CELL DIVISION IN THE GERBIL COCHLEA AFTER ACOUSTIC TRAUMA

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ABSTRACT

The recent discovery of hair cell regeneration in the avian inner ear raises the possibility that hair cell regeneration might occur in the mammalian cochlea as well. The authors used ^{3}H-thymidine labeling to detect mitotic activity in the cochleas of normal 3-week old gerbils exposed to acoustic trauma. Following an acoustic insult that caused progressively more severe damage in an apical to basal progression, ^{3}H-thymidine was injected for 5 days. Control animals were not exposed to the acoustic insult. The gerbils' cochleas were sectioned and processed for autoradiography. In the control cochleas, there were extremely rare labeled cells in the stria, the spiral ligament, and the glial cells around the acoustic nerve fibers. In the damaged cochleas, no evidence of hair cell regeneration or of any cell division within the normal sensory epithelial structures was seen. Three labeled cells were seen in intercellular spaces within the sensory epithelium; they appeared to be macrophages. Frequent cell division was seen in numerous other regions of the damaged cochleas and among glial cells adjacent to the acoustic nerve fibers. It is concluded that there is no evidence for hair cell regeneration following acoustic trauma in the gerbil, but acoustic trauma does induce cell division in numerous other areas of the cochlea.

It has traditionally been believed that hair cells in the auditory and vestibular epithelium of warm-blooded vertebrates are not produced postembryonically.[1] Recent work has shown, however, that the avian auditory epithelium is capable of regenerating hair cells to replace those lost to acoustic and ototoxic insult.[2-6] Essentially complete hair cell replacement is seen. The new hair cells develop the size and the stereocilia number and orientation appropriate to their location in the basilar papilla; and the new hair cells appear to be functional and to correct most of the hearing loss that follows the initial hair cell loss.[7]

Ruben[1] and Koburg[8] have shown that new hair cells are not produced postembryonically in the organ of Corti in the normal mouse. The discovery of hair cell regeneration in the avian auditory epithelium, however, raises the possibility that increases in mitotic activity or even hair cell regeneration might occur in the mammalian sensory epithelium after ototoxic insult. This experiment was designed to detect hair cell regeneration and other cell proliferation in the mammalian cochlea following acoustic trauma.

If mammals were capable of the complete replacement of the receptor cells that appears to occur in birds, the mammalian cochlea would always maintain a full complement of inner and outer hair cells. In both humans and other species, however, it has repeatedly been shown that permanent hair cell loss occurs with noise damage, ototoxic insult, and aging.[9-11] Therefore, if hair cell regeneration occurs in mammals, it must be partial, limited to certain specific conditions (e.g., minimal damage), or limited to younger animals. Our experimental model was chosen with these considerations in mind.

We used 3-week old gerbils to allow for the possibility that regeneration in the organ of Corti might be limited to young animals. Since regeneration might occur only after some specific amount of damage (e.g., only after minimal damage or only after maximal damage), we selected an acoustic exposure paradigm that caused graded damage to the organ of Corti, ranging from little or no visible damage in the apex of the cochlea, to complete obliteration of the organ of Corti in the basal cochlea.

We used ^{3}H-thymidine autoradiography to label dividing cells. Chemically, the radioisotope ^{3}H-thymidine is identical to thymidine, and is therefore incorporated into DNA during the synthetic (S) phase of the cell cycle. Its presence in tissue sections can be detected by coating the sections with a photo-

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graphic emulsion that is exposed by the emission of beta particles from ^{3}H-thymidine. After photographic development, exposed emulsion appears as small black grains over the nuclei of cells that were proliferating while the ^{3}H-thymidine was available.

