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APPLICATIONS OF SCANNING ELECTRON MICROSCOPY AND X-RAY MICROANALYSIS IN INNER EAR PATHOLOGY

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Abstract

Surface pathology of inner ear structures so far described in detail concern cochlear and vestibular hair cells and the stria vascularis. In man, surgical intervention into the inner ear is very uncommon and when performed is in general with the primary objective of destroying the diseased peripheral end organs. The vast majority of inner ear tissue available for use with scanning electron microscopy (SEM) is therefore obtained from animals.

The present paper reviews the progression of surface pathology caused by aminoglycoside antibiotics, acoustic overstimulation and in a guinea pig strain with genetic inner ear disease. The primary site of onset of surface pathology differs, depending on the underlying cause. Advanced surface pathology shows a similar type of morphological degeneration independent of cause. The combination of SEM and energy dispersive X-ray microanalysis (XRMA) of inner ear pathology has as yet been reported in only three studies, all concerning inner ear fluids or otoconia.

Key words: inner ear , surface pathology , aminoglycoside antibiotics , acoustic trauma , genetic inner ear disease , scanning electron microscopy , X-ray microanalysis.

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Introduction

The inner ear was first studied by surface microscopical techniques at the end of the last century (40), but the surface preparation technique using fixed temporal bones without or with embedding material (mainly epoxy resins) did not become common until 1950's and 1960's (16, 18, 28, 37). These studies are limited by the resolving power of the light microscope. Although the principles for scanning electron microscopy (SEM) have been known since 1929, the first commercial SEM was not available until 1965 (39). The normal surface architecture of cochlear and vestibular epithelia has been reported in a series of papers published during the last decade (15, 17, 27, 32, 35). However, a limited number of studies on inner ear pathology have been conducted. Pathological changes of the inner ear predominantly affect the sensory cells, in particular the stereocilia (review: 23, 46). In only a few instances is a primary effect exerted on the stria vascularis (2, 21).

The present review paper deals with principles of labyrinthine surface pathology, whether of experimental origin or inherent, with emphasis on common morphologic degenerative patterns independent of underlying cause. X-ray microanalysis of diseased inner ear tissues has so far been combined only with SEM (review: 9). The elemental analysis of pathological inner ear conditions will be reviewed. It should be emphasized that in man surgical intervention into the inner ear is extremely uncommon and is in general with the objective of destroying the diseased membranous labyrinth. Material obtained at autopsy is several hours old and shows postmortem artefacts. However, the surface structures of the organ of Corti and of the stria vascularis are quite stable and show only very small changes up to 6 hours postmortem (14). Material obtained from animal models thus comprises the major part of all studies.

Tissue preparation

Procedures of inner ear specimen preparation for scanning electron microscopy (SEM) are, in general, similar to those of surface

preparation for light microscopy (18, 37), and those of routine transmission electron microscopy (TEM) using epoxy resins for embedding (5). In light microscopy, using the surface preparation technique, different anatomical levels can be studied by shifting the focus (Figs 1-2). However, in SEM the surface only can be examined. All overlying tissues will completely obscure the view of the underlying surfaces.

To study early and subtle surface pathology, the preparation techniques used for SEM must provide for a high degree of reproducibility without causing even minor changes which can be misinterpreted as early pathological findings. The types of artefacts involved in each technique must be well known. In particular,this can be difficult where extracellular structures such as the tectorial membrane and cupula are concerned which show considerable changes depending on fixatives and/or buffers used with different ionic concentrations or different pH (29, 30). The optimal techniques and principles of SEM in inner ear research have been summarized by Lim (32) and Hunter-Duvar (27) including descriptions of the most common artefacts. With the standardized techniques at present available for SEM of inner ear tissues, even delicate pathological aberrations from normal surface morphology can be identified with great accuracy. Embryonic inner ear tissue constitutes a special problem during handling of specimens for SEM. The cryofracturing technique has proven of great value for orientation to expose these delicate structures (34).

The technique for X-ray microanalysis of the inner ear as viewed with SEM has been recently described (7, 9). So far, energy dispersive elemental analysis regarding inner ear pathology has been performed on bulk specimens only. With improved preparation techniques, especially freeze-substitution with epoxy resins, also semi-thin (2-4 μm) and thin (< 1 μm) specimens for analysis in the scanning transmission electron microscopical (STEM) system will be available in the study of inner tissues (10). This, however, is outside the scope of the present paper.

Principles in surface pathology (SEM)

General comment

The membranous labyrinth constitutes a sealed compartment in the temporal bone. In order to exert a toxic influence on the organs of hearing and balance, ototoxic substances must reach the inner ear via blood vessels or directly enter the perilymphatic space from the middle ear through the oval and/or round windows. The blood vessels are located below the sensory epithelium in the vestibular organs. Toxic substances are likely to diffuse in the tissue between hair cells and blood vessels. In the cochlea, however, all toxic drugs must diffuse through fluid-filled spaces in order to reach the sensory cells. Changes in inner ear surface morphology are often an early sign of altered cell structure and function, in particular concerning hair cells (19, 20, 44). In addition, sometimes also the marginal cells of the stria vascularis manifest early morphological changes (2, 21). SEM illustrations of inner ear pathology appear in many publications dealing with labyrinthine pathology. However, in only a very limited number of studies, have consecutive analyses of early changes and the subsequent progressive development of surface pathology been described. As yet, inner ear surface pathology has been restricted to vestibular and cochlear hair cells (20, 35, 41), and to the stria vascularis (14, 15). In man, sensory cell pathology is encountered also in "normal" inner ears, e.g. caused by ageing or noxious influences on the labyrinth earlier during life (35, 36). The gross morphological changes of surface pathology in individual cells are the same in a given tissue independent of type of noxious injury. At the onset of damage diversities are observed depending on the underlying cause. The anatomical location for the onset of damage to the sensory cells can vary considerably (1, 2, 21, 24, 33, 46). The principal pattern of surface pathology following a defined injury seems rather universal and is not confined to mammals only. In e.g., the lizard basilar papilla containing sensory cells, the surface deformities show a similar pattern as described for mammals (13).

