ELSEVIER

Hearing Research 92 (1995) 17–29

HEARING RESEARCH

Avian cochlear hair cell regeneration: stereological analyses of damage and recovery from a single high dose of gentamicin

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Received 12 May 1995; revised 7 September 1995; accepted 15 September 1995

Abstract

Hair cell regeneration after acoustic trauma has been conclusively documented in birds. Previous studies of aminoglycoside ototoxicity have typically used 5–10 day courses of drug to damage the cochlea and trigger regeneration. This long-term lesion prevented analysis of the early events of regeneration. We set out to determine how much damage would occur and how recovery would proceed after a single high-dose injection of the aminoglycoside gentamicin.

White Leghorn chicks were given a single high dose of gentamicin (100 mg/kg). Three post-injection survival groups with age-matched controls were studied: short-term (3–5 days), intermediate-term (2 weeks) and long-term (5 weeks). After sacrifice, cochleae were dissected and processed for scanning electron microscopy. Using stereological techniques, a quantitative analysis of cochlear hair cell counts along the proximal 50% of the cochlea was performed from scanning electron micrographs on 4–7 chicks from each group.

Variable degrees of damage were seen 3–5 days after the drug injection. All hair cells were lost from the proximal 20% of the cochlea in all chicks. This complete hair cell loss could extend to 50% of the cochlea. Immature appearing hair cells could be first identified by their immature stereocilia at 3 days. Immature appearing hair cells were present in greatest number in regions which had been denuded of native hair cells and in regions where partial loss occurred. Interestingly, immature appearing hair cells also occasionally appeared in adjacent areas in which there was no apparent loss of native hair cells. Two-week survivors showed an elevation in hair cell number compared to controls in regions which had sustained damage and immediately adjacent regions. This elevation implies that an overproduction of hair cells might occur as part of the regeneration response. By 5 weeks after damage hair cell numbers approximated controls.

Keywords: Regeneration; Aminoglycoside otoxicity; Chicks; Basilar papilla

1. Introduction

Hair cell regeneration after ototoxic insult has been conclusively documented in the avian inner ear. Ototoxic agents and noise exposure result in loss and subsequent recovery of hair cells in the sensory epithelium (Cotanche, 1987a; Cruz et al., 1987; Duckert and Rubel, 1990). DNA labeling studies prove that most of the recovery is due to cell division and differentiation of new hair cells (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Lippe et al., 1991). In addition, the newly regenerated hair cells approach normal number (Ryals and Rubel, 1988; Duckert and Rubel, 1993), show ultrastructural characteristics of native mature hair cells (Cotanche, 1987a; Duckert and Rubel, 1990), and even restore function (Tucci and Rubel, 1990; Hashino et al., 1988; Marean et al., 1993).

A current area of research interest is identification of the specific precursors to the new hair cells. To address this issue, the earliest mitoses after acoustic insult have been sought. In experimental paradigms of noise trauma, sensory epithelium cells were first labeled with bromodeoxyuridine between 18 and 24 h after the start of noise exposure (Girod et al., 1989; Stone and Cotanche, 1994). New hair cells are first identifiable by scanning electron microscopy (SEM) between 90 and 96 h after the onset of noise exposure (Cotanche, 1987a; Girod et al., 1989). The time between S-phase and observing hair cells by SEM

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SSDI 0378-5955(95)00190-5

during embryogenesis is similar (Cotanche and Sulik, 1984; Katayama and Corwin, 1993).

Experimental paradigms using aminoglycoside ototoxicity provide some analytical opportunities which differ from noise exposure paradigms. First, the tectorial membrane as well as the support cells underlying the hair cells are spared, while in noise damage they can be injured by the mechanical trauma imparted to the basilar membrane (Girod et al., 1989; Cotanche, 1987b). Second, in noise exposure paradigms, the location of the damage is controlled by the frequency of sound exposure. In aminoglycoside ototoxicity, hair cell loss occurs at the base of the cochlea and tends to be virtually complete, so that any hair cell observed after a sufficient survival period is almost certainly a regenerated hair cell (Cruz et al., 1987; Duckert and Rubel, 1990; Girod et al., 1991; Hashino et al., 1991; Marean et al., 1993). However, previous studies of aminoglycoside ototoxicity have typically used 5–10 day courses of the drug to damage the cochlea and trigger regeneration. These dosing intervals likely evolved in order to mimic the clinical dosing schedule for aminoglycoside use. Clearly hair cell regeneration begins during aminoglycoside administration as immature appearing hair cells can be identified by SEM 24 h after a 5-day gentamicin treatment (Duckert and Rubel, 1990). A single high-dose injection of aminoglycoside would provide an experimental paradigm in which the earliest events that trigger hair cell regeneration are more easily studied than with more extended dosing periods, where the injury to the sensory epithelium is more gradual.

In the current study, we set out to determine how much damage would occur and how recovery would proceed after a single high-dose injection of the aminoglycoside gentamicin. Three survival intervals were studied: short-term (3–5 days), intermediate-term (2 weeks) and long-term (5 weeks). Using stereological techniques, a quantitative analysis of hair cell counts along the entire sensory epithelium was performed from scanning electron micrographs of age-matched control chicks. The same type of analysis was then carried out for the damaged portion of the cochlea of experimental chicks to assess the extent of damage and the time course of recovery. In a separate group of chicks, tritiated thymidine was used to grossly determine the beginning of support cell division in response to the single high dose of gentamicin.

A preliminary report of these findings has been presented (Janas et al., 1994).

2. Methods

2.1. Scanning electron microscopy

Sixteen White Leghorn chicks received a single subcutaneous injection of gentamicin (100 mg/kg) on post-hatch days 0–1. Chicks were then divided into 3 survival groups: short-term (3–5 day; $n = 6$), intermediate term (2 weeks; $n = 6$), and long-term (5 weeks; $n = 4$) recovery periods. An additional 6 chicks served as age-matched controls, 2 each sacrificed at 5 days, 2 weeks, and 5 weeks. On the day of sacrifice chicks were decapitated and the temporal bones dissected free and immersed in fixative (3.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4). Cochleae were stored in fixative at 4°C for 24–48 h followed by post-fixation in 1% osmium tetroxide for 1 h at room temperature. Tissue was then dehydrated and final dissection completed. SEM was performed on a JEOL JSM6300 (15 kV accelerating voltage).

Tissue analysis was carried out in two phases. First, a low-magnification 50 × montage of the entire sensory epithelium was constructed for each cochlea. This provided a working data library which allowed gross identification of the region of damage induced by the gentamicin. Damage was defined as a disruption of the normal hexagonal array of mature hair cell apical surfaces as previously described (Saunders and Tilney, 1982; Cotanche et al., 1987; Cotanche, 1987a).