MATERIAL AND METHODS

Four, 21-day-old gerbil pups from the University of Washington colony (derived from breeding stock from Tumblebrook Farms, West Brookfield, Massachusetts) were subjected to acoustic injury. The acoustic insult consisted of a two octave (1460–5650 Hz) band of white noise at 130 dB SPL for 14 hours. Two control pups, aged 24 days, were not subjected to the acoustic insult. Beginning immediately after the noise exposure, the pups were injected intramuscularly with 0.2 mCi of ^{3}H-thymidine twice daily for 5 days (total dose, 2.0 mCi, approximately 130 mCi/kg body weight). The control pups received one fewer injection (total dose, 1.8 mCi). Seven to eleven days after the final injection of ^{3}H-thymidine, the pups were deeply anesthetized and their cochleas perfused with 2 percent paraformaldehyde/2.5 percent glutaraldehyde in 0.1 N cacodylate buffer with 10 mg/L CaCl. The pups were then sacrificed and their cochleas removed and kept in fixative overnight at 4°C.

The control animals were injected, sacrificed, and processed several months after the experimental animals. During this time interval, it was found to be more convenient to decalcify cochleas prior to sectioning. The processing of the noise-exposed and control cochleas was thus slightly different.

The noise-exposed cochleas were washed three times in 0.1 N phosphate buffered saline (PBS), pH 7.4, dehydrated in a graded series of methanols and acetones, and embedded in Polybed media (Polysciences, Warrington, Pennsylvania). The control cochleas were washed three times in PBS, decalcified in 10 percent EDTA for 10 days, washed again three times in PBS, and dehydrated and embedded as above. The cochleas were divided into quarters axially by two midmodiolar cuts made with a jeweler's diamond saw. To facilitate sectioning, the external bony cochlea was drilled away from the exposed face of the noise-damaged specimens under a binocular dissecting microscope (to the extent possible without damaging the membranous cochlea). This step was not necessary in the decalcified control cochleas.

Between 10 and 40 sections 3 microns thick, each containing all three cochlear turns, were taken from the exposed face of each cochlear fragment. The sections were placed on acid-washed, chrome-alum subbed slides, which were then dipped in Kodak NTB2 Nuclear Track emulsion (1:1 dilution) and exposed at 4°C for 2–4 months. One set of slides was allowed to expose for slightly more than 2 years. The emulsion was developed in D-19 developer for 4 minutes, rinsed in distilled water, and fixed in Kodak fix for 3.5 minutes at 13°C. The slides were counterstained with toluidine blue and coverslipped with DPX (Gallard-Schlesinger, Carle Place, New York). A labeled nucleus was defined as one with five or more silver grains above it. In those sections that were allowed to develop for 2 years, background grains were more frequent, and a labeled nucleus was defined as one with 25 or more grains above it.

The care and use of the animals reported on in this study were approved by the University of Washington animal care committee.

RESULTS

Cell Division in the Undamaged Cochlea

Over 200 sections, most containing all three cochlear turns, were examined from the control cochleas. No labeling was seen in the organ of Corti, the epithelium of Reissner's membrane, or the limbus or the tympanic border cells. Labeling was seen above four cells in the stria vascularis, three glial cells among the acoustic nerve fibers, and four cells in the spiral ligament. Fibrous tissue cells and vascular endothelial cells were routinely labeled within the control cochleas, confirming an adequate level of ^{3}H-thymidine within the cochleas and successful autoradiographic processing.

Characterization of Acoustic Trauma

In cochleas from the noise-damaged animals, damage to the cochlear structures was graded, with little or no damage at the cochlear apex and progressively more severe damage in the middle and basal turns (Fig. 1, A–D). Sections from the apical turn often showed no damage at the light microscopic level. Damage in the middle and basal turns ranged from slight swelling of the epithelial cells, to loss of outer hair cells but preservation of the underlying architecture, to progressive loss of the architecture of the organ of Corti, to complete obliteration of the organ of Corti and its replacement by a new epithelial monolayer on the basement membrane.

