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Hair Cell Death in a Hearing-Deficient Canary

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ABSTRACT

Cell death has been documented in bird auditory inner ear epithelia after induced damage. This cell death is quickly followed by an increase in supporting cell division and regeneration of the epithelium, thereby suggesting a possible relationship between these two processes. However, aspects of this relationship still need to be better understood. The Belgian Waterslager (BWS) canary is an ideal system in which to study cell death and subsequent cell division. In contrast to mixed breed (MB) canaries, cell division normally occurs in the auditory end organ of the BWS without any external manipulation. In addition, some of the cells in the auditory epithelium may be dying through an apoptotic-like process. In the present study two methods were used to quantify dying cells in the BWS and MB canary auditory epithelia: morphological criteria and TUNEL. Results confirm that some of the abnormal hair cells in the BWS auditory epithelium are apoptotic-like. The presence of both cell death and cell division indicates that these processes act concurrently in the adult end organ. Future studies are needed to determine if cell death is a stimulus for the observed cell division.

Keywords: hair cell, cell death, TUNEL, Waterslager

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INTRODUCTION

Death of sensory hair cells in the bird inner ear can be caused by ototoxic drugs or intense sound (Cotanche 1987; Cruz et al. 1987; Corwin and Cotanche 1988; Ryals and Rubel 1988; Hashino et al. 1992; Marean et al. 1993; also reviewed in Stone et al. 1998). After damage, the surviving supporting cells divide to produce new hair cells and the epithelium is regenerated (Girod et al. 1989; Raphael 1992; Hashino and Salvi 1993; Stone and Cotanche 1994; Tsue et al. 1994; Warchol and Corwin 1996). The close temporal association between hair cell death and subsequent cell division suggests that hair cell death may be an integral part of the signaling pathway that stimulates supporting cell division. An association between hair cell death and supporting cell proliferation in an auditory end organ without the use of an exogenous stimulus might provide further invaluable information about the production of new auditory hair cells.

The Belgian Waterslager (BWS), a strain of the common canary (*Serinus canarius*), provides an excellent system in which to study the role of cell damage in hair cell regeneration. This strain is bred for its loud low-frequency song (Gleich et al. 1994a) and has acquired a high-frequency hearing deficit as great as 20–40 dB (Okanoya and Dooling 1985, 1987; Okanoya et al. 1990). Previous studies have shown that this deficit is associated with an average 30% reduction in hair cell number in the basilar papilla (BP), the hearing end organ (Gleich et al. 1994a), compared with mixed breed (MB) canaries. Hair cells within the BWS canary BP also have a greater surface area and abnormal, irregular, or fragmented stereovillar bundles (Gleich et al. 1994a, b; Weisleder and Park 1994). Waterslagers

are also unique among birds because there is evidence of supporting cell division and new hair cell production in the adult BP (Gleich et al. 1997) in the absence of any exogenous stimulus. Simply put, the BWS canary papilla is an auditory end organ in which there are abnormal hair cells and ongoing proliferation and regeneration. Therefore, this system affords a unique opportunity to test hypotheses about the stimulus for such continued hair cell production.

The objectives of the current study were to determine if cell death occurs in the BWS canary papilla. While the 30% hair cell loss and strange morphological characteristics imply that cell death is occurring, this has not yet been directly investigated. The presence of cells undergoing apoptosis was hypothesized since a relationship between this type of cell death and division has been observed in other systems and in the bird inner ear during regeneration (Kerr et al. 1972). To test this hypothesis, hair cells that had morphological characteristics of cell death were quantified. In addition, the TUNEL assay was used to label cells with cleaved DNA typical of dying cells.

METHODS

Subjects

Mature MB canaries were purchased from a local supplier, and BWS canaries were obtained from a private breeder. All subjects were housed in a colony with continuous access to food and water prior to sacrifice. All procedures were approved by the University of Maryland Institutional Animal Care and Use Committee.

