ARTICLE

Ototoxicity-induced loss of hearing and inner hair cells is attenuated by *HSP70* gene transfer

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The most common reason for sensorineural deafness is death of hair cells (HCs). Heat shock proteins (HSPs) are molecular chaperones that participate in folding, targeting, and degrading proteins. HSP expression is increased in response to various environmental stresses to protect cells from damage. Here, we tested whether viral-mediated overexpression of HSP70 can protect HCs and hearing from severe ototoxicity (kanamycin and furosemide) in guinea pigs. Adenovirus-*HSP70* mCherry (Ad.*HSP70*-mCherry) was injected to experimental animals and adenovirus-mCherry to controls, 4 days before the ototoxic insult. Hearing thresholds were measured by auditory brainstem response before the insult and again before sacrificing the animals, 14 days after the insult. Epifluorescence immunocytochemistry showed that injection of Ad.*HSP70*-mCherry resulted in mCherry fluorescence in nonsensory cells of the organ of Corti. The ototoxic insult eliminated both outer HCs and inner HCs throughout most of the cochlea of control (adenovirus-mCherry-injected) ears and contralateral (uninjected) ears. Ad.*HSP70*-mCherry-injected ears exhibited a significant preservation of inner HCs compared to control and contralateral ears, but outer HCs were not protected. Auditory brainstem response thresholds were significantly better in Ad.*HSP70*-mCherry-injected ears than in control and contralateral ears. Our data show that *HSP70* augmentation may represent a potential therapy attenuating ototoxic inner HC loss.

INTRODUCTION

Hearing impairment is the most common sensory deficit worldwide.[1] Most cases of hearing loss are of the sensorineural type, caused by degeneration or dysfunction of cells in the cochlea. The most commonly affected cell is the hair cell (HC), which transduces the mechanical input (sound or noise) to action potentials in the auditory nerve. HC function and survival can be affected by both genetic and environmental factors. The latter include therapeutic drugs with inner ear-specific side effects (ototoxic drugs), infections, and acoustic trauma because of acoustic overexposure. The ototoxic drugs that most commonly affect the cochlea are aminoglycosides, antimicrobial drugs largely used for treating Gram-negative infections such as tuberculosis. In the organ of Corti, aminoglycoside antibiotics have been shown to interact with membranous phosphatidyl inositol diphosphate leading to caspase-mediated apoptosis.[2,3] In the guinea pig, a systemic coadministration of the aminoglycoside kanamycin and the diuretic furosemide has been shown to induce severe loss of HCs and hearing.[4,5]

Heat shock proteins (HSPs) are a family of proteins known to be induced when tissues,[6,7] including the inner ear,[8] are exposed to various types of stress such as heat, acoustic trauma, and ischemia or ototoxicity. This response to stress is called the heat shock response. HSPs are known to have cytoprotective functions, often as molecular chaperones participating in folding, targeting, and degradation of proteins and as inhibitors of apoptotic pathways.[9–12] One of the HSPs, HSP70, is a potent cytoprotective family member,

recently shown to be protective against ototoxic HC degeneration when overexpressed in cochlear and vestibular tissues using transgenesis.[13,14] Another study has shown that Ad.*HSP70* viral vector overexpressed in nonsensory cells prevents HC death induced by ototoxic insult in the utricle *in vitro*.[15] In this study, using the severe ototoxic model of kanamycin and furosemide, we tested the extent of attenuation of HC loss and threshold shift resulting from HSP70 overexpression mediated by adenovirus-*HSP70* mCherry (Ad.*HSP70*-mCherry) delivery into the scala media in guinea pigs. We found that HSP70 overexpression reduced both inner HC (IHC) loss and threshold shifts.

RESULTS

Ototoxic lesion

To characterize the outcome of the deafening protocol in our experimental setup, we compared whole mount preparations of the auditory epithelium in normal cochleae (Figure 1a) to deafened cochleae (Figure 1b,c) that were obtained 14 days after systemic treatment with kanamycin and furosemide. In the lower half of the cochlea (basal and second turns) of intact ears (normal cochlea), the sensory HCs (outer HCs (OHCs) and IHCs) and the nonsensory cells were intact (Figure 1a, second turn shown). The auditory epithelium appeared normal along the entire length of the cochlea (not shown). In contrast, the auditory epithelium in the basal cochlea of deafened guinea pigs exhibited a complete

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bilateral loss of all HCs. Despite the absence of HCs, pillar cells and Deiters cells remained intact (Figure 1b,c). The outcome of deafening in higher turns (third and apical) was similar, although the apical turn typically included several HCs that survived the insult (not shown).

Ad.*HSP70*-mCherry inoculation induces HSP70 overexpression in the cochlea

We investigated the cellular localization of HSP70 following inoculation of Ad.*HSP70*-mCherry into ears that were subsequently exposed to an ototoxic insult. Cochleae were obtained 18 days after the viral vector injection and prepared as whole-mounts, stained for HSP70 (green) and phalloidin (blue), and assessed with confocal fluorescence microscopy (Figure 2). We detected a robust mCherry expression and HSP70 immunoreactivity as expected for the hybrid HSP70-mCherry transgene.[15] Expression was observed in Deiters cells (Figure 2a–dyz) and pillar cells (Figure 2e–hyz,i-lyz), consistent with earlier demonstrated infection patterns of similar adenovirus vectors.[15] Cells that were negative for mCherry were also negative for HSP70, confirming that at this stage, aminoglycoside trauma-induced HSP70 is not detected by immunocytochemistry in these nonsensory cells. Contralateral (uninjected) ears were also stained for HSP70 immunocytochemistry and were found to be negative (not shown).

