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Round window gentamicin application: an inner ear hair cell damage protocol for the mouse

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Abstract

It is important to develop an inner ear damage protocol for mice that avoids systemic toxicity and produces damage in a relatively rapid fashion, allowing for study of early cellular and molecular mechanisms responsible for hair cell death and those that underlie the lack of hair cell regeneration in mammals. Ideally, this damage protocol would reliably produce both partial and complete lesions of the sensory epithelium. We present a method for in vivo induction of hair cell damage in the mouse via placement of gentamicin-soaked Gelfoam in the round window niche of the inner ear, an adaptation of a method developed to study hair cell regeneration in chicks. A total of 82 subjects underwent the procedure. Variable doses of gentamicin were used (25, 50, 100 and 200 μg). Saline-soaked Gelfoam, sham-operations and the contralateral, non-operated cochlea were used as controls. Survival periods were 1, 3 and 14 days. Damage was assessed on scanning electron microscopy. We found that this method produces relatively rapid hair cell damage that varies with dose and can extend the entire length of the sensory epithelium. In addition, this protocol produces no systemic toxicity and preserves the contralateral ear as a control.

Keywords: Aminoglycoside; Ototoxicity; Hair cell; Damage protocol

1. Introduction

The ototoxicity of aminoglycoside antibiotics, in particular their ability to damage the hair cells of the inner ear, became apparent shortly after the initiation of their clinical use (Hawkins et al., 1969; Schacht, 1993). Investigators in hearing research have utilized aminoglycoside-induced inner ear hair cell damage in animals to understand a variety of processes in the auditory system, including development (O'Leary and Moore, 1998), function of different elements (Dumas and Charachon, 1982; Lenoir et al., 1983), the process of cell death (Forge and Li, 2000; Cunningham et al., 2002), and hair cell regeneration (Cruz et al., 1987; Girod et al., 1991; Lippe et al., 1991). As our understanding of the auditory system progresses, damage protocols that produce rapid and relatively precise damage become increasingly useful. An ideal aminoglycoside damage protocol would avoid systemic effects and leave one ear undamaged as a control. More importantly, damage would occur in a defined, relatively rapid period of time such that the early events following the damage could be pinpointed and studied. Additionally, a damage protocol should produce consistent lesion size with defined transition zones between damaged and undamaged sensory epithelium, thereby allowing study of intermediary steps in cell death and facilitating identification of undamaged hair cells versus hair cell regeneration. Finally, a protocol that is capable of reliably producing a complete lesion across the entire sensory epithelium would allow for better comparison of apical vs. basal responses to aminoglycoside exposure.

Application methods used to date have included systemic injection and local application via *trans*-tympanic injection, endolymphatic sac injection, and intracochlear perfusion (Sullivan et al., 1987; Kimura et al., 1988; Lee and Kimura, 1991; Wanamaker et al., 1998; Dodson, 1997). Although systemic injection has been a

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particularly useful application method in some species, it has limitations in the mouse. Because the mature mouse cochlea is relatively resistant to the effects of systemic aminoglycosides, most systemic injection protocols require repeated injections of aminoglycosides to achieve inner ear hair cell damage and the amount of damage is still limited (Wu et al., 2001; Forge and Shacht, 2000). No systemic injection protocol produces complete, or near complete, lesions of the sensory epithelium in either mammals or non-mammals. The development of the single high-dose gentamicin injection protocol in the chicken (Janas et al., 1995) represented an advance in damage protocols as it resulted in a standard method for producing consistent lesions of the basal one-third of the avian sensory epithelium in a defined time period. Unfortunately, attempts at single high-dose injection protocols in the mouse are not feasible due to their lethality (Wu et al., 2001).

Direct application of aminoglycosides to the auditory structures avoids the problem of systemic toxicity, leaves the contralateral ear to serve as a within-subject control, and has the ability to produce larger lesions relatively rapidly. Unfortunately, *trans*-tympanic and endolymphatic sac injections usually fail to produce a reliable lesion size, require multiple injections or produce lesions of highly variable length (Kimura et al., 1988; Lee and Kimura, 1991; Wanamaker et al., 1998). Intra-cochlear perfusion of aminoglycosides results in a complete lesion, but appears incapable of producing a partial lesion (Dodson, 1997). A rapid, consistent damage protocol that produces partial and complete lesions without systemic toxicity in mammals would be extremely useful. Such a protocol in mice would be particularly advantageous due to the abundance of probes, antibodies, and genetic mutants available for studies in the mouse.

We present a method for induction of in vivo hair cell damage in the mouse via placement of a gentamicin-soaked Gelfoam pledget in the round window niche of the inner ear. The method is adapted from that used by Husmann et al. (1998) and Muller and Smolders (1998) in birds. This protocol results in relatively rapid hair cell damage following a single treatment and allows for consistent, dose-dependent partial lesions as well as a complete lesion of the entire sensory epithelium. In addition, this method avoids systemic toxicity and leaves the contralateral ear to serve as an undamaged control.