Hair cells

Both the cochlear and vestibular hair cells of the inner ear organs can be affected by aminoglycoside induced ototoxicity and in genetic inner ear disorders. Damage caused by acoustic overstimulation is in general confined to cochlear hair cells only (43). The sequences in development and progression of surface pathology are similar in cochlear and vestibular hair cells following exposure to aminoglycosides and in genetic inner ear damage. The surface pathology independent of underlying cause is confined primarily to the hair cells. Only when hair cells are severely degenerating and are expelled into the endolymphatic space do the adjacent supporting cells become involved in the surface changes and stretch to fill the space earlier occupied by the degenerated hair cells. In this way, the endolymphatic compartment is always sealed. In both the aminoglycoside antibiotic and the acoustic overstimulation groups, the sensory hairs are the targets of primary damage, though in slightly different patterns (Figs 3-5).

Exposure to aminoglycoside antibiotics causes primarily a disarray of the normally regular pattern of stereocilia and local attachment points of the plasma membrane between neighbouring sensory hairs. The plasma membrane at these points of contact fuses and then disappears with the formation of protoplasmic bridges between the sensory hairs. Finally, a more or less complete fusion of the sensory hairs takes place starting at the base of the hairs and proceeding distally. In early stages of fusion the axial fibril bundles of several individual sensory hairs can be observed in transmission electron microscopy (TEM) in the giant hair formed by the fusion. Further, rootlets from sensory hairs can be followed from the fused giant hair down into the cuticle. Later, however, both the hair rootlets and the hair fibril bundles disintegrate (44, 45, 47).

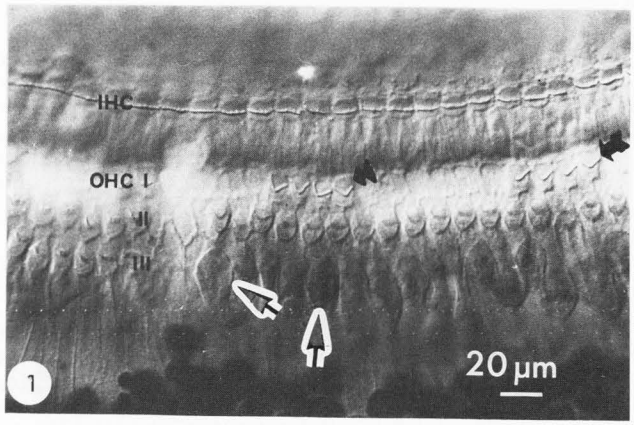


Fig. 1. Light micrograph (LM). Interference contrast. Guinea pig cochlea. Atoxyl-induced outer hair cell (OHC) degeneration in all three rows. Missing hair cells have been replaced with scarring in a regular pattern. OHC in the first row (OHC I) have been most damaged and only small groups of cells (filled arrows) remain. Many OHC in the third row (OHC III) are extremely swollen (unfilled arrows). All inner hair cells (IHC) remain.

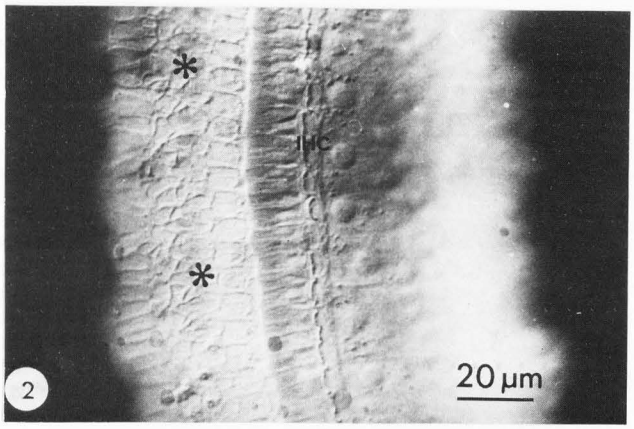
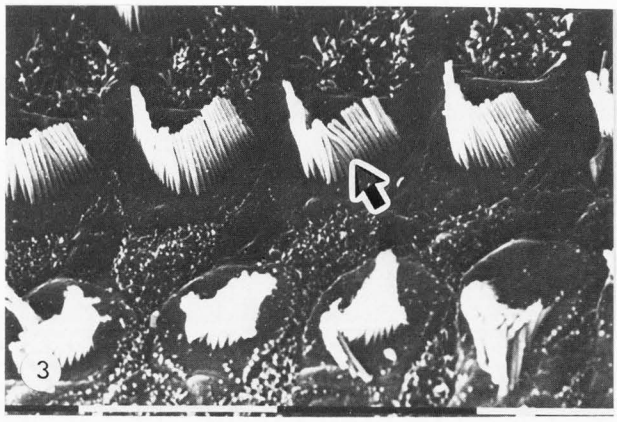
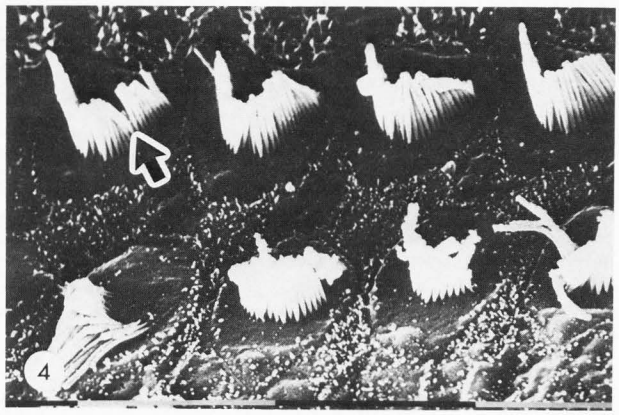
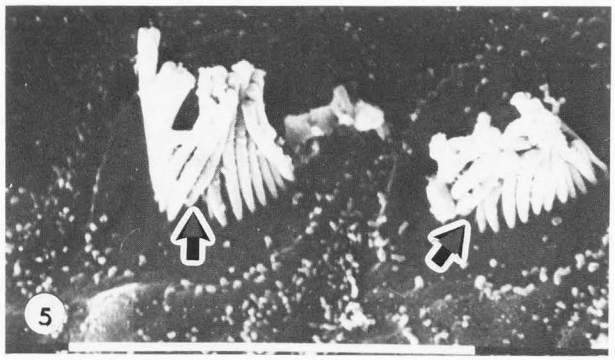


Fig. 2. LM. Interference contrast. Guinea pig cochlea. Atoxyl intoxication. All outer hair cells have degenerated and have been replaced by a regular pattern of scarring tissue (asterisks). Inner hair cells (IHC) remain including the normal pattern of stereocilia.

