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Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma *

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Hair cell regeneration following acoustic trauma to the avian cochlea has been documented using DNA labeling with tritiated thymidine. The goal of this study was to identify potential precursor cell populations for regenerating hair cells. Chicks were exposed in pairs to a 1500 Hz pure tone at 120 dBSPL for 18 h. The animals received repeated injections of ^{3}H-thymidine over a survival period of 6, 15, or 24 h, 3 days or 30 days after the completion of noise exposure. One cochlea from each animal was processed for autoradiography and the other for scanning electron microscopy. Labeled, regenerated hair cells were present by 3 days after exposure and recovery from injury was nearly complete by 30 days. Examination of animals in short survival groups suggest that two precursor populations may exist. For inferior sensory epithelial damage, cuboidal or hyaline epithelial cells appear to serve as the precursor cell population for the regeneration of both hair cells and supporting cells. With isolated superior damage, however, supporting cells may be the precursor population.

Regeneration; Hair cell; Precursors; Cochlea; Acoustic trauma

Introduction

In amphibians and some fish, auditory sensory hair cells are produced throughout life as well as in response to injury (Corwin, 1981, 1985). In both mammals and birds, on the other hand, the production of cochlear sensory hair cells is thought to cease after early embryogenesis (Ruben, 1967; Rubel, 1978). Until recently, postembryonic hair cell loss in birds or mammals was held to be irreversible and associated with a permanent hearing loss.

The potential for regeneration of hair cells in the avian basilar papilla (cochlea) was first suggested indirectly when Cruz et al. (1987) demonstrated recovery of hair cell numbers following ototoxic drug insult. At about the same time Cotanche and collaborators (Cotanche et al., 1987; Cotanche, 1987a) observed that hair cell stereocilia were replaced following acoustic trauma and attributed this to the regeneration of new hair cells. More recently, regeneration of hair cells (and supporting cells) following acoustic trauma has been conclusively demonstrated by DNA labeling with tritiated thymidine in both neonatal chicks (Corwin and Cotanche, 1988) and sexually mature quail (Ryals and Rubel, 1988). In addition, Jorgensen and Mathiesen (1988) have shown a slow rate of proliferation of hair cells and supporting cells in normal adult avian vestibular sensory epithelium. The source of these new cells in an otherwise postmitotic sensory epithelium has not been elucidated.

Studies of the lateral line sensory epithelia in amphibians suggest that supporting cells, by a process of proliferation and transdifferentiation,

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may be the precursor for new hair cells following injury (Corwin, 1986). On the other hand, studies of the olfactory sensory epithelium have demonstrated an undifferentiated basal cell which gives rise to new neuroreceptor cells (Graziadei, 1986). No study to date has addressed this issue in the avian cochlea. The present experiment was designed to identify candidate precursor cell populations which may be responsible for the regeneration of hair cells and supporting cells in the neonatal chick cochlea following acoustic trauma.

Materials and Methods

Twenty-four white leghorn chicks (age range 9–12 days) were divided into five groups of four animals based on survival time after completion of noise exposure. The remaining four animals received no noise exposure and served as controls. The sound exposure, survival time and tritiated thymidine injection period for each group is shown in Fig. 1. Tritiated thymidine is a radioactively labeled DNA precursor which, when present, is incorporated into the nuclear DNA of any cell synthesizing DNA prior to mitosis. Using standard autoradiographic techniques, cells which were proliferating during the labeling period were later identified by the presence of ^{3}H-thymidine within their nuclear DNA.

Noise exposure was performed by confining pairs of animals under a JBL 2482 power horn in a sound attenuated box for 18 h. A 1500 Hz pure tone at 120 dBSPL was generated by a Wavetek 186 function generator, attenuated (Hewlett-Packard model 350D) and amplified (RAMSA WP-9055 amplifier). The sound pressure level (120 dB SPL) was monitored at the begining, during, and at the completion of each exposure, using a Bruel and Kjaer 1 inch 4145 microphone and a Hewlett-Packard 3561A signal analyzer. Sound intensity variation within the box was ± 3 dB SPL. Harmonics were at least 40 dB below the level of the primary frequency.

The 6, 15, and 24 hour survival groups had the noise exposure briefly interrupted after 12 h for an initial injection of tritiated thymidine (ICN 24060, 60–90 Ci/mmole; I M) at a dose of 10 μCi/gram body weight. After completion of the noise exposure (18 h total), injections with ^{3}H-thymidine were repeated at the same dosage 3 or 4 times over

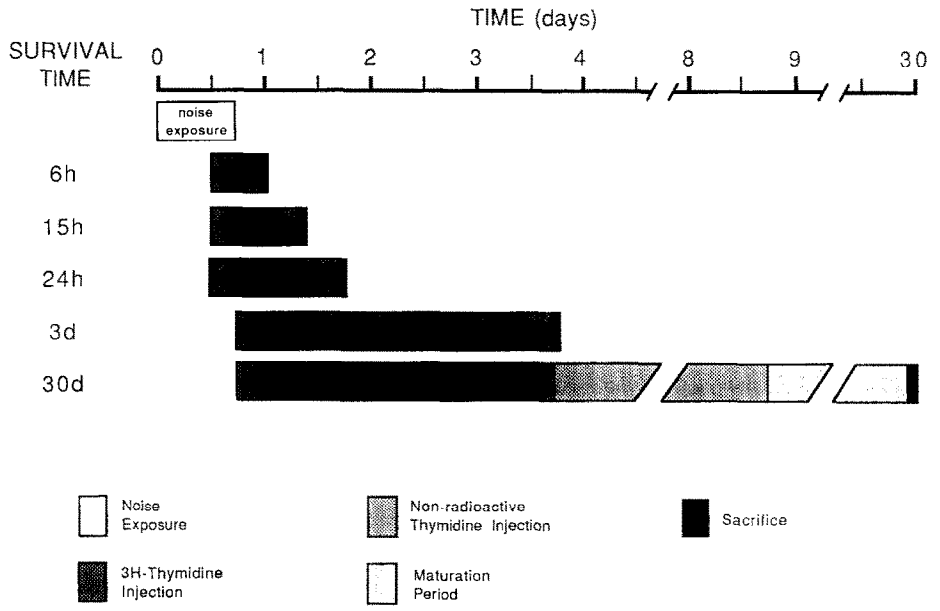

Fig. 1. Schematic representation of the experimental design. The animals were divided into five subgroups based on survival time following the completion of the noise exposure. Tritiated thymidine labeling was initiated 12 h into the noise exposure for the early survival groups ($\leq$ 24 h) and upon completion of the noise exposure for the longer survival groups ($\geq$ 3 days). $N = 4$ in each group.

each survival period. Four experimental animals were used for each survival period.

The labeling period for both the 3 day and 30 day survival groups was initiated upon completion of the noise exposure. Injections with ^{3}H-thymidine (10 μCi/g) were performed twice daily for 3 days. The animals were either sacrificed immediately (3 day survival group) or underwent termination of the labeling period by injection of non-radioactive thymidine at 100 times the radioactive thymidine dose, twice a day, for a total of 5 days. Thus, any cells proliferating during these 5 days were more likely to incorporate non-radioactive thymidine than residual ^{3}H-thymidine into their nuclear DNA. These animals were then allowed to survive for a total of 30 days following the end of noise exposure. In addition, two groups of control animals had no noise exposure, but were otherwise treated the same as the experimental animals of the 15 hour ($N = 2$) and 30 day ($N = 2$) groups.

At the end of the survival period all animals were given an overdose of pentobarbital sodium and underwent bilateral cochlear perfusion (intralabyrinthine via the round window) with 3.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4). After decapitation the external auditory canals and cochlear ducts were opened and the head was immersed in the same fixative at 4°C for 24–48 h. The left cochlea (dissected free) and right temporal bone with the cochlea exposed were post-fixed in 1% osmium tetroxide (in 0.1 M PBS) for 2 h at room temperature.

The right temporal bones were processed for scanning electron microscopy (SEM). They were dehydrated in 70% ethanol at 4°C for 72 h followed by final dissection, which included removal of the tectorial membrane. The tissue was then dehydrated through a graded ethanol series and the temporal bones were critical point dried and sputter coated to 500 Å with gold palladium. Scanning EM was performed on a JEOL 35C electron microscope (15 kV accelerating voltage).

The left cochleae, to be processed for autoradiography, were dehydrated through a graded methanol series and embedded in Polybed 812 epoxy resin (Poly Sciences). The cochleae were cut transversely on a Sorvall MT2 microtome into 3 μm serial sections. Three sections were collected at 100 μm intervals (every 50 μm through the damaged region) and mounted onto acid washed chrome alum subbed slides for both light microscopy (LM) and autoradiography (AR). The LM slides were stained with Toluidine Blue and coverslipped.

The AR slides were dipped in Kodak NTB-2 emulsion (diluted 1:1 with distilled H_2O) and stored at 4°C for 4–30 weeks. Slides were developed with Kodak D19 developer and Kodak Rapid Fixer without hardener before staining very lightly with Toluidine Blue and coverslipping. The sections were examined under brightfield illumination for the presence of labeled cells. There was negligible background deposition of silver grains in the AR specimens. The criteria for a labeled cell was 3 or more silver grains overlying the cell nucleus. Labeled cells typically had 5 to 15 grains over the nucleus after 4–6 weeks of exposure time.