To investigate the cellular localization of HSP70 following Ad.*HSP70*-mCherry inoculation into normal ears (without an ototoxic insult), we obtained ears 4 days after the inoculation and stained them for HSP70. We determined that nonsensory cells of the organ of Corti expressed
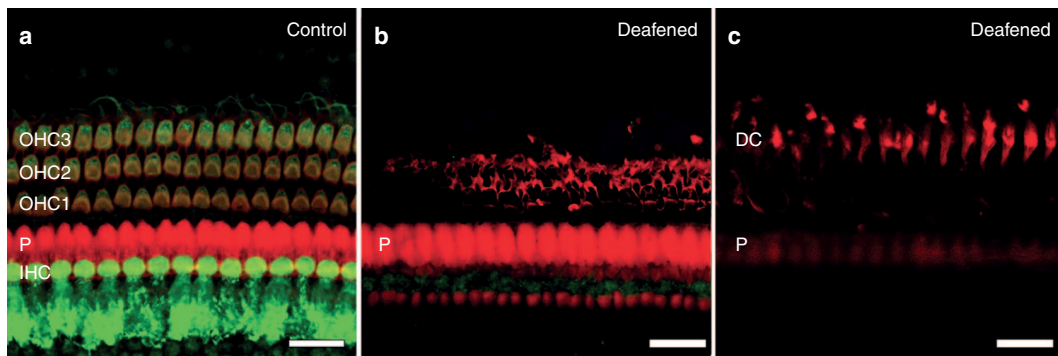

Figure 1 Confocal fluorescence microscopy of phalloidin (red) and Myosin VIIa-specific antibody (green) in whole-mounts of the auditory epithelium obtained from normal (**a**) and lesioned cochleae (**b,c**). (**a**) Control cochleae demonstrate a well-defined single row of IHC and three rows of OHC with intact nonsensory cells, as shown in the second turn. (**b,c**) In a cochlea exposed to ototoxic insult, all HCs are missing and are replaced by phalangeal scars. Pillar cells appear intact. Deiters cells appear intact. OHC1–3, outer hair cell rows 1–3; IHC, inner hair cell; P, pillar cell; D, Deiters cell. Scale bar indicates 20 μm.
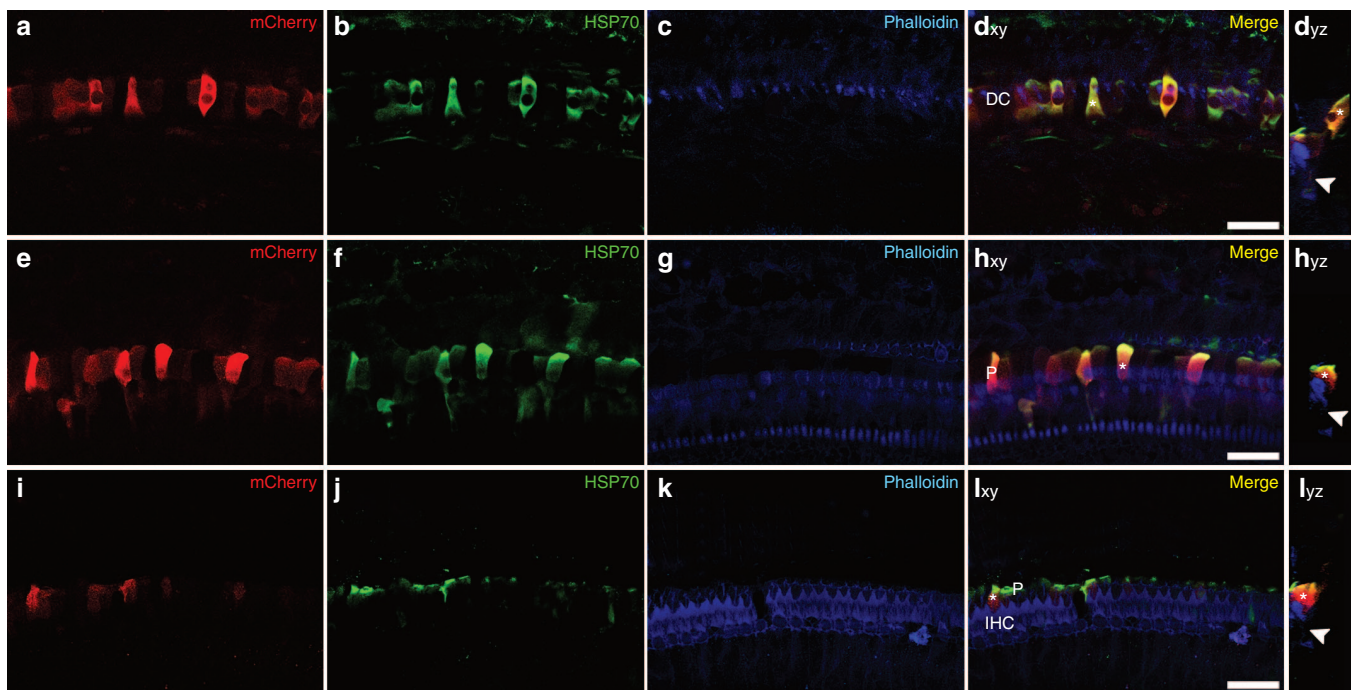