Absence of Labeling of Epithelial Elements

In over 300 sections examined from the noise-damaged cochleas, there was no labeling of hair cells, supporting cells, or any cells within the sensory epithelium proper (see Fig. 1, A–D). In three minimally to moderately damaged sections, labeled cells were seen in the sensory epithelium that were not consistent with any of the normal cellular elements (Fig. 2). One was within the tunnel of Corti, one was in the space of Nuel, and one was in the intercellular space between the inner and middle rows of outer hair cells. The identity of these cells is uncertain, but their location in the intercellular spaces and their vacuo-

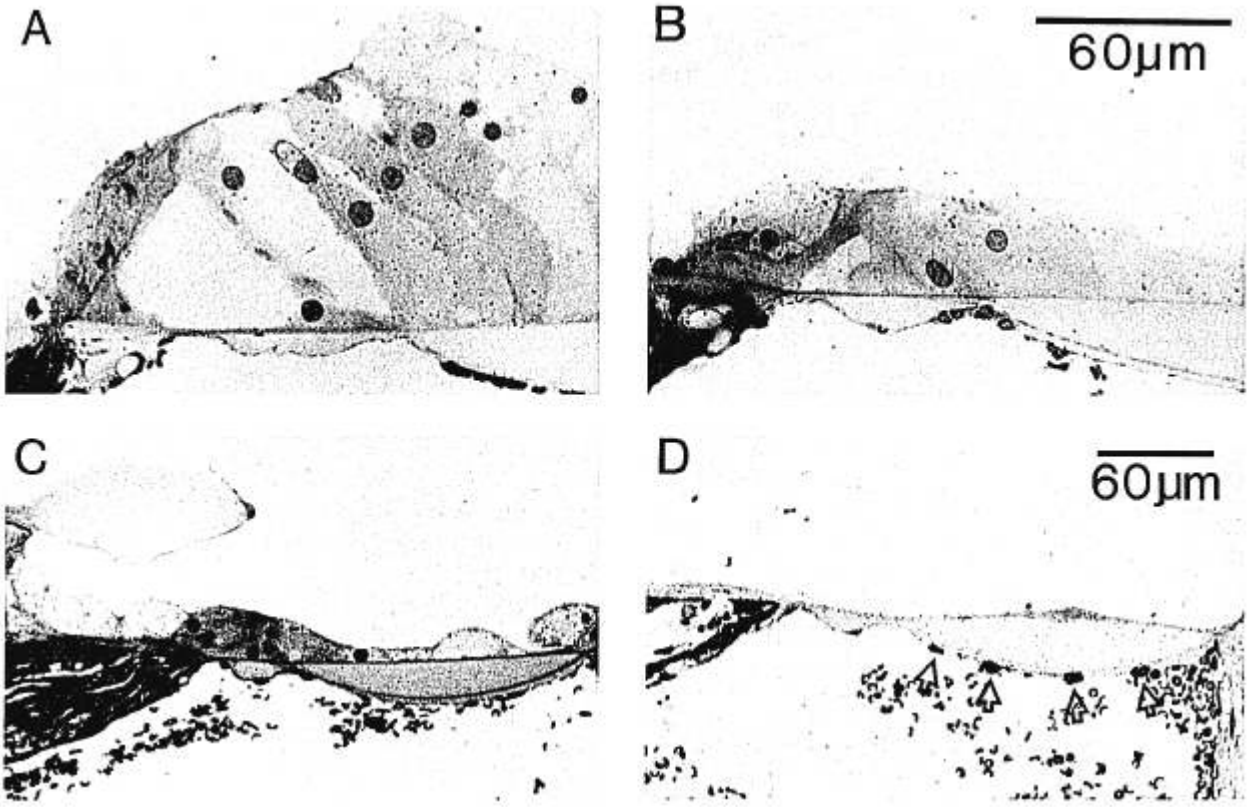

Figure 1. Damage to the gerbil organ of Corti following acoustic trauma. Progressively more severe damage was seen in an apical to basal progression. In the apical turn (not shown), there was typically little or no visible damage. In the middle and basal turns damage ranged from minimal cell swelling (A), to loss of outer hair cells with retention of the tunnel of Corti (B), to a complete loss of recognizable cellular elements and architecture (C), to complete destruction of the organ of Corti and its replacement by a new epithelial monolayer (D). In no instance was any autoradiographic labeling seen within the sensory epithelium, although tympanic border cells are labeled in 1D (*arrows*). (Bars = 60 µm. 1A is at the same scale as 1B; 1C is at the same scale as 1D.)