Results

Autoradiography

In the following discussion, experimental groups are identified by the number of hours or days that animals were allowed to survive after termination of the noise exposure (see Fig. 1). The location and extent of both the damage and labeling will be described. All animals surviving 6–24 h had similar patterns of damage. However, considerable variation in the degree of hair cell loss was seen by both autoradiography and SEM in the 3 and 30 day survival groups. Two specific patterns of injury emerged in these two groups and will be described in both the autoradiographic and SEM results.

For orientation, a cross section through the cochlea of a control animal is shown in Fig. 2a. This sample was taken from the junction between the proximal (basal) and middle thirds of the cochlea, which is the region maximally damaged by exposure to a 1500 Hz pure tone (Rubel and Ryals, 1982). Tall hair cells, which have many similarities to mammalian inner hair cells (Takasaka and Smith, 1971; Tanaka and Smith, 1978), are located on the superior portion of the sensory epithelium. Short hair cells, which have similarities to the mammalian outer hair cells, are located

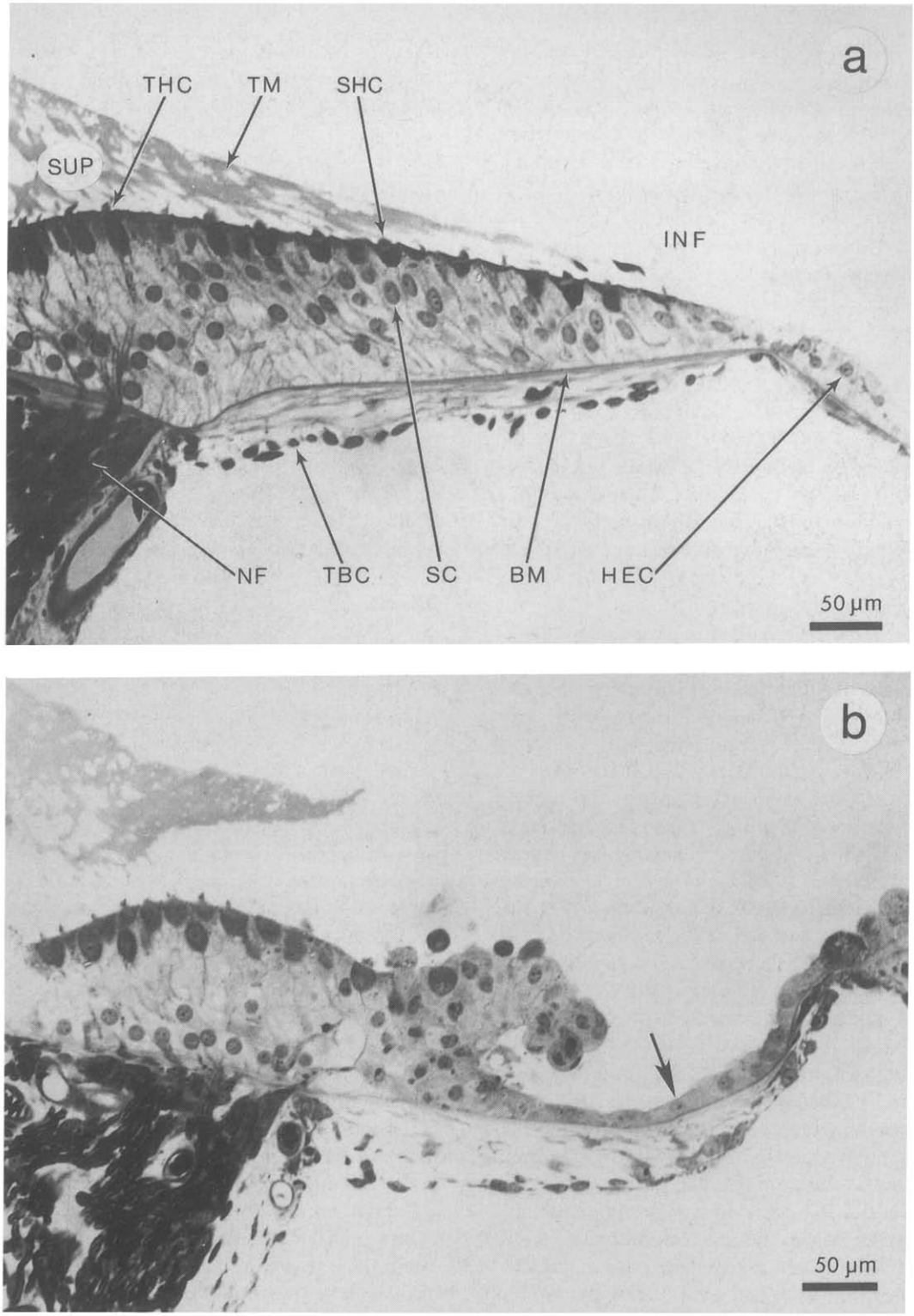

Fig. 2. Transverse light microscopic sections through the 1500 Hz region of the chick cochlea. a) Normal control animal. Superior edge (SUP), inferior edge (INF), tall hair cells (THC), tectorial membrane (TM), short hair cells (SHC), cochlear nerve fibers (NF), tympanic border cells (TBC), supporting cells (SC), basilar membrane (BM), and hyaline and cuboidal epithelial cells (HEC). b) Same region of an experimental cochlea 6 h after completion of noise exposure demonstrating extensive hair cell and supporting cell loss at the inferior edge of the sensory epithelium. Note the thin monolayer of cells spreading to cover the basilar membrane (arrow).

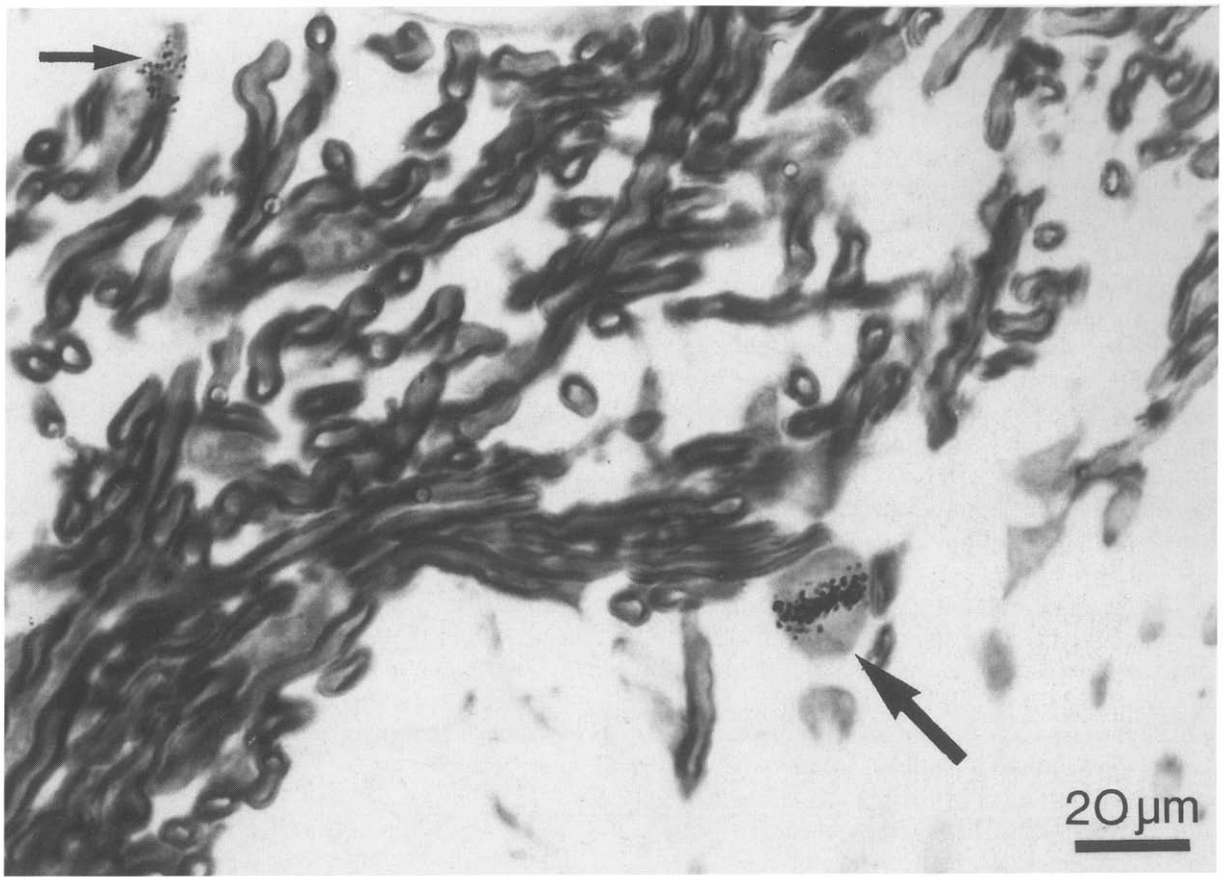

Fig. 3. Nuclei labeled by autoradiography (arrows) within the cochlear nerve fiber bundle in the 1500 Hz region 6 h after completion of noise exposure. Large arrow indicates a nucleus captured in early mitosis.

more inferiorly. Immediately adjacent to the inferior-most short hair cells are two separate cell types: the cuboidal and hyaline epithelial cells. Lining the undersurface of the basilar membrane are the tympanic border cells.

Control animals

These animals did not undergo noise exposure but were otherwise treated the same as the 15 hour and 30 day groups (Fig. 1). The cochleae demonstrated no labeling of hair cells or supporting cells. However, the following four cell types were consistently labeled in all control cochleae: 1) red blood cells (RBCs), 2) capillary endothelial cells, 3) mesoepithelial cells lining the scala tympani side of the basilar membrane (tympanic border cells), and 4) occasional tegmentum vasculosum epithelial cells. When comparing the two control groups, all four cell types were seen labeled with a higher frequency in the 15 hour group than the 30 day group, suggesting a normal turnover rate for these cell types of less than 30 days.