Figure 2 Confocal fluorescence microscopy images of whole-mounts of the auditory epithelium (second turn) lesioned with an ototoxic insult and preinjected with Ad.*HSP70*-mCherry showing expression of mCherry (**a,e,i**), HSP70 (**b,f,j**), localization of phalloidin (**c,g,k**), and merged (**d,h,l**). **a**–**d**xy images are at focal plane of Deiters cells, **e**–**h**xy and **i**–**l**xy are the same fields at a lower plane. mCherry expression is seen in Deiters cells (**a**) and pillar cells (**e,i**), corresponding to cells expressing HSP70 (**b,f,j**). **c**, **g**, and **k** show nonsensory cells labeled for phalloidin (blue) for facilitating orientation in the tissue. Merged images (**d,h,l**) confirm fusion proteins HSP70 and mCherry colocalization and show absence of HSP70 in cells negative for mCherry. **d**yz, **h**yz, and **l**yz are orthogonal sections showing Deiters cell or pillar cell (*) adjacent to the IHC (arrowheads). DC, Deiters cell; P, pillar cell; HSP, heat shock protein; IHC, inner hair cell. Bar = 20 μm.
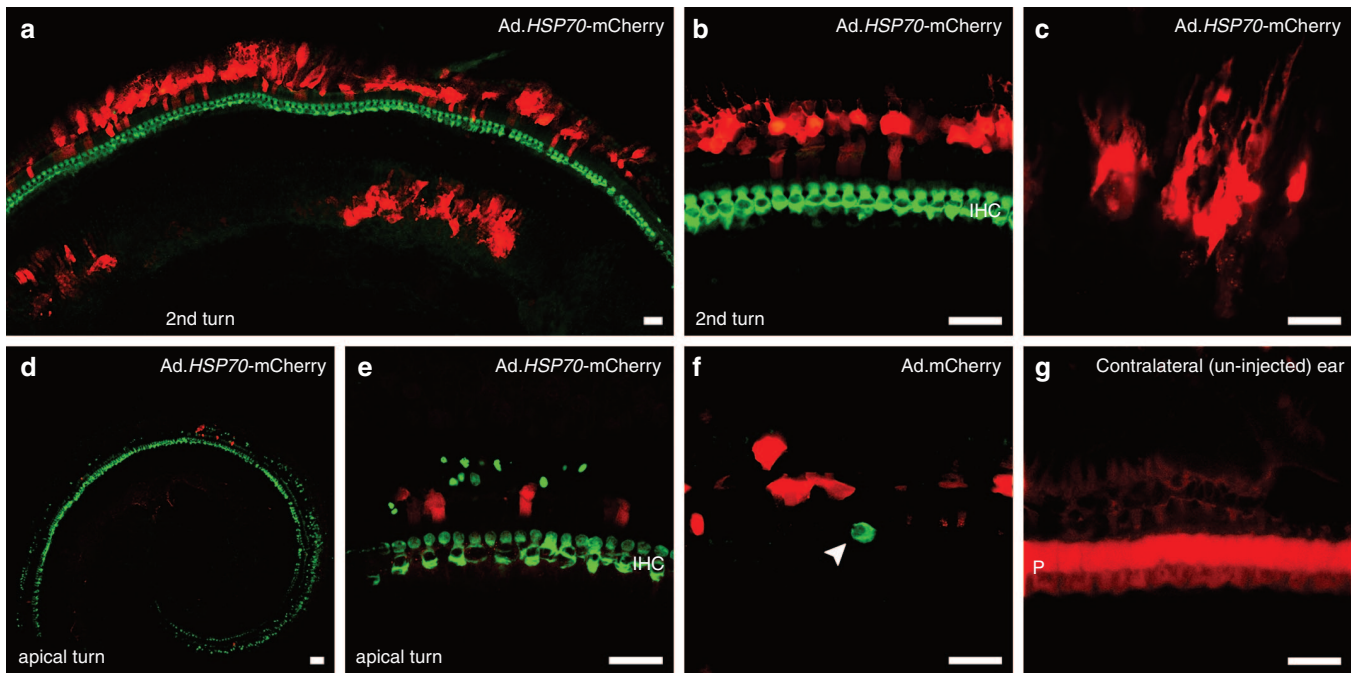
Figure 3 Confocal fluorescence microscopy images of whole-mounts of the auditory epithelium lesioned with kanamycin/furosemide and preinjected with Ad.*HSP70*-mCherry (**a–c**: second turn, **d,e**: apical turn) or control tissues (Ad.mCherry injection (**f**) and contralateral uninjected ear (**g**)). (**a**) Myosin VIIa staining (green, all panels) shows a single row of IHCs and complete lack of OHCs in a cochlea treated with Ad.*HSP70*-mCherry. mCherry fluorescence (red, panels **a–f**) is in epithelial cells lining the scala media and spiral limbus region in medial area as well as Deiters and pillar cells which invade the spaces left by the missing OHCs. IHCs are negative for mCherry. (**b**) Higher magnification of **a** shows that HSP70-mCherry expression is restricted to nonsensory cells, including pillar and Deiters cells, whereas the IHCs are devoid of transgene expression. (**c**) Higher magnification of medial area in **a** showing mCherry staining in cells in the interdental cell area and spiral limbus region. (**d**) Myosin VIIa staining shows a single row of IHCs and remaining OHCs in a cochlea treated with Ad.*HSP70*-mCherry. mCherry is in a few epithelial cells lining the scala media. The remaining OHCs and IHCs are negative for mCherry. (**e**) Higher magnification of **d** shows HSP70-mCherry expression restricted to nonsensory cells, including pillar cells. (**f**) Expression from Ad.mCherry is seen in pillar cells and Deiters cells of the auditory epithelium, as well as in one IHC (arrow head). One remaining HC stained for Myosin VIIa appears pathological. (**g**) Phalloidin (red) and Myosin VIIa (green)-stained preparation shows that all HCs are missing and are replaced by scars. Nonsensory cell elements of the organ of Corti are present. IHC, inner hair cell; HSP, heat shock protein, OHC, outer hair cell; P, pillar cell. Bar = 20 µm.