2. Materials and methods

2.1. Experimental subjects

All experiments involved adult male mice (6–10 weeks old) of the CBA/CaJ strain, obtained from Jackson Laboratory, Bar Harbor, ME. CBA/CaJ mice were chosen on the basis of their normal hearing and lack of age-related hearing loss (Zheng et al., 1999b). All experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

2.2. Damage protocol

Experimental animals underwent operative placement of a Gelfoam pledget in the left round window niche. Surgical controls underwent the same operation without placement of a pledget. The right ear was not manipulated in any of the animals and was used as a control in each subject. The pledgets contained saline or varying amounts of gentamicin. Experimental animals were allowed to survive 1, 3 or 14 days. The amount of hair cell damage was assessed via scanning electron microscopy (SEM) and immunohistochemistry with an antibody against myosin VIIa.

For surgical implantation, animals were anesthetized with 0.75 ml of Avertin I.P., (Aldrich, St. Louis, MO). They were then positioned in a metal head holder with the left ear exposed. The left external auditory canal (EAC) and surrounding fur were cleaned with Betadine. Two vertical and a connecting horizontal incision were made in the posterior, cartilagenous EAC to expose the mastoid bone posterior to the tympanic membrane, overlying the round window niche. An electrical cautery was used to remove the musculature from this bone and achieve hemostasis. Once the mastoid bone was cleaned of tissue, the round window niche and the stapedial artery were exposed by removing a portion of the mastoid bone with a small burr and forceps. Great care was taken to preserve the stapedial artery.

A small Gelfoam (Pharmacia & Upjohn, Kalamazoo, MI) pledget was soaked either in saline or various concentrations of gentamicin (Sigma Chemical Co., St. Louis, MO), and was then placed in the round window niche. Some Gelfoam protruded from the niche into the middle ear space. The inferior skin incision was then closed with 4.0 silk suture. The procedure requires $\approx$10 min to perform and is relatively easy to learn. Postoperative animals often had vestibular disturbances. We found it useful to supply food and water on the floor of the cage and occasionally supply subcutaneous saline. Any animals that exhibited logrolling or other signs of severe vestibular damage at three days postoperatively were sacrificed.

Gentamicin was selected as the ototoxic agent for use in these studies due to the well-established ability of this aminoglycoside antibiotic to damage inner ear hair cells (Hawkins and Johnsson, 1981). A stock solution of 200 mg/ml gentamicin was prepared using gentamicin sulfate, distilled water, sodium bisulphite (0.016 g/ml), and EDTA (0.5 mg/ml). All chemicals were obtained from Sigma (St. Louis, MO). The stock solution was then diluted to 100 or 50 mg/ml with saline. Either a 0.5 or 1 μl volume was used for each Gelfoam pledget, resulting

in an applied dose to the animal of 25, 50, 100 or 200 μg. Since both the concentration and the volume of the gentamicin were varied to achieve the four applied dosages (25, 50, 100 and 200 μg), all results will be reported in terms of the total dose of gentamicin applied as opposed to concentration. The pledget was prepared by using just enough Gelfoam to entirely absorb the measured dose of gentamicin, ≈2 mm^3. Saline pledgets were prepared in the same fashion with 0.5 μl of solution. Surgical control animals underwent the operation without placement of a pledget. In all experimental and control cases, only the left ear was treated and the right ear was maintained as a control.

Experimental animals were allowed to survive 1, 3 or 14 days. The number of animals in each experimental and control group is shown in Table 1. The pledgets were not removed at any point prior to sacrifice of the animal. Both left and right cochleae were harvested and assessed for hair cell damage. Post-operative day one was chosen to reflect the amount of damage produced at an early time point. Post-operative day three was chosen as the possible period for highest rate of hair cell loss based on information derived from studies on chickens. Post-operative day 14 was selected to reflect a nearly stable amount of damage, although it is recognized that some continued hair cell damage could occur. Saline controls were evaluated only in animals surviving 14 days to detect maximal damage effects.

2.3. Assessment of cochlear damage

2.3.1. Scanning electron microscopy

Animals were sacrificed via CO_2 inhalation and decapitated. The right and left cochleas were then dissected free from the surrounding temporal bone and immersed in 2.5% glutaraldehyde/1% paraformaldehyde in 0.12 M PO_4 (pH 7.2–7.4) with 1.5% sucrose. The stapes was removed and the apical tip of the bony capsule was perforated with a scalpel. The fixative was gently perfused through the labyrinth via the oval window using a syringe. The cochleas were then placed in fresh fixative for three days with agitation. Cochleas were then washed three times in PBS for 30 min and post-fixed in 1% OsO_4 in PBS for 40 min with agitation. After three additional 30 min washes in PBS, specimens were serially dehydrated in ethanol and then dried in a critical point drier (Autosamdri – 814, Tousinis Research Corporation, Rockville, MD). Each pair of cochleas was mounted on an aluminum stub. At this point, a temporal bone drill and fine dissecting instruments were used to remove the bony capsule of the cochlea, spiral ligament, stria vascularis and Reissner's membrane. This resulted in exposure of the entire organ of Corti, except for the basal hook region. Specimens were then coated in gold palladium with a Hummer VIA (Anatech Ltd., Alexandria, VA) and viewed on a JEOL JSM 6300 F scanning electron microscope.

