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Anatomic and Morphometric Changes to Gerbil Posterior Cristas Following Transtympanic Administration of Gentamicin and Streptomycin

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ABSTRACT

Many studies have sought to document ototoxic damage and to study repair and regeneration of mammalian vestibular sensory epithelia. However, linear density analysis of the sensory cells or use of methods that focus on detection of actin in the stereocilia and cuticular plates at the reticular lamina detect only the disappearance of "hair cells" as defined by a narrow set of criteria. The research presented here focuses on the effects of two ototoxic drugs (gentamicin and streptomycin). We used light microscopic analysis of semithin sections to observe changes in the distribution of sensory and supporting cell nuclei and to elucidate other, previously undetected, morphological changes that occurred within the vestibular epithelia. Age-matched untreated and vehicle-treated controls showed that the gerbil posterior crista is asymmetrical on either side of the septum cruciatum; the longer end is taller and narrower than the shorter end. In cross sections taken throughout the length of each posterior crista, the thickness of the sensory epithelium along the sides (peripheral zone) is greater than at the apex (central zone). In tissue sections of the sensory epithelium, the ratio of sensory cell nuclei to support cell nuclei is slightly over 1:1.5 in all regions except the septum cruciatum where most sensory cells are absent and supporting cells predominate. In tissue sections from the most damaged drug-treated specimens, there was a decrease in the linear density of nuclei in the sensory cell layer, with a compensatory increase in the linear density of nuclei in the support cell layer of the sensory epithelia. In these specimens, linear density of total nuclei/tissue section remained the same. In these regions, the width of the epithelium became up to 50% thinner. The ratio of sensory to supporting cell nuclei changed to 1:6. Drug exposure led additionally to a decrease in length of the cristas, but there was not a linear relationship between the change in length of the crista and length of the septum cruciatum in these shorter cristas. In drug-treated cristas, other changes included a decrease in calculated surface area and volume of the epithelia. Thus, while linear density measurements of sensory cell nuclei provide an indication of damage, there are additional anatomic changes to the cristas and caution is advised with regard to interpreting changes as "loss" of cells.

Keywords: repair, regeneration, ototoxic drugs, vestibular end organs, gerbil

INTRODUCTION

Gentamicin and streptomycin are ototoxic drugs that have long been known to affect both the cochlear and the vestibular systems in humans (Schuknecht 1957). In animal model systems, they are also ototoxic in cats (Hawkins and Lurie 1952), lizards (Bagger-Sjöbäck and Wersäll 1976), birds (Jones and Nelson 1992; Roberson et al. 1992; Cotanche et al. 1994), guinea pigs

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(Lindeman 1969, Twine 1985), and gerbils (Wanamaker et al. 1998, 1999).

Some studies have relied on scanning electron microscopy (SEM) and confocal microscopy to look at hair bundles and scar proteins along the apical lamina of various end organs of the vestibular systems from several different species (Meiteles and Raphael 1994; Baird et al. 1997; Steyger et al. 1997; Forge et al. 1998). In these studies, results showed a loss of mature hair bundles followed by an increase of immature hair bundles (suggesting a loss of sensory cells) and/or increase of scar proteins. Results from cell surface studies such as these are useful because they may be indicators of damage to sensory cells. However, focused analysis of actin in the stereocilia or scar proteins along the reticular lamina does not adequately address actual cell loss that follows ototoxic drug exposure, and it may be deceptive when the goal is to study regeneration of a new sensory cell population. In effect, it may indicate only intracellular repair if those sensory cells are damaged but not destroyed (Zheng et al. 1999).

Other studies using light microscopic analysis have quantified ototoxic damage by documenting a change in the thickness of the vestibular epithelium and/or by using limited counts of cell nuclei (Twine 1985; Lopez et al. 1997; Tsuji et al. 2000). Our recent work on gerbil posterior cristas has quantified ototoxic damage by documenting a change in nuclear density in the sensory cell layer of this vestibular epithelium (Wanamaker et al. 1998, 1999). The calculation of the number of sensory cells per unit length of basal lamina allowed us to quickly detect drug-induced changes to the sensory epithelia.

However, none of these types of studies have taken into account the possibility of more than one parameter changing at a time. All of these types of studies (i.e., analysis of hair bundles per unit surface area, number and density of cell nuclei, and thickness of epithelium) may be influenced directly by changes to the shape or size of the crista following ototoxic drug exposure. For example, changes in linear density could be accounted for in two ways: first, by a change in the absolute number of cells and, second, by a change in the length of the basal lamina, resulting in either a decrease or an increase in linear density (assuming no loss of cells).

In previous research from our lab (Wanamaker et al. 1998, 1999), we characterized linear density throughout normal and drug damaged posterior cristas. We showed a decrease in linear density of nuclei in the sensory cell layer following treatment with ototoxic drugs. However, preliminary observations of our specimens suggested that additional changes were occurring to the posterior cristas. In this article, we have further explored the morphologic changes that accompany the alterations in hair cell number within the posterior crista sensory epithelia. These alterations occur to drug-treated gerbils two weeks after drug exposure. Variables now measured include ratio of nuclei in the sensory cell layer to nuclei in the supporting cell layer; length of crista; length of septum cruciatum (SC); surface area of epithelium; length of basal lamina; and thickness of sensory epithelium at the apex and the sides of the crista.

MATERIALS AND METHODS

Twenty-one healthy Mongolian gerbils (*Meriones unguiculatus*), 31 days old, were anesthetized with 50 mg/kg ketamine and 8 mg/kg xylazine injected subcutaneously. Gerbils ($n = 3$ for each drug-treated group) received one daily transtympanic injection into their right ear either one day (1×) or five days in a row (5×). The injections were 30 μL each of either 50 mg/mL gentamicin sulfate (GM, Schering-Plough, Kenilworth, NJ) mixed with gelfoam powder (Upjohn, Kalamazoo, MI) or 350 mg streptomycin powder (SM, Pfizer, Groton, CT) per milliliter sterile saline mixed with gelfoam powder. Age-matched uninjected gerbils and gerbils injected 1× and 5× with saline/gelfoam powder were used as controls ($n = 3$ for each group of controls). All animals were used and cared for according to protocols filed with the Syracuse University IACUC.

Animals were sacrificed 2 weeks following their last injection. Gerbils were made unconscious with CO_2 inhalation and decapitated. Each bulla was removed and opened to access the cochlea and vestibular system. Small holes were made in the apex, base, and round window of the cochlea and in each semicircular canal. A fixative solution (3% paraformaldehyde and 0.1% glutaraldehyde) in 0.1 M phosphate buffer, pH 7.2 (PB), was perfused through the holes; specimens were left in the fixative overnight on a rotator at room temperature, followed by decalcification with 5% EDTA in PB for 1 week. After the cochlea and vestibular end organs were dissected free, they were dehydrated in increasing concentrations of ethanol, transferred to propylene oxide, infiltrated, oriented, and embedded in epoxy resin (Durcupan ACM, Fluka AG, Buchs, Switzerland). Specimens were placed in a 45°C oven for 1 week to harden. Each posterior crista was cut into 1-μm sections; every sixth section and seventh section were stained with methylene blue/Azure II and coverslipped with TissueTek (Miles Inc., Elkhardt, IA) mounting medium.