Non-damaged regions of experimental cochleae

The non-damaged regions of the experimental cochleae also served as control tissue. Again, no hair cells or supporting cells were labeled but the same four cell types labeled in control cochleae were also consistently labeled in these regions of all experimental cochleae. The labeling of these four cell types increased in frequency as the survival time after noise exposure increased from 6 h to 3 days. Maximal labeling was seen in the animals surviving 3 days. A marked decrease in the labeling frequency of all four cell types was observed at 30 days survival.

Six hours after exposure

The location of the lesion produced by the noise exposure was consistently at the level of the oval window, in the 1500 Hz region of the cochlea (Rubel and Ryals, 1982). The size and severity of the lesion however varied considerably among animals. At the six hour survival time, extensive damage to the inferior portion of the sensory epithelium was consistently seen (Fig. 2b). Both hair cells and supporting cells were lost in this region. The basilar membrane was without any identifiable sensory epithelium and was covered only by a thin monolayer of cells (arrow, Fig. 2b). The origin of these cells could not be conclusively identified. They may represent supporting cells surviving the noise induced damage. However, these cells had round nuclei and small amounts of moderately staining cytoplasm, unlike the oval nuclei and large amounts of minimally staining cytoplasm seen in the supporting cells normally found in this region. Morphologically these cells were very similar to adjacent cuboidal or hyaline epithelial cells. The cuboidal and hyaline epithelial cells are two cell populations found only at the extreme inferior edge of the sensory epithelium in controls (Fig. 2a). They cannot be differentiated from each other at the light microscopic level. Following noise damage, however, they appeared to have elongated and migrated superiorly on the basilar membrane in order to fill in the gaps produced by extruded or degenerating hair cells and supporting cells (large arrow, Fig. 4a). This observation was supported by the SEM findings (Fig. 9b). No labeling on the basilar membrane was seen in this region at this short survival time.

Several nuclei within the nerve bundle demonstrated early labeling, indicating proliferation within the previous 12 hours (arrows, Fig. 3). It is not clear if these labeled cells represent Schwann cell proliferation in response to injury or a separate cell population within the nerve bundle. However, these labeled nuclei were indistinguishable from non-labeled Schwann cell nuclei located in the same region. Labeling in the nerve bundle was primarily within the area of damage and was seen in all groups (6, 15, and 24 h, 3 and 30 days).

The damaged region also demonstrated labeling of RBCs, capillary endothelial cells, and occasional tegmentum vasculosum cells as described for control cochleae. The tympanic border cell population, however, was labeled with increased frequency in the region of damage as compared to the non-damaged regions of the same cochleae (small arrow, Fig. 4a).

Fifteen hours after exposure

Labeling was clearly seen over the monolayer of cells covering the basilar membrane in the region of damage (arrow, Fig. 4b). This cellular proliferation resulted in apparent increase in density of the cells forming the monolayer. At this time none of these labeled cells had structural similarities with either hair cells or supporting cells in our Toluidine Blue stained material. Instead, they were cytologically indistinguishable from the hyaline and cuboidal cells. Extensive labeling was also seen in the adjacent hyaline and cuboidal cell region. As with the 6 hour group, nuclei within the nerve bundle were also labeled in the area of damage. Tympanic border cells were again labeled with a higher frequency in the region of damage than the non-damaged regions of the same cochleae.

Twenty-four hours after exposure

For the first time, nuclei which were clearly part of the sensory epithelium were labeled. These cells were at or near the extreme inferior edge of the epithelium. The identification of the labeled cells as hair cells or supporting cells could not be made on the basis of their appearance. Labeled cells did share some characteristics with supporting cells, including minimal cytoplasmic staining and darkly staining, round to ovoid nuclei. They were found adjacent to the labeled monolayer of cells with a progression toward stratification (arrows, Fig. 5a). No supporting cells or other nuclei were labeled in the more superior portions of the sensory epithelium despite the loss of some hair cells in this region. Nerve bundle nuclei were again labeled in the region of damage, as were tympanic border cells, as previously described.

Three days after exposure

This group received ^{3}H-thymidine for the full 3 days following acoustic overstimulation (Fig. 1). Labeled cuboidal or hyaline epithelial cells were again found, although much less frequently than

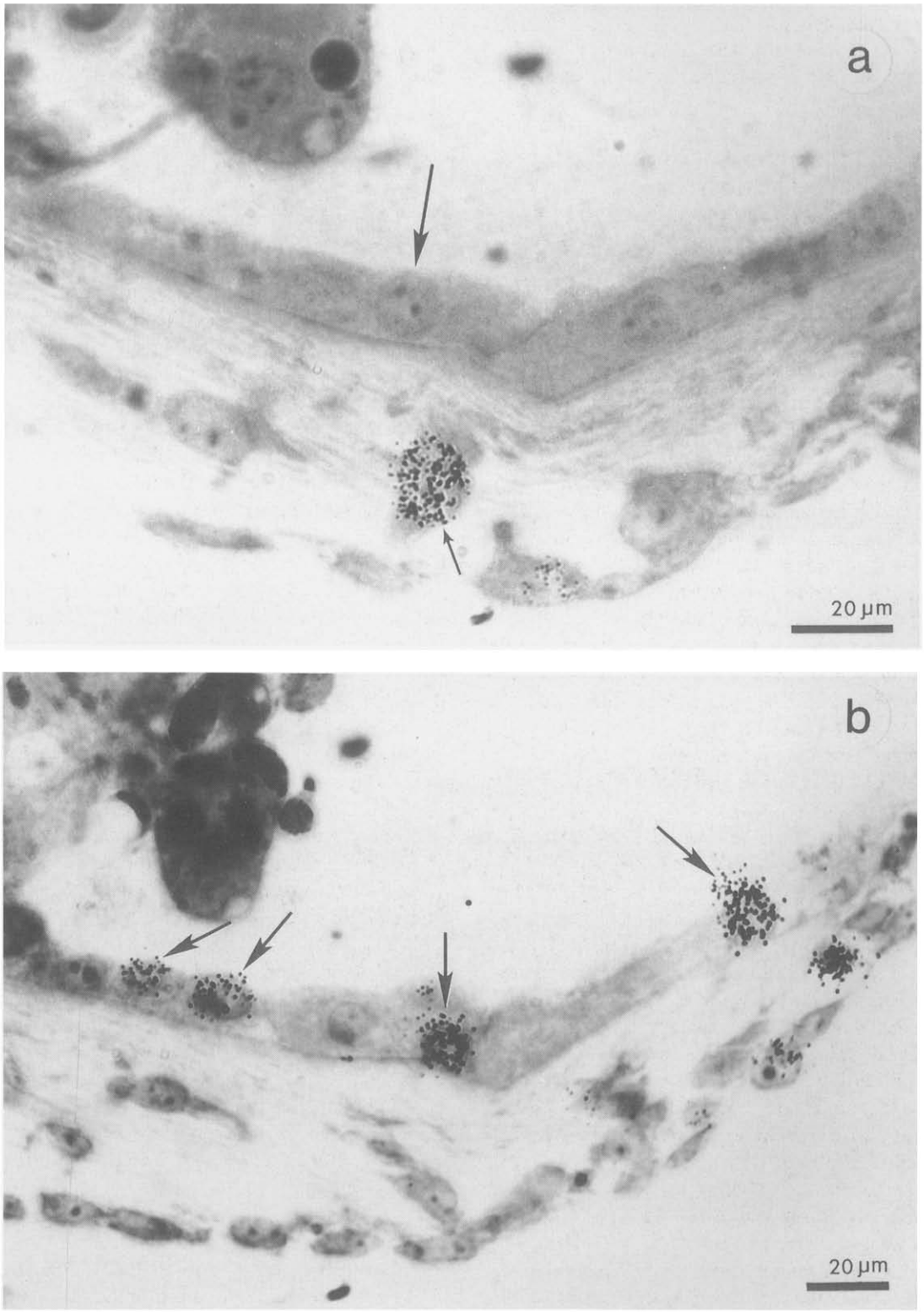

Fig. 4. Inferior border of the sensory epithelium in the 1500 Hz region of experimental cochleae. a) Six hour survival. Monolayer of thin cells with round nuclei covering the basilar membrane where hair cells and supporting cells were lost (large arrow). Labeled tympanic border cell nucleus (small arrow). b) Fifteen hour survival. Labeled nuclei (arrows) indicating mitosis within the cellular monolayer.