In the second phase of tissue analysis, adjacent 500 × photomicrographs were taken starting from the proximal tip and continuing distally. Each photomicrograph covered 220 μm of the length (proximal to distal) along the sensory epithelium. As the organ widens in cross-section distally, 2 or (rarely) 3 photomicrographs were required to image the entire width (inferior to superior) at the more distal 220 μm segments. Care was taken to ensure that the same 220 μm linear segment was encompassed in both the superior and inferior aspects of these regions. Photomicrographs were taken from the proximal tip to distal end of all control sensory epithelia. This required 16–22 photomicrographs per cochlea. One cochlea of each of the experimental chicks was imaged from the proximal tip to approximately half of the total length, since damage never extended beyond this point in any of the chicks. The original $4 \times 5''$ photomicrograph negatives were then enlarged to $8 \times 10''$ photographs. In this fashion a library of 1000 × photos allowed for ease of recognition of hair cell apical surfaces and stereocilia bundles.

Separate counts of mature and immature appearing hair cells were made from the high-power photomicrographs. Mature appearing hair cells were defined as those cells with a broad surface area and relatively condensed stereocilia bundle. Mature hair cells comprise the normal hexagonal array of the control cochlea. In the most proximal sections of the sensory epithelium in both the control and experimental chicks, all the mature hair cells were counted directly. This was done when the total number of cells was less than 200 per photomicrograph. In more distal 220 μm segments, the total number of mature hair cells could be as high as 1000 per segment. In these regions, a stereological method was used to estimate the number of mature hair cells in both the control and experimental cochleae (Gunderson et al., 1988).

For the stereological analyses a 2×2 cm grid was copied onto a transparency. This was then placed in random orientation on the 8×10 photomicrograph. Two separate counts were made. First, the total number of intersections of the 2×2 cm grid squares which fell onto the surface of the sensory epithelium were counted. This provided an estimate of the total area of sensory epithelium encompassed in the photomicrograph (AREA). Next, an unbiased counting rule was applied to determine the number of mature hair cells in each 220 μm segment of sensory epithelium. For this procedure, the number of mature hair cells in every third grid square which fell onto the sensory epithelium was counted. A random number was generated between 1 and 3. The first grid square in which hair cells were counted was either the first, second or third square from the upper left-hand corner of the grid of 2×2 cm squares, randomly determined. The total number of grid squares in which hair cells were counted ranged from 10 to 30 per photomicrograph. All mature hair cells which fell completely within the grid square, as well as those touching the upper side or the right-hand side of the grid square were counted. Hair cells touching the bottom or left-hand side of the grid square were not counted. The total number of mature hair cells per grid square ranged from as low as 3 in the inferior regions to as high as 22 in the superior regions of the epithelium, reflecting differences in size of hair cell apical surface and parallax caused by curvature of the sensory epithelium. The mean number of mature hair cells per grid square was then calculated for each photomicrograph (MEAN).

The total number of mature hair cells per 220 μm segment of the sensory epithelium (TOTAL) was then calculated. For each photomicrograph the total number of mature hair cells was calculated by:

AREA (no. of intersections) $\times$ MEAN = Total number of mature hair cells for that photomicrograph.

Since each 220 μm segment of the sensory epithelium occupied 1, 2 or (rarely) 3 photomicrographs, the above totals had to be summed to estimate the TOTAL number of mature hair cells per 220 μm segment of the sensory epithelium. For the control chicks, a running sum (cumulative total) of the number of mature hair cells along the entire length of the sensory epithelium was calculated (see Fig. 1). In addition, the number of hair cells in each 220 μm segment of length (TOTAL) was averaged for the 2 chicks at each of the 3 survival times. For the experimental chicks, which ranged in number from 4 to 6 for each survival time, the mean and variance of the total number of mature hair cells (TOTAL) were calculated for each 220 μm segment of length. In experimental animals, this was done for only the proximal half of the sensory epithelium, since we never observed damage in the distal half. The reliability of this procedure was assessed by repeating the counts on 2 of the control animals. The repeat counts were

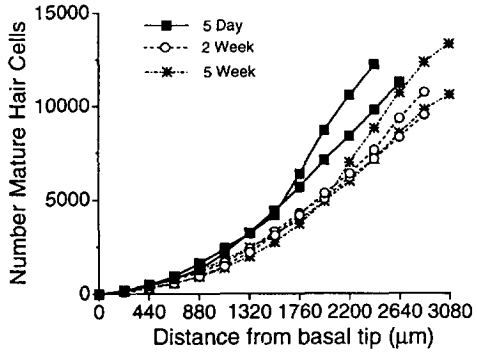

Fig. 1. Running sum of the total number of mature hair cells along the entire length of the cochlea for each of the 6 control chicks. The cochleae of 5-day chicks ranged from 2200 to 2420 μm in length while the cochleae of 5-week chicks were both 3080 μm in length. The average total number of hair cells was 11260. The range was from 9502 to 13294.

within 3% of the original counts on one sample and within 5% on the other, giving us confidence in the applicability of this stereological procedure for estimating the number of hair cells from surface preparations of the avian sensory epithelium.

In addition to the above analysis, a separate count was made in the experimental chicks of all the immature appearing hair cells for each 220 μm segment. Immature appearing hair cells have apical surfaces which appear like the apical surfaces of hair cells in stages of embryonic development (Cotanche, 1987a). They have a smaller surface area than mature hair cells, and their stereocilia occupy a greater percentage of the total surface area than do the stereocilia of a mature hair cell. Although the stereocilia of immature hair cells are wider and longer than the microvilli of support cells, they do not have the length and orientation specificity of a mature stereocilia bundle (Cotanche and Sulik, 1984; Cotanche, 1987a). In all 220 μm segments, the number of immature appearing hair cells was counted directly from the photomicrographs. The number of immature appearing hair cells was then graphed for each segment of the 3-, 4-, and 5-day survivors as well as tabulated for the short-, intermediate-, and long-term experimental groups.

