1. Introduction

First described by Niikawa [1] and Kuroki [2] in 1981, Kabuki syndrome is a congenital disorder that, in some cases, is associated with the chromosomal abnormalities, ring chromosome X and paracentric inversion of 4p (46XX inv(4)/p12pten) [3–5]. Its clinical features include moderate to severe mental retardation, postnatal growth retardation, unusual facial features, fingertip pads and minor skeletal, dermatoglyphic, urogenital and cardiac abnormalities [3]. Typical facial anomalies involve arched eyebrows with sparse or dispersed lateral halves, long palpebral fissures, eversion of the lower lateral eyelids, long eyelashes and large, prominent ears. To date, more than 90 Japanese

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