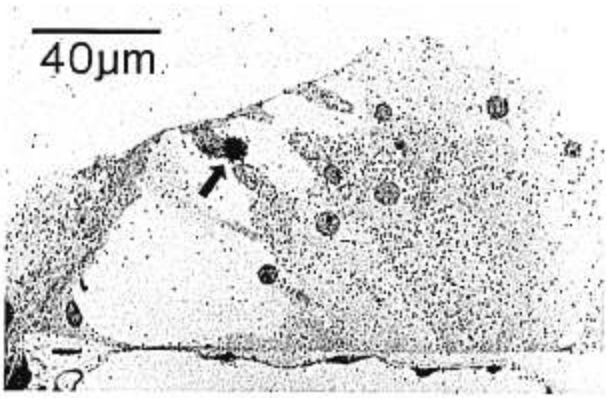

Figure 2. ^{3}H-thymidine-labeled nucleus in the space of Nuel within the organ of Corti following acoustic trauma. Its light microscopic appearance and location is not consistent with any of the normal cellular elements of the organ of Corti. A total of three labeled cells were seen in intercellular spaces within the organ of Corti in over 300 sections examined. It was difficult to identify these cells with certainty because of the intense labeling, but as all three were in intercellular spaces and had vacuolated cytoplasm, it was believed that they were most likely macrophages. Background labeling is high as the emulsion was exposed for slightly more than 2 years. Bar = 40 µm.

lated cytoplasm suggested that they were macrophages. Their nuclei could not be seen because of the intense autoradiographic labeling.

Cell Division in the Damaged Cochlea

Although no labeling was present within the sensory epithelium, labeling was noted in many of the other cochlear structures in the damaged cochleas (Figs. 3–8). Labeled cells were observed most commonly among the acoustic nerve fibers coursing toward the modiolus (Fig. 3). In the middle and basal turns of the damaged cochleas, almost every section had from one to ten labeled cells among the acoustic nerve fibers. In the upper turn, where damage to the cochlea was often not apparent by light microscopy, about half the sections had labeled cells in this region, usually one or two per section.

Labeled cells were common in the stria vascularis (Fig. 4). Typically one to three labeled cells per section were present in the stria in the middle and basal turns. Labeled cells were occasionally seen in the stria in the upper turn. The labeled cells in the stria were most commonly basal cells or capillary

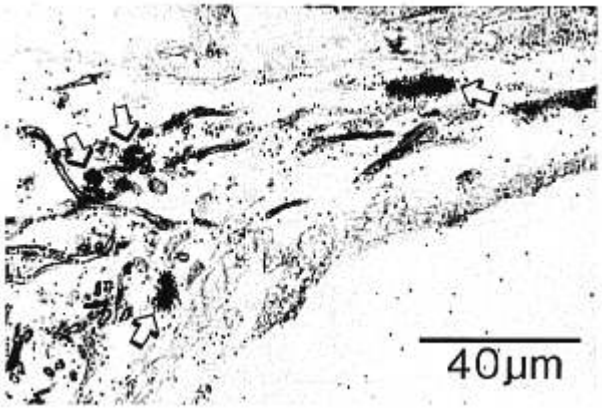

Figure 3. ^{3}H-thymidine-labeled nuclei in the glial cells around the acoustic nerve fibers in noise-damaged gerbil cochlea. Bar = 40 µm.