2.2. Tritiated thymidine

Once the time frame of damage and regeneration in the single-dose gentamicin paradigm was established from SEM, tritiated thymidine was used to confirm that mitosis was triggered by the single high-dose gentamicin injection and that the newly generated cells differentiate into hair cells. It is important to note that the goal of this analysis was not a detailed analysis of the kinetics of the proliferative response. It was, instead, to insure that a mitotic process was induced and to begin estimating the time at

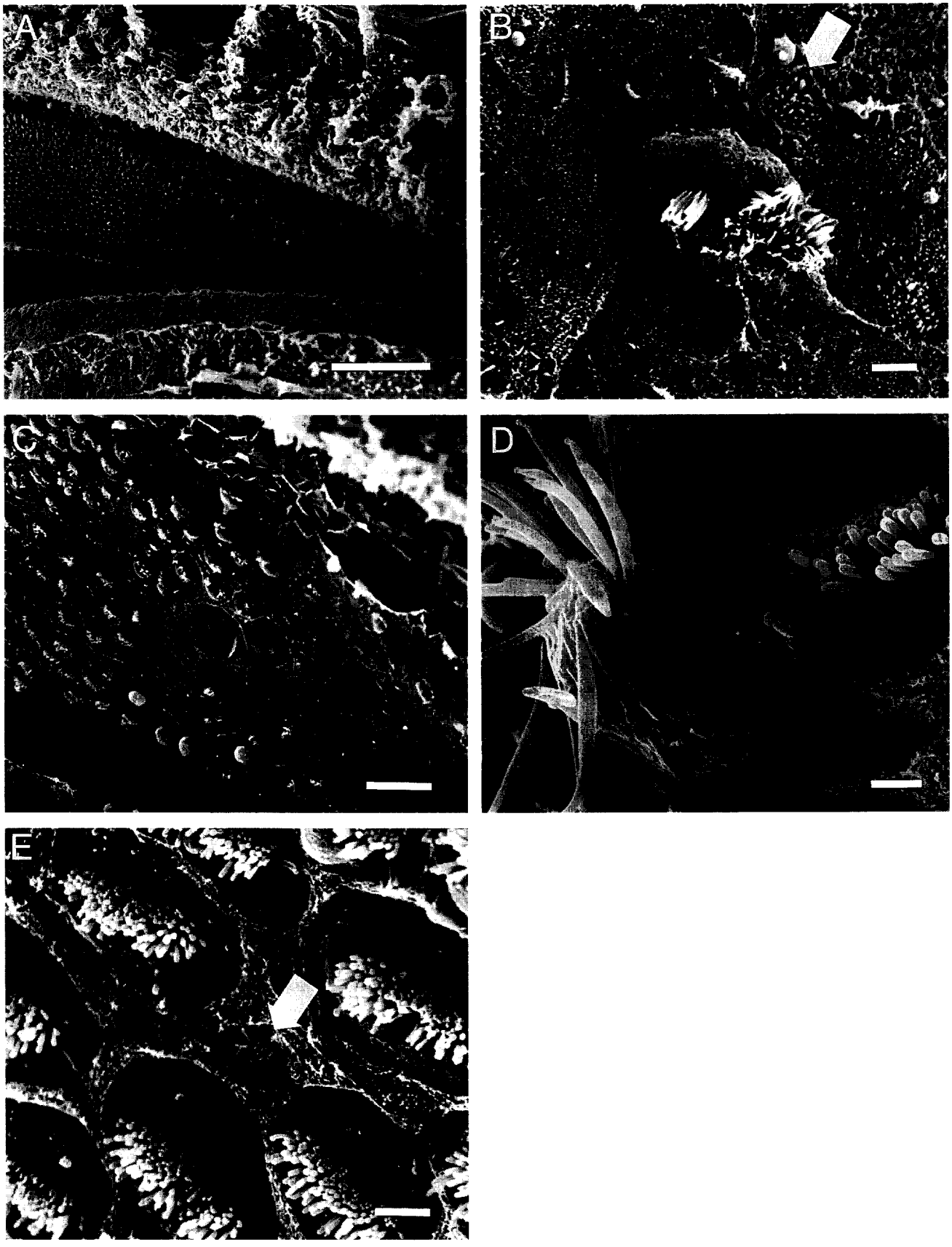
A
B
C
D
E

Table 1
Number of immature appearing hair cells seen per 220 μm segment along the proximal 1320 μm of all 3 experimental survival times

	Segment distance from base (μm)									
	220		440		660		880		1100	1320
3–5 day ($n = 6$)	18.8	(0–48)	62.2	(0–174)	68.0	(3–171)	33.5	(0–102)	0.4 (0–2)	0.3 (0–1)
2 week ($n = 6$)	0.5	(0–2)	0.83	(0–3)	0.5	(0–2)	0.5	(0–2)	0	0
5 week ($n = 4$)	1.25	(1–2)	1.0	(0–4)	0.25	(0–1)	1.25	(0–5)	0	0

Means (and ranges) are shown.

which it was first seen following this paradigm of ototoxic insult. Thus, we utilized a 'pulse-chase' procedure, and only two labeling periods were examined.

Four chicks each received 100 mg/kg subcutaneous gentamicin. Two chicks received injections of tritiated thymidine (ICN; 60–80 Ci/mmol) at a dose of 10 μCi/g body weight at 8 h and 10 h after the gentamicin injection. A chase of non-radioactive thymidine at 100 × the radioactive thymidine dose was then given at 12 h and 14 h. The other 2 chicks received the initial injections of tritiated thymidine at 18 h and 20 h after the gentamicin injection followed by the non-radioactive thymidine at 22 h and 24 h. Thus in each chick a 4 h 'pulse' of radioactive thymidine was 'chased' by an excess of cold thymidine. Chicks were then allowed to survive a total of 4 days. At the end of the survival period the temporal bones were dissected free, immersed in 3.5% glutaraldehyde in 0.1 M PBS, pH 7.4, and stored at 4°C for 24–48 h. In the second stage of dissection, the entire cochlea was carefully dissected from the temporal bone. Cochleae were then dehydrated through a graded ethanol series and embedded in Spurr resin, and sectioned transversely into 3 μm serial sections. All sections from the proximal tip extending to half the total length were preserved and mounted onto acid-washed, chrome alum subbed slides for both light microscopy and autoradiography. All slides were then processed by our standard protocol for autoradiography (Girod et al., 1989). Counter-staining was done with Toluidine Blue. Negligible background was encountered in the autoradiography slides.

Slides were inspected at the light microscope level (63 × oil immersion). The presence of damage was indicated by loss of hair cells and the location of labeled nuclei was noted. The criterion for a labeled cell was 5 or more silver grains overlying the cell nucleus. Labeled cells

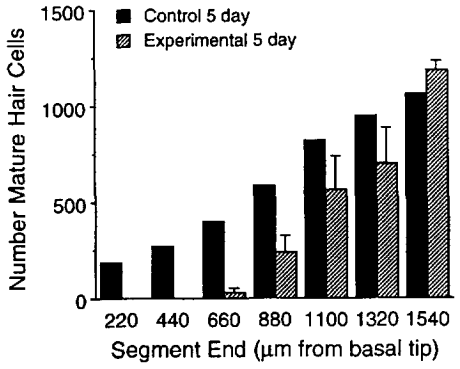

Fig. 3. Mean number of mature hair cells per 220 μm segment along the proximal 1550 μm in both control (solid; $n = 2$) and experimental (striped; $n = 6$) chicks at survival times of 3–5 days. Standard error bars are shown for the 6 cochleae of the experimental group.