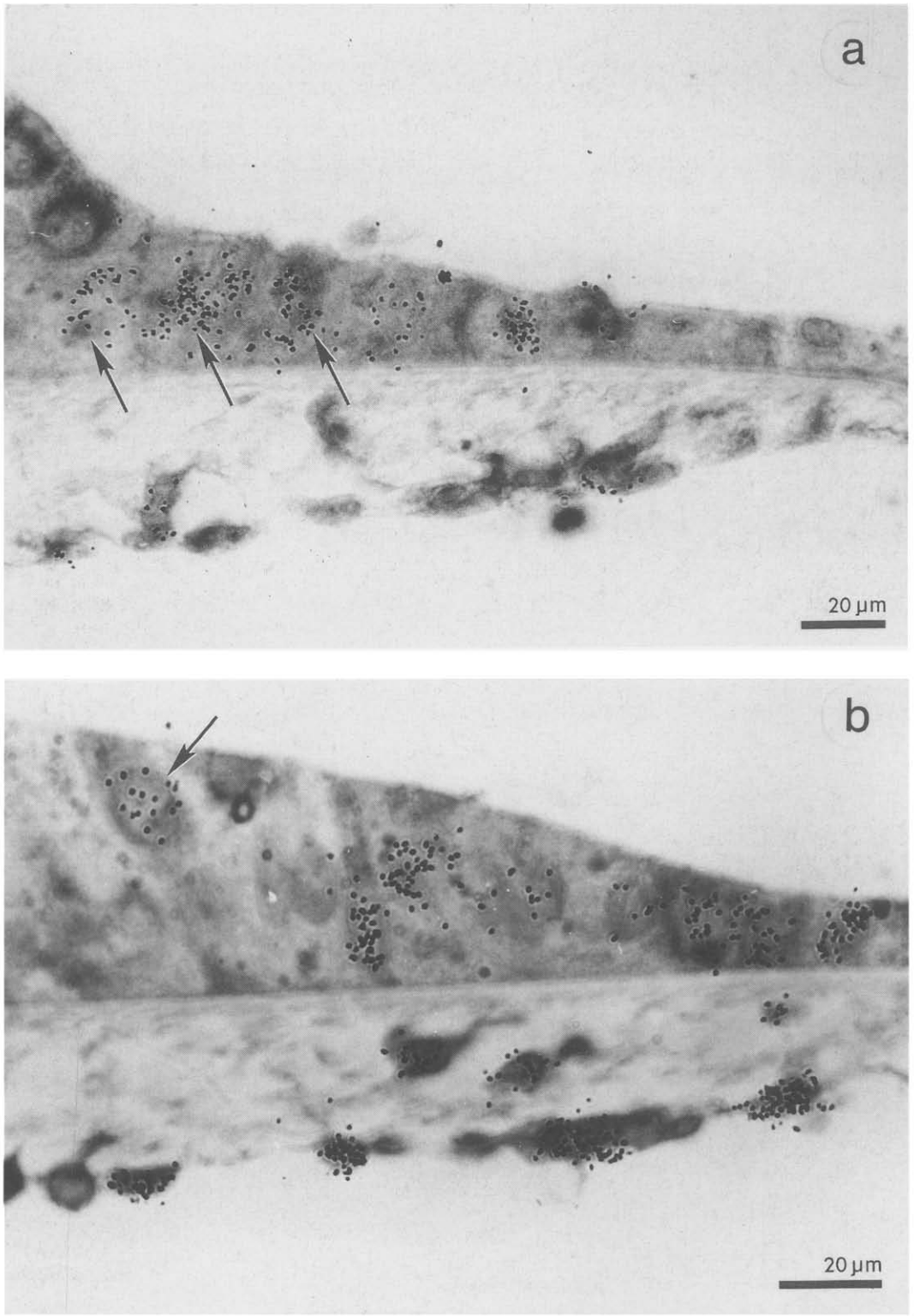

Fig. 5. Inferior border of the sensory epithelium in the 1500 Hz region of experimental cochleae. a) Twenty-four hour survival. Multiple labeled nuclei (arrows) in the now stratified epithelium covering the basilar membrane. b) Three day survival after inferior hair cell loss. Labeled regenerated hair cell (arrow) with lightly staining cytoplasm and a large round nucleus adjacent to the region of active proliferation. Plane of focus is on the overlying silver grains putting the cells partially out of focus.

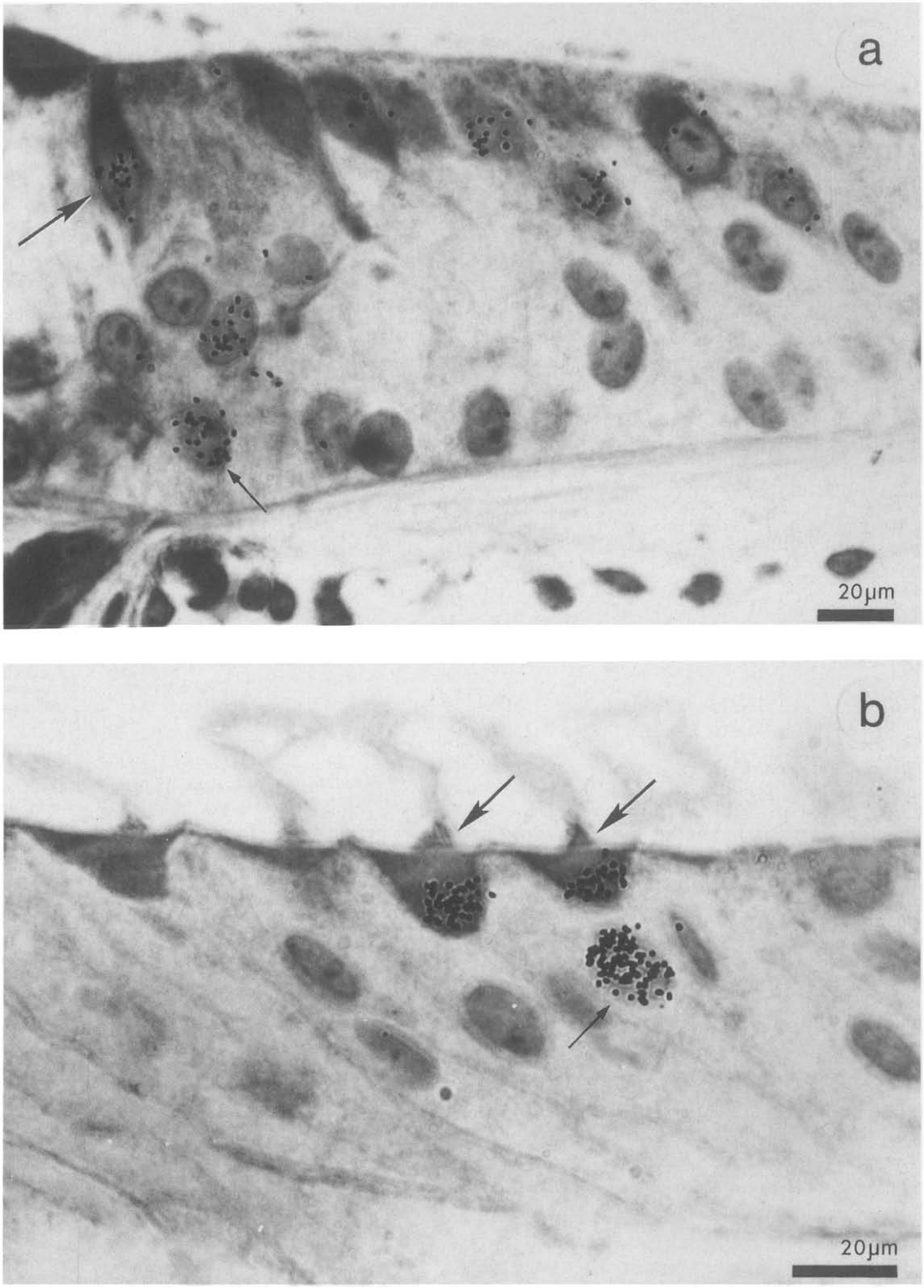

Fig. 6. Superior portion of the sensory epithelium in the 1500 Hz region of experimental cochleae. a) Three day survival after less severe, superior hair cell loss. Somewhat more mature appearing (compared to Fig. 5b) labeled regenerated hair cell (large arrow). Note labeled supporting cells underneath the regenerated hair cells (e.g. small arrow). b) Thirty day survival. Labeled regenerated short hair cells (small arrows) are indistinguishable from adjacent non-labeled hair cells. Labeled mature supporting cell (large arrow) underneath labeled hair cells. There is no evidence of residual damage.