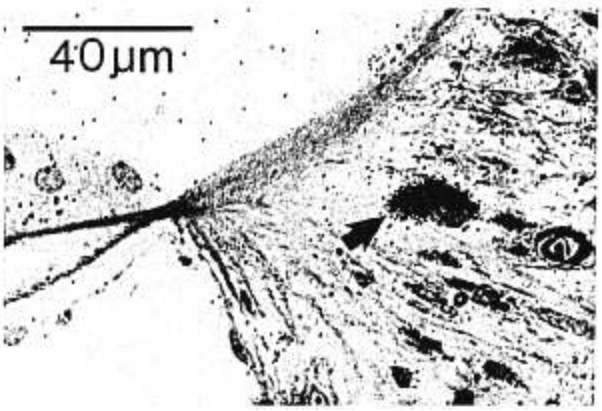

Figure 5. ^{3}H-thymidine-labeled nucleus in the spiral ligament in noise-damaged gerbil cochlea. Bar = 40 µm.

endothelial cells, but marginal (chromophil) and intermediate (chromophobe) cells were also often labeled.

Labeled nuclei were seen among the tympanic border cells in the middle and basal turns in about every fourth section (Figs. 1D and 8). Labeled tympanic border cells were much less common in the undamaged upper turn (about one labeled cell in every 30 sections). Labeled nuclei were seen in the stroma of the spiral ligament in approximately every other section (Fig. 5).

Labeled cells were present on Reissner's membrane in about one section in ten and in the stroma of the limbus in about one section in twenty (Figs. 6 and 7). No labeling was seen in these locations in the undamaged upper turn. No labeling of the limbus epithelium was seen.

In those areas where the organ of Corti was completely obliterated by acoustic trauma and replaced by an epithelial monolayer, approximately every tenth nucleus in this monolayer was labeled (Fig. 8).

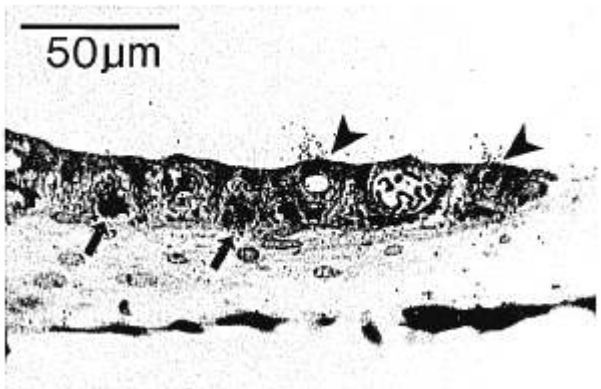

Figure 4. ^{3}H-thymidine-labeled nuclei in the stria vascularis in noise-damaged gerbil cochlea. Basal cells (*arrows*) and marginal (chromophil) cells (*arrowheads*) are labeled. Intermediate (chromophobe) cells and capillary endothelial cells were also labeled in many sections (not shown). Bar = 50 µm.

DISCUSSION

Since the discovery of hair cell regeneration in the avian inner ear, a considerable body of knowledge has accumulated about this process. It is known that the avian auditory epithelium is normally very nearly quiescent,[12] that the auditory epithelium regenerates hair cells to replace those lost to acoustic[4,5,13,14] and ototoxic[2,6,15] insult, that this regeneration results in an essentially normal number of hair cells following even very severe injury,[16] and that the regenerated hair cells appear to be functional.[17,18]

In the avian vestibular epithelium, there is an ongoing production of new sensory hair cells[19,20] and the rate of hair cell production is dramatically increased to replace hair cells lost to ototoxic insult.[21] It appears that the ongoing hair cell production in the vestibular epithelium serves to replace hair cells lost to a low level of "natural" cell death, as there is evidence that the hair cell number in the vestibular epithelia does not increase over the first 2 postnatal months.[21]

The discovery of hair cell regeneration in the avian auditory epithelium raises the question of