We developed a method for quantifying the extent of damage in the spiral organ of Corti. We divided the cochlea into two complete turns, 360° each, and excluded the hook region from quantification (Fig. 1). All damage was measured in terms of the number of degrees from the apex that the lesion spanned. Thus, if a lesion spanned the entire basal turn of the cochlea as well as the basal quarter of the apical turn, it was considered to be 450° of damage. This amount was recorded separately for inner and outer hair cells. Damage was recorded in two categories: (1) stereocilia fusion, (2) stereocilia loss. A segment of the cochlea was considered damaged if five of ten consecutive cells exhibited stereocilia fusion or loss.

2.3.2. Immunohistochemistry

In addition to analysis of damage using scanning electron microscopy, damage was assessed in several mice utilizing immunohistochemical labeling of hair cells and light microscopy. Two animals that received 25 μg of gentamicin and one animal that received 200 μg of gentamicin were sacrificed on postoperative day 14 by overdose of sodium pentobarbitol. Cochleas were dissected away from the auditory bulla, and the stapes was removed from the oval window. A scalpel blade was

Table 1
Number of animals in each treatment group

Survival period (days)	Surgery control	Saline control	25 (μg)	50 (μg)	100 (μg)	200 (μg)
1	0	0	4	9	4	6
3	3	0	5	3	6	5
14	7	6	7	5	7	4

Survival periods were 1, 3 and 14 days. Surgical controls received the operation without placement of a pledget. Saline controls received a saline-soaked pledget. The applied dosages of gentamicin were 25, 50, 100 and 200 μg.

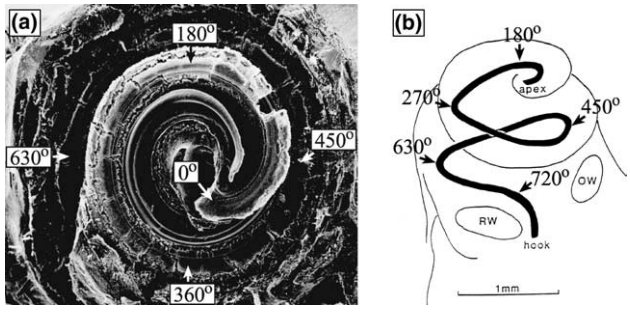

Fig. 1. Division of the cochlea into two 360° turns. Example of left cochlea on SEM (a) and in diagram (b) demonstrating how the cochlea is divided into two turns totaling 720°. The hook region is not visible on this preparation and therefore was not included in our analyses. Extent of damage is reported as the total number of degrees of damage relative to the apex. *Diagram in B obtained from* Harvey (1989).

used to create a small opening in the bone at the apex, through which the cochleas were perfused with 4% paraformaldehyde. Cochleas were then immersion fixed in 4% paraformaldehyde for 2 h at room temperature. Following fixation, cochleas were washed with PBS and decalcified in 0.5 M EDTA for five days. Decalcified cochleas were washed and bisected in the mid-modiolar plane.

Hair cells were labeled with rabbit antiserum directed against myosin VIIa (generously provided by Dr. Tama Hasson). Bisected cochleas were processed in microcentrifuge tubes on a nutator at 4 °C as follows: endogenous peroxidase was quenched by incubation in 3% hydrogen peroxide/30% water/50% PBS/10% methanol for 2.5 h. Blocking was carried out overnight in 5% bovine serum albumin/0.2% normal goat serum/0.2% Triton X-100. Anti-myosin VIIa was diluted 1:30 in blocking solution, and cochleas were incubated in primary antiserum for four days. Following extensive washing, cochleas were incubated overnight in biotinylated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) diluted 1:100 in blocking solution. Cochleas were incubated in ABC (Vector Labs) overnight. Cochleas were then incubated in 0.05 M Tris buffer (pH 7.4) followed by DAB (0.25 mg/ml, Sigma) in 0.05 M Tris buffer/0.005% H_2O_2 overnight.

Following completion of tissue processing, half-cochleas were carefully dissected into half-turns using a microscalpel and iridectomy scissors. The stria vascularis and modiolus were removed along with the osseous spiral lamina. The resulting cochlear half-turn whole mounts were mounted in glycergel (Dako, Carpinteria, CA) and coverslipped.

2.4. Statistics

The extent of hair cell damage was determined for each animal as described above. Means for each group were compared using ANOVA (Statview 4.5; Abacus Concepts, Berkeley, CA).

3. Results

3.1. Summary of hair cell damage characteristics from SEM analyses

We observed hair cell damage as defined by stereocilia fusion or stereocilia bundle loss in all gentamicin-exposed cochleas. Contralateral cochleas processed along with experimental cochleas from each animal appeared completely normal. For each gentamicin dose and exposure period, damage occurred first at the basal region of the cochlea and proceeded toward the apex. All transition zones from damaged to undamaged tissue were toward the apical end of the damaged region. This reliable progression of damage from base to apex resulted in a definable lesion area with no interrupted or intermittent damage (Fig. 2). In other words, the damage did not stop in a specific area and then start again in a more apical area. This characteristic of aminoglycoside-induced damage has been previously documented in both systemic and direct application, in a variety of animal species (Husmann et al., 1998; Matz and Lerner, 1980). There was one exception to this finding. There were some animals ($n = 6$) in the 14 day survival group that demonstrated limited hair cell damage at the extreme apex despite having an area of healthy appearing stereocilia basal to the extreme apex. Stereocilia fusion, when present, occurred at the apical aspect of the lesion with complete stereocilia loss present more basally. Both assessments of damage, stereocilia fusion and loss, were used for analyses.