Light microscopic analysis of hair cell damage

Abnormal hair cells, including those with characteristics of cell death, were quantified in four BWS and two MB canary papillae. Birds were decapitated after being deeply anesthetized with a combination of ketamine (60 mg/kg, IM) and xylazine (30 mg/kg, IM) to a level that maintained slow shallow respiration. The ears were perfused with fixative (2% paraformaldehyde/2% glutaraldehyde in 0.1M phosphate buffer) before being removed and postfixed by immersion in 1% osmium tetroxide. The tegmentum vasculosum and tectorial membrane were left *in situ*. Following postfixation, the basilar papillae were embedded in Polybed 812 and sectioned at 2.0 μm. For two BWS and one MB canary, every third section was saved and examined. For the other two BWS and one MB canary, three sections were saved and examined every 100 μm of tissue. Sections were counterstained with toluidine blue, coverslipped, and viewed with a 100× oil immersion objective. Every hair cell in every available section was examined and compared in the BWS and the MB canary. All hair cells with characteristics of cell death were quantified. Dying hair cells were defined as containing condensed chromatin, darkened cytoplasm, and sometimes an unusual number of vacuoles. Abnormal hair cells were also quantified. These hair cells were defined by an unusual overall shape and exhibited characteristics outside the normal variation observed in MB canary hair cells. Adjacent sections were examined if the complete morphology of a hair cell could not be determined from one section alone.

TUNEL labeling of dying cells

The TUNEL assay was used to label apoptotic cells with internucleosomal cleavage of DNA. Six BWS and four MB canary papillae were isolated from a second group of transcardially perfused subjects, fixed for 10 minutes with 2% paraformaldehyde in 0.1M phosphate buffer, and placed in 0.01% collagenase in 0.1M phosphate buffer containing 1.5% Triton X-100 (PB) for 45 minutes.

Apoptotic cells were detected in whole mount end organs using the TUNEL assay as described in the Apoptag manual (Intergen Company, Purchase, NY). After 5 minutes in 33% glacial acetic acid in 100% ethanol, endogenous peroxidases were blocked by 0.09% H_2O_2 in PB for 5 minutes. This was followed by labeling of cleaved DNA with digoxigenin-conjugated nucleotides using terminal deoxynucleotidyl transferase (TdT). The tissue was further treated with a peroxidase-conjugated digoxigenin antibody, and labeled cells were stained dark brown after application of the substrate, diaminobenzidine (DAB; Sigma 4293, Sigma, St. Louis, MO). Positive controls were obtained using DNase I to cleave DNA in all cells prior to the TdT reaction. Tissue from four BWS canaries and four MB canaries was dehydrated and then embedded in plastic (Immunobed, Polysciences, Inc., Warrington, PA) and serially sectioned at 5 μm. All sections were examined using differential interference microscopy and an 100× oil immersion lens. Cells labeled by the TUNEL assay were quantified within the sensory epithelium. Cells that were labeled in two adjacent sections were considered the same labeled cell. The distance from the neural side of the sensory epithelium of every TUNEL-labeled hair cell was measured using a reticule and a 40× lens. Similarly, the complete width of each sensory epithelium was measured in every fourth section and the mean values were used to create Figure 4.

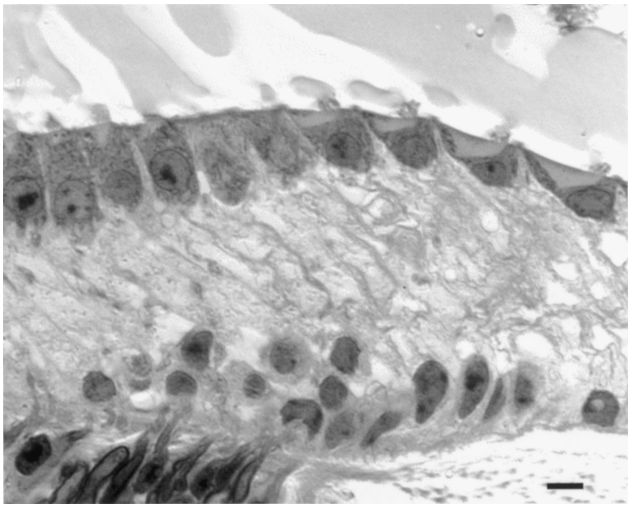

FIG. 1. A cross section through the mixed breed canary basilar papilla. None of abnormal cells observed in the Belgian Waterslager were observed in the mixed breed canary tissue. Scale bar = 5 μm.