For each specimen, the end boundaries of the sensory epithelium within the crista were established based on the region where sensory and supporting cell nuclei first appeared in a double layer. The SC was identified as the region with an arrowhead profile in cross section, few sensory cells, and many vacuoles within the sensory epithelium. The total length of the

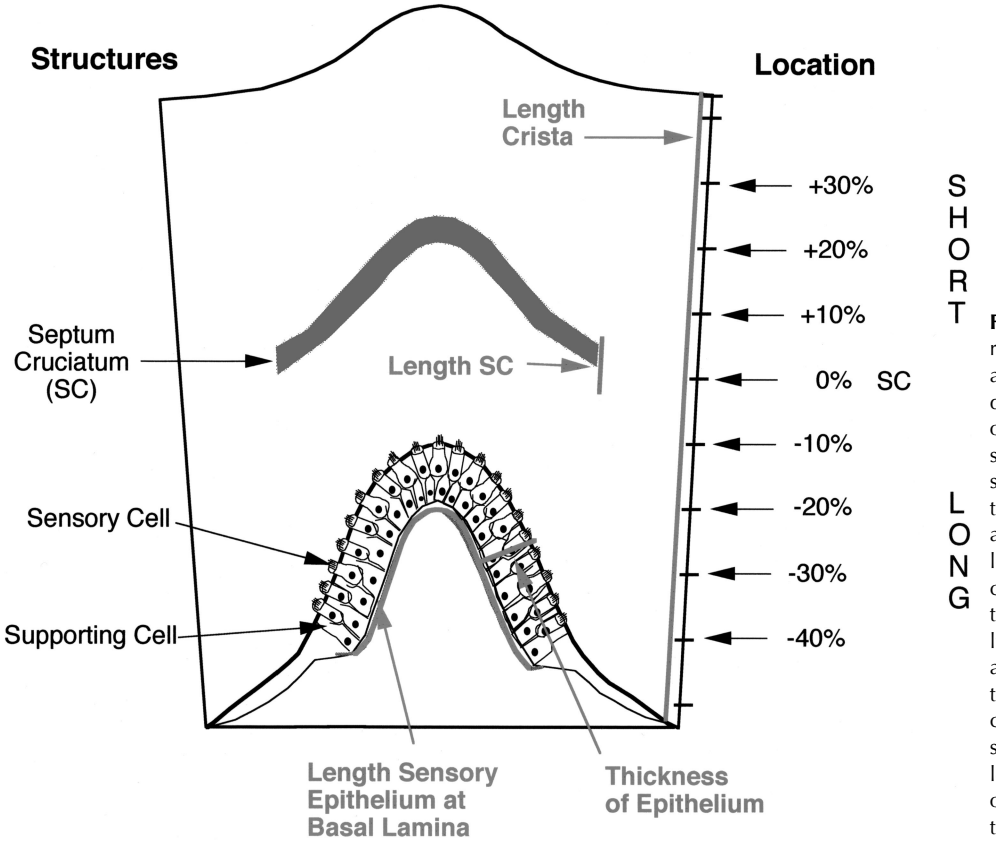

FIG. 1. Diagram of gerbil posterior crista. The septum cruciatum, an area devoid of sensory cells, divides the crista into two regions of different length and shape. Measurements were obtained from tissue sections taken perpendicular to the long axis of the crista. Sampling at specific intervals was based on locations at 10% increments of total crista length (normalized to the septum cruciatum). The locations analyzed are denoted by the positive and negative numbers, which refer to % distance away from the septum cruciatum. Items measured in tissue sections include length of crista, length of septum cruciatum, length of basal lamina, and thickness of the sensory epithelium.

SC and the crista were established based on the number of 1-μm sections containing these structures, and the number of sections within a 10% increment was determined for each crista. Thus, for these studies, entire cristas were sectioned to analyze similar regions across all specimens.

We normalized our data to the SC in order to compare linear density (number of nuclei per micron of basal lamina) of sensory and supporting cells along the length of the crista. The midpoint of the SC was set as the zero point; sections were then analyzed at positions corresponding to 10% increments (relative to the entire length of the crista) on either side of the SC (see Fig. 1). Microscopic images (40× objective) of the selected sections at the specific locations were captured on a Macintosh Quadra 950 computer using Color Snap software; images for publication were taken with 40× oil objective lens on a Zeiss Axioskop using TMax 400 film.

Methods used to distinguish "sensory" from "support" cells and the sensory from nonsensory epithelia have previously been presented in detail (Wanamaker et al. 1998, 1999). Nuclei were classified as belonging to either "sensory" or "support" cells based upon their location in the sensory epithelium relative to the basal lamina. We recognize that differentiating sensory and nonsensory cells based on nuclear position alone may not give totally accurate counts, especially in light of previous evidence that nuclear position can change following aminoglycoside treatment. However, other methods that work in control specimens, such as labeling the sensory cells with antibodies to calmodulin, do not reliably identify sensory cells following aminoglycoside treatment (Ogata and Slepecky 1998). In that light, we are careful never to describe the cells as sensory or supporting, rather as cells that have their nuclei in a particular layer. Additionally, we have not quantified changes to type I and type II hair cells, since even in controls it is difficult to differentiate them unless serial-section transmission electron microscopy is used to probe for nerve terminals and stereocilia.

Nuclei were included in the count of cells if they had a diameter equal to or greater than 4.5 μm, were spherical or elliptical in shape, and contained a visible nucleolus. In order to evaluate change in the length of the basal lamina in drug-damaged specimens versus controls, average basal lamina lengths were determined using NIH Image software (NIH, Bethesda, MD) following calibration with a stage micrometer on these same tissue sections. Additional measurements included thickness (width) and area of the sensory epithelia.

"Sensory cell" and "supporting cell" linear densities were at 10% locations along the length of each crista. The number of nuclei in the sensory and supporting cell layers in each cross section of the sensory epithelium was divided by the length of the basal lamina as

measured on the section. Data from two sections, 6 μm apart, were averaged for each data point at each location. "Total" cell linear density refers to the total number of nuclei in the sensory epithelium (sensory plus support cell layers) per micron of basal lamina. This method differs slightly from those used by others estimating cell number/density with correction factors (Merchant et al. 2000) or stereology based on the disector principle (Fernández et al. 1995; Lopez et al. 1997) to eliminate the bias of double counting of cells. However, we chose to use it for several reasons. First, unlike true stereological methods that are based on the use of randomly oriented sections, our specimens must be oriented in a very precise manner for sectioning in order to interpret our data. Second, in a specimen with an unsymmetrical shape, it is not possible to take a section and call it representative if we know that there exist variations that depend on the location of the tissue section. Third, because of the large number of sections to be cut and the precise locations (not known at the time of sectioning) to be sampled, it would be impossible to maintain complete serial section sets in order for us to use the disector method. Since our goal was to compare normal with damaged specimens and not to determine absolute numbers of cells, we calculated the linear density measurements on sections separated far enough (6 μm) that the same nuclei would not be counted twice. Since data sets were compared using similar methods, any systemic bias should cancel out.