As seen in Fig. 2 and usually found using conventional protocols, outer hair cells were more susceptible to gentamicin-induced ototoxicity than inner hair cells. Additionally, outer hair cell row three was more susceptible than row two, and row two was more susceptible than row one (Matz and Lerner, 1980). There was no specimen in which the area of inner hair cell damage was greater than that of the outer hair cell damage. The contralateral cochlea of each animal was evaluated using SEM. These cochleas were found to have no evidence of hair cell damage (Fig. 3).

3.2. Dose–response relationship

At all survival times, the amount of hair cell damage increased with increasing doses of gentamicin. Fig. 4 shows this relationship for both outer and inner hair cells, when damage is reported as the total number of degrees along the cochlear spiral in which we observed fused stereocilia or loss of stereocilia bundles on post-operative day one. There is a clear trend toward increasing amounts of damage with increasing dose of gentamicin for both outer and inner hair cells, although the highest dose of 200 μg did not result in as much damage as anticipated. Data were statistically evaluated by ANOVA. The effect of gentamicin dose was significant for outer hair cells ($p < 0.01$). For inner hair cells, the dose effect was not significant ($p = 0.06$). This is largely due to the fact that the damage seen at 200 μg was not as robust as that at 100 μg. When the 200 μg group is removed from the analysis, the dose effect is significant for the inner hair cells ($p < 0.05$). The inner hair cell damage in the 25, 50 and 100 μg groups does increase with increasing gentamicin dose.

A clear dose–response relationship was also seen in animals allowed to survive for three days post-operatively (Fig. 5). The effect of gentamicin dose was significant for outer hair cell damage ($p < 0.01$). The trend continued for inner hair cell damage, however it was not statistically significant ($p = 0.08$). This figure also shows

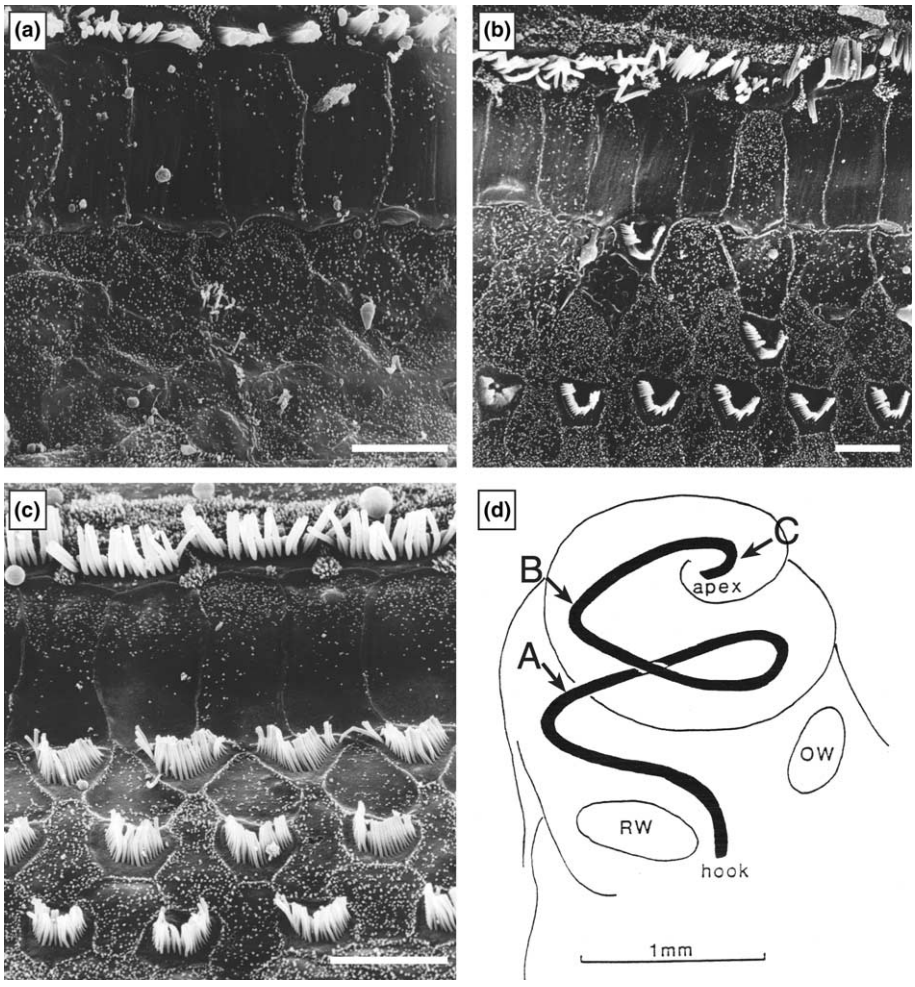

Fig. 2. Characteristics of gentamicin-induced hair cell damage. Representative photomicrographs from an animal that survived three days after receiving a Gelfoam pledget that contained a 50 μg dose of gentamicin. These SEMs demonstrate the increased susceptibility of outer hair cells to gentamicin-induced damage and the progression of damage from base to apex. (a) SEM of the sensory epithelium 610° from the apex. Outer hair cell stereocilia bundles are largely absent, and some inner hair cell stereocilia are fused. (b) More apically in the same animal (260° from apex). Some outer hair cell stereocilia are preserved. (c) Close to the apex in the same animal. Both outer hair cell and inner hair cell stereocilia are preserved. (d) Diagram demonstrating where the SEM images in (a), (b) and (c) were obtained from the cochlea. *Scale bars in A, B, C* = 5 μm.