DAPI labeling

In order to view dying cells within the whole-mount BWS papillar sensory epithelium, DAPI was used to label nuclei in papillae from two BWS canaries that were processed for TUNEL. Tissue was placed in 1% Triton X-100 PB for 20 minutes and then nuclei were stained with DAPI (1:1000 of a 1 mg/ml solution; Sigma) for 20 minutes. Papillae were then mounted in Antifade (Molecular Probes, Eugene, OR) and viewed using fluorescent microscopy.

RESULTS

Morphological evidence of cell death

Some hair cells in the BWS canary papilla showed characteristics that were distinctly different from hair cells in the MB canary papillae (Fig. 1). These hair cells showed evidence of cell death including very dark staining nuclei and cytoplasm (Fig. 2) which were never seen in the MB canary sections examined. Of the 1,527 hair cells sampled in the BWS tissue, 8.9% had this dark appearance (Table 1). Detail of the intracellular structure of these cells was sometimes difficult to see through dark staining. It was not possible to ascertain if the nucleus was fragmented. Such cells did exhibit circular areas that were not stained resembling vacuoles. In many cases these cells had an overall shape that was similar to the shape of normal hair cells in the same region, and ciliary bundles could often be distinguished (Fig. 2A). Some of these dark cells, however, were small and thin, and contained a small amount of cytoplasm that sometimes consisted of only a thin line near the apical surface (Fig. 2B). This thin appearance was not likely to be due to artifact since adjacent sections did not show evidence of the remainder of a normally sized cell. Most of these dark cells were in a traditional hair cell position, but a few were partly or completely outside the epithelium, as if in the process of extrusion (Fig. 2C, D). Similar to their *in situ* counterparts, many of these extruded cells possessed a normal ciliary bundle. Despite the relatively large number of dark cells *in situ*, only about 1% of all hair cells from BWS tissue were extruded (Table 1). All of these extruded cells had this dark appearance.

In addition to these clearly degenerating cells, there were also many hair cells in the BWS papillae with abnormal shapes but with no grossly discernable cytoplasmic or nuclear evidence of death (e.g., Fig. 2E). Many of these abnormal hair cells were very thin and in some cases a nucleus could not be discerned (Fig. 2F). These thin cells were usually seen in groups located in the short-hair-cell region of the papilla. Other examples of oddly shaped cells included hair cells that seemed excessively large or unusually circular, but these were rare and found in isolated cases. Taken together, abnormal hair cell profiles constituted 23.2% of all hair cells counted in the BWS papillae (Table 1). The remaining 68.2% of hair cells were categorized as normal. Abnormally shaped hair cells were also observed in the MB canary papillae but these made up less than 1% of the cells. These percentages do not consider more subtle abnormalities that could not be detected using light microscopic analysis.

TUNEL labeling in the basilar papilla

The results from the TUNEL labeling paralleled the morphological data. Dying cells were labeled in the BWS canary BP while there was no labeling in the MB canary papillae. There was an average of six TUNEL-labeled cells in each BWS papilla (Table 2). All labeled cells were identified as hair cells; their nuclei were located in the hair cell stratum of the epithelium and many possessed a clearly identifiable hair cell bundle (Fig. 3). Some of these dying cells were unusually small, contained very little cytoplasm, and were similar to the thin cells observed in the morphological analysis. Although we did see some cells extruded into the overlying lumen, none of these cells were labeled by the TUNEL assay. Positive controls treated with DNase I prior to processing resulted in all nuclei being labeled (data not shown). In addition, labeled cells were observed in the underlying connective tissue in MB control tissue.