The ratio of nuclei in the sensory cell layer to nuclei in the supporting cell layer was calculated using sensory and supporting cell nuclei linear density data. Total surface area values for each sensory epithelium was calculated by integrating the sums of the average length of the surface epithelium (basal lamina) at each 10% interval multiplied by the number of 1-μm-thick sections within each 10% interval. Cross-sectional area refers to the area of sensory epithelium within each tissue section. This data could be used to infer volume for each crista (data not shown), which takes into account both the thickness and the length of the epithelium. Analysis of variance (ANOVA) followed by a Student Newman–Keuls multiple-range test were conducted on the data. All sections were analyzed without knowledge of treatment.

RESULTS

Lengths measured and analyzed are indicated in Figure 1. They included length of the crista, length of the SC, length of the basal lamina, and thickness of the epithelium. We previously determined that the gerbil posterior crista is not symmetrical in terms of length on either side of the SC (Fig. 1), being divided into a long and a short end. We have now further analyzed each of these ends and found that they are morphologically different as well. The longer (−40%) end of the crista (Fig. 2A) is taller and more narrow in profile than the shorter (+30%) end (Fig. 2B). The shift in profile occurs gradually along the length of the crista, with the tall end fusing with the ampullary wall and the short end eventually flattening out completely.

Microscopic observation of each posterior crista from the control ears (Fig. 3A) showed cells with homogeneously staining cytoplasm and the sensory and supporting cell nuclei forming separate rows. The supporting cell nuclei formed a relatively orderly layer along the basal lamina, while the nuclei in the sensory cell layer were staggered. This difference in level within the epithelium did not appear to correspond to the distribution of type I versus type II cells, based on the obvious presence of calicyal nerve endings.

Posterior cristas from the 1×GM ears displayed gaps between cells, shrinkage of cells, and vacuolization within cells. In some sections, nuclei appeared to have migrated toward the apical surface or the basal lamina, leading to disruption of the orderly two-layered arrangement. While the nerve terminals were still visible, they were swollen, leaving a clear area around the type I cells. In the posterior cristas from the 5×GM, there were very few nuclei in the sensory cell layer, while the nuclei in the supporting cell layer were greater in number and crowded together. The cytoplasm of the remaining cells stained more darkly and homogeneously, suggesting active protein synthesis in the supporting cells. The nerve terminals surrounding the type I hair cells were no longer visible.

Posterior cristas from 1×SM ears revealed variability in response to the transtympanic application of the drug. The least damaged specimen appeared similar to the control specimens, with homogeneously staining cytoplasm, two layers of nuclei with staggering in the sensory layer, and visible nerve calyces. In the two more damaged specimens, the rows of nuclei were present in the peripheral region but there was jumbling and crowding in the central region. Few stereocilia were obvious at the light microscopic level and nerve calyces were no longer visible. The few nuclei present in the sensory cell layer were closer to the supporting cell layer, with little staggering of position. Posterior cristas from the 5×SM ears also showed a range of damage. The least damaged specimen appeared similar to the most damaged 1×SM ears, with loss of sensory cell nuclei and stereocilia, crowding at the central region, and thinning of the epithelium. The other two specimens showed severe damage (example shown in (Fig. 3B), with few to no cells in the sensory cell layer, darkly staining cells interpersed between lightly staining cells, no visible nerve calyces, and marked thinning of the epithelium, especially at

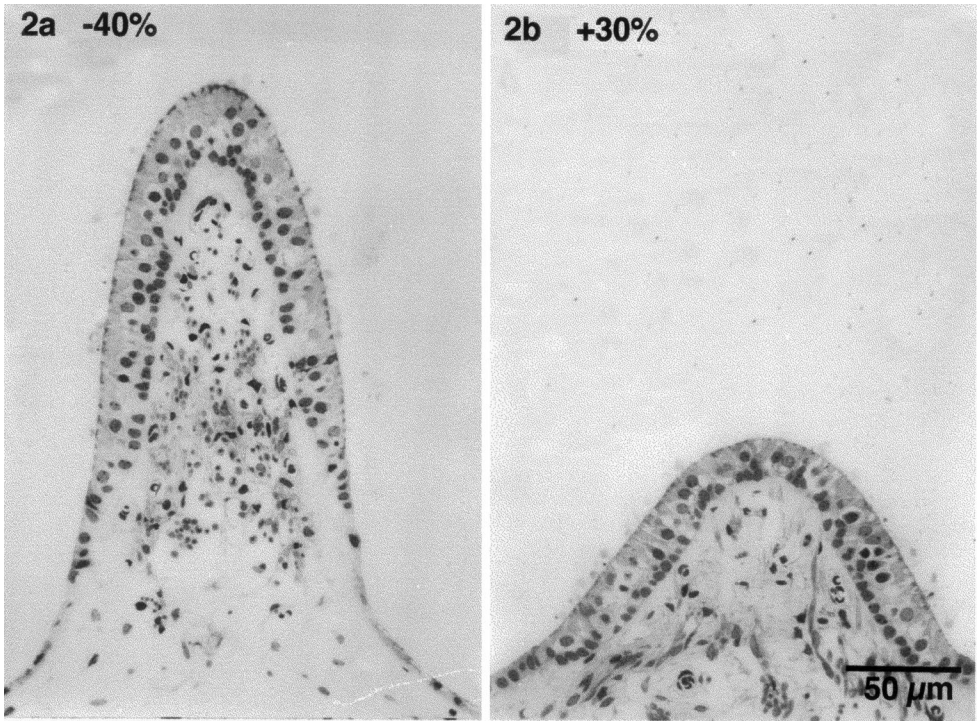

FIG. 2. Light micrographs of representative 1-μm-thick sections from a control, 1× saline control, posterior crista. The shape of the crista changes progressively from one end to the other. **A** A section from the −40% region (the long end of the crista); its profile is tall and narrow. **B** A section from the +30% region (the short end); its profile is less tall and wider.