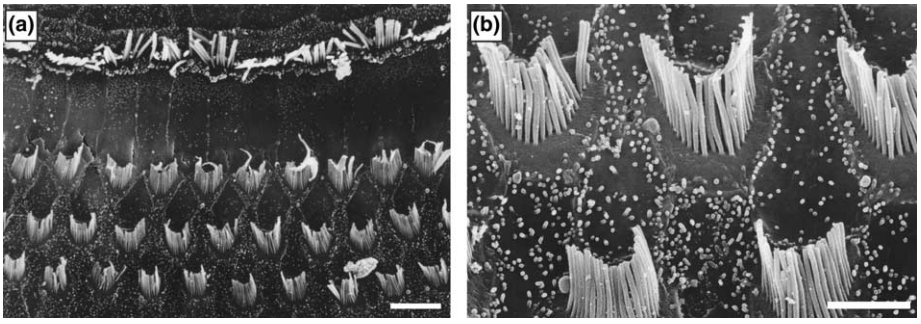

Fig. 3. Untreated, contralateral control cochlea is undamaged. SEM of the contralateral, control (right) cochlea harvested from an animal that received the maximum dose of gentamicin (200 μg) to the left round window niche and survived 14 days. Both lower (*A*) and higher (*B*) magnification reveal intact stereocilia. *Scale bar for A* = 10 μm *and B* = 5 μm.

that by day three, a 100 μg dose of gentamicin resulted in complete outer hair cell damage for all subjects ($n = 6$). The 200 μg group is not included in the analysis of hair cell damage at day three due to the fact that only one of the five animals in this group had a damage pattern that could be unequivocally quantified. Promi-

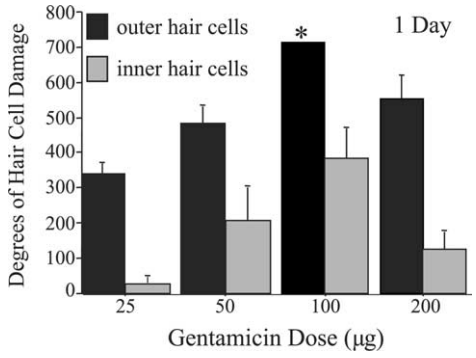

Fig. 4. Dose–response relationship for inner and outer hair cell damage at one day survival. For outer hair cells there is a significant increase in the area of damage as gentamicin dose increases ($p < 0.01$). For inner hair cells there is a similar trend ($p = 0.06$). Error bars represent SEM. *Note: There was complete damage to the outer hair cells in all 4 subjects at the 100 μg dose, resulting in no error bars.

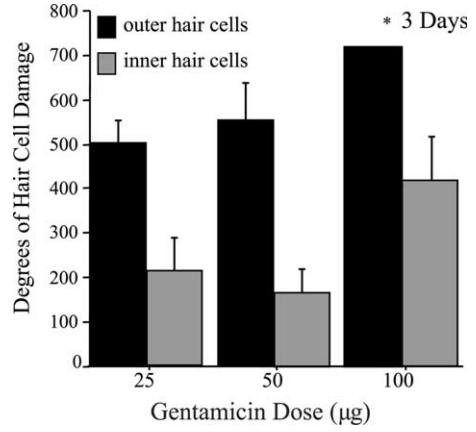

Fig. 5. Dose–response relationship for inner and outer hair cell damage at three days survival. For outer hair cells there is a significant increase in the area of damage as gentamicin dose increases ($p < 0.05$). For inner hair cells there is a similar trend ($p = 0.08$). Error bars represent SEM. *Note: At the 100 μg dose there was complete damage to the hair cells in all subjects, resulting in no error bars. The 200 μg treatment group is not included as the $n = 1$. This was due to the inability to analyze the amount of damage for most specimens at this time point by SEM due to cellular extrusion debris.

nent and widespread hair cell extrusion was observed at this time point and at this dose. This caused four of the specimens in this group to have a thick layer of cellular debris, making definitive quantification of damage impossible via SEM (Fig. 6). The non-operated cochleas from these animals were processed simultaneously and had no evidence of damage or sub-optimal fixation. The one subject that could be analyzed on SEM had complete stereocilia bundle loss for both outer and inner hair cells throughout all turns.