HSP70 in a pattern that was colocalized with mCherry expression (see Supplementary Figure S1). None of the cells positive for mCherry and HSP70 had a shape resembling that of a HC.

Another control involved investigating the tissue response and cellular localization of HSP70 following adenovirus-mCherry (Ad. mCherry) inoculation into normal ears without an ototoxic insult. Ears used for this control were obtained 4 days after the inoculation surgery and stained with antibodies to HSP70 (green) and phalloidin (blue) (see Supplementary Figure S2). We detected mCherry expression in pillar cells. These cells remained negative for HSP70, suggesting that the surgical trauma was insufficient for inducing HSP70 expression in them. The procedure did result in a lesion that involved loss of some OHCs but did not eliminate IHCs. Remaining IHCs were devoid of transgene expression.

Ad.*HSP70*-mCherry inoculation protects IHCs

We characterized the expression and distribution of Ad.*HSP70*-mCherry in the auditory epithelium and the impact of this expression on the extent of HC survival in cochlear tissues collected 18 days after the viral vector injection. Whole-mounts of the lesioned auditory epithelium assessed with confocal fluorescence microscopy showed that mCherry was present in epithelial cells lining the scala media. Robust expression was seen in pillar cells, Deiters cells, and interdental cells, mostly in the second turn. OHCs were absent, but the row of IHCs appeared intact and lacking mCherry expression (Figure 3a). Higher magnification analysis of the field shown

in Figure 3a confirmed localization of mCherry expression to pillar and Deiters cells, whereas surviving IHCs are devoid of transgene expression (Figure 3b). Robust mCherry staining was also seen in medial areas that include interdental cells in the spiral limbus region (Figure 3c). In addition, little or no mCherry expression was detected in apical turns (Figure 3d). Higher magnification analysis of the field shown in Figure 3d confirmed localization of mCherry expression to pillar cells (Figure 3e) and Deiters cells (seen in a lower focal plane, not shown), whereas surviving HCs are devoid of transgene expression. Overall, the data on mCherry distribution with the current method of injection points to viral-mediated gene expression in the middle portion of the cochlea (second turn), close to the region of injection, with a lesser spread of viral-mediated gene expression to the extreme apical and basal ends of the cochlear duct.

Two controls were used: contralateral ears that received no virus injection (systemically deafened only) and ears that were injected with a control vector, Ad.mCherry. In both groups of controls, the vast majority of HCs were absent in the first two cochlear turns (Figures 3e,f and 4). In the apical two turns of the cochlea, a few OHCs as well as some IHCs survived the insult (Figure 3d,e). These cells appeared to have atypical spherical morphology, possibly indicative of a degenerative process.

HC survival with Ad.*HSP70*-mCherry versus controls

The percentage of missing IHCs and OHCs was assessed in kanamycin–furosemide treated ears that received adenovirus injections
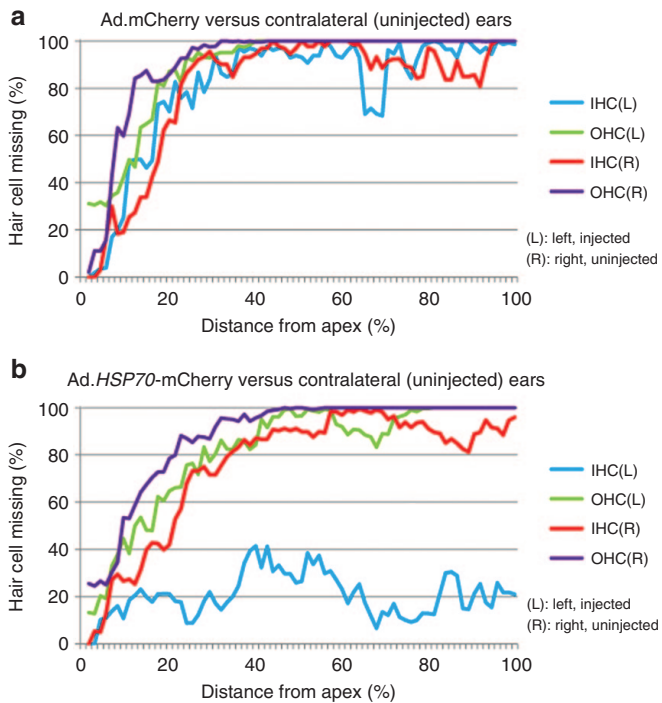
a Ad.mCherry versus contralateral (uninjected) ears

b Ad.*HSP70*-mCherry versus contralateral (uninjected) ears

Figure 4 Cytocochleograms showing percentage of missing IHCs and OHCs assessed in deafened ears that were injected with Ad.*HSP70*-mCherry versus the contralateral control ears (with no further manipulation) (**a**) and Ad.mCherry (control viral vector) versus the contralateral control ears (**b**). (**a**) In both Ad.mCherry-injected and contralateral ears, kanamycin/furosemide eliminated nearly all IHC and OHCs in the basal half of the cochlea. In the apical two turns, the elimination of IHC and OHCs is incomplete. (**b**) IHC survival is substantially enhanced in the Ad.*HSP70*-mCherry-injected ears compared to both controls (**a**). Ad.*HSP70*-mCherry did not reduce the loss of OHCs because of kanamycin/furosemide. The number of mCherry animals =7. The number of *HSP70*-mCherry animals =12. IHC, inner hair cell; HSP, heat shock protein, OHC, outer hair cell.

(control viral vector) and in the contralateral control ears (uninjected ears) (Figure 4a,b). In the basal half of the cochlea (first two turns), IHCs and OHCs were nearly completely eliminated by the ototoxic lesion in Ad.mCherry-injected and contralateral ears (Figure 4a). In contrast, cochleae injected with Ad.*HSP70*-mCherry demonstrated a partial preservation of IHCs cells throughout the cochlear duct, with loss ranging from 5 to 40% (Figure 4b).