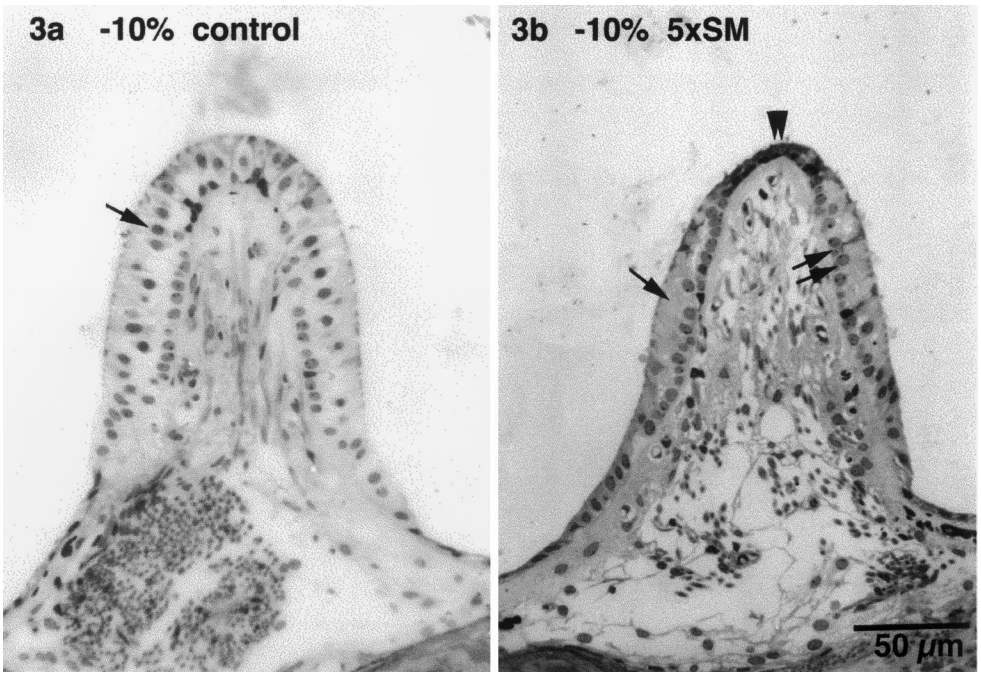

FIG. 3. Light micrographs of 1-μm cross sections from the −10% region of posterior cristas. **A** Age-matched 0× control. **B** 5×SM two weeks post-treatment. Compared with controls, the 5×SM-treated specimens showed fewer nuclei in the sensory cell layer (single arrows) with a compensatory increase in nuclei in the supporting cell layer adjacent to the basal lamina (double arrows). Cristas from the drug-treated specimens also show a decrease in the thickness of the sensory epithelium (arrowheads). Basal lamina length remains unaffected by drug treatment.

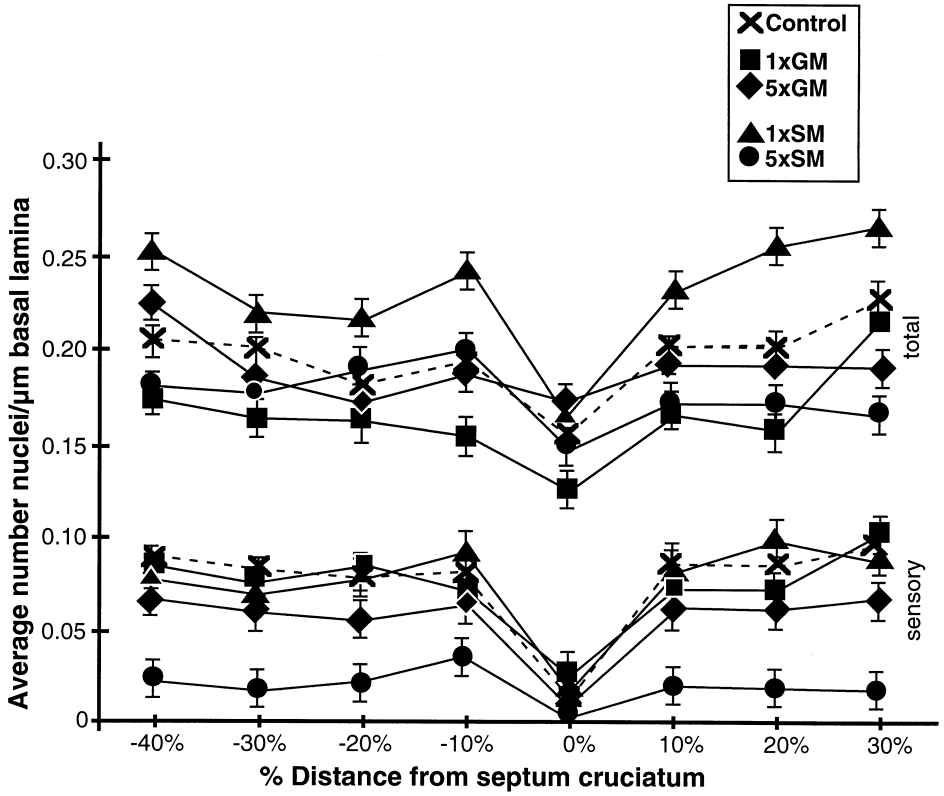

FIG. 4. Average linear density (number of nuclei per micron of basal lamina) of nuclei in sensory cell layer and total number of cells at each of the specific locations sampled for each specimen ($n = 9$ controls; $n = 3$ for each treated group). Error bars represent standard error of the mean. Linear density of nuclei in sensory cell layer showed a statistically significant decrease following 5×SM treatment, yet total linear density did not change significantly. Results suggest that either there are more supporting cells or that sensory cells may have lost the characteristics by which they are normally classified by light microscopy (midlevel nuclei and apical surface stereocilia).

the central region. In one preparation, nuclei were staggered and there were cells of differing shapes. In the other preparation, the supporting cell nuclei were lined up neatly along the basal lamina.

Average linear density of nuclei located in the sensory cell layer and average linear density of the total number of nuclei located within the sensory epithelium were plotted as a function of location along the length of the crista, relative to the SC (Fig. 4). When the number of nuclei per micron of basal lamina in the sensory cell layer was quantified, results showed some variability in the controls, with the 0×Control having the lowest values followed by the 1× and 5×Controls. The 0×Controls were significantly lower than 5×Controls but not significant versus 1×Controls; the 1×Controls were significantly lower than the 5× Controls. When the total number of nuclei per micron of basal lamina was quantified, results showed that there were no significant differences between the 0× and 1×Controls or the 1× and 5×Controls. The difference between the 0× and 5×Controls did reach significance ($p < 0.05$ for all analyses).

For the GM-treated specimens, there were significant differences ($p < 0.05$) between the number of nuclei in the sensory cell layer per micron of basal lamina for the 1×GM versus 0× and 1×Control groups, with the 1×GM group having greater numbers of nuclei in the sensory cell layer per micron of basal lamina. There were statistically significant reductions in the 5×GM group compared with all three controls as well as the 1×GM group. The total number of nuclei per micron of basal lamina remained relatively constant. There were no significant differences in total nuclei per micron of basal lamina between the 1×GM group and the control groups or between the 5×GM group and the control groups.