The dose–response relationship was quite clear in the 14 day survival group. This was true whether damage was assessed by the number of degrees of stereocilia bundle loss, or the total number of degrees of hair cell damage (stereocilia fusion and bundle loss). Fig. 7 shows that total hair cell damage increases with increasing gentamicin dose for both outer and inner hair cells. The effect of gentamicin dose is significant for both outer and inner hair cells ($p < 0.01$). All animals in the 200 μg dose group demonstrated complete outer hair cell stereocilia bundle loss (resulting in no standard error). Fig. 7 also demonstrates a ''ceiling'' effect with respect to the amount of outer hair cell loss. Since a complete lesion of the entire sensory epithelium is represented by 720° of damage, it is apparent that animals in even the lowest dose group (25 μg) approach complete outer hair cell damage by 14 days survival. Complete outer and inner hair cell damage was achieved in three of the four animals that received 200 μg of gentamicin, and four of the seven animals that received 100 μg of gentamicin.

Both the saline controls and the sham-operated controls exhibited mild to moderate amounts of outer hair cell damage (Fig. 7). However, the 200, 100 and 50 μg treatment groups were significantly different than the saline controls and the sham operation controls for outer hair cell damage ($p < 0.05$ for all). The 25 μg group was statistically different than the sham operation group ($p < 0.01$). Saline controls had significantly more damage than sham operation controls ($p < 0.01$). Both saline and sham controls had mild amounts of inner hair cell damage. Again, the 200, 100 and 50 μg gentamicin treatment groups had significantly greater damage than the saline and sham controls, while the 25 μg group had significantly greater damage than the sham control for inner hair cell damage ($p < 0.05$ for all).

3.3. Time-dependent effects

There was a clear progression of lesion size from post-operative day 1 to day 14. Given the large extent of damage even at early time periods with the highest doses, this progression was not evident at the 200 and 100 μg doses. Data for the 25 μg dose of gentamicin best exemplified the trend of increased damage with increased survival time. Fig. 8 shows this effect for inner and outer hair cells. ANOVA revealed a significant main effect for both hair cell types ($p < 0.05$). However, there was not a statistically significant difference between inner hair cell damage at 3 and 14 days. This may be due to the increased variability in damage seen at low dose on day three of survival. Alternately, it may suggest that while outer hair cell damage continues to progress past post-operative day three, inner hair cell damage stabilizes by this time period.

3.4. Immunohistochemistry

To confirm the relationship between loss of stereocilia bundles as assessed by SEM and loss of hair cells, we examined the effects of this damage protocol using an antibody against myosin VIIa. The number of degrees of

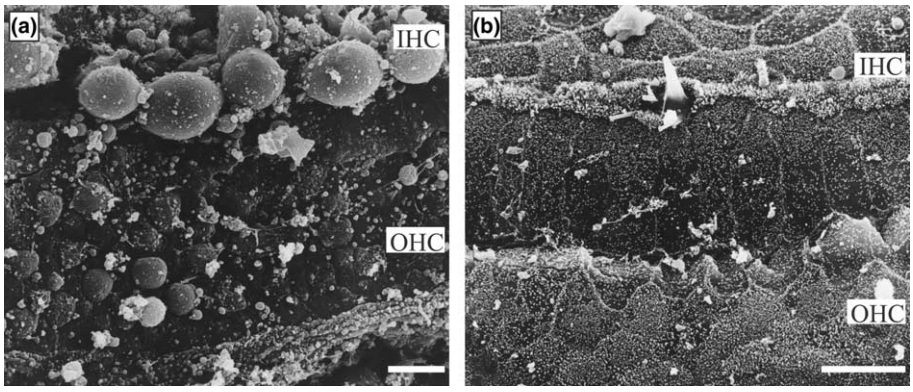

Fig. 6. Example of cellular extrusion debris at three days survival after high dose gentamicin application and the resolution of the debris by 14 days survival. SEMs of hair cells from animals that were sacrificed 3 days (a) and 14 days (b) following treatment with a 200 μg dose of gentamicin. The three day animal exhibits extensive cellular debris typical of that seen in high doses with this protocol, making quantification of the extent of hair cell damage difficult. The 14-day animal exhibits a sensory epithelium that is essentially denuded of stereocilia bundles. *Scale bar in A* = 5 μm *and B* = 10 μm.

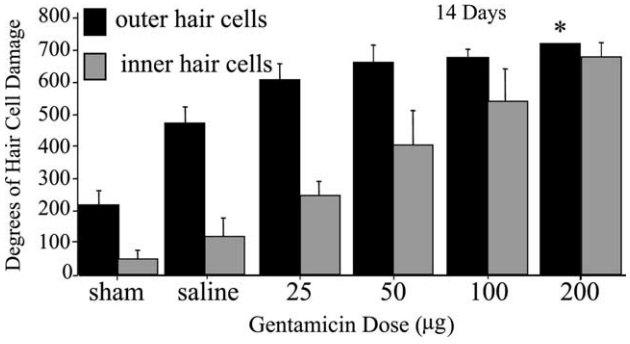

Fig. 7. Dose–response relationship for inner and outer hair cell damage at 14 days survival. There is a significant increase in the area of damage as gentamicin dose increases for both outer hair cells and inner hair cells ($p < 0.01$ for each). Damage is seen in both the saline and operated controls. Error bars represent SEM. *Note: At 200 μg of gentamicin, there was complete damage to the outer hair cells in all subjects, resulting in no error bars.