In the mCherry group, the amount of HC loss was not significantly different from the contralateral control ears for IHC (L: 96.3%, R: 95.3%, *P* = 0.48) or OHC (L: 99.2%, R: 99.9%, *P* = 0.34). The Ad.*HSP70*-mCherry group also did not have a significant difference between left and right ears in OHC (L: 89.8%, R: 96.9%, *P* = 0.19); however, the Ad.*HSP70*-mCherry-treated ears did have significantly less IHC loss (L: 34.7%, R: 87.0%, *P* = 0.0002). Preservation of IHCs cells was found throughout the cochlear duct. Thus, although OHCs were not protected, injection of Ad.*HSP70*-mCherry protected IHCs throughout the cochlea.

Auditory brainstem response (ABR) thresholds in Ad.*HSP70*-mCherry injected ears versus controls
ABR thresholds were measured at 4, 8, and 16 kHz. Baseline thresholds were established before injection of the viral vectors (Ad.*HSP70*-mCherry or Ad.mCherry control). ABRs were measured again before sacrificing the animals, 14 days following the ototoxic
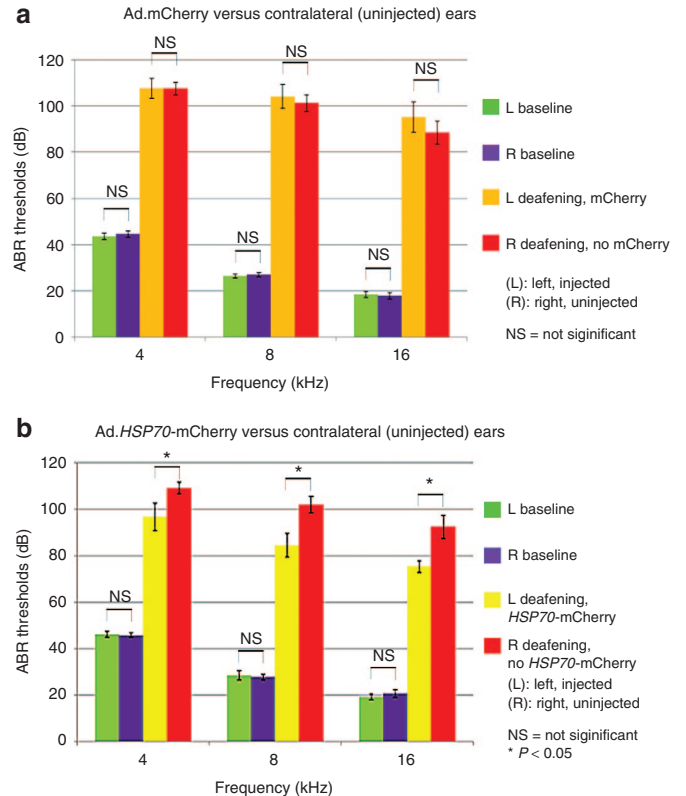

a Ad.mCherry versus contralateral (uninjected) ears

b Ad.*HSP70*-mCherry versus contralateral (uninjected) ears

Figure 5 Comparison of ABR thresholds between injected ears and contralateral ears for animals receiving Ad.mCherry (**a**) and Ad.*HSP70*-mCherry (**b**). ABR thresholds were measured at 4, 8, and 16 kHz, before injecting the viral vectors (baseline) and immediately before sacrificing the animals (14 days following the ototoxic insult). (**a**) At baseline, threshold differences between the injected ears and uninjected ears are not significant (*P* = 0.92). Two weeks after deafening, ABR thresholds were elevated in all animals and not significantly different between the Ad.mCherry-injected ears and contralateral ears (*P* = 0.80). Even at 16 kHz, where the differences in ABR threshold were greatest, the difference between injected and uninjected ears was not significant (*P* = 0.42). (**b**) At baseline, before injection, the difference in ABR threshold between ears of the animals that would receive Ad.*HSP70*-mCherry injections were not significant (*P* = 0.86). Two weeks after deafening, both ears had higher thresholds, but the Ad.*HSP70*-mCherry-injected ears had significantly lower thresholds than contralateral ears at all three frequencies tested (*P* ≤ 0.033). In addition, ABR thresholds were significantly better in Ad.*HSP70*-mCherry-injected ears than for mCherry only (*P* = 0.013, orange bars in **a** versus yellow bars in **b**). Protection was not significant at 4 kHz (*P* = 0.16), but was significant at both 8 kHz (*P* = 0.012) and 16 kHz (*P* = 0.0027). mCherry animals *n* = 7, *HSP*-mCherry animals *n* = 12. Error bars indicate the standard error of the mean. ABR, auditory brainstem response; HSP, heat shock protein.

insult. For comparison, ABR thresholds were also obtained for contralateral (uninjected) ears (Figure 5a,b). At baseline, there were no significant differences in ABR thresholds between injected and uninjected ears. Similarly, when the second ABRs were measured after deafening, thresholds in contralateral ears were not significantly different from Ad.mCherry (control)-injected ears at any of the three frequencies tested (Figure 5a). In contrast, when compared with the contralateral ears, the ears injected with Ad.*HSP70*-mCherry exhibited significantly better thresholds at 4, 8, and 16 kHz (Figure 5b). Although the protection was partial, the data show that ABR thresholds were significantly better in Ad.*HSP70*-mCherry-injected ears than in control ears and contralateral ears, despite the extremely severe lesion model. ABRs were measured in the high-frequency

areas of the guinea pig cochlea and did not appear to correlate well with IHC survival, which was mostly noted in the middle regions of the cochlea. Evaluation of the effect of HSP70 overexpression and IHC preservation on hearing thresholds is complex because of the distortion of frequency mapping inherent in the severe lesions induced throughout the cochlea.