Although a formal reconstruction of the BWS sensory epithelium was not performed, hair cells labeled by TUNEL did not appear to be concentrated in either the basal or the apical end of the epithelium. Figure

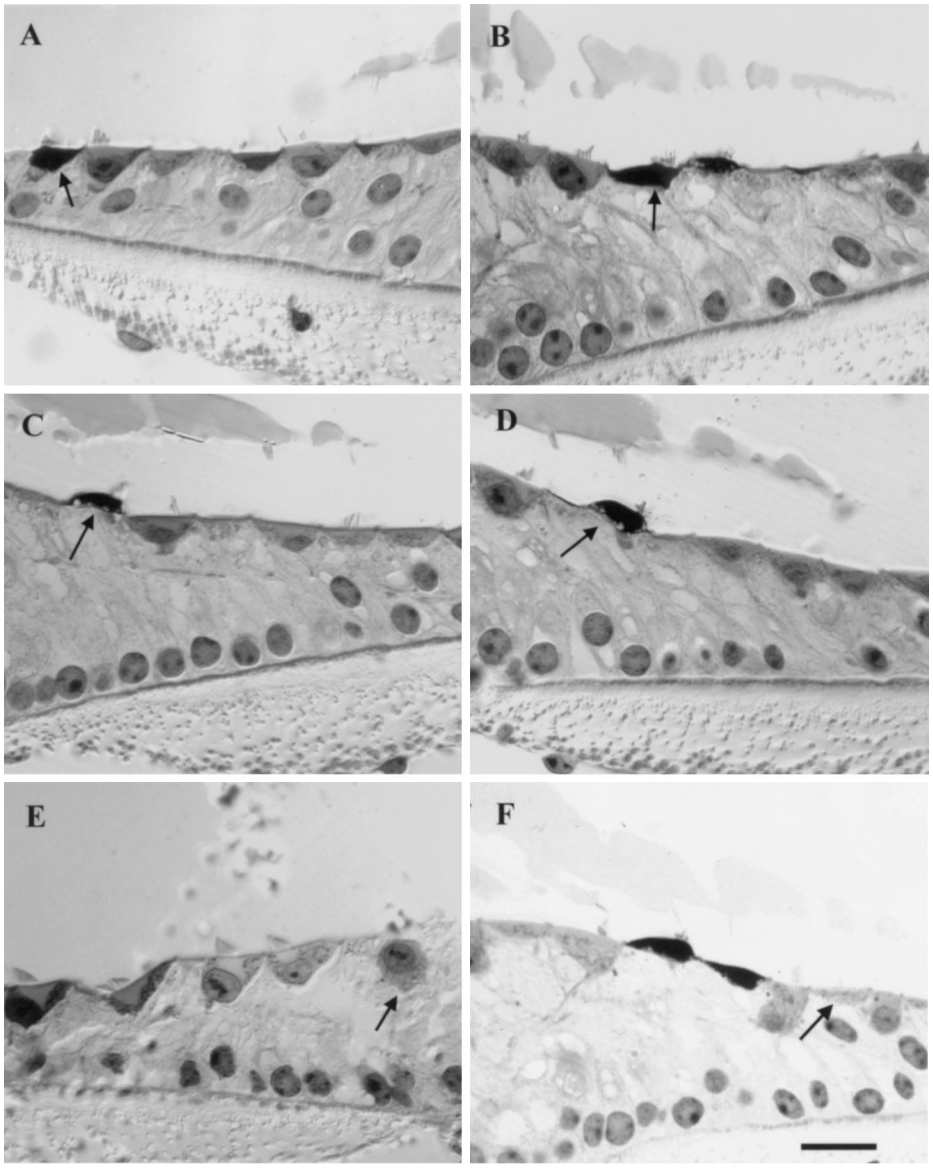

FIG. 2. Abnormal hair cells observed in cross section of the Belgian Wateslager basilar papilla. **A.** Some dark cells had an overall shape similar to normal hair cells in the same region while others (**B**) were thin and contained a small amount of cytoplasm. **C, D.** Some dark cells appeared to be in the process of extrusion. **E, F.** Other abnormal hair cells were also observed. Scale bar = 5 μm.

4 illustrates the distribution of hair cells labeled by the TUNEL assay.