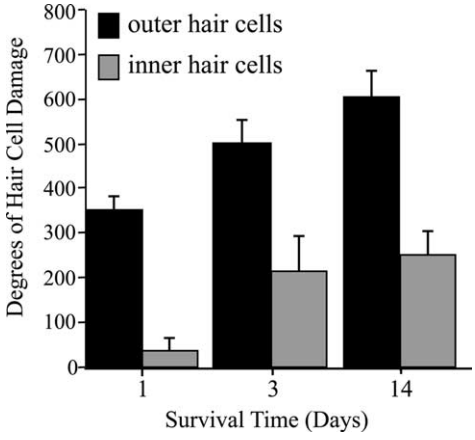

Fig. 8. Progression of damage over 14 days in animals that received 25 μg of gentamicin. Both outer hair cell and inner hair cell damage significantly progressed between 1 and 14 days survival ($p < 0.05$). Error bars represent SEM.

hair cell loss was calculated for three animals at 14 days survival utilizing light microscopy and immunohistochemistry. Representative results are shown in Fig. 9. Immunohistochemistry on control cochleas revealed normal staining of both outer and inner hair cell bodies throughout the length of the cochlea (Fig. 9(a)). Absence of staining was interpreted as a loss of the hair cell body. The amount of damage seen with this method was consistent with the measurements obtained using SEM. One animal that was treated with 25 μg of gentamicin had 470° of outer hair cell loss and 130° of inner hair cell loss, as evidenced by a lack of myosin VIIa staining up to those points along the cochlea. The other had 360° of outer hair cell loss and 90° of inner hair cell loss (Fig. 9(b)). The one animal that received 200 μg of gentamicin and was sacrificed on postoperative day 14 showed no evidence of surviving inner or outer hair cell bodies on light microscopy, again consistent with the SEM findings (Fig. 9(c)).

4. Discussion

Experimental protocols to induce hair cell damage in mammals, particularly mice, have been limited by systemic toxicity, the need for repeated applications to produce damage, lesion variability, and lack of rapid hair cell damage following a single application. Our data show that the placement of a gentamicin-soaked Gelfoam pledget at the round window niche of the mouse inner ear is an effective method for production of relatively rapid, dose-dependent hair cell damage. This technique can produce lesions along the entire length of the sensory epithelium and avoids systemic toxicity

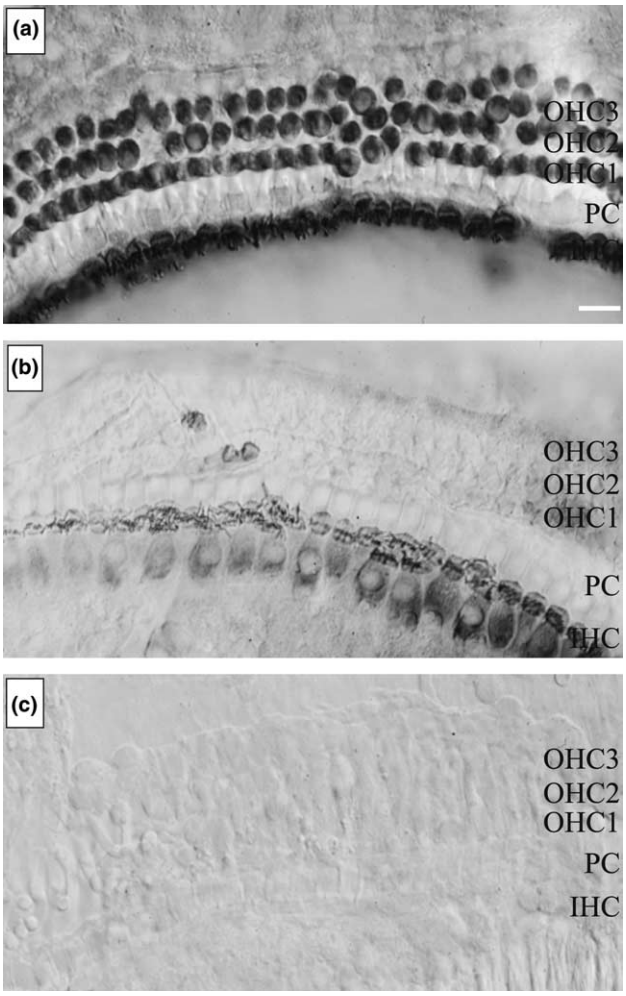

Fig. 9. Immunohistochemistry using the hair cell marker anti-myosin VIIa. (a) Sensory epithelium from a contralateral, control cochlea revealing normal staining of the three rows of outer hair cells (OHC) and single row of inner hair cells (IHC). (b) Basal sensory epithelium (360° to 450° from apex) from an animal treated with 25 μg of gentamicin and sacrificed on post-operative day 14. There is very little staining in the OHC region, but the IHCs are visible, consistent with damage quantification on SEM. (c) Sensory epithelium from an animal treated with 200 μg gentamicin and sacrificed on post-operative day 14. There are no apparent IHC or OHC. Again, this is consistent with damage quantification by SEM. *Scale bar in A* = 10 μm.

while leaving the contralateral ear unaffected to serve as a control. Damage proceeds from base to apex and affects outer hair cells prior to inner hair cells. The transition from damaged to undamaged sensory epithelium occurs at the apical end of the damaged region, and regions of undamaged hair cells are usually not observed in areas more apical to an area of complete damage. These characteristics of aminoglycoside-induced damage allow for a definable lesion area, making studies of "damaged", "undamaged" and "transition" regions possible. The one exception to these observations is the six animals that showed a very short segment of damage and hair cell loss at the extreme apex, despite healthy appearing hair cells more basally. Interestingly, other researchers have also found that in addition to the well-known pattern of outer and inner hair cell damage extending from base to apex, damage may also occur at the extreme apex. Hequembourg and Liberman (2001), in a study of age-related cochlear degeneration, found an apical focus of spiral ganglion cell loss in the C57Bl/6 mouse. However, they did not find accompanying hair cell loss.