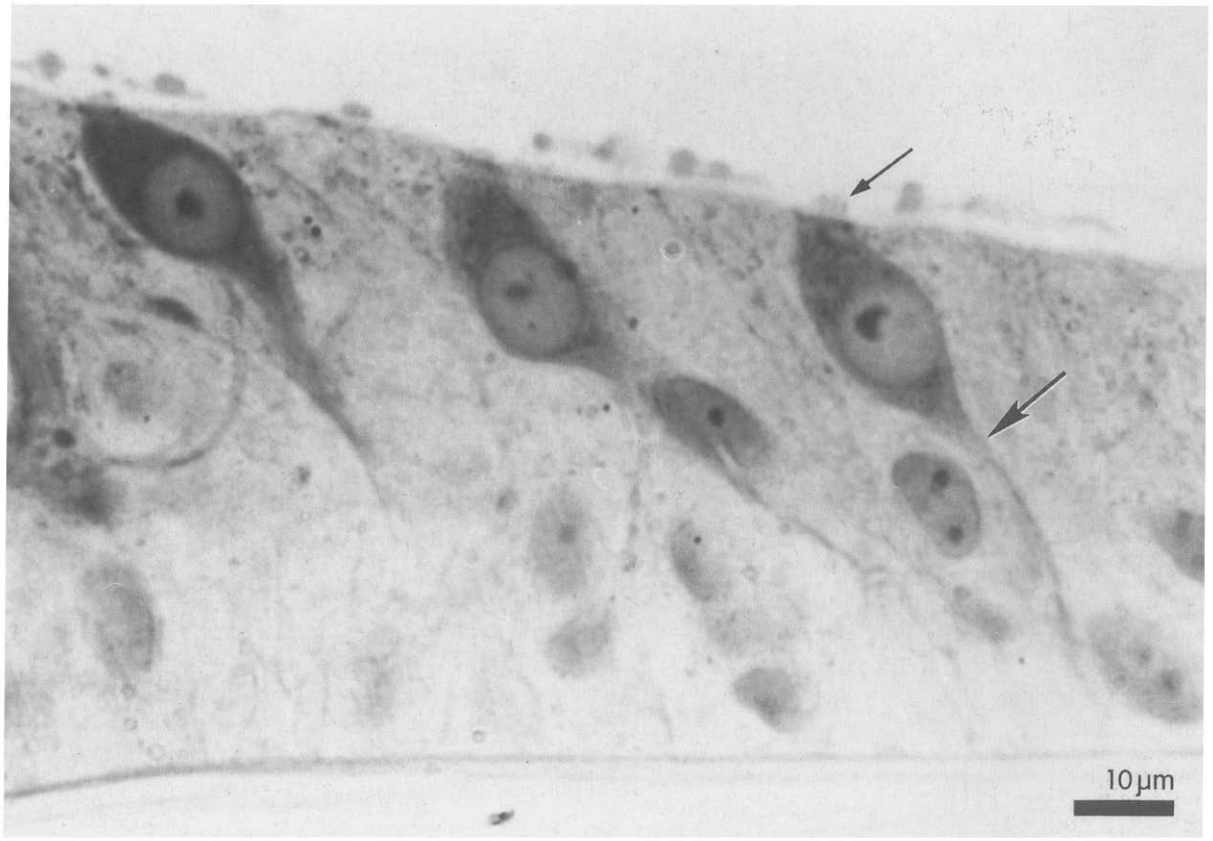

Fig. 7. Immature regenerated hair cells seen 3 days after noise exposure. Plane of focus is now on the cells and not the overlying silver grains (as are Figs. 3–6). The cells have a unique appearance including a tall spindle-shaped cell body with lightly staining cytoplasm, a large round nucleus and very short stereocilia (small arrow). Processes seen at the cell bases may represent innervating axons or trailing cytoplasm from cell migration to the lumen (large arrow).

in the 15 or 24 hour groups. For the first time, labeled cells which could be positively identified as immature sensory hair cells were seen (Fig. 5b, and 6a). They demonstrated a unique appearance including a tall spindle-shaped cell body, light staining cytoplasm, a large round nucleus and a very small apical surface with short stereocilia (Fig. 7). All labeled hair cells in this group had a similar characteristic appearance; thus regenerated hair cells at this level of maturity were readily identified by light microscopy alone, *without* the use of autoradiography. There were some hair cells present with this same appearance which had no labeling. These probably represent regenerated hair cells as well (see Discussion).

The immature regenerated hair cells seen in this group had processes which appeared to extend to the basilar membrane. Some of these could be axons innervating the new cells while others were clearly cytoplasmic processes left behind as the cell migrated to the lumenal surface (large arrow, Fig. 7). Transmission electron microscopic analysis, which will be reported in a subsequent paper, has confirmed this conclusion.

Several nuclei within the supporting cell layer were also labeled at this time. These cells were located both underneath and adjacent to the labeled hair cells. They had the ovoid nucleus with minimal cytoplasmic staining characteristic of mature supporting cells.

Of note is the fact that most of the cochleae in this group demonstrated a more superiorly located region of less severe hair cell loss, as opposed to the inferiorly located region of extensive loss seen

in earlier groups. Less severe hair cell loss was located in a narrow strip at the transition zone between tall hair cells and short hair cells. The more inferior portions of the sensory epithelium were relatively undamaged. Regenerating hair cells and supporting cells were clearly seen in this superior region of damage as well (Fig. 6a). In these cochleae no labeled cuboidal or hyaline epithelial cells were seen, suggesting they were not involved in the replacement of superiorly located hair cells and supporting cells. Supporting cells were consistently labeled in a position beneath or near most labeled hair cells, suggesting a possible relationship as hypothesized by Corwin and Cotanche (1988). In addition, labeled hair cells appeared more mature in cochleae with only mild superior damage (Fig. 6a) than in cochleae with inferior damage (Fig. 5b). This suggests that regeneration may occur earlier when damage is less severe and was supported by the SEM findings (Figs. 9e and 9f).

Labeled nuclei in the nerve bundle were again seen in these animals. These labeled cells were predominantly in the area of damage but were occasionally seen in non-damaged regions as well. As before, tympanic border cell labeling was also predominantly in the region of damage.

Thirty days after exposure

This group of animals received 3 days of ^{3}H-thymidine injections after the sound exposure followed by 5 days of injections with an excess of nonradioactive thymidine. They were then allowed to survive for another 22 days (a total of 30 days survival; Fig. 1). Therefore all labeling occured in the first three days after the sound exposure. In these animals, no residual damage was evident by light microscopy. Many labeled, mature-appearing hair cells and supporting cells were seen in the 1500 Hz region of the cochlea. These labeled cells were otherwise indistinguishable from adjacent, non-labeled cells (Fig. 6b).

Like the majority of the 3 day group, most of the cochleae in this group demonstrated labeling patterns consistent with prior loss of superior hair cells. Others had most of the labeled cells located on the inferior edge. In the former group, some labeled hair cells and supporting cells were located at the inferior border of the sensory epithelium, but most were located more superiorly, near the junction between the superior cartilaginous plate and the basilar membrane. Labeling included both tall hair cells and short hair cells.

Of note, labeling of cuboidal or hyaline epithelial cells was not present in any of the 30 day survival cochleae regardless of whether hair cell labeling was superior or inferior. Recall that labeled hyaline or cuboidal epithelial cells were present shortly after noise exposure as seen in the early survival groups (at least with inferior hair cell loss), while in the 30 day group only labeled hair cells and supporting cells remain. Thus when damage was inferior the labeled hyaline and cuboidal cells either differentiated, migrated away, or died during the 30 day survival period.

Labeling of nuclei in the nerve bundle, as well

Fig. 8. Scanning electron micrographs of the sensory epithelium in the 1500 Hz region of the chick cochlea. a) Normal control cochlea. Superior edge (sup) and inferior edge (inf). Hyaline and cuboidal epithelial cell region (arrows). b) Six hour survival. The surviving sensory epithelium has receded superiorly (arrows). Box region is magnified in Fig. 9b. c) Twenty-four hour survival. Note the apparent increase in cell density at the inferior edge of the surviving sensory epithelium (arrows). Box region is magnified in Fig. 9c. d) Three day survival with region of more severe, inferior hair cell loss (arrows). e) Three day survival with a less severe, superior band of hair cell loss (arrows) and inferior hair cell survival. f) Thirty day survival. Note mild disorganization of the normal hair cell mosaic pattern and very small residual scar (arrow).

Fig. 9. Scanning electron micrographs of the sensory epithelium in the 1500 Hz region of the chick cochlea. a) Normal control cochlea. b) Six hour survival. Hyaline or cuboidal epithelial cells may have migrated superiorly (arrows) as the surviving sensory epithelium receded. c) Twenty-four hour survival. Increased cell density (arrows) at the inferior edge of the surviving sensory epithelium. d) Three day survival with a fibrinous network of regenerating tectorial membrane (arrows). e) Three day survival with an inferior region of hair cell loss. Note the embryonic appearing stereocilia of a regenerated hair cell (arrows). f) Three day survival with a region of superior hair cell loss. Contrast the stereocilia of regenerated hair cells (arrows) to the adjacent mature hair cell.