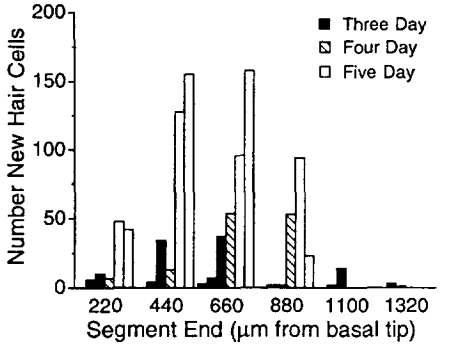

Fig. 4. Total number of new immature appearing hair cells per 220 μm segment along the proximal 1320 μm for each of the 6 chicks in the 3–5-day experimental group. When no immature appearing hair cells could be identified in a segment a zero value was assigned; thus for these segments a bar does not appear on the histogram. The number of immature appearing hair cells increases dramatically over the additional 48 h encompassed in this survival interval.

Fig. 2. Scanning electron photomicrographs of the proximal aspect of the cochlea of a chick surviving 4 days after the single high-dose gentamicin injection. A: Low-power overview demonstrates complete loss of apical surfaces of native mature hair cells at proximal region with preservation of normal appearance at more distal regions (bar = 100 μm). B: Proximal region at higher magnification shows the microvillus surfaces of the support cells. Dying hair cells can be identified and appear to be extruded from the surface. Many new immature appearing hair cells (arrow) are present (bar = 2.0 μm). C: The 'transition zone' represents that 200 μm segment in which the proximal aspect shows complete loss of native mature hair cells and the distal aspect shows grossly normal hair cell appearance (bar = 20 μm). D: Higher magnification of the 'transition zone'. Some hair cells seem to be extruded while others appear grossly normal (bar = 1 μm). E: Immature appearing hair cells (arrow) can be identified in the interstices between the existing hair cells in the 'transition zone' (bar = 2 μm).

typically had greater than 10 silver grains over the nucleus in the 8-week exposure period.

The care and use of animals reported on in this study were approved by the University of Washington Animal Care and Use Committee, and approved by the NIH (Grant DC00395: Ontogeny of Vertebrate Sensory Processes).

3. Results

3.1. Control chicks

The hair cells for each 220 μm segment along the complete length of the cochlea were counted for each of

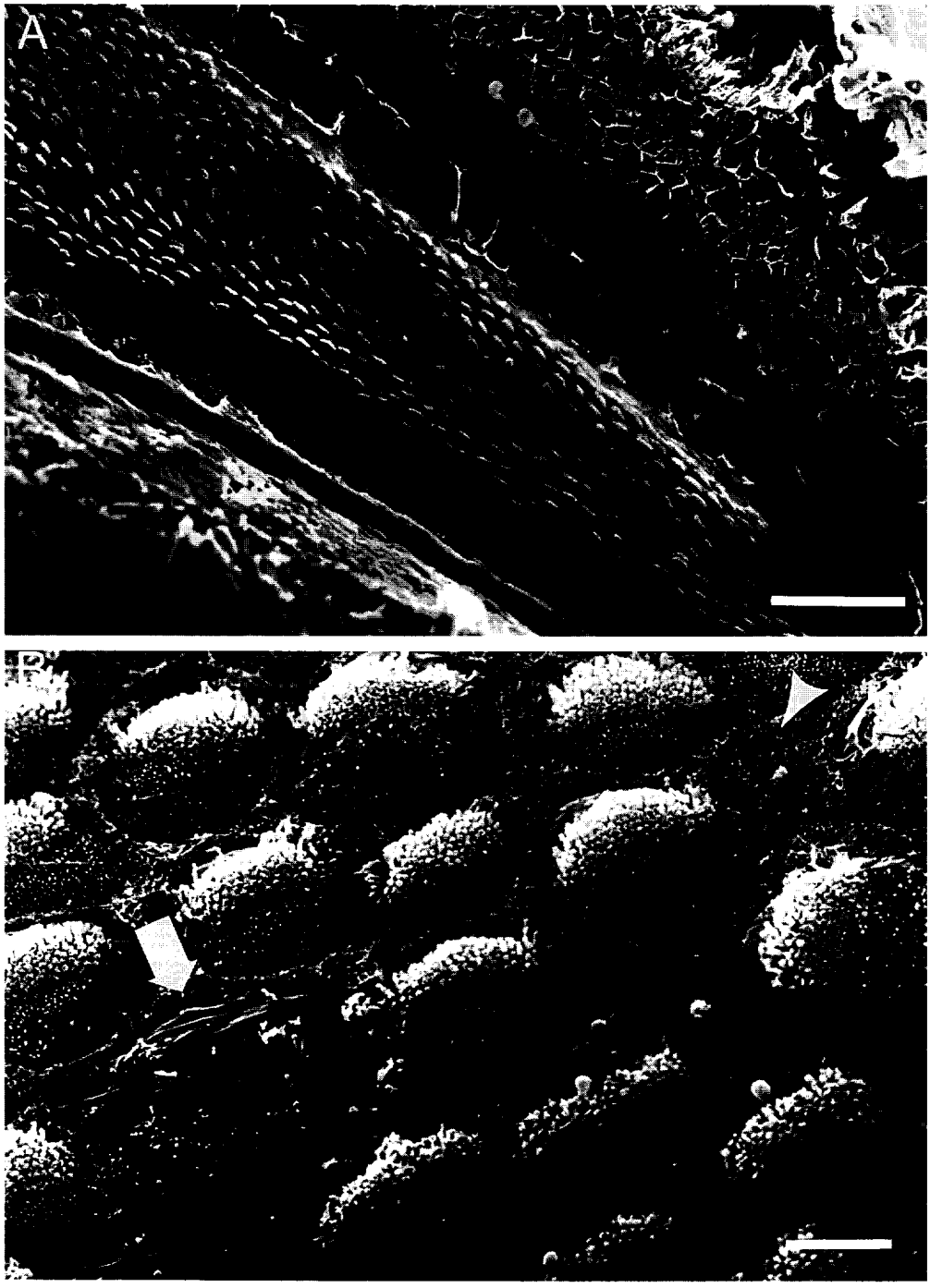

Fig. 5. Scanning electron photomicrographs of the proximal aspect of the cochlea of a chick surviving 2 weeks after the single high-dose gentamicin injection. A: Low-power overview demonstrates repopulation of the surface of the cochlea with relatively mature appearing hair cells (bar = 50 μm). B: Previously damaged hair cell, characterized by a large apical surface area and lack of stereocilia bundle (large arrow). The apical surfaces of the hair cells show microvilli characteristic of immature hair cells. Immature appearing hair cell apical surfaces (arrowhead) can still be identified in the interstices between the more mature appearing cells (bar = 10 μm).

the 6 control chicks. The running sum of hair cells along the length was then calculated to provide the total number of hair cells for each cochlea (Fig. 1). The total number of hair cells ranged from 9502 to 13 294, with a mean of 11 260. The length of the cochlea increased slightly over the ages examined. The two 5-day controls were 2200 μm and 2420 μm long, respectively. Both 5-week control cochleae were 3080 μm long. Though the length of the cochlea did increase somewhat over age, no consistent change in hair cell number was seen with increasing age.