To understand the significance of cellular and molecular changes following hair cell damage, it is useful to be capable of temporally relating those changes to the eventual changes in cellular integrity. This is particularly true when very early events following hair cell damage are of interest. Because systemic aminoglycoside injections in mammals are limited by toxicity, lower doses given over an extended period of time are often necessary before hair cell damage is obtained (Wu et al., 2001; Forge and Shacht, 2000; Sullivan et al., 1987). This results in gradual injury to the sensory epithelium and interferes with attempts to pinpoint the stages of cellular change in time and to study the earliest events following damage. We feel that the protocol presented here, by producing significant damage after a single application of gentamicin, will allow greater precision in examination of early events.

A variety of local aminoglycoside application techniques have been utilized in mammals. Most have resulted in either an inability to produce a lesion of consistent size or an inability to produce anything less than a complete lesion (Kimura et al., 1988; Lee and Kimura, 1991; Dodson, 1997; Wanamaker et al., 1998). *Trans*-tympanic injection of gentamicin in gerbils resulted in a lack of hair cell damage after one injection and produced highly variable amounts of damage after five injections, with one animal showing virtually no hair cell loss (Wanamaker et al., 1998). Kimura et al. (1988) found cochlear damage following repeated administration of *trans*-tympanic gentamicin in guinea pigs, but no specific lesion size was noted and no studies with a single injection were performed. Lee and Kimura (1991) performed injections of gentamicin into the endolymphatic sac of guinea pigs and found cochlear damage following a single injection, but the size of the lesion was extremely variable. Intra-cochlear perfusion of aminoglycosides in guinea pigs results in destruction of all cochlear hair cells (Dodson, 1997), but the method does not appear to allow for subtotal lesions and may be difficult to perform in mammals as small as the mouse. The ability to achieve subtotal damage is necessary because the cellular and molecular information obtained from tissue adjacent to the site of damage is often important (Bhave et al., 1995).

Not all of the hair cell damage we observed can be attributed to the effects of gentamicin. Both the sham-operated controls as well as the controls with placement

of saline-soaked Gelfoam exhibited hair cell damage. In fact, the average amount of outer hair cell damage at 14 days survival for the saline-soaked Gelfoam controls was ≈450°. The average damage for sham-operated controls was significantly less at 200° ($p < 0.01$). Average inner hair cell damage was relatively minimal at ≈100° for saline and 40° for sham controls. Despite the relatively large amount of outer hair cell damage with the saline controls, gentamicin doses of 50, 100 and 200 μg produced significantly greater damage. There are several possible explanations for the excessive damage seen in our "control cochleas". The temporal bone drilling that is required to gain access to the round window niche may induce acoustic and/or mechanical damage to the cochlea. This may explain the damage found in the sham-operated controls. However, such acoustic trauma could also cause damage in the contralateral cochlea via bone conduction, and we did not find this to be the case. The drilling and/or the placement of the Gelfoam at the round window membrane may also create a perilymphatic fistula. Perilymphatic fistula may also explain the occasional loss of hair cells at the extreme apex observed in some of the subjects, since perilymphatic fistula can result in hair cell damage at the basal turn of the cochlea as well as the extreme apex (Flint et al., 1988). Additionally, Gelfoam itself may contain one or more compounds that are capable of crossing the round window membrane and causing hair cell damage. Ultimately, the source of the hair cell damage distinct from the effect of gentamicin remains unknown. We find that gentamicin in this study does produce damage above and beyond that produced in the controls, and this damage does occur in a dose-dependent manner.

It is possible that leaving the Gelfoam in place throughout the study period may act as a sustained-release mechanism for the gentamicin over an extended period, interfering with the pinpointing of damage in time. We suspect that this is not the case. Husmann et al. (1998) found no significant differences in the amount of damage in chicks whether the pledget was left in place for 30 min or five days. Yet, further studies such as analysis of the concentration of gentamicin remaining in the Gelfoam at various time periods, assays of gentamicin concentration within the endolymph at various time periods or timed removal of the Gelfoam pledget will be required to further address this issue.

This study relies primarily on SEM of the surface of the sensory epithelium to quantify hair cell damage and loss. However, loss of stereocilia is not synonymous with death of the hair cell (Zheng et al., 1999a). To examine whether loss of stereocilia correlated with loss of hair cell bodies, several damaged cochleas were evaluated at the light microscope level by immunohistochemistry using an antibody against myosin VIIa. Analysis of the amount of damage using immunohistochemistry was consistent with our results using SEM. Thus, at least at later time points, SEM evidence of lack of stereocilia is consistent with an interpretation of hair cell death.

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