DISCUSSION

The data presented indicate that viral-mediated overexpression of HSP70 in nonsensory cells can protect IHCs from a severe ototoxic insult. Preservation of IHCs was accompanied by significant protection of hearing thresholds. Because OHCs were not protected in this model of severe ototoxicity, the protection of hearing was incomplete.

HSP70, a well characterized member of the HSP family, has recently been shown to preserve HCs in explanted utricles that were exposed to ototoxic aminoglycosides or cisplatin.[13,16] In addition, mice that are genetically designed to overexpress HSP70 exhibit reduced HC death and better preservation of hearing when exposed to an ototoxic insult.[14] Earlier studies on gain and loss of function support a critical role for HSP levels in protection of the inner ear from ototoxic drugs and noise.[17–20] Our aim was to test the protective capacity of elevated levels of HSP70 *in vivo*. The aim is motivated by the need to enhance our understanding of the mechanism of protection and prepare the foundations for its future use in clinical applications.

We have selected a severe ototoxic lesion as the trauma model, because it eliminates the entire population of HCs in the lower part of the cochlea, allowing for clear comparative assays when assessing protection. We injected kanamycin, an aminoglycoside antibiotic that is known to cause substantial ototoxic trauma in mammalian HC systems[21] and the loop diuretic furosemide which augments the extent of the lesion caused by kanamycin.[4,5] This combination of drugs leads to permanent loss of HCs and profound deafness within 1 or 2 days.[5,22] Our data demonstrate protection of IHCs along with partial protection of hearing thresholds, suggesting that elevated levels of HSP70 in the cochlea can reduce the extent of lesion caused by the ototoxic drug cocktail.

To further improve the extent of protection, it is necessary to elucidate the mechanism of the protective action of the HSP. It is important to determine whether the mode of action of HSP70 is influencing the HCs directly or indirectly via the nonsensory cells. Using a viral vector with a reporter gene, we observed HSP70-mCherry expression in nonsensory cells, especially in the region of the cochleostomy in the second turn of the cochlea. This feature of adenovirus gene transfer into the cochlea, leading to gene expression in the nonsensory cells of the organ of Corti and not in the HCs, has been noted in several animal models including rat, guinea pig, and mouse.[15,23,24] There are two, nonmutually exclusive possibilities for nonsensory cells transduced with the Ad.*HSP70*-mCherry to exert a protective effect on HCs. One possibility is that the direct influence is mostly on the nonsensory cells. Along this line, we speculate that nonsensory cells, known to "execute" injured HCs[25,26] and later phagocytose them,[27] are inhibited by the upregulation of HSP70, thereby leading to rescue of the HCs. The alternative mechanism is that nonsensory cells secrete HSP70 which acts on the HCs in a paracrine fashion. *In vitro* experiments showing rescue of HCs when incubated in media conditioned by heat-shocked utricle cultures or when exposed to soluble HSP70 added to the media support the notion that diffusible HSP70 can induce protective effects on HCs.[15] Putative receptors and downstream mediators of this protection are unknown at present. In addition, a possible link between HSP70 upregulation and cell death prevention may be derived from other systems where HSP70 has been shown to be a potent modulator of cell death pathways.[28,29]

Our data show that *HSP70* gene transfer protected IHCs against an extremely severe ototoxic insult, whereas OHCs were not protected. Several reasons can potentially contribute to the lack of OHC protection. First, the surgical procedure for viral vector inoculation into the scala media, by itself, causes a severe trauma leading to OHC loss. When combined with the ototoxic insult, the two stresses can augment each other and act in a detrimental fashion that is beyond the rescue by HSPs. This may also explain why the extent of protection with Ad.*HSP70*-mCherry is lower than that seen in an earlier study that used transgenic mice overexpressing HSP70. The difference in the extent of protection between the transgenic model and our surgical approach may be related not only to the more severe ototoxic insult used in our work, but also to the fact that in the mouse study, overexpression was in the HCs.[14] In addition, the presumed widespread distribution of HSP70 expression driven by a chicken β-actin/cytomegalovirus promoter/enhancer in the transgenic line may underlie greater protection throughout the cochlea in the transgenic model. *In vitro* studies of utricles exposed to ototoxic drugs also exhibited partial protection of HCs after transfection with an adenovirus vector expressing HSP70.[15]

Although results obtained from cochlear versus vestibular organs and between *in vivo* versus culture systems are not completely comparable, it is likely that the variability seen in these studies is related to the severity of the ototoxic insults, the efficiency of transfection with the viral vector, the side effects of the surgical procedure, or the presence of the viral vector.

In this study, the most efficient gene expression was in the second turn, where the virus was injected. The extent of transfection in flanking regions, apical and basal to the site of injection, was variable but typically present. The protection of IHCs was most efficient in the area of injection, suggesting that proximity of gene expression to the site of rescue is important. To rescue HCs throughout the cochlea, it would be necessary to have a practical way to achieve gene expression from base to apex. The current data do not provide clear evidence to distinguish between a paracrine mechanism for IHC rescue and the possibility that the HSP inhibits the scarring activity of nonsensory cells, thereby rescuing the HCs.

The ototoxic insults induced a near complete loss of HCs throughout most of the cochlea. Moreover, the scala media injection surgery that we used in this study also induces severe damage of HC and hearing loss, as confirmed here (not shown) and demonstrated earlier.[23] It would be important to determine whether a less severe cochlear lesion such as acoustic trauma would be more amenable to HSP70 protection, and whether a less severe ototoxic lesion may also yield better protective outcome.