3.2. 3–5 Day survival

Cochleae from this group showed variable degrees of damage from the single 100 mg/kg dose injection of gentamicin. The characteristic proximal to distal (basal to apical) spread of damage was seen (Fig. 2A,C). The most proximal regions showed loss of the normal hexagonal array of hair cell apical surfaces, leaving the microvilli of the support cells to now provide the surface architecture. Hair cells were often seen in the process of being extruded from the surface (Fig. 2B). That is, remnants of hair cells, often with disrupted but recognizable stereocilia bundles, could be identified adherent to the surface of the organ (Fig. 2D). A 'transition zone,' between the proximal region denuded of native hair cells and the distal normal appearing region, could be identified (Fig. 2C). In the distal part of the transition zone the mature appearing hair cells showed grossly larger apical surfaces, and more space was evident between the cells. This transition zone was approximately 200 μm in length and occurred between 660 and 1320 μm from the proximal tip in all chicks.

All cochleae showed total loss of mature hair cells that extended at least 440 μm from the proximal tip. The greatest degree of damage was seen in a 3-day survivor which showed complete loss of hair cells to 1100 μm from the proximal tip. Two of the 6 experimental chicks in this group showed complete loss of hair cells from the base to 440 μm distally. The remaining chciks displayed intermediate values. A histogram showing the average number of hair cells per 220 μm segment for the 6 experimental chicks and the 2 age-matched controls is provided in Fig. 3. In the 660 μm through 1320 μm segments, native mature hair cells are present in fewer number than in controls, indicating that some of the hair cells in these regions have been lost.

New hair cells (immature appearing hair cells, on the basis of stereocilia bundle appearance) could be identified throughout the damaged segment in the 3–5-day chicks. New hair cells were identified in regions almost totally denuded of hair cells (Fig. 2B) as well as in the transition zone, including the most distal part of this zone, where most of the cells appeared grossly normal (Fig. 2E). In the transition zone, new hair cells appeared to arise in the interstices between existing mature cells.

Fig. 4 shows the number of immature appearing hair cells for chicks surviving 3–5 days after the gentamicin injection. As the survival interval increased from 3 to 5 days, an increase in the number of new cells was seen at all distances from the proximal tip. In the 3-day survivors, immature appearing hair cells numbered less than 50 for each 220 μm segment. For the 5-day survivors, greater than 100 immature appearing hair cells often could be identified per 220 μm segment.

Table 1 summarizes the number of immature appearing hair cells in all segments for the proximal 1320 μm of all 3 experimental groups. For the 3–5 day survivors, immature appearing hair cells were present throughout the length of the damaged segment, with the greatest numbers in the 440 μm and 660 μm segments. For the more long-term survivors, immature appearing hair cells were seen sporadically within the proximal 1100 μm.

3.3. 2-Week survival

By 2 weeks the cochleae of experimental chicks typically showed an impressive return to normal appearance. The broad apical surfaces of relatively mature appearing hair cells once again comprised the surface architecture (Fig. 5). However, some evidence of the previous damage remained. The apical surfaces of the hair cells still showed some microvilli, the apical surfaces were variable in size and the hair bundles did not show their normal precise orientation. In addition, a small number of large irregularly shaped cells, occasionally with two sets of disoriented stereocilia, were seen. These cells were not included in the hair cell counts presented below; at low magnification (e.g., Fig. 5A) these regions appeared as disruptions of the normal mosaic of hair cell apical surfaces (holes). Finally, immature appearing hair cells could still be seen, but were more uncommon than in the earlier survival periods.

A histogram providing the counts of mature appearing hair cells in experimental and age-matched controls at this survival interval is shown in Fig. 6. It is important to note

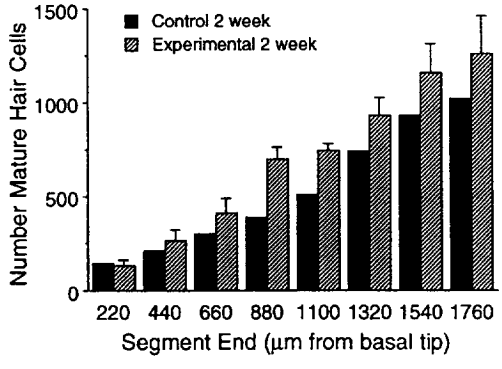

Fig. 6. Mean number of mature hair cells per 220 μm segment along the proximal 1760 μm of both control (solid; $n = 2$) and experimental (striped; $n = 6$) chicks at survival times of 2 weeks. Standard error bars are shown for the 6 cochleae of the experimental group.

that in every 220 μm segment, with the exception of the most proximal segment, there were more hair cells in the experimental chicks than in controls. This is especially apparent in the 880 μm and 1100 μm segments. These segments fall within the transition zone noted earlier.

Immature appearing hair cells were still present in the 2-week survivors (Table 1). The mean number of immature appearing hair cells in the 2-week survivors was less than 1 per 220 μm segment. This is much reduced compared to the number of new cells per segment seen in the 3–5 day survivors.