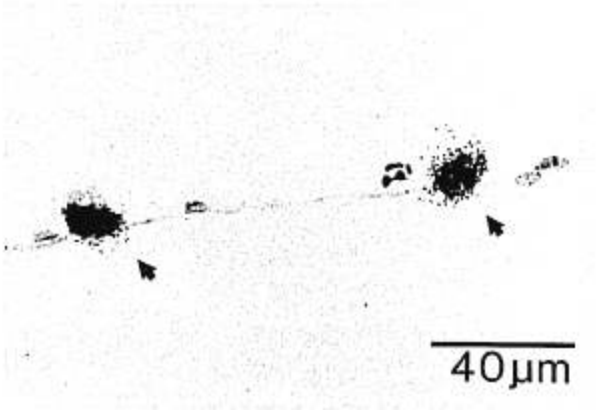

Figure 6. ^{3}H-thymidine-labeled nuclei on Reissner's membrane in a noise-damaged gerbil cochlea. Bar = 40 µm

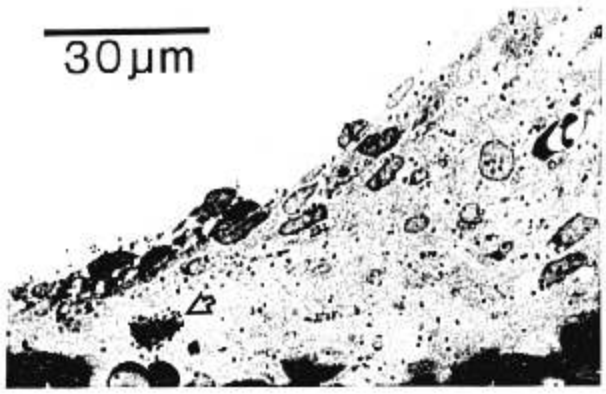

Figure 7. ^{3}H-thymidine-labeled nuclei in the limbus stroma in a noise-damaged gerbil cochlea. No labeling was seen in the limbus epithelium. Bar = 30 µm.

whether hair cell regeneration might also occur in the normally quiescent mammalian auditory epithelium. Since it is known that in humans and other mammals there is a permanent decrease in hair cell number with acoustic trauma, ototoxic insult, or aging,[9-11] it is illogical to postulate complete hair cell regeneration in mammals. It is possible, however, that limited hair cell regeneration might occur in young animals, or following a limited insult. The present experiment was designed to test this possibility, and to detect cell division in other areas within the gerbil cochlea following acoustic trauma.

Cell Division in the Undamaged Gerbil Inner Ear

In the control animals, cell division was seen only in the stria vascularis, the spiral ligament, and the Schwann cells around the acoustic nerve fibers. Cell division was exceedingly uncommon in these structures; only four strial cells, four spiral ligament cells, and three Schwann cells were labeled in over 200 sections examined. In a study of the terminal mitoses in the developing murine cochlea, Ruben[1] found terminal mitoses continuing through postnatal day 7 (the oldest animals he examined) in six cell types: the stria vascularis, the Schwann cells, the spiral ligament, the limbus stroma, the epithelium of the basilar membrane (tympanic border cells), and in Reissner's membrane. Koburg[8] reported cell division in adult mice in the spiral ligament, the stria, the Schwann cells, the epithelium of the basilar membrane, the epithelium and stroma of the limbus, and on Reissner's membrane. In light of these reports, the authors carefully examined every section from the control animals in an attempt to identify labeling in these areas, but were unable to identify labeling in the tympanic border cells or the limbus or on Reissner's membrane. However, at least one labeled fibroblast or vascular endothelial cell was identified in more than 90 percent of the control sections, to exclude the possibility that the autoradiography in these sections was improperly processed. The absence of cell division in the limbus stroma and epithelium, on Reissner's membrane, and in the tympanic border cells in the present study may be attributable to a difference in potential for cell division in inner ear epithelial tissues between the mouse and the gerbil. It is more likely, however, that the failure to identify cell division in these tissues simply reflects an extremely low rate of cell division in these tissues when undamaged.