Results from TUNEL and DAPI-stained whole-mount tissue also indicated that dying cells within the BWS papilla were located in the hair cell layer (Fig. 5). In addition, dying hair cells were pyknotic; their nuclei were fragmented and they contained condensed chromatin, which is a feature of apoptosis, a physiologically induced form of cell death.

DISCUSSION

Cell death in the BWS canary papilla

These results are the first to demonstrate cell death in an auditory end organ from a bird that has not been subject to external damage and confirm similar preliminary results from our lab (Presson et al. 1996; Wilkins et al. 1998). The only other examples of cell death in an auditory end organ have been as a consequence of aminoglycoside treatment or sound damage (e.g., Nakagawa et al. 1997). Cell death has also been reported by this lab and others in the normal chicken utricle, a vestibular end organ (Jørgensen and Mathiesen 1988; Roberson et al. 1992; Kil et al. 1997; Wilkins et al. 1999).

Strict classification of cell death types is becoming less clear. Some traditional characteristics used to identify dying cells as apoptotic have also been discovered to be present in other types of cell death such as necrosis. For example, in some rare cases, cleavage of DNA has been documented and detected by the TUNEL assay in necrotic cells (Nishizaki et al. 1999; Gold et

TABLE 1

Hair cells with abnormal and/or apoptotic morphology

Cell type	*Mean No. in MB papilla [n = 2 (%)]*	*Mean No. in BWS papilla [n = 4 (%)]*
Normal appearance	4595.5 (99.7%)	260.3 (68.2%)
Apoptotic[a] *in situ*	0 (0%)	30.3 (7.9%)
Apoptotic extruded[a]	0 (0%)	2.8 (0.7%)
Abnormal[b] *in situ*	1 (<%)	88.5 (23.2%)
Mean No. hair cells sampled in each epithelium	460.5	381.9

[a]Apoptotic cells contained condensed chromatic, darkened cytoplasm, and sometimes contained an unusual number of vacuoles.
[b]Abnormal cells had an unusual shape or characteristics atypical for hair cells.

TABLE 2

Apoptotic and S-phase cells observed in each BP from the MB and BWS

	Mean No. of apoptotic hair cells ± S.D.[a]	*Range of apoptotic hair cells[a]*	*Mean No. of S-phase cells (± S.D.)[b]*	*Range of S-phase cells[b]*
MB	0 ± 0	0	0.9 ± 1.1	0–3
BWS	6.0 ± 5.1	1–13	6.2 ± 5.6	0–14

[a]Apoptosis was basilar papilla.
[b]The same TUNEL-labeled hair cell is basilar papilla.

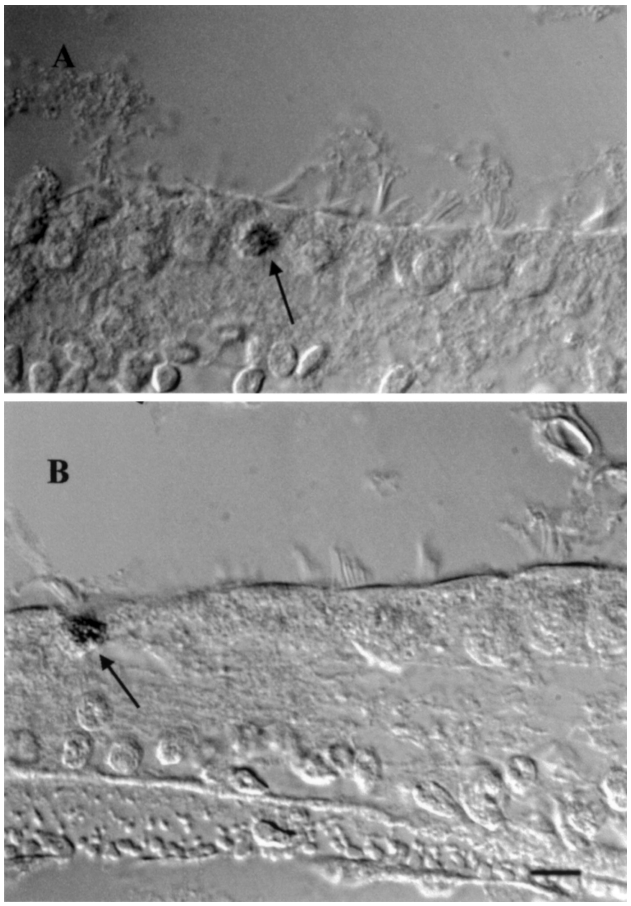

FIG. 3. TUNEL-labeled hair cells in the Belgian Waterslager basilar papilla shown in cross section. Scale bar = 5 μm.