For the SM-treated specimens, there was variability in the amount of damage across animals. Because of this, the 1×SM ears as a group did not exhibit statistically significant reductions in the number of nuclei in the sensory cell layer per micron of basal lamina when compared with the controls. Among the 5×SM gerbils, there was also variability in nuclei in the sensory cell layer per micron of basal lamina; however, in this case, there were statistically significant reductions ($p < 0.05$) for the 5×SM group compared with all three control groups as well as with the 1×SM group. Neither the 1×SM nor the 5×SM group showed statistically significant changes in total number of nuclei per micron of basal lamina.

Calculation of the ratio of sensory cells to supporting cells showed that, in specimens from control inner ears (Fig. 5A), there were 2 nuclei in the sensory cell layer for every 3 nuclei in the supporting cell layer. Following exposure to 5×SM, the group for which there were statistically significant differences from the control groups, there were shifts in the ratio such that,

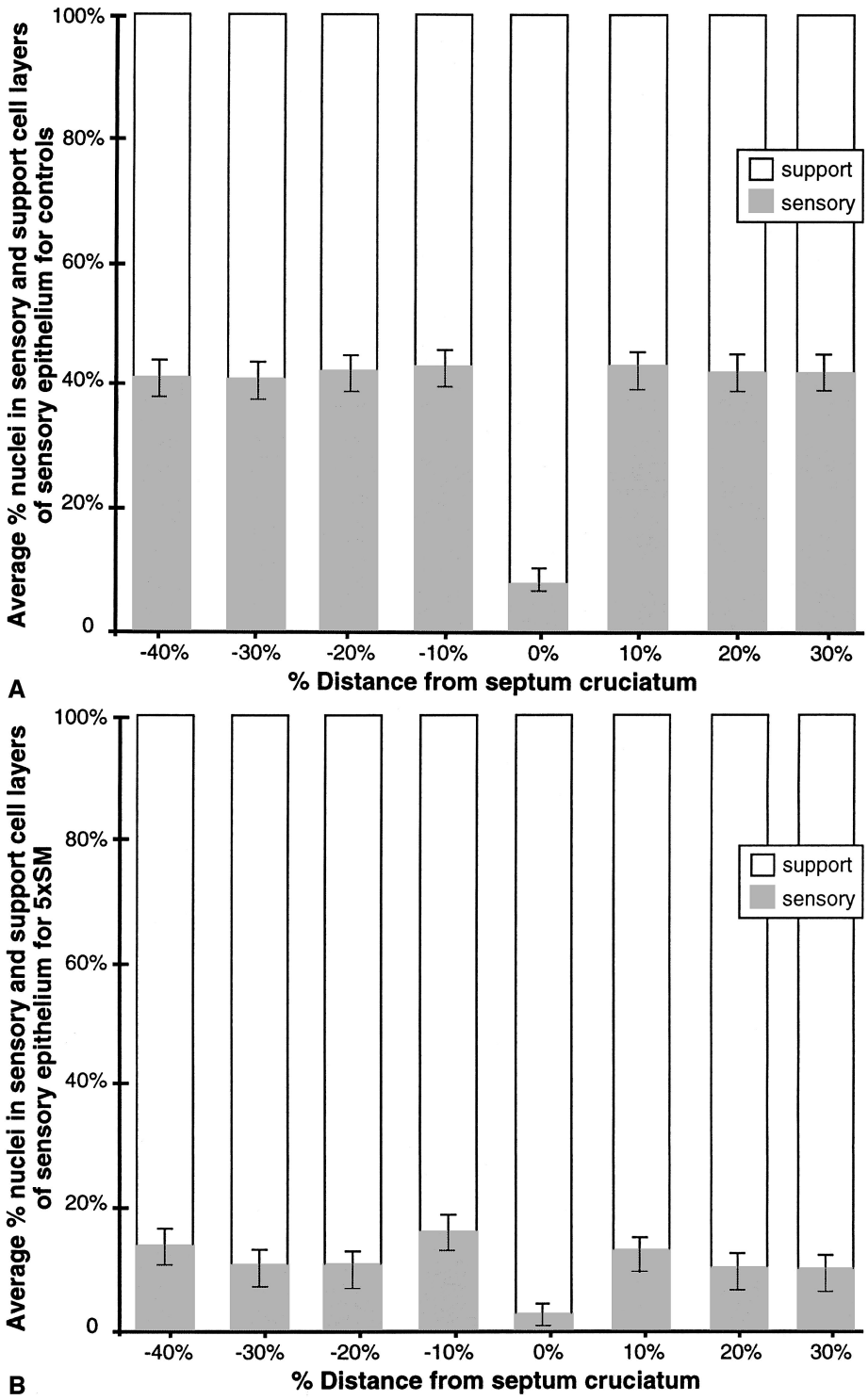

FIG. 5. Average ratio of nuclei in the sensory cell layer to nuclei in the support cell layer at each of the specific locations sampled for control and 5×SM-treated specimens. Error bars represent standard error of the mean. **A** In controls ($n = 9$), the ratio of nuclei in the sensory cell layer to nuclei in the supporting cell layer approximately 2:3. **B** In 5×SM-treated specimens ($n = 3$), the ratio changed to 1:6 (range 1:5–1:9) in areas on both sides of the septum cruciatum (while the total number of nuclei per micron of basal lamina remained the same; see Fig. 4).

for each nucleus in the sensory cell layer, there were 6 or more nuclei in the support cell layer (Fig. 5B).

However, these were not the only changes observed. Analysis of average crista length and average SC length across treatments (Fig. 6) showed decreases. Crista length, based on the total number of sections obtained from each specimen, ranged from 354 to 581 μm in the control and treatment groups. The average crista length for the controls was 489 μm. The 5×SM treatment group was the only group that had a statistically significant decrease in overall crista length, and for this group the average crista length was 372 μm. The decrease occurred on both sides of the crista (Fig. 7). The length of the septum cruciatum was found to range from 25 to 49 μm (5.4%–11.6% of total crista length) in controls

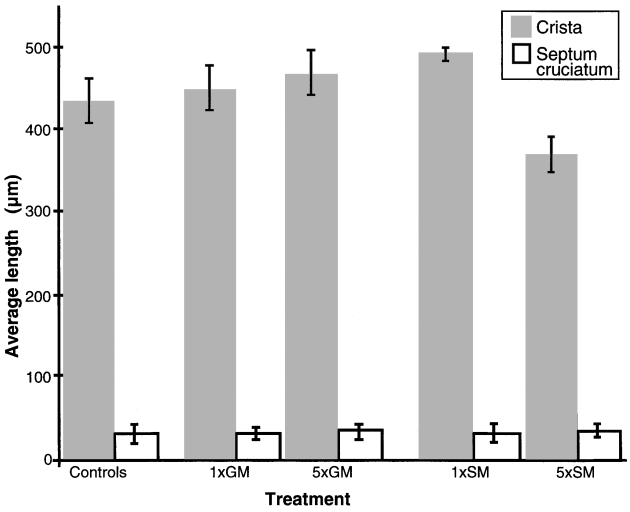

FIG. 6. Average lengths of the cristas (μm) and average lengths of septum cruciata (μm) across specimens (n = 9 controls; n = 3 for each drug-treated group). Error bars represent standard error of the mean. A statistically significant decrease in average crista length was observed in 5×SM-treated specimens; septum length did not vary.

