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Diffusible factors regulate hair cell regeneration in the avian inner ear

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ABSTRACT Damage to the avian inner ear results in up-regulation of mitotic activity resulting in regeneration of hair cells. The objective of this investigation was to determine whether the damaged inner ear epithelium releases a soluble mitogen that is responsible for the up-regulation of proliferation. The sensory epithelium from normal and drug-damaged avian inner ears was cultured alone or in the presence of other cultures. As previously shown in vivo and in vitro, damaged organs displayed increased supporting cell proliferation compared with undamaged organs, leading to eventual morphologic and functional recovery. When damaged organs were cocultured with an undamaged organ, proliferation was increased in the undamaged tissue. When undamaged organs were cultured together, proliferation was decreased. These results indicate that a soluble factor released from the damaged inner ear epithelium stimulates proliferation and suggest the release of a factor from normal tissue that suppresses mitotic activity. Thus, reparative hair cell regeneration in the inner ear appears to be regulated by a balance between proliferative and antiproliferative paracrine factors.

Over the past 6 yr it has become evident that hair cells in the mature avian inner ear can regenerate after the sensory epithelium is damaged by loud noise or ototoxic drugs (1–5). This regenerative capacity leads to nearly normal numbers of hair cells, and the regenerated hair cells assume phenotypes characteristic of their normal counterparts (6–15). Furthermore, the regenerated hair cells become appropriately innervated and appear to restore hearing and balance function to the organism (16–20). Recent reports suggest that mature mammals also have limited capacity for hair cell regeneration in at least the vestibular epithelium (21, 22). Indirect evidence indicates that the supporting cells within the inner ear sensory epithelium (5, 23, 24), and possibly a nonsensory cell type adjacent to the auditory receptor epithelium (25), are potential hair cell precursors.

A major question that must now be addressed to achieve therapeutic value for these findings is the identity of cellular and molecular signals that regulate precursor cell proliferation and hair cell differentiation in the regenerating inner ear. Two possible mechanisms responsible for initiating new hair cell production have been proposed. (i) The mechanical loss of a hair cell may uncouple the adhesive interaction of membrane-spanning glycoproteins that link hair cells with their supporting cell neighbors (26, 27). This disruption of a putative inhibitory heterophilic coupling could provide a signal directly to neighboring cells to re-enter the cell cycle and proliferate to provide a new hair cell replacement. The regenerative signal would persist until the new hair cell was differentiated enough to express the membrane-bound ligand necessary to reestablish the heterophilic coupling.

(ii) The second hypothesis involves a paracrine mechanism, whereby damaged hair cells, scavenging phagocytic cells, and/or loss of receptor neural activity causes the release of a mitogenic substance (26). This mitogen could then diffuse from the damaged region to stimulate progenitor cells. Similarly, sensory epithelial damage could decrease production of an antiapoptotic factor. In support of the diffusible factor hypothesis, several growth factors have been shown to influence proliferation in the developing inner ear (28–31).

Historically, the first step toward identification of mitogenic and differentiation factors has come from studies involving cocultures of stimulating and target tissues (32–35) and/or studies using conditioned medium (36–39). In the present report we have adopted a similar strategy by examining the ability of regenerating inner ear epithelium to stimulate proliferation in tissue from control animals. Using a culture system known to maintain the hair cell regeneration process in vitro (40), we show that cellular proliferation in the avian vestibular sensory epithelium from normal control animals is up-regulated when this tissue is grown in vitro with regenerating vestibular end-organs that have been previously damaged in vivo by aminoglycoside (streptomycin) treatment. This finding indicates that a soluble factor, released from regenerating vestibular organs, can trigger increased proliferation in the normal vestibular sensory epithelium. In addition, we show that cellular proliferation in tissue from control organs is inhibited in cocultures with other control organs, suggesting the existence of a soluble mitotic inhibitor.

MATERIALS AND METHODS

White Leghorn chickens were hatched and grown for 7–15 days before experimental use. The birds were divided into two groups: an experimental group that was treated with the ototoxic aminoglycoside antibiotic streptomycin and an age-matched control group that received no injections. Aminoglycoside-treated birds were given daily injections of streptomycin i.m. (1200 mg/kg; Sigma) for five consecutive days, which is known to cause loss of both type I and type II vestibular hair cells (5). One day after the last injection, both damaged and control birds were sacrificed by decapitation. The utricles were dissected out and placed into chilled Basal Medium Eagle containing Earle’s balanced salt solution and 0.5% D-glucose (GIBCO).

Utricles from control and aminoglycoside-treated postnatal chickens were cultured individually or in groups. The utricles were placed into specifically arranged 50-μl drops of chicken plasma (Cocalico Biologicals, Reamstown, PA) on...