al. 1994). However, when the TUNEL assay is used in conjunction with other methods to identify cells with apoptotic characteristics, researchers can be more confident in their classification. In the present study, morphological data from light microscopic analysis of dying cells was used to confirm the presence of apoptotic-like cells detected using the TUNEL assay.

Two methods that identify apoptotic-like cells

Two methods were used to identify dying cells in this study: cellular morphology and the TUNEL assay. Both methods confirm that hair cell death occurs in the BWS canary BP but not in the MB canary papilla. However, there were some disparities between the results obtained with the two methods. First, a greater number of cells with apoptotic characteristics were detected morphologically (mean = 33) than with the TUNEL assay (mean = 6) (Tables 1 and 2). This difference is most likely underestimated because every tissue section was not analyzed for the morphological portion of this study while the TUNEL tissue was analyzed in full. In addition there was a lack of TUNEL labeling in cells completely extruded into the overlying lumen of the BWS papilla despite observations of labeled thin cells in the process of extrusion.

This discrepancy could be due to the limited period of time during which the TUNEL assay labels apoptotic cells. Apoptosis can be characterized by the presence of cleaved DNA which is detected by the TUNEL assay (Gravieli et al. 1992). Apoptosis is also characterized by chromatin condensation (Arends and Wyllie 1991) which was the main morphological determinant of apoptosis used in the current study and may occur within a slightly different time window than DNA cleavage. Therefore, it is possible that the TUNEL assay labeled only a subset of apoptotic cells in the BWS papilla and the morphological data may be more inclusive.

Cell death and the hearing deficit

Although the origin of the BWS hearing deficit above about 2.0 kHz is known to be in the auditory epithelium (Gleich et al. 1995), the major factor has yet to be determined. Other studies (Gleich et al. 1994b, 1995; Weisleder et al. 1996) have hypothesized that the lack of hair cells and the abnormal hair cell bundles are most likely the source of the high-frequency

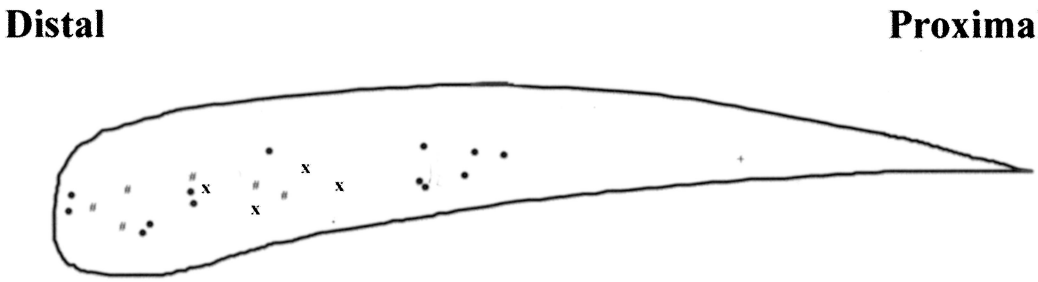

FIG. 4. Distribution of all TUNEL-labeled cells along the basilar papilla of the Belgian Waterslager canary. Data from all subjects is represented. Each dying cell is represented by a small dot.