Acoustic overstimulation causes first a disarray of the regular pattern of stereocilia followed by fusion of adjacent sensory hairs. This fusion starts at the base of the sensory hairs (Figs 6-8). In some cases, the stereocilia can undergo autolysis leaving only stumps on the cuticular plate (26). Following exposure to aminoglycoside antibiotics or acoustic overstimulation, severe morphological changes of the hair cells are characterized by bulging of the cuticular plate, ballooning of cells into the endolymphatic space followed by phalangeal scarring (Figs 9-12)(38).







Figs 3-5. Scanning electron micrographs (SEM). Guinea pig cochlea following intoxication with gentamicin. Initially a disarray of stereocilia occurs (unfilled arrows). Attachments between individual stereocilia occur at this stage of aminoglycoside induced hair cell damage. Scale mark: 10 µm.
3. Second coil. Outer hair cells (OHC) in rows I-III.
4. Second coil. OHCs I-II.
5. Third coil. OHC I.

In contrast to aminoglycoside antibiotics and noise trauma, the first sign of an altered surface morphology in genetic deafness occurs as a slight bulging of the cuticular plate (Figs 13-15). The geometrical array of the stereocilia is primarily not changed. However, the base of the sensory hairs can be minimally elevated. With the progression of damage a severe bulging of the cuticular plate occurs (Figs 16-17). The stereocilia become irregular in size and diameter but the sensory hairs are still separate. Finally, fusion of stereocila starts at their basal end causing the formation of giant hairs followed by extrusion of the degenerating cells (Fig. 18).

During hair cell degeneration in genetic deafness with subsequent cell extrusion, the tight junctions to adjacent supporting cells remain intact (42). The pathology is confined to a bulging cuticular plate and sensory hair fusion. It appears likely that a similar pattern of junctional preservation can be expected following ototoxic drugs and noise trauma. With increasing age of the scarring in the organ of Corti the non-sensory part degenerates and finally only a layer of flat microvilli-covered cells covers the basilar membrane. In the vestibular organs, extensive hair cell loss results in a less regular pattern of supporting cell scarring which remains unchanged over a very long period of time (12).

In general, a difference in vulnerability occurs between the different hair cell types (cochlea: inner hair cells, outer hair cells, vestibular organs: hair cells type I and type II) (23). In the cochlea, aminoglycoside antibiotics in guinea pig cause primarily outer hair cell degeneration in the basal coil whereafter the damage progresses apicalwards affecting the two inner rows of the second coil and the innermost (first) row in the two apical coils. The inner hair cells, which are less vulnerable than outer hair cells, follow a similar base to apical damage pattern (23, 46). In the cristae ampullares, the central part of the crista appear more severely damaged whereas the periphery of the crista, as well as the area close to the planum semilunatum show less damage. The type I sensory cells are more severely damaged than are type II hair cells (44). The order and degree of hair cell damage in the cochlea following acoustic overstimulation is, from maximum, in first row outer hair cells to minimum in third row outer hair cells. Inner hair cells are those least affected (26). In contrast to the rather sharply demarcated cochlear hair cell damage caused by aminoglycosides and acoustic overstimulation the depopulation pattern in genetic deafness is considerably more diffuse. Hair cell loss is primarily most pronounced basally in the cochlea whereafter the degeneration progresses apicalwards. Damage is initially most extensive in the third row of outer hair cells with fewer missing hair cells in the second and least in the first row. Inner hair cells are lost very late (20). Concerning vestibular hair cells the earliest and most severe changes occur in type I hair cells in the striolar regions (41).

Stria vascularis

Studies on the normal surface structure are few (2, 21, 25, 31) (Fig. 19) as on surface pathology (2, 21, 25). This is probably because strial changes are very uncommon in ototoxicity. In TEM investigations, however, it has been shown that some changes can occur in ageing and following exposure to aminoglycoside antibiotics, mercury and noise trauma (4, 22).

The principles for the onset and progress of the surface pathology of the stria vascularis are similar following exposure to both atoxyl (2) and ethacrynic acid (21) (Figs. 20-23). First, the number of microvilli and depressions on the marginal cell surface is reduced. A slight bulging of marginal cells occurs but the cell borders still remain distinct and intact. As pathology progresses, enlarged bulbous microvilli (protoplasmic protrusions?) are identified and normal microvilli are lost. Thereafter the cell borders become indistinct. Up to this point, the surface pathology of the stria vascularis seems reversible. Animals treated with ethacrynic acid show a stepwise normalization of both surface pathology and intracellular changes (21, 3). In contrast, atoxyl exposure can cause giant herniations including several adjacent degenerating strial epithelial cells from the strial surface (2). As free-floating material can be found in the endolymphatic space, it is assumed that this debris emanates from the stria vascularis.

