Comparison of the Quantity of Cochlear Melanin in Young and Old C57BL/6 Mice

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**Objective:** To elucidate the functional relationship between cochlear melanin and aging.

**Design:** Melanin has been described in the cochlear labyrinth and has been suggested to protect the cochlea from various types of trauma. The quantity of melanin has been shown to change with aging in several organs; however, to our knowledge, aging changes in the cochlea have not been documented. Therefore, we chemically quantified cochlear eumelanin and pheomelanin contents and compared these in young and old C57BL/6 mice using high-performance liquid chromatography. Because melanin deposits in the cochlea present most extensively in the stria vascularis, we morphologically examined the stria using transmission electron microscopy.

**Subjects:** Cochleae from an inbred strain of C57BL/6 male and female mice; 6 at the age of 10 weeks and 5 at the age of 100 weeks were studied.

**Results:** The quantities of cochlear eumelanin and pheomelanin were 421 and 480 ng per cochlea in young mice, and 2060 and 765 ng per cochlea in old mice, respectively. Under transmission electron microscopy, the number of pigmented granules seemed to be greater in older mice compared with younger mice, especially in marginal cells.

**Conclusion:** To our knowledge, our findings are the first quantitative evidence to show an age-related overexpression of cochlear melanin and an alteration in the proportion of eumelanin and pheomelanin with aging, suggesting a possible otoprotective function of eumelanin against age-related cochlear deterioration.

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**Melanin has been described in the cochlear labyrinth,** and it has been suggested that its function is to protect the cochlea from various types of trauma, including the effects of ototoxic drugs, such as aminoglycoside antibiotics and noise-induced sensorineural hearing loss. The quantity of melanin in various organs has been shown to vary with age (eg, age-related reduction of melanin in the hair bulb and shaft, epidermis, retinal pigmented epithelial cells, and certain areas of the central nervous system; and age-related increase in senile lentigo). In a light microscopic study of human temporal bones, cochlear melanin was reported to increase with aging; however, to our knowledge, there have been no quantitative studies of age-related alterations of cochlear melanin.

There are 2 basic types of melanin, eumelanin and pheomelanin, in mammalian neural crest–derived melanocytes. Each of them has been suggested to possess different chemical and biological properties (eg, eumelanin may principally scavenge toxic reactive oxygen species [ROS] and play an otoprotective role, whereas pheomelanin may produce toxic ROS and exert toxic effects on the cochlea). Studies regarding cochlear melanin, therefore, should be designed to evaluate the quantity and proportion of both types of melanin.

The stria vascularis (SV) has been reported to have the most extensive quantity of melanin deposits in the cochlea, and many studies have focused on melanin deposits at this site. While the function of strial melanin on age-related strial degeneration has not been determined, we anticipated that strial melanin could have a protective effect on strial aging, because ROS has been suggested to be one mechanism associated with age-related cochlear degeneration and melanin has been demonstrated to be able to scavenge ROS. The C57BL/6 inbred strain of mouse develops extensive hearing loss by the age of 24 months, and the cochlear pathological features with aging of this strain have been characterized by early degeneration of the organ of Corti and spiral ganglion cells or the spiral ligament. Endocochlear potential declines little with age in this strain. We assumed that it would be beneficial to study an animal model whose SV remains functional with aging, like the C57BL/6 mouse, to determine if the lack of damage to the SV might be related to an increase in
striatal melanin. In this study, we, therefore, quantitatively compared cochlear eumelanin and pheomelanin contents in young and old C57BL/6 mice using high-performance liquid chromatography (HPLC). We also examined the SV in these young and old mice using transmission electron microscopy to address ultrastructural changes of melanin at this particular site.

**METHODS**

**SUBJECTS**

Cochleae from C57BL/6 male and female mice, 6 at the age of 10 weeks and 5 at the age of 100 weeks, were studied. All aspects of animal care and experiments were approved by the Nagoya University Animal Research Committee. The mice were housed at 22°C with free access to food and water and with 12-hour light and dark cycles. They were raised and maintained by mating in a closed colony in a quiet vivarium at the institute. All mice were deeply anesthetized with sodium pentobarbital, and both sides of their otic capsules were removed. One side was processed for evaluation for HPLC and the other for transmission electron microscopy. Both cochleae from one older mouse were damaged and were not available for HPLC analysis.

**QUANTITATIVE ANALYSIS OF EUMELANIN AND PHEOMELANIN**

For HPLC evaluation, the stapes was dislocated, the bony labyrinth at the apical turn was opened, and cold fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.4) was injected by perilymphatic perfusion. Fixation was continued by immersion for 2 hours. The lateral walls of the basal turn of the cochlea, containing the SV and spiral ligament, were dissected and rinsed in phosphate buffer, postfixed with 2% osmium tetroxide in phosphate buffer for 1 hour, rinsed again in phosphate buffer, dehydrated in a graded series of alcohol and propylene oxide, and embedded in epoxy resin. All fixation and dehydration procedures were performed at 4°C. Specimens were sectioned along a midmodiolar plane at about 100 nm, stained with 2% uranyl acetate and lead citrate, and examined with a transmission electron microscope (model H7100; Hitachi, Tokyo, Japan).