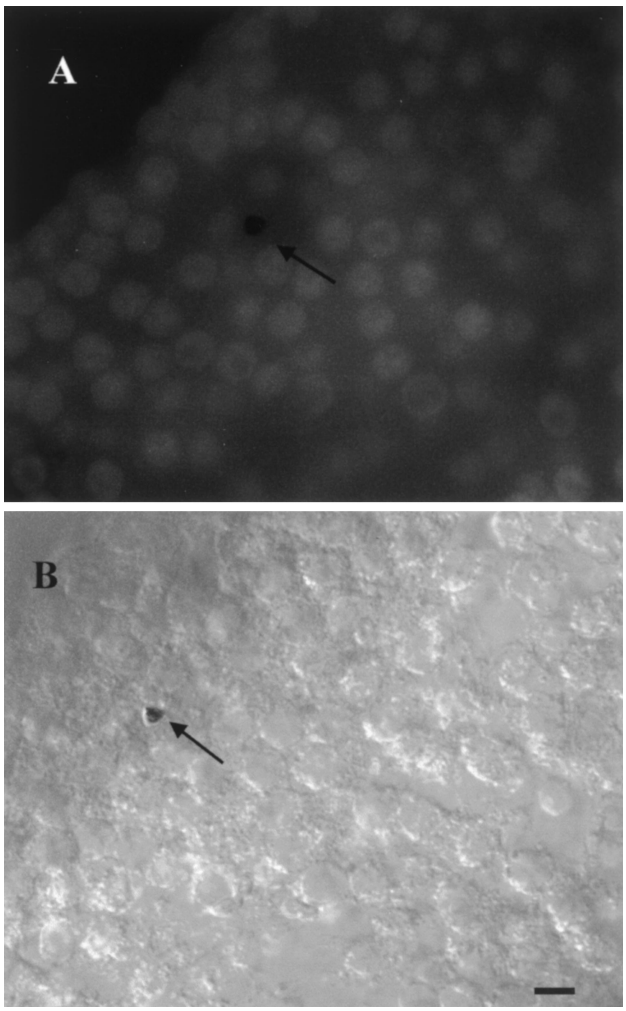

FIG. 5. A, B. Whole mount of the Belgian Waterslager basilar papilla. The same TUNEL-labeled hair cell is shown in both panels and indicated by the arrows. DAPI labels all the nuclei in **A** and shows that the TUNEL-labeled cell is within the hair cell layer. Scale bar = 5 μm.

hearing deficit. We suggest that the location of dying hair cells along the basilar papilla may also contribute to this deficiency. Dying hair cells might be concentrated in the basal or proximal end of the papilla where hair cells are stimulated by high-frequency stimuli. However, Figure 4 shows that there appears to be a smooth distribution of TUNEL-labeled cells along the length of the BWS papilla, and most dying cells are located on the abneural side of the epithelium among short hair cells. Moreover, dying hair cells were not observed more often at the basal end than at the apical end of the epithelium. This may indicate that although apoptosis is not the cause of the BWS hearing deficit, it may contribute to the effects of structural abnormalities such as the lack of hair cells and abnormal bundles.

Supporting cell division and cell death

Several studies suggest that the rate of cell death in hair cell epithelia is similar to that of supporting cell division. In the chicken (*Gallus gallus domesticus*) inner ear, the normal utricle exhibits apoptotic levels that are similar to mitotic indices (Jørgensen and Mathiesen 1988; Roberson et al. 1992; Kil et al. 1997), and a direct correlation has been established between apoptosis and supporting cell division (Wilkins et al. 1999). In addition, a similar correspondence has been seen between the degree of hair cell loss and the number of supporting cells that enter S phase and divide in the chicken BP; more severe hair cell damage induces more supporting cells to divide (reviewed in Cotanche 1997). Other studies have also indicated that cell death is temporally (Hashino and Salvi 1993; Stone and Cotanche 1994) and spatially (Warchol and Corwin 1996) related to new hair cell production in regenerative paradigms using the avian inner ear.

The results from this study also support the hypothesis that cell death and division are coupled processes in inner ear epithelia. A previous study by Gleich et al. (1997)) showed evidence of supporting cell division and continued hair cell production in the BWS papilla. The S-phase marker bromodeoxyuridine (BrdU) was incorporated into 6.2 ± 5.6 cells per epithelium (Table 2 in Gleich et al. 1997). It is interesting to note that the mean number of BrdU-labeled cells (6.2) found by Gleich et al. (1997) is very similar to the mean number of dying cells detected in the present study using the TUNEL method [6.0 ± 5.1 (see Table 2)].