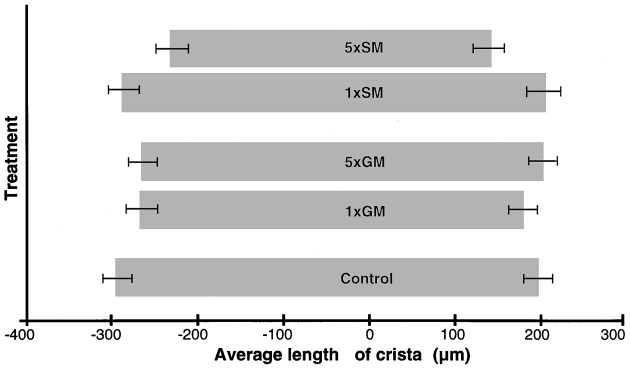

FIG. 7. Average length of cristas (μm) normalized to the septum cruciatum (n = 9 for controls; n = 3 for each drug-treated group). Error bars represent standard error of the mean. The 5×SM treatment group shows a statistically significant decrease in length; the change in length occurs at both ends of the crista.

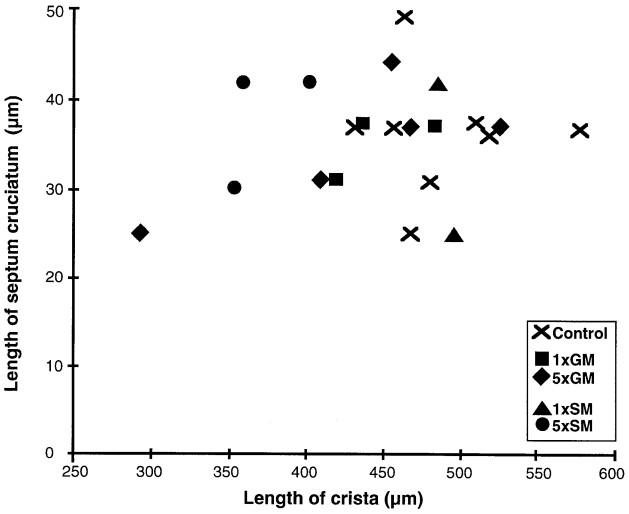

FIG. 8. Length of septum cruciatum (μm) versus length of crista (μm) for individual specimens (n = 9 controls; n = 3 for each drug-treated group). While some drug-treated cristas were shorter than controls, there was no linear relationship between the length of the septum cruciatum and length of the crista.

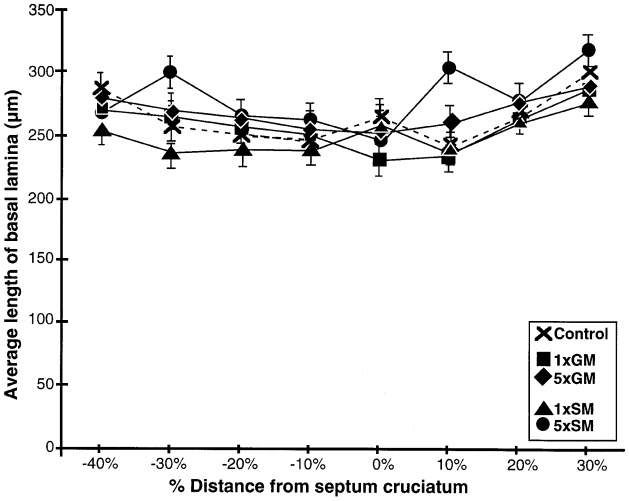

FIG. 9. Average length of basal lamina (μm) as measured in tissue sections at selected locations along the length of the sensory epithelia. Error bars represent standard error of the mean. Basal lamina length remains consistent across normal and all drug-treated specimens (n = 9 for controls; n = 3 for each drug-treated group).

and treatment groups. A comparison of crista length versus septum length across treatments (Fig. 8) showed no linear correlation between length of the crista and septum length.

Although the 5×SM group had statistically significant changes in the number of nuclei in the sensory cell layer per micron of basal lamina and in the length of the crista, the basal lamina length in tissue cross sections remained consistent across treatments (Fig. 9). Measurement of thickness of the sensory epithelium from control specimens (Fig. 10A) demonstrated that the sensory epithelium was wider at the sides (peripheral zones) than at the apex (central zone). Drug-treated cristas (Figs. 10B and C) showed a decrease in sensory epithelium thickness, with changes occurring to both the central and the peripheral zones. The apex appeared to be more sensitive to damage than the sides in the least damaged specimens treated with gentamicin, but both regions were thinner in the more damaged specimen following streptomycin treatment. In the 5×SM group, the decrease was statistically significant in both regions.

Based on a decrease in crista length, basal lamina length, and epithelial thickness, calculations predict a decrease in both surface area (Fig. 11) and cross-sectional area (Fig. 12). Both decreases are statistically