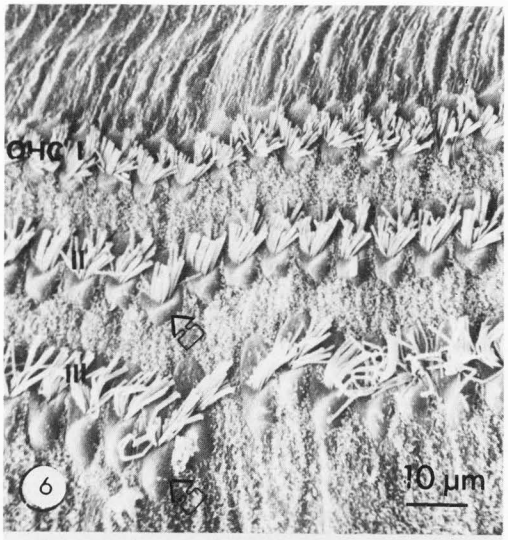
In the first study on strial surface pathology, Anniko (2) reported a primary atoxyl-induced lesion in the apical part of the cochlea. Ethacrynic acid, however, shows asymmetry of damage in the opposite direction, the base being most affected. Substances entering the stria vascularis from the blood, as do ethacrynic acid and atoxyl, would be expected to affect all turns equally. A speculative explanation is that there is a progressive change in the physiological condition of marginal cells in the basal - apical direction. Some minor differences of the normal surface morphology between the apical and basal turns were reported by Forge (21). A physiological variation possibly can result in differing responses of various parts of the cochlea to different ototoxic materials.

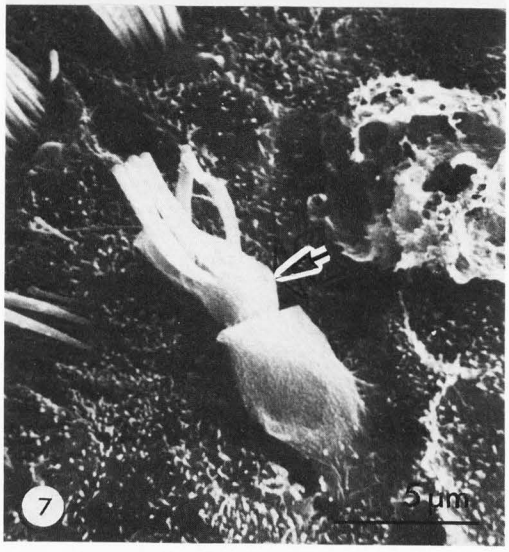
Figs 6-8. SEM. Guinea pig cochlea following exposure to noise.

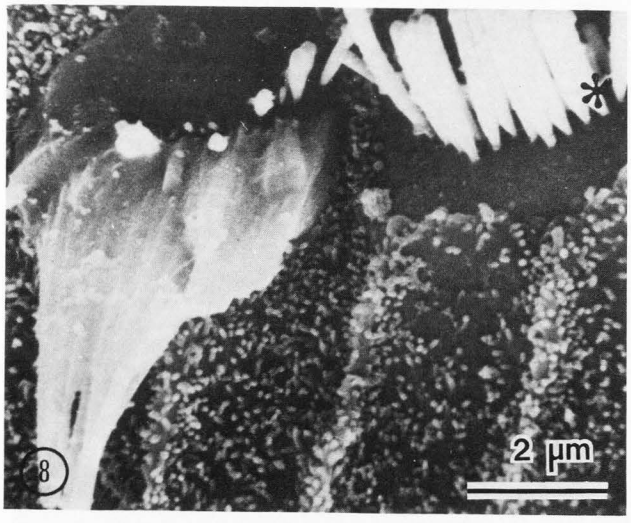
6. Normal pattern of stereocilia on the inner hair cells (IHC). Considerable disarray of stereocilia on outer hair cells (OHC) in all three rows (I-III). The cuticular plates are slightly protruding (arrows).

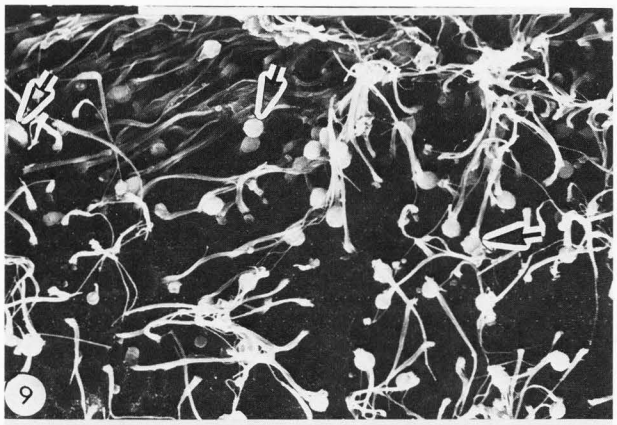
7. OHC-III with fusion of stereocilia close to the cuticular plate (unfilled arrow).

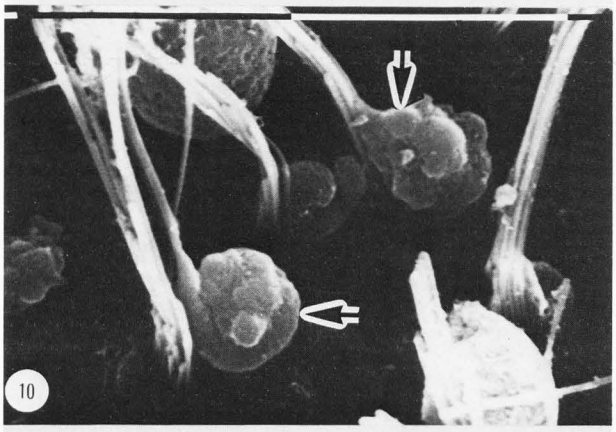
8. Inner hair cells (IHC). One IHC (asterisk) shows only disarray of stereocilia while an adjacent IHC reveals fusion of all stereocilia which also have lost their rigidity and are bent down towards the adjacent supporting cell surface.

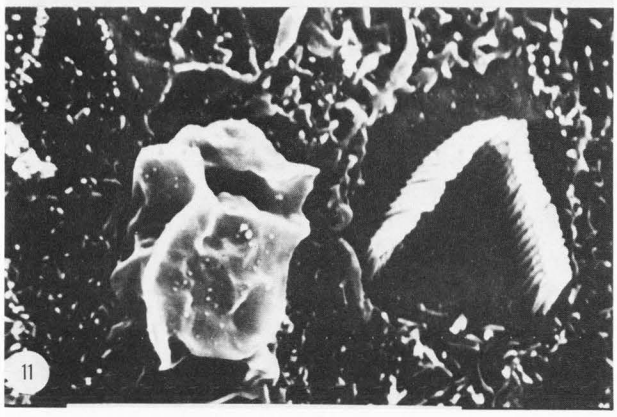












Figs 9-10. SEM.Crista ampullaris of the guinea pig following exposure to gentamicin. Many hair cells remain but show ballooning of the sensory cell surface (unfilled arrows) and various degrees of sensory hair fusion. Scale marks: 10 µm.