3.4. 5-Week survival

At 5 weeks the surface architecture was again dominated by the apical surfaces of mature appearing hair cells

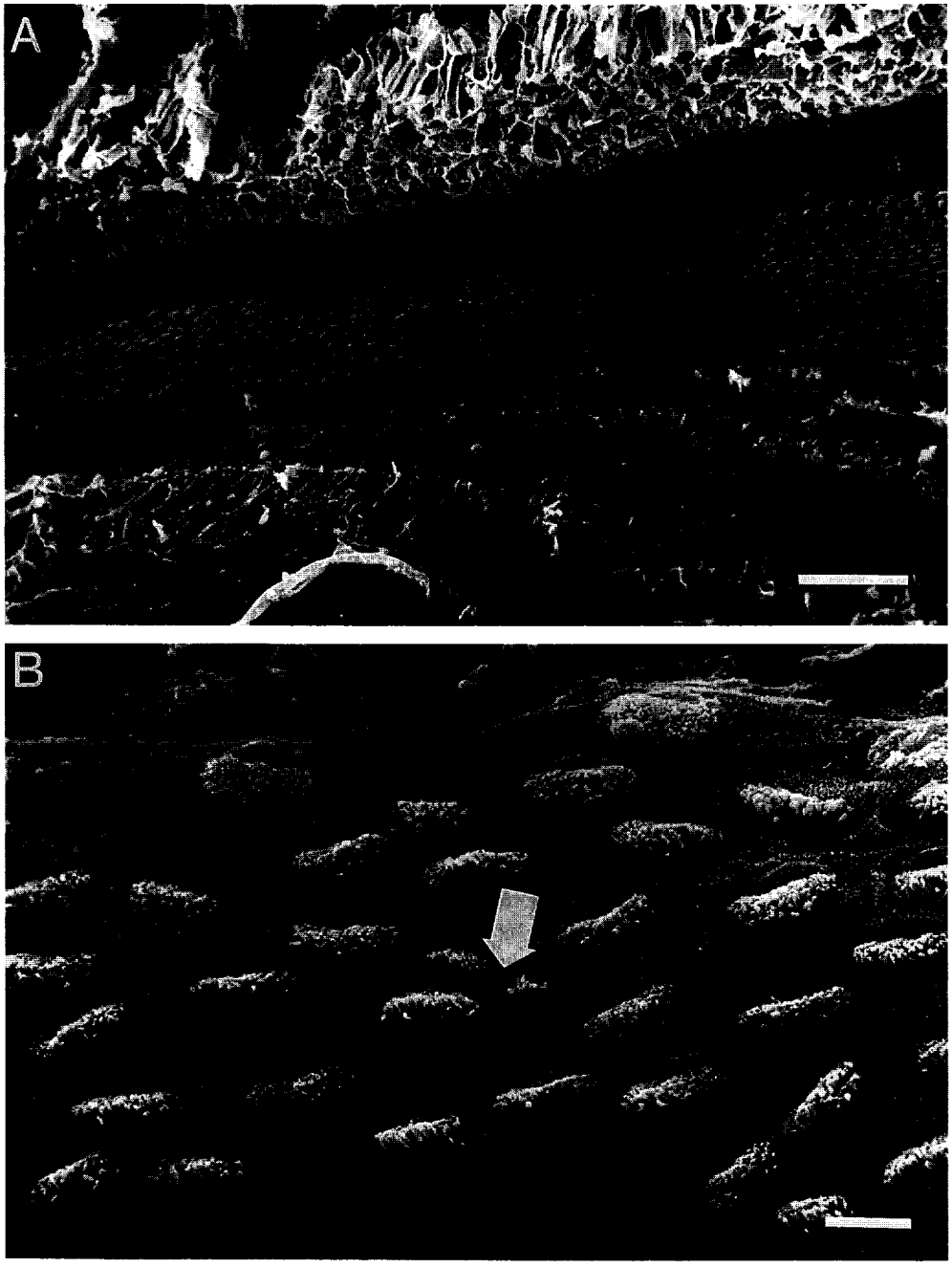

Fig. 7. Scanning electron photomicrographs of the proximal aspect of the cochlea of a chick surviving 5 weeks after the single high-dose gentamicin injection. A: Low-power overview demonstrates repopulation of the surface of the cochlea with relatively mature appearing hair cells (bar = 100 μm). B: Evidence of prior damage remains in that there is variation in the size of the apical surfaces of the hair cells and microvilli have not yet resorbed. The arrow points between two immature appearing hair cells in the interstices between the more mature appearing hair cells (bar = 5 μm).

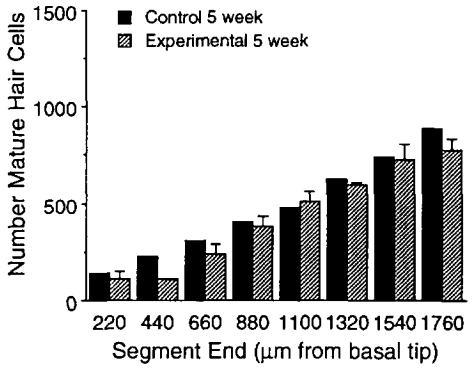

Fig. 8. Mean number of mature hair cells per 220 μm segment along the proximal 1760 μm of both control (solid; $n = 2$) and experimental (striped; $n = 4$) chicks at survival times of 5 weeks. Standard error bars are shown for the 4 cochleae of the experimental group.

(Fig. 7). Some evidence of previous damage remained. For example, stereocilia often did not show the same precision of alignment as native mature hair cells. Apical surfaces with both stereocilia and microvilli, as well as regions with expanded supporting cells and no evidence of hair cells (e.g., see top margin of Fig. 7B), were seen, though fewer in number than in the 2-week survivors.

Counts of the mature appearing hair cells at this period showed a return to numbers comparable with controls (Fig. 8). The 440 μm and 660 μm segments had somewhat fewer cells than did controls. Of note, the 440 μm experimental segment here is based on data from only 2 specimens. Dissection damage rendered tissue loss in this segment of 2 specimens, thus error bars are not provided.

An ongoing low-level of immature appearing hair cells was still present at the 5-week survival time (Table 1).

3.5. Tritiated thymidine

All cochleae processed for autoradiography were sectioned from approximately 200–1000 μm from the proximal tip. All cochleae showed loss of hair cells to approximately 800 μm from the base. Chicks that received the tritiated thymidine at 8 and 10 h after the gentamicin injection showed no labeled support cells or hair cells in the cochlea despite excellent labeling of endothelial cells in the surrounding tissues. The chicks that received the radioactive label at 18 and 20 h after the gentamicin injection showed abundant labeling of support cells and occasional hair cells throughout the damaged region (Fig. 9). Labeled cells appeared to be spread uniformly throughout the segments of length and were also present in grossly equal distribution between the superior and inferior aspects of the cochlea.