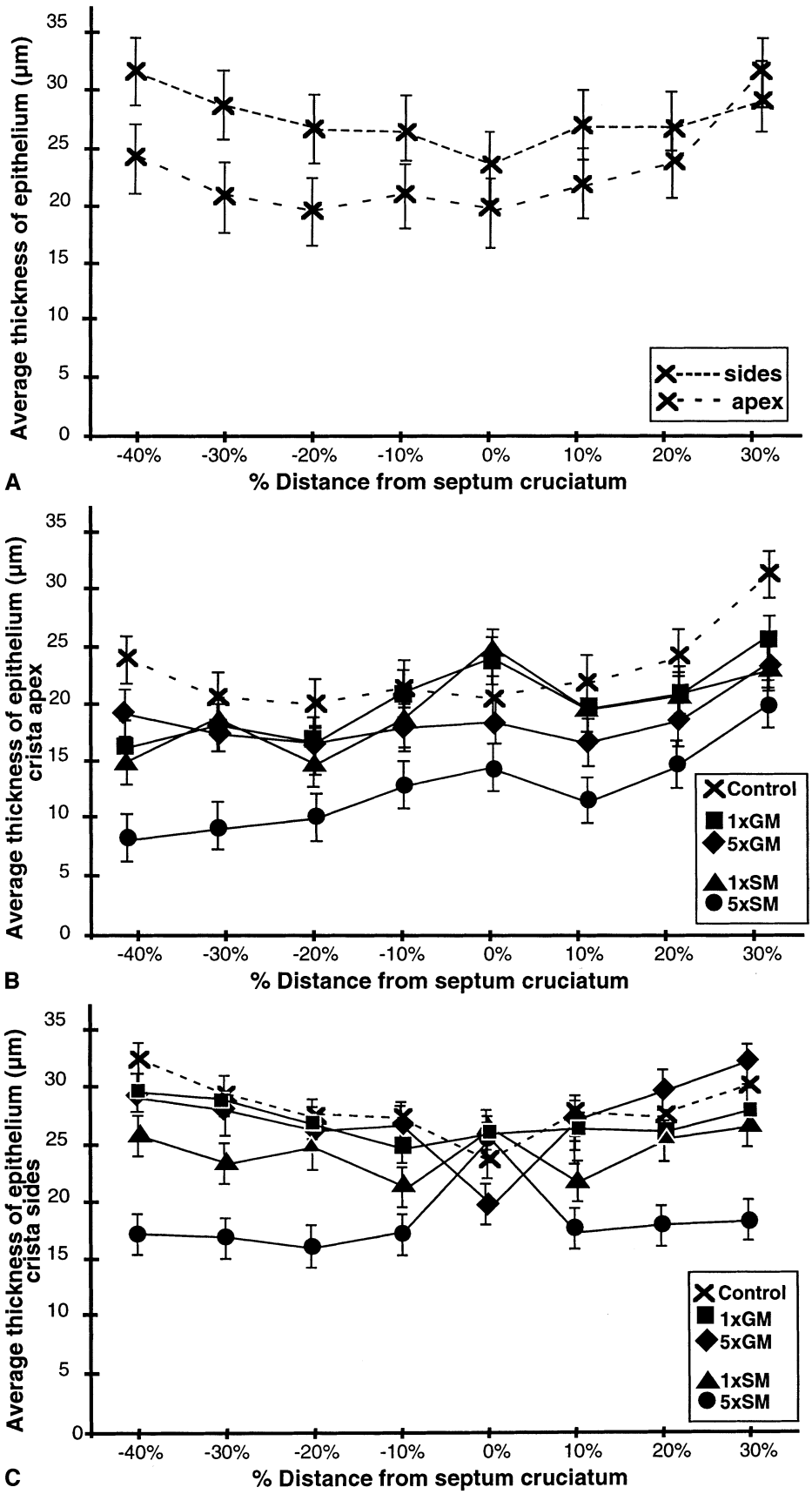

FIG. 10. Average thickness (μm) of sensory epithelia in the apex (central) and side (peripheral) regions of the cristas as measured on tissue sections at selected locations along the length of the cristas. Error bars represent standard error of the mean. **A** In control cristas (n = 9), the vestibular sensory epithelium is thicker along the sides than at the apex. **B** In the apex region of drug-treated cristas (n = 3 for each drug-treated group), there were changes in the thickness of the apex of all drug-treated groups, with a statistically significant decrease in the thickness epithelium for the 5×SM group. **C** Along the sides, the epithelium was thinned more from the streptomycin treatment than from the gentamicin treatment, with a statistically significant decrease for 5×SM.

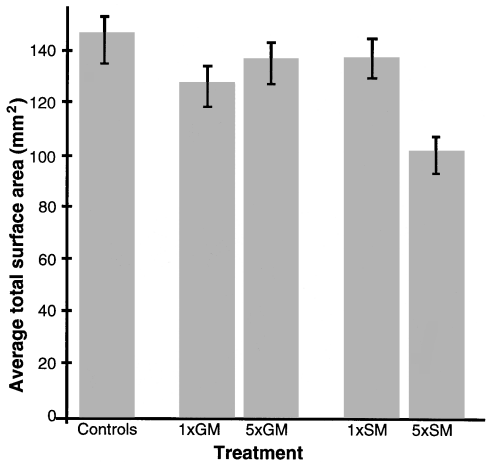

FIG. 11. Average total surface area (mm^2) for posterior cristas. Calculated from measurements of basal lamina length in tissue sections at each of the specific locations sampled and of the number of 1 μm sections within each 10% increment of total length for each specimen ($n = 9$ controls, $n = 3$ for each treated group). Error bars represent standard error of the mean. The decrease in total surface are is statistically significant for the 5×SM specimens.

significant for the 5×SM group. In addition, these calculations can be used to infer a decrease in volume treatment (data not shown).

DISCUSSION

The goal of the anatomic and morphometric analyses performed on these gerbil posterior cristas was to help us better understand and recognize changes to sensory epithelia following exposure to ototoxic drugs. Our choice of gentamicin and streptomycin for transtympanic application was based on the fact that both of these aminoglycoside antibiotics have been used clinically in treatment protocols presented in the literature. However, there are few animal studies in which these drugs have been tested using the same route of administration and analyzing the same end organ (Wanamaker et al. 1998, 1999).

We have characterized the normal gerbil posterior crista as an asymmetric structure with a septum dividing the crista into two regions differing in size and morphology. The long end is taller and narrower than the short end. The sensory epithelium is made up of at least two layers of cells: The supporting cells have nuclei near the basal lamina while the sensory cells have nuclei in the mid- to apical regions of the epithelium.

The sensory cells can be additionally classified into type I and II cells based on their shape, stereocilia arrangement and length, nuclear position, and innervation (Lindeman et al. 1981). Based on cell shape, type I cells can be further differentiated into short, intermediate, and long type I cells in the gerbil (Kevetter et al. 1994). It has been estimated that the ratio of type I to type II cells in the crista is 2.4:1 for humans (Merchant et al. 2000); 1.5:1 for guinea pigs (Lindeman et al. 1981); 1:1 for chinchillas (Fernández et al. 1995); 3:1 for squirrel monkey (Fernández et al. 1995); and 8.3:1 for gerbils (Kevetter et al. 1994). Because our drug treatment paradigm caused migration of sensory cell nuclei into the supporting cell layer and loss of calyx nerve endings—both used to identify type I hair cells—we did not make an attempt to quantify type I and type II hair cell changes. However, in control specimens it appears the type I cells clearly outnumbered the type II cells, both in the apical and the peripheral parts of the cristas. The epithelium is thicker at the sides (peripheral regions) than at the apex (central region). Within tissue sections, there is a ratio of 2:3 for nuclei in the sensory to support cell layers.

Following ototoxic drug exposure using a transtympanic administration paradigm, we have shown a change in the ratio of nuclei in the sensory cell layer to nuclei in the supporting cell layer of the posterior cristas in tissue sections without a loss in the total number of nuclei. While it has been previously reported that ototoxic drug administration can yield a decrease in sensory cells without an apparent decrease in supporting cells (Hawkins and Lurie 1952), our data provide quantitative evidence for a shift in the appearance of sensory cells to cells with supporting cell characteristics *in vivo*. This supports *in vitro* findings reported by others suggesting that hair cell nuclei survive ototoxic injury while losing their surface characteristics (Zheng et al. 1999; Ryan 2000).