Fig. 11. SEM. Organ of Corti of the guinea pig following exposure to gentamicin. One outer hair cell in the second row has a normal surface structure while the adjacent hair cell is severely degenerated with bulging of cell content into the endolymphatic space. Scale mark: 10 µm.

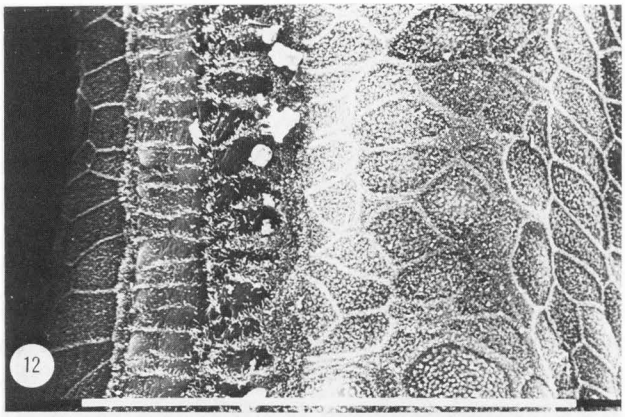
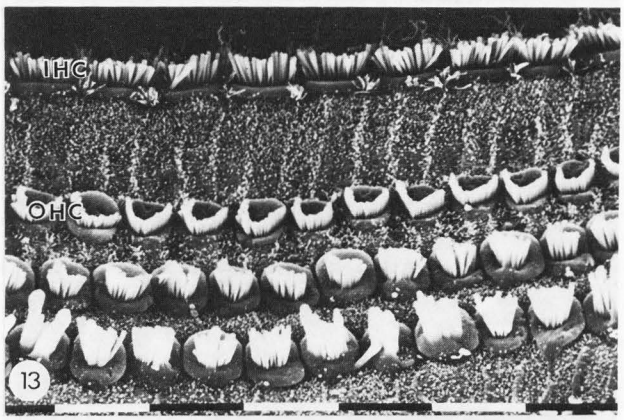
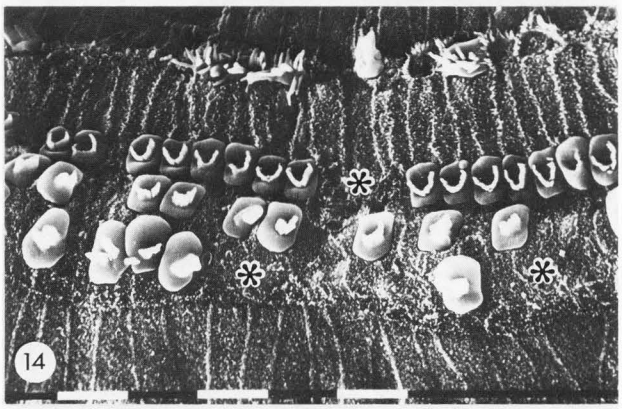
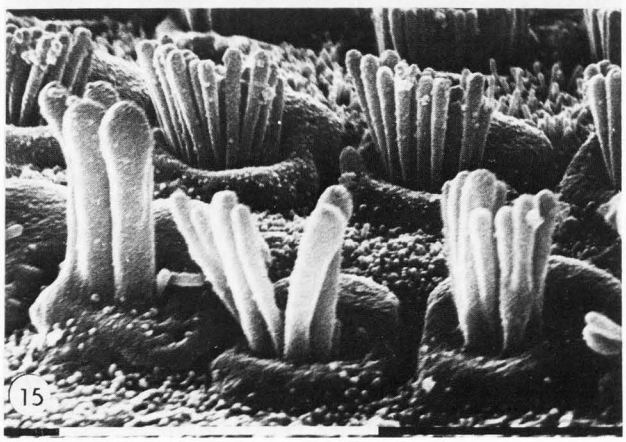


Fig. 12. SEM. Organ of Corti in the guinea pig following intoxication with gentamicin. Complete degeneration of hair cells which have been replaced with scarring tissue. Scale mark: 0.1 mm.







Figs 13–15. SEM. Organ of Corti of the waltzing guinea pig.

Fig 13. 2 week old guinea pig. Disarray of stereocilia on inner hair cells (IHC). Many outer hair cells (OHC) in the first row have a normal surface morphology whereas sensory hair fusion and bulging of the cuticular plate are obvious in the second and third rows of hair cells. Scale mark: 10 μm.

Fig 14. 8 week old guinea pig. Many inner and outer hair cells have degenerated and have been replaced with scarring tissue (asterisks). Scale mark: 10 μm.

Fig. 15. Two week old guinea pig. Early degenerative changes are manifested by sensory hair fusion starting close to cuticule. The cuticular plate has lost its rigidity and is slightly bulging into the subtectorial space. Scale mark: 10 μm.

Fig. 16. Crista ampularis of 6 week old waltzing guinea pig. The sensory cells show signs of early degeneration: slight bulging of the cuticular plate and sensory cell fusion starting basally. Scale mark: 2 μm.