Our data were generated using a scala-media inoculation. This procedure, by itself, is invasive enough to cause a lesion in the auditory epithelium.[23] When a viral vector is developed that can transduce the auditory nonsensory cells via a less-invasive approach such as scala tympani inoculation, a better protective effect can possibly be accomplished. For any potential clinical applicability of the HSP70 protection, it will likely be necessary to use a noninvasive procedure for inner ear drug delivery, possibly using a small molecule that will mimic the HSP action.

In conclusion, we have demonstrated that viral-mediated overexpression of HSP70 can protect IHCs from a severe ototoxic insult,

in vivo. A moderate yet significant improvement in ABR thresholds was also observed. These results support the development of novel protective approaches based on augmenting HSP-related pathways for preserving HCs and hearing in human patients.

MATERIALS AND METHODS

Animals and groups

All animal experiments were approved by the University of Michigan, University Committee on the Use and Care of Animals, and performed using accepted veterinary standards. We used young adult pigmented guinea pigs from our breeding colony at the University of Michigan. At the beginning of the experiment, all animals had normal Preyer's reflex and weighed 300–400 g. A total of 22 guinea pigs were enrolled in this study. Nineteen animals were deafened bilaterally with kanamycin and furosemide (see in what follows): left ears were used for the experimental group and right ears were used as contralateral controls. These animals were divided into two groups for virus vector treatment: Ad.*HSP70*-mCherry injection (*N* = 12) and Ad.mCherry injection (*N* = 7). Additional control animals included Ad.*HSP70*-mCherry injection with or without an ototoxic insult and Ad.mCherry injection without ototoxic insult. Both ears of these animals (left, injected and right, uninjected) were stained with HSP70-specific antibodies and analyzed for mCherry and HSP70 using fluorescence microscopy.

Adenovirus

Animals in the experimental groups received an adenoviral vector serotype 5 (dE1/E3) with gene inserts driven by the cytomegalovirus promoter (Vector BioLabs, Malvern, PA). Stock viruses included Ad.mCherry and Ad.*HSP70*-mCherry. The viral vector titer of the injected solution was 5.3×10^{10} PFU/ml. The viral suspension was stored at −80 °C and thawed on ice before use. A volume of 1.5 µl was injected into the scala media, as described in the subsequent section. More details about the viral vector are described in an earlier study.[15] In the current *in vivo* study, control ears were either left ears that received a control inoculation (Ad.mCherry) or right (contralateral) ears that received no inoculation.

Surgery

Before surgery, the following devices were prepared. A polyethylene tube was connected to a fine polyimide tube at one end, and the other end was connected to a 30-G needle attached to a 5-µl Hamilton syringe filled with sterile water. Fast green dye FCF (Sigma-Aldrich, St. Louis, MO) was added to the vector solution to trace it during the injection process. The animals were anesthetized with ketamine HCl 40 mg/kg (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine 10 mg/kg (AnaSed, Shenandoah, IA), and the left submandibular area was prepared for surgery. Lidocaine HCl (1%, 0.5 ml) was injected subcutaneously in the submandibular area for local anesthesia. The animals were placed in the supine position on a thermoregulated heating pad. The temporal bone was exposed via a submandibular incision and opened to visualize the medial aspect of the cochlea from apex to base. The pigment in the stria vascularis was used as a landmark for accessing the scala media. The otic capsule was perforated with a hand drill at the level of the second turn of the stria vascularis. The tip of the polyimide tube was inserted into the cochlea and advanced through the stria vascularis into the scala media. Viral vector (1.5 µl at 0.5 µl/min) was injected into the scala media endolymph, using a motorized pump to control the infusion speed. Successful injections into the cochlea were confirmed by the observation of Fast green. After the inoculation was complete, the tube was left in place for an additional 2 minutes. After the tube was removed, the cochleostomy and bulla wall were sealed with tissue adhesive (Vetbond, 3M) and carboxylate cement (Durelon, ESPE, Norristown, PA). The muscle incision was closed with a 5-0 Vicryl suture and the skin incision was closed with 5-0 Ethilon suture. The procedures took ~45 minutes to complete. Afterwards, the animals were allowed to recover from anesthesia, and their pain was controlled with carprofen (Rimadyl, 4 mg/kg, subcutaneously , Pfizer, New York, NY).

Administration of kanamycin and furosemide

Four days after the first surgery, animals were deafened using a surgical procedure. Under anesthesia (as mentioned earlier), the animals were placed in the supine position on a thermoregulated heating pad. The jugular vein was exposed and cannulated by 27-G needle. Furosemide (100 mg/kg; Hospira,

Lake Forest, IL) was injected intravenously into the jugular vein as described previously.[30] When the inoculation was complete, the incision was closed in the same manner as described therein. Immediately afterwards, a single dose of kanamycin (400 mg/kg dissolved in saline (Sigma-Aldrich, Cat. No. K-1377)) was injected subcutaneously. Postoperative pain was controlled with carprofen (4 mg/kg, subcutaneously).