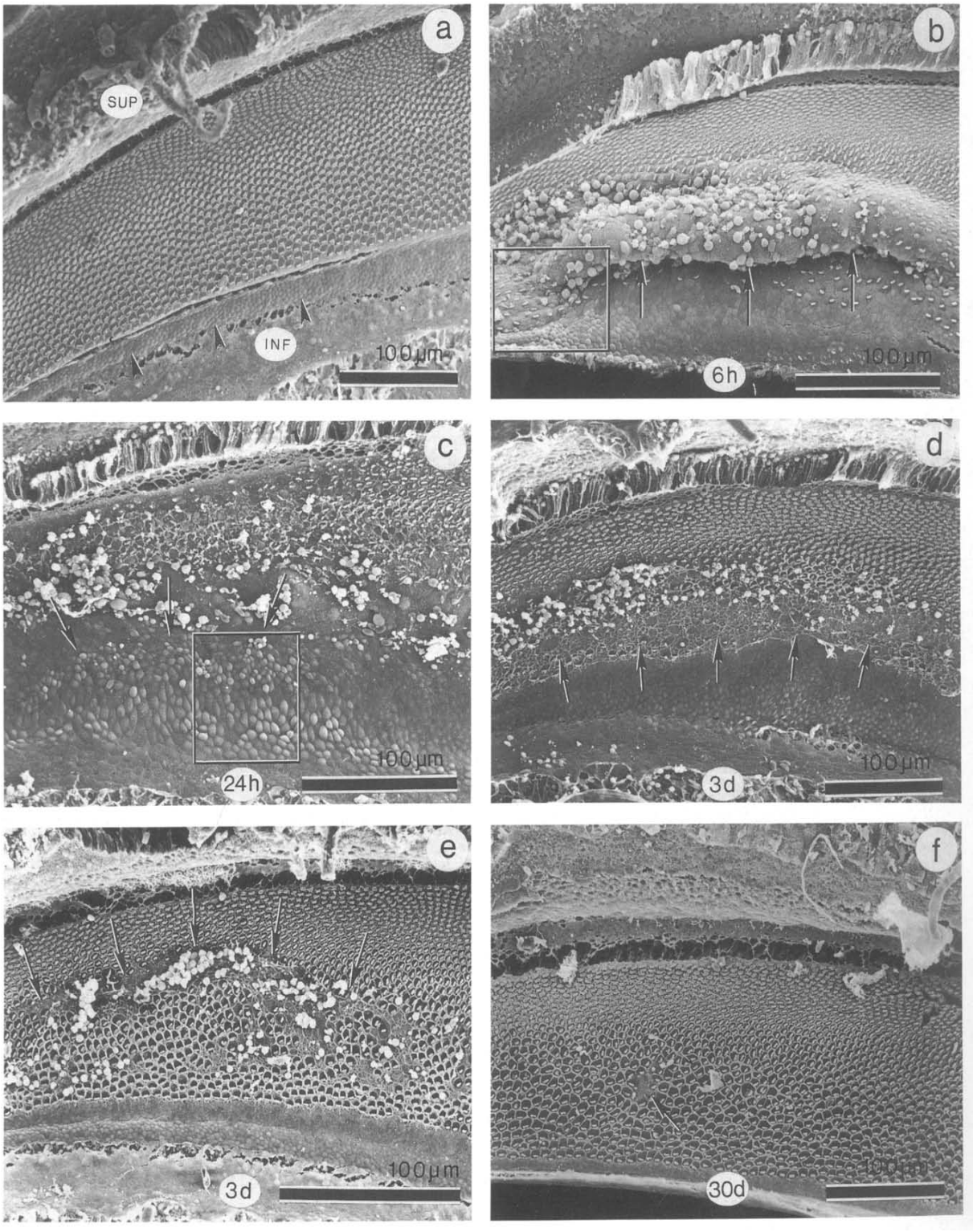

Fig. 8.

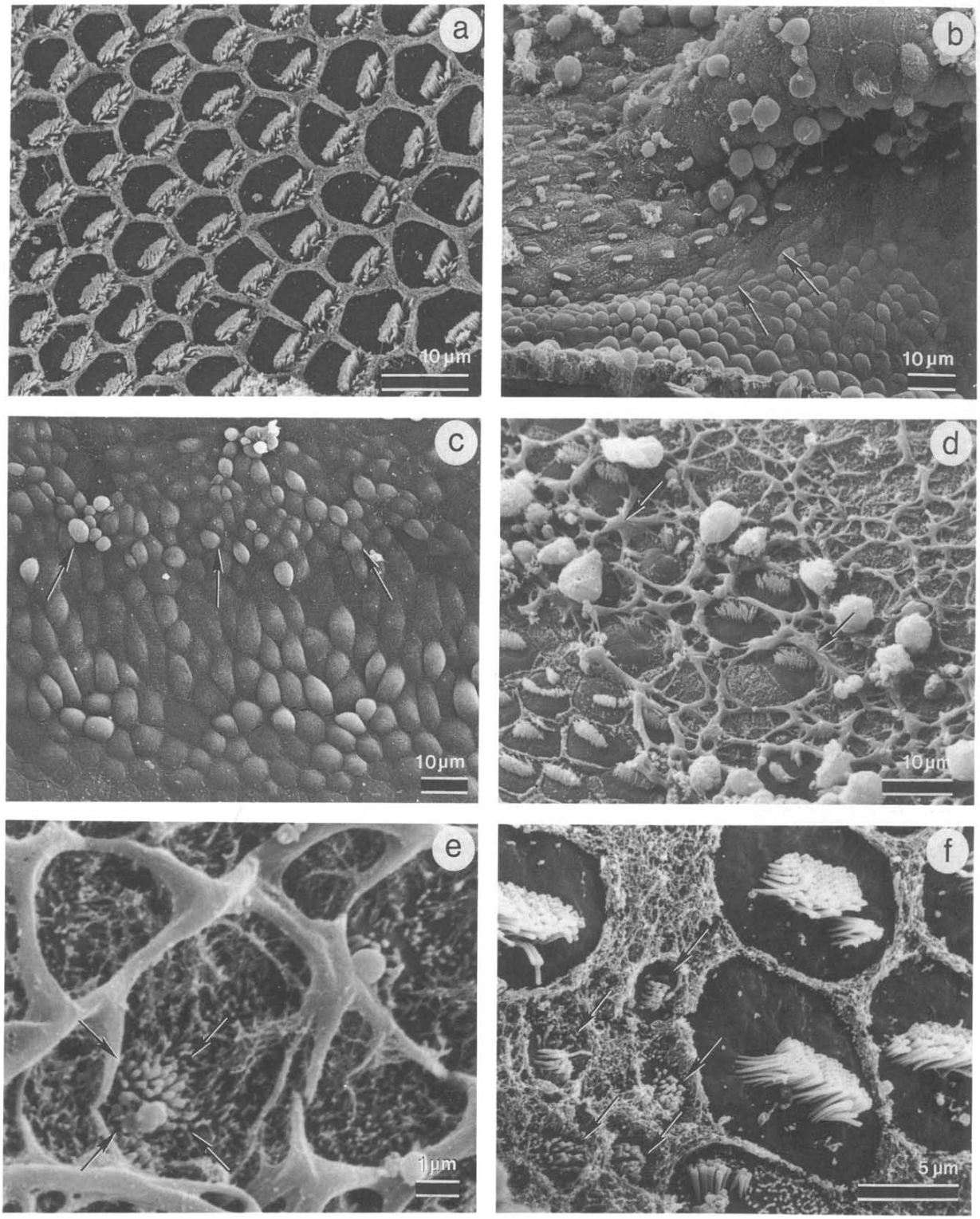

Fig. 9.

as tympanic border cells, RBCs, capillary endothelial cells and tegmentum vasculosum cells was infrequent in this group.

Scanning electron microscopy

At least two cochleae from each of the 6 hour, 24 hour, 3 day and 30 day survival groups were examined by SEM for comparision with the light microscopic findings. Removal of the tectorial membrane allowed examination of the apical surface of all hair cells. A discrete, well circumscribed lesion, disrupting the normal mosaic pattern of the hair cells in the 1500 Hz region (Saunders and Tilney, 1982; Cotanche et al., 1987; Cotanche, 1987a) was seen in all but the 30 day survival group. This latter group demonstrated no significant residual damage.

As with the AR specimens, considerable variability in the amount and position of hair cell loss was observed in the SEM specimens. The majority of cochleae with evidence of hair cell loss demonstrated an inferior semicircle of extensive loss with evidence of sublethal hair cell injury at the borders (Fig. 8b). However in the 3 day group, some of the cochleae had less severe loss with an inferiorly based semicircle of sublethal injury and actual hair cell loss observed only in a narrow strip at the superior-most border of the lesion (Fig. 8e). This marked difference in location of hair cell loss between animals probably represents interanimal variation in susceptability to noise damage. Cotanche et al. (1987) reported similar patterns of superior hair cell loss when lower intensity noise was used (115 dB SPL) and more extensive, inferior hair cell loss with higher intensity noise (125 dB SPL).

The pattern of injury and phases of apparent recovery will be described for each survival group. Figs. 8a and 9a show the 1500 Hz region of a normal cochlea for comparison.

Six hours after exposure

The lesion shown in Fig. 8b is representative of the inferiorly-based, well circumscribed, semicircular area of hair cell loss seen in this group. Short hair cells were lost primarily, but the lesion extended superiorly to include loss of a few tall hair cells. The inferior margin of the surviving sensory epithelium had an abrupt, curled-up edge (arrows, 8b), below which the vast majority of hair cells and possibly supporting cells had been lost. In some areas, the cells remaining in the inferior region of injury appeared to be continuous with the cuboidal and hyaline epithelial cells (Fig. 9b). Some of the hyaline or cuboidal cells were elongated superiorly into the region of sensory cell loss (arrows, Fig. 9b). This is suggestive of superior hyaline or cuboidal cell migration consistent with our observations in AR tissue.

Hair cells immediately adjacent to the region of hair cell loss demonstrated various degrees of damage. This has been well described elsewhere (Saunders and Tilney, 1982; Cotanche et al., 1987; Cotanche, 1987a) and includes loss, elongation, and fusion of stereocilia, blebbing of the apical surface adjacent to the tallest row of stereocilia, and shrinkage of the apical surface area with supporting cells filling in the resulting gaps. In addition, some hair cells showed a ballooning of the entire apical surface including the stereociliary bundle. Other cells were almost entirely ballooned into the scala media with only severely disfigured stereocilia present. This ballooning phenomenon presumably represents ongoing hair cell loss and accounts for the spheres of cellular debris that remained in the scala media at the edges of the lesion.

It was noted during the dissection of these specimens that there was no tectorial membrane present over the region of the lesion. The loss of the tectorial membrane following acoustic overstimulation has been previously described by Cotanche (1987b).

Twenty-four hours after exposure

The location and pattern of hair cell loss in this group (Fig. 8c) was very similar to that described for the 6 hour group. Inferior hair cell and supporting cell loss was again replaced by the apparent expansion of the adjacent cuboidal or hyaline epithelial cell population. These cells appeared to have proliferated and increased in density in the region immediately adjacent to the remaining sensory epithelium (arrows, Fig. 9c). This correlated well with the pattern of AR labeling in both the 15 hour and the 24 hour groups.

There was no suggestion of hair cell regeneration by SEM at this time.

As with the 6 hour group, there was evidence of ongoing hair cell loss at the edges of the lesion. Also, there was no remaining tectorial membrane over the lesion.