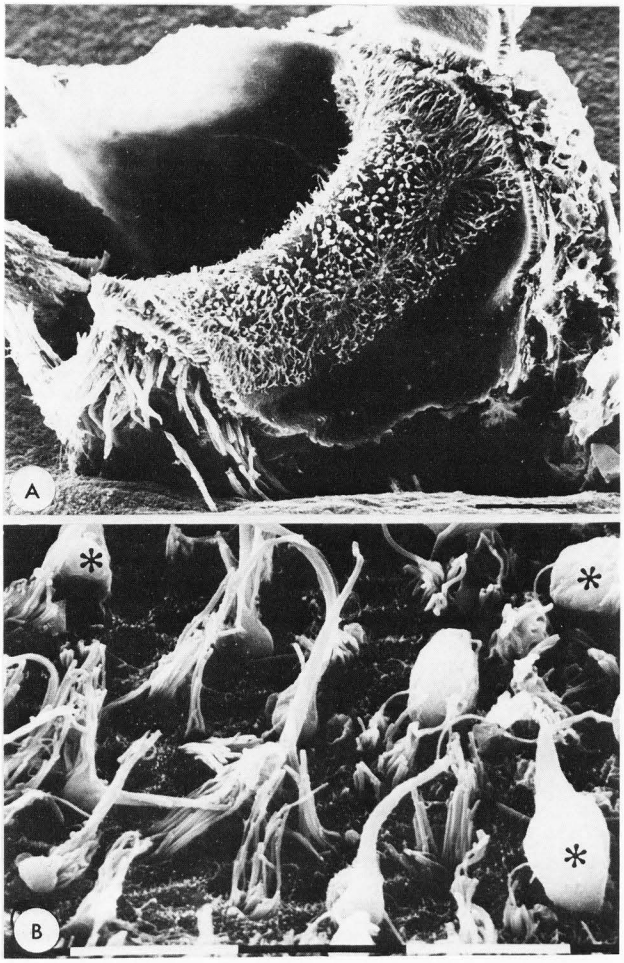


Fig. 17 A-B. SEM. Six month old waltzing guinea pig. A. Crista ampularis. The most advanced hair cell changes are confined to the central area of the crista. Scale mark: 0.1 mm. B. Macula utriculi. Moderate to severe morphologic cell degeneration. Many hair cells have an advanced sensory hair fusion and protrusion of cell content (asterisks) into endolymphatic space. Scale mark: 10 μm.

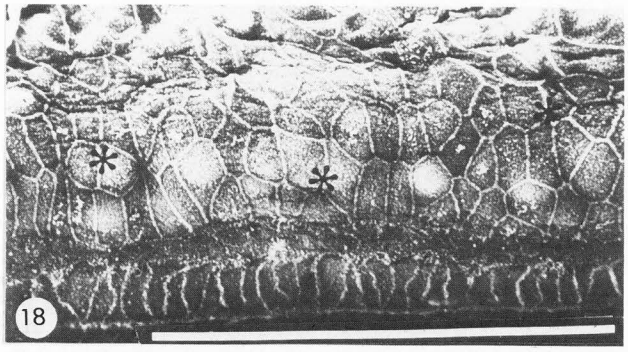


Fig. 18. SEM. Organ of Corti of 6 month old waltzing guinea pig. Complete sensory cell degeneration. Scarring tissue has replaced the sensory cells. Individual scarring cells are indicated with asterisks. Scale mark: 0.1 mm.

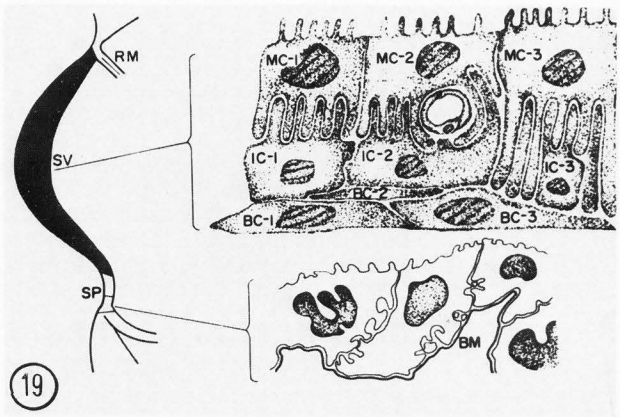


Fig. 19. Schematic drawing showing the principal cell shape of the epithelial cells covering the stria vascularis (SV) and the spiral prominence (SP) in the guinea-pig cochlea. MC, marginal cell; IC, intermediate cell; BC, basal cell; BM, basal membrane; RM, Reisner's membrane.

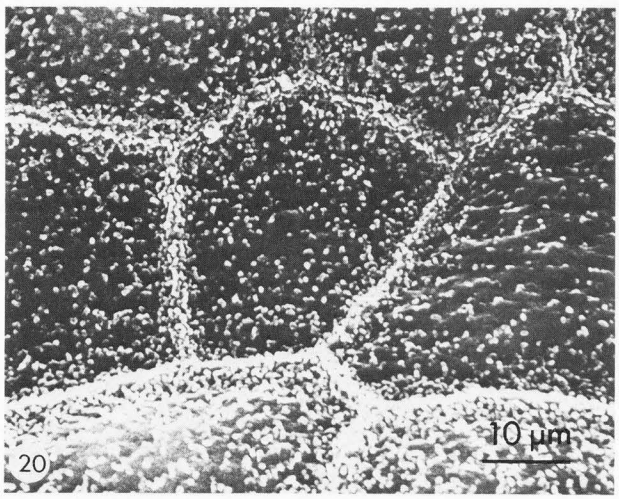


Fig. 20. SEM. Normal surface morphology of the stria vascularis. One cell has the form of pentagon. The individual cell surface is covered with microvilli.

Combination of SEM and X-Ray Microanalysis

Energy dispersive X-ray microanalysis (XRMA) is a new tool in inner ear research (9) and applications of this technique have focused on normal embryonic and adult conditions (Figs 24-25). In only three studies, all by Anniko et al. (6, 8, 11), have pathological conditions been analyzed (Figs 26-27). In one study (6), it was shown that in the endolymph of some deaf mutants with a shaking-waltzing behaviour (mouse strains: Kreisler, Shaker-1, and Shaker-2) the characteristic K-high and Na-poor composition is lacking in the adult animal. To measure the elemental composition of fluid-filled spaces at least semi-thick (> 16 μm) specimens were needed to give the same excitation volume at each

microprobe analysis. In this study, cryosectioned and freeze-dried specimens were used. For technical considerations see (7).