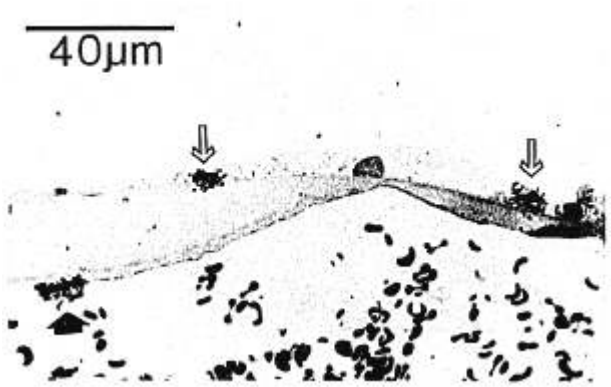

Figure 8. ^{3}H-thymidine-labeled nuclei on new epithelial monolayer (*open arrows*) and in a tympanic border cell (*solid arrow*) in a section from the basal turn of a noise-damaged gerbil cochlea in which the organ of Corti was completely obliterated. Bar = 40 µm. Labeled tympanic border cells can also be seen in Figure 1D.

Absence of Evidence of Cell Division within the Organ of Corti

The noise exposure used caused minimal damage to the organ of Corti in the upper turn, moderate to severe damage in the middle turn, and profound damage in the lower turn. This pattern is consistent with previous studies of noise damage in the mammalian cochlea.[22] In no circumstance was there any evidence of hair cell regeneration, or indeed of any cell division, within the sensory epithelium proper. These data suggest that the capacity for epithelial regeneration, retained in the inner ear of lower vertebrates and birds, has been lost or is actively inhibited within the mammalian organ of Corti.

Two limitations of this model should be noted. The insult used (brief, severe acoustic overstimulation) causes significant mechanical trauma to the entire acoustic epithelium. It is possible that this insult damages potential hair cell precursors and thus prevents regeneration. These results do not rule out regeneration following insults that cause more hair cell-specific injury, such as ototoxic insult or long-term exposure to low-level noise. Secondly, the method used for detecting hair cell regeneration, ^{3}H-thymidine labeling, presupposes that new hair cells arise only via cell division. New hair cells arising from direct transdifferentiation of precursor cells, without preliminary cell division, would not be detected by ^{3}H-thymidine labeling.

The absence of hair cell regeneration in the auditory epithelium does not rule out ongoing production of hair cells or regenerative potential in the mammalian vestibular epithelia. The gross structure of the mammalian cochlea and the arrangement of the sensory epithelium is substantially different from the avian auditory end-organ.[23] The mammalian vestibular organs and sensory epithelia, in contrast, are little different from the avian system or indeed from other lower vertebrate species.[24] It would be plausible, therefore, that the mammalian vestibular epithelium might retain some regenerative capacity even though the auditory epithelium does not.

Two recent reports have suggested that there is, in fact, an extremely low rate of hair cell regeneration in the damaged mammalian vestibular epithelium. In one of these studies, Forge et al performed scanning electron microscopy on guinea pig vestibular epithelia following ototoxic insult and demonstrated morphologically immature hair cell bundles, suggesting hair cell regeneration was taking place.[25] In the other, Warchol et al harvested vestibular organs from guinea pigs and from humans (at translabyrinthine surgical procedures), maintained them in culture and subjected them to aminoglycoside insult. After 4 weeks, there was autoradiographic evidence of supporting cell proliferation and some of the new cells had characteristics of immature hair cells.[26] Taken together, these reports suggest that the mammalian vestibular epithelium retains at least some capacity to replace lost sensory cells. This contrasts with our results, which suggest that the mature mammalian auditory epithelium has lost the capacity to replace hair cells following damage caused by noise exposure.