Three days after exposure

Two patterns of hair cell loss were seen in this group. A lesion similar to those seen in the earlier survival groups was seen in one of the three cochleae (Fig. 8d). Residual cellular debris and blebbing remained at the superior edge of the lesion. A honeycomb network of fibrinous material was seen covering the area of hair cell loss and surrounding the surviving hair cells at the edge of the lesion (arrows, Fig. 9d). This material did not lift off with the dissection of the tectorial membrane and appeared to be partially regenerated tectorial membrane (Cotanche, 1987b).

The first SEM evidence of hair cell regeneration was seen in this group. Small bundles of thickened microvilli were discernible between the supporting cell microvilli at the inferior edge of the damaged region amongst the fibrinous network (arrows, Fig. 9e). These had the appearance of embryonic hair cell stereocilia (Cotanche and Sulik, 1984; Tilney et al., 1986; Cotanche, 1987c) and presumably represent the apical surfaces of immature regenerating hair cells. This SEM appearance and stereocilia maturation has previously been described by Cotanche (1987a) and correlates with the tall spindle-shaped, labeled hair cells seen by AR.

The other two cochleae in this group demonstrated a very different pattern of hair cell loss. The lesion was again a well circumscribed, inferiorly based semicircle. However hair cell loss was less severe and occurred mainly in a thin band along the superior rim of the lesion and not inferiorly as seen previously (arrows, Fig. 8e). This pattern of injury is more typical of the findings reported for lower intensity noise exposure (115 dB SPL) by Cotanche et al. (1987).

Despite this contrast in location of hair cell loss, the response to injury was very similar. Regeneration of the tectorial membrane appeared the same and the embryonic-appearing stereocilia of regenerating hair cells were observed in both regions (superior and inferior) of hair cell loss. However, the regenerated stereociliary bundles were clearly more mature in appearance in the cochleae with less severe superior hair cell loss (Fig. 9e). This again suggests hair cell regeneration may occur earlier after noise exposure when the degree of damage is less severe.

Thirty days after exposure

Nearly complete regeneration of the sensory epithelium was seen in this group. The 1500 Hz region of the cochleae demonstrated minor disorganization of the normal mosaic pattern but appeared otherwise normal (Fig. 8f), with one exception. One cochlea had a very small area of apparent scar where three or four hair cells were missing (arrow, Fig. 8f). There was no evidence of residual damage to the stereocilia of any cells. In addition, there was no evidence of tectorial membrane loss at the time of dissection, suggesting complete regeneration of the TM.

Discussion

Background

The conclusive documentation of hair cell and supporting cell regeneration in the avian cochlea following acoustic trauma has resulted in speculation as to the source of these new cells (Corwin and Cotanche, 1988). Potential candidates for precursor populations include hair cells, supporting cells, and a yet unidentified latent stem cell population. There is precedence in the literature for both supporting cells and stem cell populations playing a major role in the production of receptor cells and supporting cells during development and in response to injury.

The olfactory sensory epithelium, like the auditory sensory epithelium, is derived from an ectodermal placode. It contains both supporting cells and neuroreceptor cells plus an undifferentiated basal cell population. These basal cells have been shown to proliferate and differentiate into neuroreceptor cells as a result of continual turnover and in response to injury (Graziadei, 1986). The supporting cells however, undergo mitosis with self-replacement on a continual basis and in response to injury. Thus, the undifferentiated basal cell is

capable of regenerating only the neuroreceptor cells and not the entire sensory epithelium.

The auditory organ of amphibians, rays, sharks, and some fish may also have an undifferentiated stem cell population. In these animals, hair cells and supporting cells are added throughout life to the periphery of the ever-expanding sensory epithelium. Corwin (1981) postulated a stem cell population located at the periphery of the sensory epithelium which gives rise to progeny capable of differentiation into both hair cells and supporting cells.

These same studies, however, also provided evidence for ongoing supporting cell proliferation with a basal turnover rate. New supporting cells and occasional hair cells were seen in the central area of the sensory epithelium where no stem cells could be identified. Further studies in the lateral line organ of amphibians have demonstrated the complete regeneration of a neuroblast organ (containing both hair cells and supporting cells) by the mitosis of only supporting cells (Balak and Corwin, 1988).

Summary of results

Two patterns of noise-induced injury to the sensory epithelium were observed in this study. The findings suggest that the regeneration process may be different for each pattern of injury so they will be discussed separately.

In the first pattern of injury, hair cell loss was predominantly at the inferior edge of the basilar membrane. In this position, the autoradiographic results would suggest that a latent precursor cell population may exist for the regeneration of both hair cells and supporting cells. Following noise damage, hair cells and supporting cells near the inferior edge of the cochlea degenerated and were extruded. The exposed basilar membrane initially had only a thin monolayer of covering cells. The exact origin of this monolayer could not be determined. However, by light microscopy these cells appear similar to cuboidal or hyaline epithelial cells and SEM images suggest they have migrated superiorly to cover the denuded basilar membrane. This monolayer then rapidly proliferated and the new cells appeared to differentiate into both hair cells and supporting cells. This sequence of events was well supported by the SEM results.

The second pattern of injury was much less severe and involved isolated superior hair cell loss. Unfortunately, this occured only in later survival groups (3 and 30 days) making identification of early events impossible. In this setting, only labeled supporting cells and hair cells were seen. Labeled hair cells were usually associated with one or more labeled supporting cells.

Candidate precursor cell populations

Of the three potential precursor populations (hair cells, supporting cells and a latent stem cell), two may well play a role in hair cell regeneration. Post-embrionic, mature neurosensory cell division has not been described in the literature. In keeping with this, our results demonstrate regeneration in the absence of surviving hair cells. Thus, the hair cell does not appear to be capable of self-regeneration.

When hair cell loss was inferior, both supporting cells and stem cells (hyaline or cuboidal cells) are potential precursor populations. Regeneration appeared to occur from a thin monolayer of cells covering the basilar membrane following the loss of the sensory epithelium. The origin of this monolayer could not be unequivocally determined with our methods since independent cytological markers for the different cell types have not been identified.

Surviving supporting cells may account for this cellular monolayer despite the apparent loss of both hair cells and supporting cells immediately after the noise exposure. However, the cells comprising the monolayer were morphologically very different from any supporting cell normally found in this region. The cells in the monolayer 6 h after noise exposure had round nuclei, moderate cytoplasmic staining, very little cytoplasm and were very few in number. In contrast, the supporting cells seen in this region in control cochleae (Fig. 2a) had oval nuclei, minimal cytoplasmic staining, large quantities of cytoplasm and were abundant in number. Thus the surviving supporting cells would have had to undergo extensive morphologic changes during and immediately following the noise exposure. Although this seems unlikely, it cannot be excluded.

The third and most promising potential precursor for inferior regeneration were the hyaline

or cuboidal epithelial cells serving as a latent stem cell population. By light microscopy the cells of the monolayer were very similar to the cells found in the immediately adjacent hyaline and cuboidal cell region of normal cochleae (Fig. 2a). By SEM the hyaline or cuboidal cells appeared to have migrated superiorly to fill in the gap caused by the lost sensory epithelium. In addition, the earliest cell labeling (i.e. cellular proliferation) was seen in both the cellular monolayer and in the adjacent hyaline and cuboidal cell region. However, by 30 days after noise exposure there was no labeling of hyaline or cuboidal cells. Only mature hair cells and supporting cells were labeled. Thus, the early proliferation of hyaline or cuboidal cells was followed by 1) differentiation into hair cells and supporting cells or, less likely, 2) migration of these cells completely out of the region or 3) cell death (in the absence of ongoing trauma).

Studies of the normal avian cochlea describe two distinct cell populations located immediately adjacent to the inferior-most border of the sensory epithelium (Takasaka and Smith, 1971). Cuboidal epithelial cells are non-specialized cells which rest on the basilar membrane and reach the lumenal surface of the scala media. One to two rows of cuboidal cells separate the inferior-most hair cells and supporting cells from a second cell population: the hyaline epithelial cells.

Hyaline epithelial cells line the inferior basilar membrane and extend inferiorly to the fibrocartilaginous plate. Electron microscopic studies by Takasaka and Smith (1971) have identified some unique characteristics of the hyaline cells. The hyaline cells are cuboidal in shape, rest on the basilar membrane and extend to the lumenal surface. The midportion of the hyaline cell bodies are narrowed, creating an extracellular space. The hyaline cells send processes into this space which also contains a dense network of ACh-E positive efferent nerve fibers. No synaptic terminals have been identified in this region. However, unusual swellings containing many vesicles are seen on the nerve fibers. The functional relationship between the hyaline epithelial cells and the efferent innervation of the cochlea is not known. However, these specializations of nerve fibers and the hyaline cells suggest the hyaline cells may be part of the efferent innervation of the cochlea.

In the present study, hyaline cells could not be differentiated from the nonspecialized cuboidal cells. Therefore it was unclear which was apparently responsible for the regeneration of the inferior sensory epithelium. It seems possible that the cuboidal cells were lost due to damage, along with the adjacent sensory epithelium, with the surviving hyaline cells undergoing proliferation. If this were the case, the efferent innervation of the cochlea (via communication with hyaline cells) may play an important role in regeneration. This needs to be futher evaluated with transmission electron microscopy and by the blocking or elimination of these axons.

The regeneration process was clearly different in regions of isolated superior hair cell loss. Unfortunately, this less severe pattern of hair cell loss was not observed in any of the early survival groups (< 3 days). Therefore, it was not possible to reconstruct the sequence of early events following noise damage, as we have attempted for the inferior regions. Noteworthy was the complete absence of labeling of the hyaline or cuboidal cells. Thus these cells do not appear to serve as a precursor population for regeneration in this region.