**RESULTS**

**QUANTITATIVE ANALYSIS OF EUMELANIN AND PHEOMELANIN**

In our study, the detection limits for pyrrole-2,3,5-tricarboxylic acid and 4-amino-3-hydroxyphenylalanine were calculated to be 0.3 and 0.4 ng per cochlea, respectively. The amounts of eumelanin and pheomelanin in the young and old mice were higher than the detection limits. The cochlea of the old mice contained about 5 times the amount of eumelanin compared with that of young animals (2060 vs 421 ng), whereas the amount of pheomelanin in old mice was less than 2 times the amount in young mice (765 vs 480 ng).

**TRANSMISSION ELECTRON MICROSCOPY**

The most apparent ultrastructural alterations with aging were loss of the basolateral infoldings of marginal cells and loss of the dendritic processes of the intermediate cells. There were few mitochondria remaining within the processes of the intermediate cells and a reduction of interdigitations between the marginal and intermediate cells. In the older mice, there was dilatation of the interstitial spaces between the marginal and intermediate cells with some amorphous material scattered throughout. These changes were patchily distributed in the SV of the basal turn and varied in degree among specimens. In the basal and marginal cell layer, however, interconnections at tight junctions between these cells were well preserved, even in areas of severe pathological alterations. No membrane blebbing from the luminal surface was observed in either group. Granular and spherical profiles of inclusions were sparsely distributed in the SV of the basal turn in young and old mice. They were of medium to high electron density and varied in shape, size, and quantity. The SV from the older mice seemed to contain more granules than the SV from the younger mice (Figure 1 and Figure 2A); however, the ultrastructural appearance did not seem to differ with aging. Granules in marginal cells

![Figure 1](https://www.archoto.com/132/ARCH_OTOLARYNGOL_HEAD_NECK_SURG_VOL_133_FEB_2007_www.archoto.com/132/image66x579to293x747)
were generally located adjacent to intermediate cells and inside of their lobules. Granules in the marginal cells occasionally demonstrated a lysosome-like appearance (Figure 2B). In intermediate cells, the granules were located within the cytoplasm, near the nuclei (Figure 2C). Granules were also sporadically present in basal cells.

**COMMENT**

Oxidative stress due to damage from accumulation of ROS, a natural product of aerobic metabolism, has been suggested to be one mechanism associated with age-related cochlear degeneration. Studies have suggested a correlation between ROS and melanin in the cochlea. There are 2 types of melanin, eumelanin and pheomelanin, in melanocytes derived from the neural crests of mammals. Eumelanin is thought to principally scavenge ROS and may, therefore, play an otoprotective role in the cochlea, whereas pheomelanin may produce ROS and exert toxic effects on the cochlea. By using HPLC, we demonstrated that the cochleae of old mice contained about 5 times the amount of eumelanin compared with the cochleae of young mice; however, the amount of pheomelanin content in old mice was less than 2 times the amount in young animals. Because the cochlea has been suggested to be exposed to increased levels of free radicals with aging, the predominant overexpression of cochlear eumelanin with aging, compared with pheomelanin, might support the potential of cochlear melanin to protect the cochlea against the age-related hyperproduction of ROS.

Although it was not possible to distinguish the subtypes of melanin morphologically, we did observe an increased expression of spherical granules of high electron density in the SV in old mice. These granules had similar structural characteristics to melanin granules that have been identified to be heterogeneous, to be extremely electron dense, and to have numerous tiny vesicles. Because the SV possesses the largest quantity of melanin deposits in the cochlea, we would expect the proportion of the melanin subtype in the SV to correlate with the ratio obtained in our quantitative evaluation. Because the SV is an area that contains numerous mitochondria, and although the mitochondrial respiratory chain is the primary intracellular source of ROS, it seems reasonable to assume that the SV is the area most profoundly involved in the action of eumelanin against the age-related hyperproduction of ROS in the cochlea.

In this study, age-related strial degeneration of this strain was patchily distributed and was not apparent in some areas of the stria. While older mice seemed to contain more granules than younger mice, the ultrastructural characteristics did not seem to change with aging. Although we cannot rule out the possible contribution
of environmental factors to the age-related alteration of cochlear melanin, exogenous factors, including environmental noise exposure, diet, and ototoxic chemical intake, were controlled. To our knowledge, our findings are the first quantitative evidence to show an age-related overexpression of cochlear melanin and an alteration in the proportion of eumelanin and pheomelanin with aging, suggesting the possible otoprotective potential of cochlear eumelanin against age-related cochlear deterioration.

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REFERENCES