Abbreviations: BL, basal layer; LL, luminal layer; SC, single control utricle cultured alone; SD, single streptomycin-damaged utricle cultured alone; GC, group (seven) control utricles cultured together; GD-C, single control utricle cocultured with six streptomycin-damaged utricles; GD-F, six streptomycin-damaged utricles cocultured with one single control utricle.

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round glass coverslips as shown in Fig. 1. Organs were oriented such that the sensory epithelium faced away from the coverslip. The excess chicken plasma was aspirated and replaced with 25 μl of sterile thrombin at 50 units/ml (Sigma). After 3–5 min, a clot was formed, thus attaching the utricular explants to the glass. The coverslip was placed into an individual well of a 24-well tissue culture plate that was filled with 0.75 ml of the above culture medium supplemented with 1% fetal bovine serum (GIBCO). In cocultures, all utricles were separated by at least 1 mm. At the start of the culturing period, 1 μCi of sterile tritiated [3H]methylthymidine (1 mCi/ml; 6.7 Ci/mmol; 1 Ci = 37 GBq; NEN) was added to the culture medium in each well to label cells that passed through S phase of the cell cycle during the experiment. These culture conditions are known to maintain good sensory epithelial morphology and to maintain the hair cell regeneration process in vitro (40). The culture well plates were placed on a slow-moving nutator (Clay Adams) and incubated at 37°C in a 5% CO2/95% air environment. Inspection of the cultures at the end of the culture period revealed that the organs remained attached to the coverslips within the fibrin clots, and no cellular “bridges” were formed between the individual cocultured organs.

Four utricular explant paradigms were compared (see Fig. 1): SC, SD, GC, and GD-C cocultured with GD-P. Neither the culture medium nor [3H]thymidine was replenished during the 2 days of in vitro growth. After 48 hr in vitro, all cultures were immersion-fixed with 3.5% glutaraldehyde/phosphate-buffered saline, pH 7.4, overnight and postfixed in 1% OsO4 for 1.5 hr. The cultures were then dehydrated in graded ethanol and propylene oxide. Before final embedding in Spurr’s resin (Polysciences), individual utricular explants were separated from the attached glass coverslips with a thin razor blade. Semithin serial sections (3 μm) were cut through half of each utricular explant; these sections were mounted onto acid-washed chrom/alum-coated slides and dipped in a 50% aqueous solution of Kodak NTB-2 nuclear track emulsion. Emulsion-coated slides were stored in the dark at 4°C for 2–5 days before development in Kodak D-19 developer and fixer. The sections were lightly counterstained with toluidine blue before placing under coverslips.

Tissues were analyzed to determine the mean linear density of [3H]thymidine-labeled cells and unlabeled cells in the sensory epithelium. In each utricular explant, two autoradiographic sections were randomly chosen from every 12 serial sections (36-μm intervals) through half the organ. This procedure resulted in 16–24 sections analyzed per organ. In each section, two randomly chosen 100-μm-wide strips of sensory epithelium were analyzed by using phase-contrast microscopy. [3H]Thymidine-labeled cells were identified by the presence of five or more silver grains overlaying the cell nucleus. As shown in Fig. 3B, the sensory epithelium was artificially bisected into basal (BL) and luminal (LL) layers. The BL consisted of all cell nuclei within two nuclear diameters (=13 μm) of the basement membrane. The LL was composed of the remaining luminal one-half to two-thirds of the epithelial thickness. In the vestibular sensory epithelia, the BL contains exclusively supporting cell nuclei, whereas hair cell nuclei make up 93% and 94% of the nuclei found in the LL from control and aminoglycoside-damaged explants, respectively (23). Thus, counts of nuclei in the LL allow us to estimate the number of hair cells present in control and damaged tissue. The layer location of each labeled and unlabeled nucleus in the analyzed epithelium was recorded. The mean linear density of labeled (L) and unlabeled (UL) cells in the LL and BL could then be determined for each utricle. From these values, the LL cell linear density (LL + UL), the BL cell linear density (LB + UL), the total labeled-cell linear density (LL + LB), the proliferation index (LL + LB)/(LL + UL + LL + LB), and the total cell linear density (LL + UL + LB) were calculated for each explant. Mean values of the above variables were determined for each coculturing paradigm. Altogether, 11–30 organs from each utricular coculturing paradigm (SC, SD, GC, GD-C, and GD-P) were analyzed in five separate replications of the experiment.