4. Discussion

Experimental paradigms using noise exposure have classically been employed to study the earliest changes in the cochlea following ototoxic insult. Noise exposure pro-

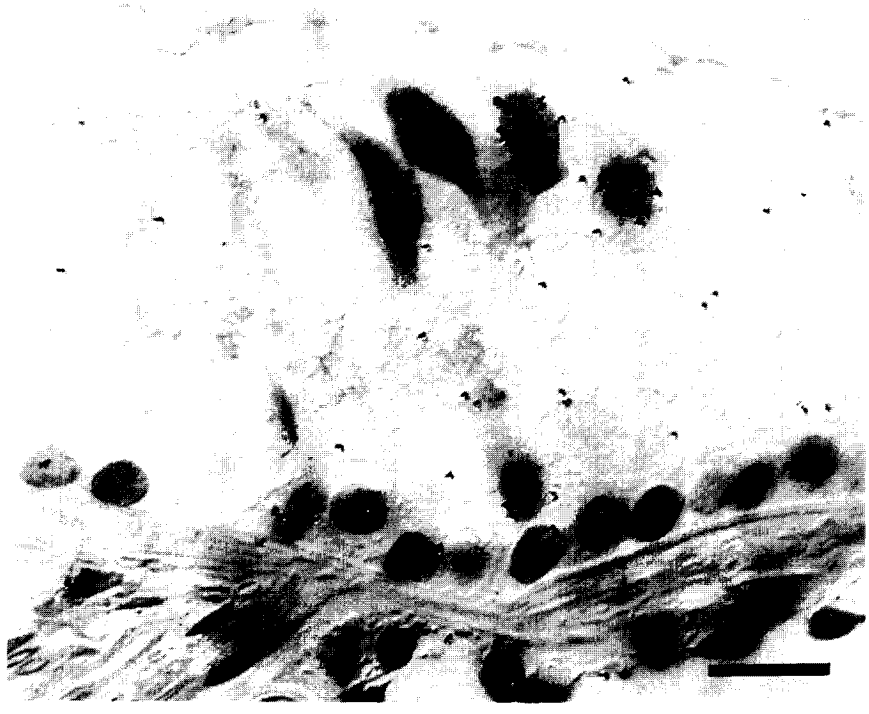

Fig. 9. Cross-section of the cochlea of a chick surviving 4 days after single high-dose gentamicin injection. The chick was treated with tritiated thymidine at 18 and 20 h after the gentamicin injection. Loss of hair cells along the surface of the epithelium shows that damage did occur. Immature appearing labeled hair cells are seen in the damaged segment (bar = 10 μm).

vides a paradigm in which the start and stop times are clearly definable. Immature appearing hair cells have been identified by SEM between 90 and 96 h after the onset of an 8-h noise exposure (Girod et al., 1989). More recent work (Stone and Cotanche, 1992) has shown that whether the noise stimulus is presented for 4, 12, or 24 h, the immature appearing hair cells appear on the surface of the cochlea 96 h after the onset of noise. In addition, cell division is triggered by 4- and 24-h noise exposures with labeled nuclei first detected between 18 and 24 h after the onset of the exposure (Stone and Cotanche, 1994). These studies imply that the onset of the ototoxic insult is important in timing the regeneration process and that some precursors to new hair cells are stimulated to divide within a short time (< 24 h) after the initiation of the trauma.

Previous aminoglycoside studies have used 5–10 day courses of drug to damage the cochlea (Cruz et al., 1987; Tucci and Rubel, 1990; Lippe et al., 1991; Hashino et al., 1991, 1992; Duckert and Rubel, 1990, 1993). This long-term drug administration paradigm is disadvantageous for analyses of the earliest regenerative responses. We felt that a single high-dose injection of aminoglycoside would provide an experimental paradigm in which the earliest events triggered by aminoglycoside injury could be studied more easily, in combination with the other advantages of aminoglycoside treatment.

A unique quantitative analysis of the chick cochlea is presented in this paper. Stereological technique has had wide application throughout the biological sciences to provide an unbiased estimate of the population of cells contained within a structure. Previous analyses of hair cell counts along the chick cochlea have most commonly relied on sampling throughout its length (Ryals and Rubel, 1988) or on estimates based on the density of cells (Cotanche and Dopyera, 1990).

Stereological techniques allow one to simply and easily estimate the total number of cells present, and low-power SEM montages are well suited to stereological analysis.

4.1. Hair cell counts

An unbiased estimate of the total number of hair cells in the chick cochlea was made for each of 6 control chicks. The mean total was found to be 11 260. This is in remarkable agreement with the number provided by the comprehensive work of Tilney et al. (1986). In this paper, Tilney et al. state that "... the total number of hair cells per cochlea ($10\,405 \pm 529$) is already visible in a 10-day embryo and the growth of the cochlea is a result of the growth in size and surface area of the hair cells." This paper also states that the length of the cochlea on the date of hatching is 2600 μm and in chicks just beyond 5 weeks of age (39 days) the length has increased to 3200 μm. Again our results of 2400 μm at 5 days and 3080 μm at 5 weeks are in close agreement. Ryals et al. (1984) noted a similar increase in the length of the entire cochlear duct between hatching and 5 weeks of age. The number of hair cells did not show any consistent variation with age; the lowest (9502) and highest (13 291) values occurred in a 2-week and 5-week chick, respectively. Our results thereby reaffirm the proposal that growth of the chick cochlea results from an increase in size without a corresponding increase in number of hair cells.

4.2. Regeneration

Damage was consistently seen in the chicks 3–5 days after the single 100 mg/kg dose of gentamicin. Nearly all hair cells were consistently lost for the proximal 440 μm, or 20% of the cochlear length. Including the 'transition zone,' in which cell loss was scattered and normal appearing cells were intermixed with abnormal cells, the total cochlear damage region was about 30% of the length. Considerable variation in response was encountered, however. The minimal damage extended in 1 chick to only 660 μm, whereas 1 chick showed damage to 1320 μm or 55% of the total length. The factors responsible for this variability are not understood, but there was no consistent variation with age of the subject. In our previous studies of both noise exposure (Ryals and Rubel, 1985) and aminoglycoside damage (Girod et al., 1991), we also encountered considerable variability. Some investigators have noted this variation (Cotanche et al., 1991). However, in many reports the actual amount of hair cell loss has not been adequately quantified. In previous studies, 5–10 day courses of aminoglycoside are reported to have resulted in damage to the proximal 30–65% of the cochlea (Lippe et al., 1991; Duckert and Rubel, 1993). It is somewhat surprising that a single injection of the gentamicin could trigger a similar degree of hair cell loss as a long course of gentamicin in some chicks.

Immature appearing hair cells could be clearly identified by SEM at 3 days (72 h) after the single high-dose gentamicin injection. This is slightly sooner than the time periods required in the noise exposure paradigm, where new hair cells are first identified by SEM between 90 and 96 h after the onset of noise exposure (Cotanche, 1987a; Girod et al., 1989; Stone and Cotanche, 1992).