Even the amount of individual variability observed in the range of labeled S-phase and TUNEL-labeled cells is similar between these two studies (Table 2: column 3 vs. column 5). Although it is tempting to assume a direct correlation between the number of S-phase cells and the number of dying cells, the methods used to collect the two data sets are not exactly the same. However, the striking similarity between these two types of cells is noteworthy and seems to suggest that there might be a causal relationship between the processes of cell death and division in the BWS basilar papillar epithelium. Although hair cell production continues in the adult BWS papilla, abnormal dying hair cells are still observed even in the adult. Therefore, given the close relationship between supporting cell division and apoptosis described above and the static number of receptors in the BWS papilaa, the observation of apoptotic hair cell death was not surprising.

In the current study, there was no evidence of apoptosis in the MB canary papilla using morphological or molecular criteria. There is also a lack of supporting cell division in the MB papilla, it occurs very rarely (Table 2), as shown by Gleich et al. (1997). However, supporting cell division increases sharply after hair cell death is induced by sound damage (Gleich et al. 1997), indicating that the direct relationship between hair cell death and cell division is maintained. These data from both the BWS and MB papillae support the hypothesis that there is a potential relationship between apoptotic cell death and cell division in these end organs. More experiments are needed to determine if there is indeed a causal relationship.

Evidence from other systems also supports the hypothesis that apoptotic cell death and cell division are intertwined processes in various tissues (Kerr et al. 1972). For example, apoptotic cells are found in the liver and small intestinal epithelia (Potten 1991a, b; Fan et al. 1998), and increasing the number of apoptotic cells increases the level of cell renewal in these tissues. Molecular studies in the liver have also furthered the understanding of the connection between the division and death signaling pathways. Many typical S-phase- and mitosis-associated molecules are expressed in apoptotic cells in the regenerating liver, and often the reverse is true (Fan et al. 1998). In addition, proteins such as retinoblastoma are being identified as molecules that participate in both the apoptotic and mitotic signaling pathways (Fan et al. 1998). The identification of these types of molecules could explain how these two counteractive processes control the balance between death and division and how they act during regenerative periods and in tissues with high cell turnover such as the chicken utricle or the BWS canary papilla.

Fewer hair cell progenitors in the Waterslager?

After damage is induced in the chicken basilar papilla, supporting cell division continues until new hair cells are generated and the epithelium is completely repaired (Girod et al. 1989; Raphael 1992; Hashino and Salvi 1993; Stone and Cotanche 1994; Tsue et al. 1994; Warchol and Corwin 1996). Only then does the end organ return to its previous quiescent state. Since cell division and death are constantly occurring in the BWS papilla, it is of interest to consider why the epithelium is never completely repaired and why the 30% deficit in hair cell number is never corrected. One possibility is that fewer supporting and hair cell progenitors are produced during embryogenesis in the BWS papilla. This would result in an end organ that develops relatively normally but has fewer hair cells than other canary strains. Many of these hair cells could develop abnormally but still be maintained within the epithelium. According to the current hypothesis, some of the hair cells within the BWS papilla would become apoptotic. Dying hair cells would be replaced, but the production of new cells might be just sufficient to replace those hair cells that have died; complete repair of the epithelium would never occur. As a consequence, there would be no net gain of hair cells over the life of the bird and the 30% deficit would never be reduced. Further research on embryogenesis in the BWS canary should allow this hypothesis is to be tested.

CONCLUSIONS

The BWS canary papilla is an unique model in which to study the effects of hair cell death upon supporting cell division. The present results demonstrate apoptotic cell death in a hearing end organ without any induction of damage. Since supporting cell division results in the production of new hair cells during regeneration as well as in normal inner ear epithelia, identification of a mitotic stimulus could potentially allow for the manipulation of hair cell production. The current study supports the hypothesis that there is a relationship between cell death and supporting cell division in inner ear epithelia and demonstrates that further research using the BWS papilla could more clearly determine the mechanisms whereby cell death may be a mitotic stimulus.

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