Labeled Cells in Intercellular Spaces within the Organ of Corti

Three labeled cells were seen in the sensory epithelium, all in mildly to moderately damaged areas. All were within intercellular spaces, and none were consistent in appearance or location with any normal cellular elements. Their nuclei could not be seen because of intense autoradiographic labeling. Because they displayed a moderately vacuolated cytoplasm, it was believed that they were probably macrophages. Jones and Corwin[27] have reported phagocytic cells in both the normal and regenerating lateral line, and Corwin et al[28] report that phagocytic cells are seen in areas of damage in explants of auditory epithelium in both birds and mammals. It is certainly reasonable to suppose that a damaged inner ear in an active inflammatory state would attract phagocytic cells.

Post-Traumatic Cell Division in the Nonsensory Tissues of the Inner Ear

Evidence of cell division was seen in numerous areas in the cochlea, including the cells surrounding the acoustic nerve fibers, the stria vascularis, the spiral ligament, the tympanic border cells, the limbus, the epithelium of Reissner's membrane, and (in those sections in which the organ of Corti was completely obliterated) the new epithelial monolayer covering the basilar membrane. Labeling was most common among the cells surrounding the acoustic nerve fibers coursing between the organ of Corti and the modiolus. These cells are presumably glia that had undergone division in response to nerve fiber degeneration. These labeled glia were present even in sections in which there was little or no evidence of damage to the cochlea at the light microscopic level. Labeling among the acoustic nerve fibers was more common, however, in areas of severe damage to the organ of Corti.

Labeling was also commonly seen in the stria vascularis, most often in areas where damage to the organ of Corti was more severe. Several authors have shown changes in strial blood flow or capillary permeability during or following acoustic trauma. After severe trauma, the stria may undergo atrophy.[29] The cell division seen in this experiment probably represents repair in a mildly to moderately damaged stria vascularis.

Labeling was common in the stroma of the spiral ligament. Cell division in the spiral ligament also probably represents repair in a structure damaged mechanically or following metabolic changes induced by acoustic insult.

The cell division seen on Reissner's membrane, in the stroma of the limbus and the tympanic border cells is difficult to explain based on the literature, because few if any previous studies have specifically commented on damage to these structures after acoustic insult. It is known that there are extensive metabolic and ionic changes in the endolymph and perilymph during acoustic trauma, including, for example, changes in sodium and potassium concentration, changes in lactate concentration (possibly secondary to increased anaerobic metabolism), and many others.[30] It is probable that during the extended acoustic trauma in this experiment some of the epithelial and support cells lining the different cochlear spaces died as a result of these metabolic stresses. The cell division seen in these areas probably represents an epithelial repair process. Since Koburg[8] has reported that a very low rate of cell division persists in these structures through adulthood, it might be expected that these tissues would retain an ability to repair themselves by increasing the rate of cell division following damage. Similarly, the labeled cells on the basilar membrane in those areas in which the organ of Corti was completely obliterated presumably represent epithelial ingrowth on a denuded structure. Sobkowicz et al[31] have described similar epithelial ingrowth over damaged areas in developing cochleas injured mechanically *in vitro.*

Similarities between Patterns of Cell Division after Trauma in the Avian and Mammalian Cochlea

Significant similarities exist between the patterns of cell division in the gerbil and chick cochlea after acoustic insult.[13] In the chick cochlea, as in the gerbil, labeled cells among the acoustic nerve fibers were prominent. Tympanic border cell division was noted in undamaged chick cochleas, and was greatly increased following trauma. In those regions of the chick cochlea where the sensory epithelium was obliterated, there was an epithelial monolayer covering the basilar membrane and the cells in this monolayer were frequently labeled. In the chick, cells in this monolayer may serve as precursors to new supporting and hair cells, while in the mammal this regenerative capacity appears to have been lost. Whether the provision of exogenous growth factors and/or differentiation factors can stimulate hair cell regeneration in the damaged mammalian auditory epithelium, as suggested in one recent report,[32] remains to be definitively elucidated. If in fact hair cell regeneration can be induced in mammalian species, treatment of sensorineural deafness and vestibular dysfunction in humans might become possible.

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