Our results from our drug-damaged posterior cristas also show similarities to other studies in which the regions of damage were characterized. In our specimens with the least damage, the apex of the cristas appeared to be more sensitive (as indicated by a thinning of the epithelium) than the sides. Following systemic exposure of guinea pig cristas to ototoxic drugs, it was previously shown that the central regions were most sensitive (Lindeman 1969; Watanuki and Meyer zum Gottesberge 1971; Igarashi 1973; Lopez et al.). However, in our most damaged specimens, the central and peripheral regions were both significantly thinner than control specimens. This agrees with the data from cultured explants from mature gerbils exposed to aminoglycosides. Under these conditions, the central regions are preferentially susceptible, but there is a spread to the periphery with time (Li and Forge 1995).

We did not quantify damage to type I and type II hair cells because we could no longer differentiate them (even from the supporting cells) following our drug delivery paradigm. However, it has been reported that type I hair cells can be more sensitive to aminoglycosides than type II cells, and hair cells in general

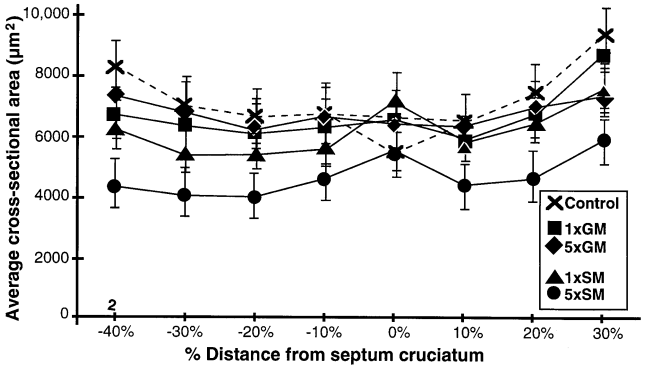

FIG. 12. Average cross-sectional area (mm^2) of sensory epithelia in tissue sections at each of the specific locations sampled, calculated from measurements of basal lamina length and average epithelium thickness for each specimen ($n = 9$ controls; $n = 3$ for each treated group). Error bars represent standard error of the mean. The decrease in cross-sectional area is statistically significant for the 5×SM specimens.

are more sensitive than supporting cells (Wersall and Hawkins 1962; Lindeman 1969; Lopez et al. 1997).

Our data also document a decrease in the length of the crista following administration of ototoxic drugs. It could be argued that this shortening is an artifact resulting from shrinkage of the crista that may occur during dehydration. The shrinkage results in an apparently shorter specimen based on number of sections per specimen. However, since the length of basal lamina in tissue sections of both our normal and drug-damaged specimens remains almost constant, this means that the crista would have to shrink in only one direction which suggests that this is not a contributor to the decreased length. A decrease in length of the crista implies a decrease in the total number of cells, assuming a constant nuclear density, and would lead directly to a decrease in surface area of the crista.

The calculated decrease in cross-sectional area of drug-damaged cristas can be explained as a function of the change in thickness of the epithelium as measured along the sides and apical regions of each tissue section. We have seen a statistically significant reduction in thickness of the epithelium of over 50% for 5×SM-treated specimens versus controls. The fact that the length of the basal lamina remains constant and undistorted in sections from normal as well as severely damaged specimens suggests that the changes in the crista do not result from shrinkage of the underlying connective tissue. Thus, these changes probably reflect only local changes within the epithelium.

Interestingly, no changes were noted in the length of the SC, and no linear relationship was observed between SC length and crista length, suggesting that the SC does not change following exposure to drugs. A possible explanation for the observed changes in crista, but not SC, length is that the administration of high levels of aminoglycoside affected only the sensory cells with a decrease in the volume previously occupied by their cell bodies. This would account for the loss of the nuclei in the sensory cell layer, thinning of the epithelium, and shortening of the crista, with no change to the SC where no sensory cells are present.

Several mechanisms could account for the change in the distribution of nuclei in the vestibular epithelium that we have seen in some of our specimens. (1) The sensory cells may have pulled back their apical processes and stereocilia and their nuclei may have migrated to the support cell layer. (2) An overall loss in sensory cells has already resulted in a series of mitotic divisions such that all lost cells have been replaced and their nuclei are located in the supporting cell layer. However, none of the data presented in this article would suggest mitosis. In all studies where mitosis has been implicated in repair, the number of cells replaced is limited and never close to the number seen in controls. Additionally, studies using the same animal model and drug administration paradigm found limited evidence for BrdU labeling of mitotic cells in gerbil utricles (Ogata et al. 1999). (3) Some of the nuclei could be a result of macrophage infiltration. However, macrophages would have a morphology distinctly different from vestibular epithelial cells and would have left the epithelium by two weeks after the drug administration (Bhave et al. 1998). (4) There may be nearly complete loss of sensory cells, leaving only the supporting cells that migrate toward the SC. In regions outside of the SC, shrinkage of the sensory epithelium would skew cell density data in the supporting cell layer. However, our results that show no change in the length of the basal lamina do not support this.

Using our present methods we plan to investigate early changes in the crista to determine if any of the above-mentioned factors could contribute to the changes we have seen at two weeks postexposure. The improved understanding of the changes that we have obtained by measuring these additional parameters will help us to differentiate between protection/repair of sensory cells and regeneration of new sensory cells.

CONCLUSIONS

Our analysis of tissue sections shows that, following transtympanic administration of gentamicin or streptomycin, the linear density of total nuclei in the gerbil posterior crista sensory epithelium does not change. An increase in the number of nuclei in the support cell layer offsets the decrease of nuclei in the sensory cell layer. Although a change in the number of nuclei in the sensory cell layer or a change in the linear density of nuclei in the sensory cell layer is a clear marker for damage, these are not the only changes

occurring in the crista. Additional analysis suggests that the crista is smaller, the surface area is reduced, and the ratio of nuclei in the sensory cell layer to nuclei in the support cell layer of the sensory epithelium is reduced for treated animals.

It is clear that one needs to be careful in interpreting data and selecting regions to study for regeneration. Cristas from some animal species have a SC, dividing them into parts that differ in length, shape, and perhaps function. If there are regional differences in the end organs, sections from similar locations need to be compared when analyzing the sensory epithelium. A thinner epithelium does not necessarily indicate that cells have been lost. We have found that the crista epithelium is thinner in regions where there has been no change in crista length, and the linear density of the total number of nuclei within the epithelium has remained normal, suggesting repair rather than regeneration. Furthermore, our results show that the use of methods that only examine the apical surface or the stereocilia of the sensory cells may overlook other changes to the crista, since we have shown that, although "sensory cells" are lost, there is compensation such that total nuclei are conserved.

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