For statistical analysis, the mean LL cell linear density, BL cell linear density, total labeled cell linear density, total cell linear density, and proliferation index for each explant served as an individual observation. These values were subjected to an ANOVA using SuperAnova (Abacus Concepts, Berkeley, CA). Prior to doing ANOVA comparisons, a logarithmic transformation of the dependent variable, the F value comparing coculturing paradigms was computed. Post hoc comparisons, where appropriate, used the Fisher’s Protected Least Significant Differences or Tukey’s Honestly Significant Differences tests.

In addition to these quantitative analyses, each organ culture was examined for overall quality. Organs were rated on a scale from 1 (poor) to 4 (excellent) according to morphologic tissue quality (number and morphology of hair cells and supporting cells, presence of inclusions, vacuoles, or extracellular spaces, and evenness and integrity of the sensory epithelium). The ratings were produced by light microscopic observations of tissue sections by an observer who was unaware of the group from which each section was taken (blind analysis). The tissue from any organs in which a rating of 1 (poor) was obtained was not quantitatively analyzed.

All experimental procedures were approved by the University of Washington Animal Care Committee.

RESULTS

After 2 days in culture, the sensory epithelium of the streptomycin-treated utricles had reliably fewer hair cells than the sensory epithelium of undamaged utricles (P < 0.001; Fig. 2A, SD or GD-P vs. SC, GC, or GD-C). For example, mean hair cell density (± standard deviation), approximated by the linear density of cells in the sensory epithelial LL, was 8.7 (± 0.96)/100 μm and 6.9 (± 0.59)/100 μm for SC and SD groups, respectively. The nonsensory, supporting cell population, located primarily along the BL, appeared unaffected by the aminoglycoside treatment, and the linear density of cells in the BL was similar across groups (Table 1). In all cultured utricles, labeled supporting cells and hair cells were evenly distributed throughout the sensory epithelium, mostly in singles or pairs, but occasionally in small clusters (Fig. 3). As seen in Fig. 2B, the mean proliferation index was three times greater in the explanted drug-damaged utricles (SD) compared with the cultured control utricles (SC) (P < 0.001, SD vs. SC). A similar labeling index was observed in the mean labeled-cell linear density (Table 1; P < 0.001, SD vs. SC). This in vitro up-regulation of reparative proliferation after in vivo aminoglycoside-induced sensory epithelial damage is quantitatively consistent with that observed previously in

![Fig. 1. Coculturing arrangements for utricular explants obtained from control (○) and streptomycin-treated (●) neonatal chickens. Four coculturing paradigms were used that yielded five groups of explants for quantitative analysis: SC, single control utricle; SD, single streptomycin-damaged utricle; GC, seven control utricles cocultured together; and GD-C, single control utricle cocultured with six streptomycin-damaged utricles (GD-P).](image-url)
vivo and in vitro (23, 40) and is known to eventually lead to recovery of the vestibular sensory hair cell population (5). For example, Weisleder and Rubel (5) found a 3.8-fold increase in mitotic activity in ampullae from streptomycin-treated animals compared with controls.

The hypothesis that there is a diffusible factor released from damaged organs that stimulates proliferation of supporting cells is best evaluated by comparing proliferation in undamaged epithelia cocultured with drug-damaged tissue (GD-C) and cocultured control utricles (GC). This comparison reveals a 320% increase in the proliferation index (P < 0.001; Fig. 2B, GD-C vs. GC) and a 280% increase in the linear density of [H]thymidine-labeled cells (P < 0.001; Table 1, GD-C vs. GC). In addition, the control utricles cocultured with drug-damaged tissue (GD-C) showed an 80% increase in the proliferation index above that observed with control utricles cultured alone (SC) (P < 0.01; Fig. 2B, GD-C vs. GC) (Fig. 2C). Finally, neither the proliferation index nor the linear density of proliferating cells are reliably different when control organs and damaged organs cultured together are compared (GD-C vs. GD-P).

An alternative explanation of these results is that when control organs are cocultured with damaged organs, hair cells or support cells in the epithelium from control animals are damaged, and this leads to up-regulation of proliferation. Three additional analyses argue against this interpretation. (i) The total cell density in utricles from control animals (GD-C) cocultured with damaged tissue was virtually identical to that in utricles from control animals cultured alone (SC) or cocultured (GC) (Table 1). (ii) As seen in Fig. 2A, hair cell density was similar in utricles from control animals in these three groups (SC, GC, and GD-C). Finally, the blind ratings of the histological quality of the cultures yielded similar scores for SC, GC, and GD-C groups. The mean tissue quality grading (standard deviation) was 3.3 (0.5), 3.3 (0.4), and 3.3 (0.6) for groups SC, GC, and GD-C, respectively. It should also be noted that some damage to organs may have occurred while they were removed from the animal and placed in vitro. Because this procedure was similar for all groups, it cannot account for the group differences we report.