For studies on otoconia the SEM technique is a prerequisite for X-ray microanalysis. The aim is to analyze intact (non-sectioned, non-divided) otoconia which have a size of several microns. In this way it is possible also to analyze different parts of individual otoconia. Elemental analysis of otoconia from mutant species (Shaker-1 and Shaker-2 mice) has shown approximately the same elemental composition as in normal CBA/CBA animals (8). Statistically, some minor changes in the concentrations of Na, Mg, K and Ca occurred. It is, however, unlikely that the shaking-waltzing behaviour of Shaker-1 and Shaker-2 mutants should derive from a minimal derangement of the elemental composition of otoconia. Concerning human otoconia (11) the elemental compositon in Trisomy-18 did not deviate from normal conditions. In contrast, in normal old age the concentration of P increases considerably which may indicate a chemical change in Ca binding in ageing otoconia.

Comments

Although the mode of insult is different between acoustic trauma, antibiotic ototoxicity and a type of genetic inner ear disorders, the end result is commonly hair cell degeneration and subsequent sensorineural hearing loss. Changes of sensory hairs, softening and modification of the cuticular plate and eventually cell extrusion giving rise to supporting cell scarring are commonly found in surface pathology. The underlying cause(s) at the subcellular level is/are only partially known. These mechanisms can include the apical cytoskeleton, the plasma membrane or the anchoring between the cytoskeleton and the plasma membrane. It has to be noted that independent of which of the three main types of trauma the inner ear organs are exposed to, the primary target is the hair cell. Differences in vulnerability occur between hair cell types.

Only two substances have as yet been reported to cause surface pathology of the stria vascularis: atoxyl and ethacrynic acid. In spite of the different primary target areas (basal and apical, respectively), the structural changes are identical on the surface of the stria vascularis.

SEM permits the analysis of a large number of cells and cell types in the membranous labyrinth. Because of the very complex coiling of inner ear structures, the SEM technique is superior to any other technique in revealing early surface pathology confined to a small number of cells. During more advanced pathological changes, a rather wide range of morphological changes can often be found in the same inner ear organ. Thus, the progression in surface pathological changes can be followed.

SEM can be combined with a microprobe for elemental analysis. Since the electron beam penetrates one or more cell layers, the structures below the surface are unidentified. The structures included in the excitation volume to obtain an X-ray spectrum must, however, be clearly defined. Therefore, the combination of SEM and X-ray microanalysis in pathology has so far been restricted to only a few well defined areas: fluid-filled spaces and otoconia. To progress in microprobe analysis this has to be combined with the STEM system to improve the spatial resolution. The excitation volume will then include only the area of interest.

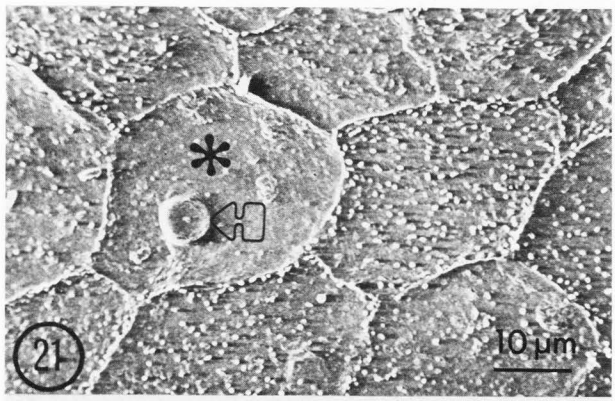
Figs. 21-23. SEM. Stria vascularis of the guinea pig following intoxication with atoxyl.
Fig. 21. Many strial cells have a normal surface morphology with a large number of small microvilli. However, one cell (asterisk) has lost these microvilli and a larger protrusion has formed (arrow).
Fig. 22. A large protrusion (asterisk) from the surface of several strial cells. Adjacent cells appear rather normal.
Fig. 23. Severe morphologic degeneration of the surface morphology. Microvilli are lost and individual cell borders cannot be identified with certainity.

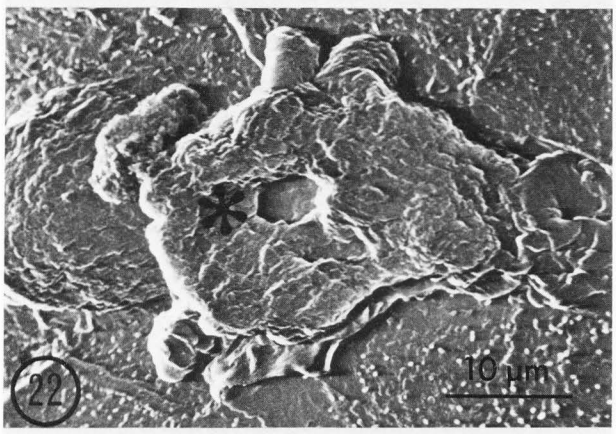
Fig. 24. Diagram showing the increase of the relative peak intensity of potassium (K) in the endolymphatic space from the 13th gestational day (otocyst stage), passing birth (star) until reaching adult conditions 8 days after birth (mouse). Analysis has been performed in SEM as square analysis of 16 µm thick freeze-dried cryosections (see reference 9). The great variation in relative peak intensity 4 days after birth is related to great variations in the morphologic maturation of the stria vascularis (considered as a source for producing endolymph) at this stage of development.

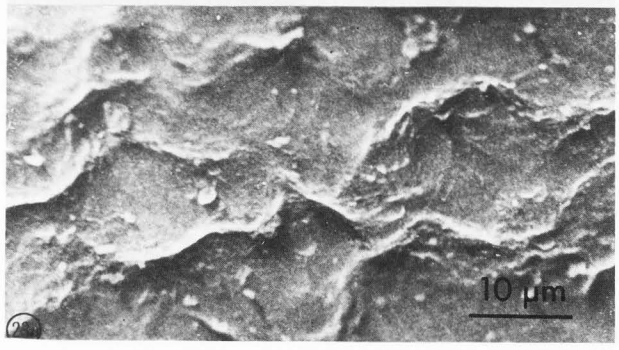
Fig. 25. Elemental histogram from endolymph of the newborn guinea pig. SEM of a 16 µm thick freeze-dried section. Peaks for Cl and K are obvious but also peak for Na, P and S occur. Full scale 2000 (Fig. A) and 200 (Fig. B), respectively.