The number of immature appearing cells increased 2–3-fold over days 3–5 (72–120 h). This is again slightly sooner than what is reported in the noise exposure studies. In a recent study, the bulk of the proliferation occurred between 48 and 96 h after the onset of noise exposure (Stone and Cotanche, 1994) as evidenced by uptake of S-phase nuclear markers. In embryonic development hair cells require approximately 48 h after DNA incorporation to have recognizable stereocilia extending from their apical surfaces (Cotanche and Sulik, 1984; Katayama and Corwin, 1993). Therefore these cells would be expected to show stereocilia bundles in increasing numbers on their apical surface between 96 and 134 h in the noise exposure

paradigm, slightly later than what we encountered with a single high dose of gentamicin.

The location of the immature appearing hair cells in this study also proved to be intriguing. The characteristic proximal to distal spread of damage seen with aminoglycoside ototoxicity leads to a more proximal region completely denuded of cells and a more distal portion where the cells remain normal. Bridging these is the 'transition zone' in which obvious cell loss is juxtaposed with regions in which the cells appear normal. The first immature appearing hair cells in this study were seen both in regions completely denuded of cells as well as in the distal regions of the transition zone, among a group of normal appearing cells. Immature appearing hair cells were not seen in the far distal regions, where hair cell morphology appeared completely normal. The appearance of newly regenerated cells in grossly undamaged regions has been described previously in the noise exposure literature. Nuclei labeled with the S-phase marker bromodeoxyuridine could be identified in areas where no hair cells remained, as well as in adjacent regions where some surviving cells were present (Raphael, 1992). In a previous study using gentamicin, the region of hair cell labeling coincided with the area of major hair cell loss, and labeling did not occur in the distal undamaged regions (Lippe et al., 1991).

The presence of immature appearing hair cells in the region of the transition zone where cells appear normal raises two interesting possibilities. First, it implies that adjacent hair cell loss may not be necessary for the production of new hair cells. This technique is unfortunately not sensitive enough to rule out a one-for-one hair cell loss with hair cell replacement model as previously discussed (Raphael, 1993). Second, it lends support to the hypothesis that a diffusible factor may be present which stimulates hair cell regeneration. Recent in vitro work on the vestibular epithelium from our laboratory has suggested that a diffusible factor which up-regulates supporting cell proliferation may be released from damaged epithelium (Tsue et al., 1994).

Analysis of the 2-week survivors revealed a return of hair cell numbers to normal or above normal within the most proximal regions of the cochlea. In previous work in which long-term (5–10 day) courses of aminoglycoside were used, the most proximal regions consistently showed partial repopulation of cells in the first 2 weeks of recovery (Cruz et al., 1987; Girod et al., 1991). In some cases, damage progressed distally over the first 4 weeks after completion of the injections (Girod et al., 1991), and full recovery was not reached until 4–20 weeks after the drug exposure (Girod et al., 1991; Duckert and Rubel, 1993). It is interesting to note that Marsh et al. (1990) did not find full recovery of hair cell density in the damaged region of the chick sensory epithelium at 15 days after noise exposure. In our single-dose paradigm, the extent of damage was likely determined within the first week, as 2-week survivors showed impressive recovery in number of hair cells. The extent of damage in the single-dose paradigm is comparable to the 5–10-day drug exposures, yet the time course to recovery is more prolonged in the longer injection paradigms. This suggests that the repeated doses may have a prolonged effect in the proximal end, and may have some implication for understanding the relationship of length of gentamicin administration and extent of recovery in patients.

Immature appearing hair cells, though few in number, could be seen in both the 2- and 5-week survival groups, implying but not proving that cell division is still occurring. In the previous long-term studies, new cells could be identified up to 20 weeks after the completion of the injections (Girod et al., 1991; Duckert and Rubel, 1993). It is unclear whether these late mitoses are part of the normal time course of regeneration, or whether the gentamicin or a persistent metabolite provides some ongoing damage to the cochlea, triggering further regeneration.

The in-depth quantification of the hair cells in this study revealed that in the region corresponding histologically to the transition zone a greater number of hair cells was seen in the 2-week survivors than in controls. This increase was not seen in the 5-week survivors, and implies that an overproduction of hair cells may occur as part of the regeneration response. Interestingly, in previous work in which chicks were given a 10-day course of gentamicin, an increase in the number of hair cells compared to controls was seen at two different time points (Girod et al., 1991). First, at 1 week, the region 25% from the proximal tip showed an abnormally high number of hair cells. This could well correspond to the transition zone in our study. Second, after 20 weeks, the most distal regions showed a persistent increase in the number of hair cells. As yet, cell counts have only been made for the proximal one-half of experimental cochleae.

The fate of these 'extra' hair cells is not known. It is well known that active cell death occurs as a normal process in a variety of developing and mature tissues (Tomei and Cope, 1991). Though cell death is found during changes in shape in the formation of the otocyst (Tomei and Cope, 1991), it is not clear whether it occurs during the embryonic development of hair cells. Tilney et al. (1986) indicated that the total number of hair cells remains constant in the chick cochlea after embryonic day 10. Work investigating earlier time periods shows that the first recognizable stereocilia appear at 6–7 days on cells at the distal end of the cochlea and progress to the proximal end, but counts at these times are not available (Cotanche and Sulik, 1984; Tilney et al., 1986).

In order to verify that cell division was involved in the repopulation of the cochlea after the 100 mg/kg gentamicin injection, an initial study using the S-phase nuclear marker tritiated thymidine was carried out. Good agreement was seen with the SEM data in that cochleae serially sectioned for autoradiography showed preservation of the support cells and tectorial membrane but virtually com-

plete loss of hair cells to approximately 600–800 μm from the proximal tip. The chicks that received the radioactive label during the period from 18 to 22 h after the 100 mg/kg injection of gentamicin showed many labeled support cells as well as labeled hair cells when allowed to survive for 4 days. On the other hand, chicks exposed to radioactive thymidine from 8 to 12 h and allowed to survive for 4 days showed no labeled hair cells or sensory epithelium support cells. These data agree with what has been observed following noise damage (Girod et al., 1989; Raphael, 1992; Hashino and Salvi, 1993; Stone and Cotanche, 1994). Comparison with these studies suggests that the 18–22 h time period coincides with the onset of S-phase (Bhave et al., 1995).

Acknowledgements

This work was supported by NIH Grants DC00395 (E.W.R.), DC00018, NS09090 (J.D.J.), and by a Resident Research Award from the American Academy of Otolaryngology Head and Neck Surgery (J.D.J.). Our sincerest thanks to Dale Cunningham for his outstanding technical support and expertise in scanning electron microscopy, to Diana Lurie for photomicroscopy assistance, and to Mark Walter for clerical and editorial assistance. We would also like to thank Dr. Robert Bolender in the Department of Biological Structure for his help in the development of the quantitative analysis.

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