An unexpected result of this study is indirect evidence for an inhibitory factor released from undamaged (control) epithelium, which can prevent or suppress proliferation. When utricles from control animals were cocultured, there was a 57% reduction in proliferative activity compared with control utricles cultured alone (P < 0.05; Fig. 2B, GC vs. SC). It is unlikely that nutrient depletion was responsible for this decrease in proliferation because the coculture conditions can support 5.6 times more proliferative activity in the cocultured aminoglycoside-damaged utricles (group GD-P) than observed in the cocultured control utricles (group GC). Additional, although weaker, evidence for a mitotic inhibitor is provided by the comparison of groups SD and GD-P, where inclusion of tissue from a control animal resulted in a 22% reduction in proliferative activity (P < 0.01; Fig. 2B).

**DISCUSSION**

This study supports the hypothesis that one or more soluble factors are released by regenerating avian vestibular tissue that can act as paracrine triggers to up-regulate ongoing supporting cell proliferative activity in the normal avian vestibular system and thereby provide the source for new vestibular hair cells. The results also suggest the existence of a second factor that suppresses proliferation in normal tissue. The balance between these opposing factors appears to regulate the extent of epithelial proliferation, a rate-limiting step in the production of new hair cells. Neither the source nor the identity of either factor is currently known, but phagocytic cells or damaged/dying hair cells are likely potential sources of a mitogen because their activity or presence is most likely proportional to the extent of epithelial damage. Presumably, either hair cells themselves or supporting cells could be sources of the proposed antimitotic agent. At this time, we have no information on the events that regulate the final phenotype of newly produced cells. Cell–cell contacts and/or circulating growth factors may regulate differentiation of hair cell versus supporting cell phenotype.

The fact that vestibular structures are highly conserved across virtually all classes of vertebrates, including mammals

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**Table 1. Analysis of total cell, supporting cell, and thymidine-labeled cell linear densities in the utricular cultures**

<table>
<thead>
<tr>
<th></th>
<th>SC (n = 11)</th>
<th>SD (n = 12)</th>
<th>GC (n = 24)</th>
<th>GD-C (n = 12)</th>
<th>GD-P (n = 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell density</td>
<td>30 (0.74)</td>
<td>28 (0.36)</td>
<td>31 (0.49)</td>
<td>30 (0.74)</td>
<td>28 (0.53)</td>
</tr>
<tr>
<td>Supporting cell density</td>
<td>21 (0.52)</td>
<td>21 (0.29)</td>
<td>22 (0.36)</td>
<td>20 (0.51)</td>
<td>21 (0.45)</td>
</tr>
<tr>
<td>Labeled-cell density</td>
<td>0.26 (0.04)</td>
<td>0.73 (0.09)</td>
<td>0.12 (0.02)</td>
<td>0.46 (0.04)</td>
<td>0.57 (0.03)</td>
</tr>
</tbody>
</table>

The mean density values for each coculture paradigm are given as cells per 100 μm (SEM).
Fig. 3. Autoradiographs demonstrating new sensory epithelial cell production in cocultures of chick utricular explants grown in vitro for 2 days in the presence of the proliferation marker, tritiated thymidine. Labeled cells have white-colored silver grains overlying their nuclei (Nomarski differential-interference contrast optics). (A) Sensory epithelium from a control utricle cocultured with six other control utricles (group GC). Note the normal numbers of hair cell nuclei in the sensory epithelium. A single supporting cell nucleus (arrow) is heavily labeled with silver grains, indicating that DNA replication occurred in this control utricular culture. (B) Sensory epithelium from a control utricle (GD-C) cocultured with GD-P-regenerating utricles. The sensory epithelium has normal morphology and contains the normal complement of hair cells in the LL and supporting cells in the BL. Three labeled cells (arrows) indicate the up-regulated cellular proliferation in the sensory epithelium compared with GC utricles. (C) Sensory epithelium from a streptomycin-treated utricular explant (GD-P) grown with five other treated utricles and one control utricle. Note the paucity of hair cells and thinning of the sensory epithelium secondary to the in vivo aminoglycoside treatment. Up-regulation of sensory epithelial proliferation is due to streptomycin-induced damage and is demonstrated by the three labeled epithelial cells (arrows). (Scale bar applies to A–C.)
(41, 42), suggests that similar mechanisms regulating hair cell production may exist in the mammalian inner ear. Additional investigations should fully isolate and characterize these soluble factors, and determine their influence in the chicken cochlea and the inner ear of other species. Identification and careful control of their concentration in the human inner ear could potentially yield the potential to restore sensory elements after hair cell loss due to injury or disease.

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