Tissue preparation and immunocytochemistry

After their final ABR assessment, animals were sacrificed and their temporal bones were removed. Samples were fixed in 4% paraformaldehyde in phosphate buffer for 2 hours, rinsed, and the area of the auditory epithelium dissected for whole-mount preparations. Myosin VIIa antibody and phalloidin were used to assess HC survival. HSP70 antibody and phalloidin were used to assess presence of HSP70 immunoreactivity and localize it in the tissue. Myosin VIIa and phalloidin immunostaining, whole-mounts were permeabilized in 0.3% Triton X-100 (Sigma-Aldrich) for 10 minutes and then blocked against nonspecific binding of secondary antibody with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in phosphate buffered saline (PBS) for 30 minutes. Tissues were reacted with primary antibody, rinsed, and incubated with the secondary antibody. The primary antibody was a rabbit antibody specific for Myosin VIIa (Invitrogen, Grand Island, NY; Cat. No. 51-2800) diluted 1 : 200 in blocking buffer, for 1 hour at 4 °C. Secondary antibody was a goat-antirabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR), diluted 1 : 200 in PBS for 30 minutes. To double stain for F-actin in right ears, we used Alexa Fluor 594 phalloidin (Molecular Probes), diluted 1 : 400 for 30 minutes.

For HSP70 immunostaining, whole-mounts were simultaneously permeabilized and blocked against nonspecific binding of secondary antibody with a solution of 2% bovine serum albumin (Sigma-Aldrich), 0.8% normal goat serum and 0.4% Triton X-100 in PBS for 2 hours at room temperature. Tissues were reacted with primary antibody, rinsed, and incubated with the secondary antibody. The primary antibody was a rabbit antibody specific for HSP70 (Santa Cruz Biotechnology, TX) diluted 1 : 250 in blocking buffer. Tissue was incubated with the primary antibody overnight at 4 °C, allowed to reach room temperature, and then rinsed 1 hour later. Secondary antibody was a goat-anti rabbit Alexa Fluor 488, diluted 1 : 500 in blocking solution. It was applied for 2 hours at room temperature. To double stain for F-actin, we used Alexa Fluor 350 phalloidin, diluted 1 : 250, for 1 hour. Specimens were mounted on glass slides using Prolong Gold antifading reagent (Molecular Probes).

Whole-mounts were examined and recorded using a Leica DMRB epi-fluorescence microscope (Leica, Eaton, PA), with CCD Cooled SPOT-RT3 digital camera (Diagnostic Instruments, Sterling Heights, MI) and/or Olympus FluoView 500 Laser Scanning Confocal Microscope (Olympus, Japan).

Assessment of HC survival

HC survival was evaluated in whole-mounts stained with Myosin VIIa-specific antibody (green) and phalloidin to stain actin of injected and contralateral ears 14 days after deafening. Tissues were viewed in a Leica DMRB epifluorescence microscope with ×50 objective lens (Leica). HC counts were analyzed using the KHRI Cytocochleogram Program, version 3.0. In each field of view, a 0.20-mm scale in the ×10 eyepiece was superimposed on the centers of the pillar cells. The percentage loss of HCs for each 0.20-mm segment was calculated for each row. To compare between specimens differing in total cochlear length, each specimen's data were used to calculate the percent of HC loss in 75 intervals (each representing 1.33% of that specimen's cochlea). Each row was analyzed separately, and then the average of the three OHC rows was computed for the final intervals. For graphical comparison of HC loss between treatment groups, the average OHC and IHC loss was calculated for each interval and plotted against percentage of distance from the apex. In animals that did not receive Ad.*HSP70* injection, this graphical analysis showed a general tendency for HC loss to approach 100% at a distance from the apex of ~40% of the total length and also showed large differences between adjacent counting frames located apically of that point. Because this complex curve could not be well approximated by a simple linear or logarithmic models, we computed the average loss in a window composed of eight frames spanning about 10% of the total length located 35–45% of the distance from the apex. This window encompasses the region most likely to reflect protective effects and yet reduces the chance that comparisons are biased by arbitrary selection of a particular segment. Statistical evaluation of differences between ears in this window was performed using the paired sample *t*-test, comparing the mCherry and *HSP*-mCherry groups. Survival of

IHC and OHC was evaluated separately. The α-level judged significance was adjusted by the sequential Bonferroni criterion.

ABR measurement

ABRs were assessed in all animals at the onset of the experimental sequence. ABR recordings were collected at frequencies of 4, 8, and 16 kHz for both ears of each animal. Before the ABR testing, animals were anesthetized intramuscularly with ketamine (58.8 mg/kg), xylazine (2.4 mg/kg), and acepromazine (1.2 mg/kg) and placed on a thermoregulating heating pad to maintain body temperature. Additional anesthetic (ketamine and xylazine) was administered if needed to maintain anesthesia depth sufficient to insure immobilization and relaxation. ABRs were recorded in an electrically and acoustically shielded chamber (Acoustic Systems, Austin, TX). Tucker Davis Technologies (TDT) System III hardware and SigGen/BioSig software (TDT, Alachua, FL) were used to present the stimulus and record responses. Neural activity in response to brief tone bursts was measured using needle electrodes inserted subcutaneously ventral to each pinna and at the vertex of the skull. Each tone burst was 15 milliseconds in duration, with 1 millisecond rise/fall times, presented at 10 bursts per second through an EC1 driver (TDT, aluminum enclosure made in-house), with the speculum placed just inside the tragus. Up to 1,024 responses were averaged for each stimulus level. Responses were collected for stimulus levels in 10 dB steps at higher stimulus levels and at 5 dB steps near threshold. Thresholds were interpolated between the lowest stimulus level where a response was observed, and 5 dB lower where no response was observed. For statistical analysis of ABR data, analysis of variance (ANOVA) was used to test for differences in thresholds between Ad.*HSP70*-mCherry-injected versus uninjected sides analyzing all tested frequencies simultaneously. Subsequently, ANOVA was used to evaluate threshold differences at each frequency, separately, with sequential Bonferroni criterion used to correct for the number of posthoc tests in each comparison and maintain a table-wide $\alpha < 0.05$. Both sets of analyses were repeated to test for differences between sides after deafening. In addition, differences in thresholds between injected and uninjected sides after deafening were compared using paired sample *t*-tests.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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