It is possible that cells situated within the nerve bundle, which are labeled as early as 6 h after noise exposure, are migrating along the nerve fibers to the sensory epithelium to serve as a stem cell population. This seems unlikely, however, since these cells do not appear adequate in number to account for the degree of regeneration observed. More likely, these are Schwann cells proliferating in response to transynaptic axonal injury.

Supporting cell loss appeared to be minimal in our cases with less severe, superior hair cell loss. Supporting cells were, however, consistently labeled beneath the regenerated hair cells (small arrow, Fig. 6a). For this reason, supporting cells appear to be the most logical precursor population for superior hair cell regeneration.

Supporting cells in the superior region of the sensory epithelium had different nuclear morphology (shape and position) than those in the inferior regions (Fig. 2a). It is entirely possible that there may be two or more supporting cell types which have yet to be structurally and functionally differentiated. This could explain why

superior supporting cells may have been hair cell precursors while inferior supporting cells apparently are not.

Thus, it appears that hyaline or cuboidal epithelial cells probably serve as a precursor population for inferior hair cell and supporting cell regeneration while supporting cells probably serve as the precursor for superior hair cell regeneration. This is analogous to the model Corwin (1981) described for the shark auditory epithelium. In that model a peripheral stem cell population (for continual production) and central supporting cells (for replacement after injury) give rise to both hair cells and supporting cells. Further work needs to be done to confirm the superior supporting cells as a precursor population in the avian cochlea.

Model for regeneration

Based on these findings we have postulated a model involving four basic phases of recovery for the inferior portion of the sensory epithelium following noise damage. These phases are described below and are summarized in Fig. 10.

Phase I: Migration The cuboidal or hyaline epithelial cells, adjacent to the inferior edge of the lost sensory epithelium, thin out and migrate superiorly to provide coverage of the basilar membrane (0–6 h after noise exposure).

Phase II: Proliferation The cuboidal or hyaline epithelial cells immediately adjacent to the surviving sensory epithelium undergo mitosis, an increase in cell number, and eventual stratification (15–24 h after noise exposure).

Phase III: Differentiation The region of proliferation undergoes further development with the production of immature, regenerated hair cells (24 h–3 days after noise exposure).

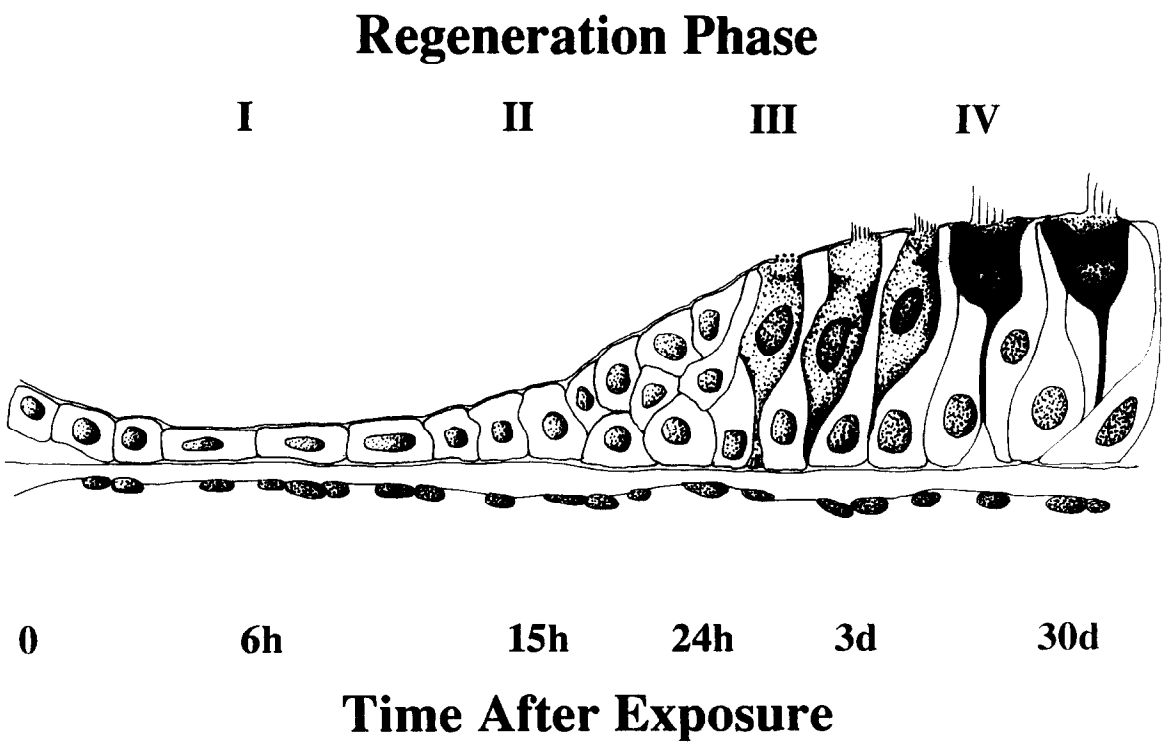

Fig. 10. Schematic representation of the proposed phases of regeneration of the inferior sensory epithelium following noise exposure. Normal (pre-exposure) hyaline and cuboidal epithelial cells are shown on the inferior basilar membrane at the left. The superior normal sensory epithelium is to the right. Time after noise exposure is on the x axis. Phase I: Migration of hyaline of cuboidal cells to cover basilar membrane exposed by lost hair cells and supporting cells. Phase II: Proliferation of hyaline or cuboidal cells to increase cell number. Phase III: Differentiation of proliferating cells into hair cells and supporting cells. Phase IV: Maturation of the regenerated sensory epithelium. (Schematic format after Corwin, 1981; Fig. 12).

Phase IV: Maturation The regenerating sensory epithelium matures, resulting in hair cells and supporting cells characteristic of adult animals (3–30 days after noise exposure). During this period the destroyed portion of tectorial membrane is reconstituted as well.

Non-labeled regenerated hair cells

In the 3 day group, the labeled immature hair cells were readily identifiable on the basis of their characteristic shape and cytoplasmic appearance. A small proportion of the hair cells with this same appearance were not labeled by ^{3}H-thymidine. These non-labeled immature hair cells were found only in the region of damage and were always adjacent to labeled immature hair cells. Thus, these cells were undoubtedly regenerated hair cells which were not labeled because: 1) mitosis occurred prior to the initiation of labeling; 2) they were produced by direct differentiation without mitosis; or 3) not enough ^{3}H-thymidine was incorporated into the nuclear DNA to produce labeling.

In the earlier survival groups, labeling was initiated 6 h prior to the termination of noise exposure. No labeling of cuboidal or hyaline epithelial cells was seen until 15 h survival and labeling of sensory epithelial cells was seen only after 24 h. Thus it seems unlikely these non-labeled regenerated hair cells are the result of early mitosis. Since most regenerated hair cells are labeled, direct differentiation seems to be unlikely as well but its occurence on a small scale cannot be ruled out. Most probably these cells reflect relative troughs in circulating ^{3}H-thymidine resulting from our intermittent injection protocol.

Non-sensory cell proliferation

Four non-sensory cells types were labeled in both control and experimental cochleae. These were red blood cells, capillary endothelial cells, tympanic border cells, and an occasional tegmentum vasculosum cell. Labeling frequency increased with survival time (and therefore increasing labeling period) up to 3 days. Labeling of these cell types was also present but infrequent in the 30 day group.

This temporal pattern of labeling is easily explained for red blood cells, which are known to have a high basal turnover rate. The maximum labeling is seen in the group with the longest labeling period immediately before sacrifice (3 day survival). In the 30 day survival group there is a 27 day period where no labeling is occuring. During this time labeled red blood cells are cleared from the blood stream and replaced by new, non-labeled cells. The result is fewer labeled red blood cells at 30 days survival compared to 3 days survival. The same temporal labeling pattern was observed for capillary endothelial cells, tympanic border cells, and tegmentum vasculosum epithelial cells. This suggests these cell types have a relatively high basal turnover rate as well. It seems more likely however that the labeling in these cell types is diluted amongst cells by continued proliferation rather than the total clearance of labeled cells over time (as with red blood cells).

Tympanic border cells had a higher frequency of labeling in the region of damage than non-damaged regions of the same cochleae or control cochleae. This suggests a transient increase in the rate of tympanic border cell proliferation in response to local stimulation or injury. Alternatively, this increase in the damaged region could be due to a transient decrease in degradation rate.

Future studies

Although these findings suggest that the cuboidal or hyaline cells are one precursor population for hair cell regeneration, the evidence is largely indirect and circumstantial. This unfortunately is the major limitation of current cell labeling techniques and attempts to identify better cellular markers are underway. The precursor cell type for isolated superior hair cell loss remains uncertain. Supporting cells in this region seem likely but early labeling of the supporting cells prior to hair cells has not been conclusively documented. Further studies using a single pulse of ^{3}H-thymidine may be useful to clarify this issue. In addition, the specific cellular triggers responsible for the proliferation of precursor cells following injury need to be elucidated. This may be best achieved with cell culture technology and could have significant clinical implications.

The functional status of the regenerated hair cells following noise trauma has yet to be conclusively determined. McFadden et al. (1988) pro-

vided evidence for recovery of threshold shifts following noise exposure in chicks which may be partially due to hair cell regeneration. More recently, Tucci and Rubel (1989) have shown that regenerated hair cells following aminoglycoside ototoxicity are functional. In addition, evidence for innervation is currently being studied using transmission electron microscopy to identify synapses on regenerated cells. These studies suggest that following trauma, the avian cochlea is capable of regenerating hair cells which are innervated and functional (Rubel et al., 1989).

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