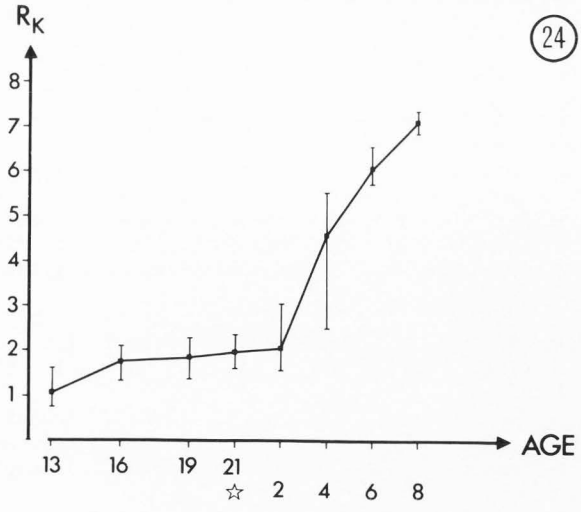
Fig. 26. Elemental histogram from endolymph of a 16 days old Shaker-1 mouse. SEM of a 16 µm thick freeze-dried section. Full scale 500. Peaks for Cl and K are identified but approximately in the same range as the peak for S. Also S and Na are identified. This indicates that a deterioration of endolymphic electrolytes occurs prior to morphological changes in the cochlear duct.

Fig. 27. Elemental histogram from the endolymph of the 40th gestational day guinea pig. Full scale 500. The elemental composition is immature. See reference 9. Distinct and high peaks for Cl and K have not yet developed. This histogram of immature endolymph is similar to the histogram from an animal with genetically induced deterioration of endolymph (Fig. 26).


21
10 µm


22
10 µm


10 µm


R_K
8
7
6
5
4
3
2
1
13
16
19
21
☆
2
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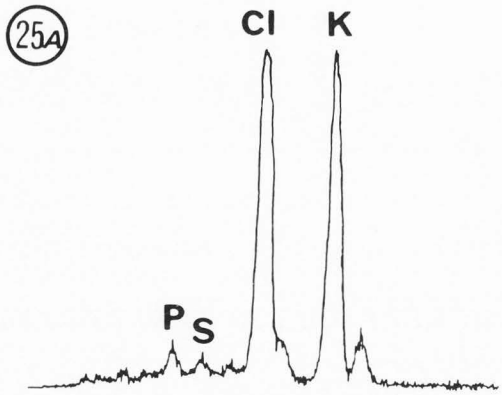
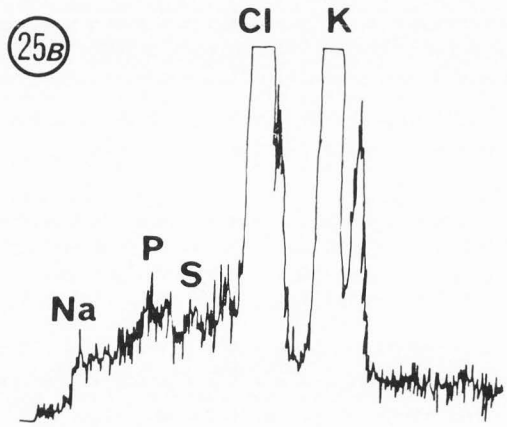
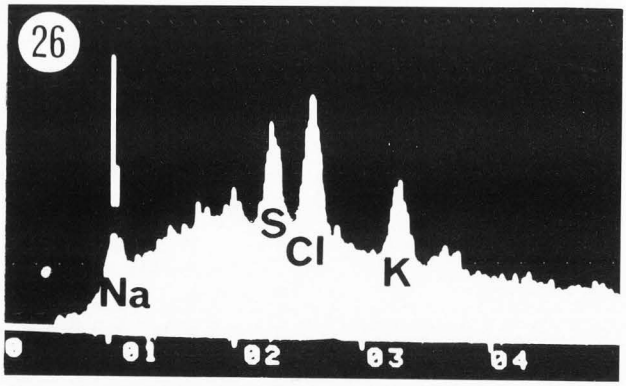
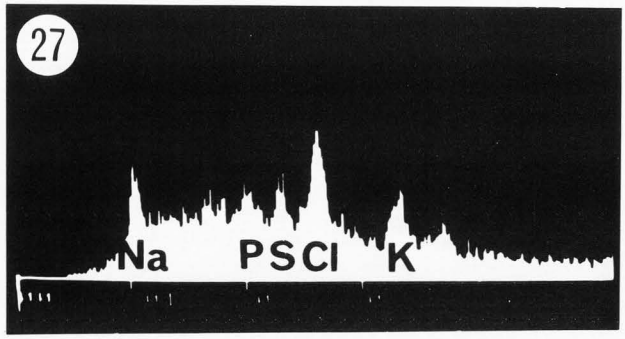
6
8
AGE

24


25A
Cl
K
P
S


25B
Cl
K
P
S
Na


26
S
Cl
K
Na
01
02
03
04


27
Na
PS
Cl
K

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Discussion with Reviewers

A.F Ryan: To explain the differences in drug-induced pathology of stria vascularis which you observed across cochlear turns, you suggest a progressive change in the physiological condition of the marginal cells in the basal-apical direction. Since the normal physiological condition of the marginal cells is still a matter of considerable speculation, what is meant by this? Also, can it really be assumed that drug access to the stria vascularis is uniform in all cochlear turns, given the variation in size and vascularization of the epithelium from base to apex?

Authors: In a recent study it was shown that an electrochemical gradient exists in endolymph from the base to the apex of the cochlea involving potassium and chlorine concentrations (48). This is likely to reflect differences in the capacity between the different turns to maintain the specific ionic composition of endolymph. Discussions on drug access to and endolymph in different regions of the cochlea can so far be only speculative.

K. Horner: You have indicated that ionic composition of the endolymph during development in normal and pathological models can be monitored with X-ray microanalysis. Could this technique be used to detect short term ionic changes associated with anoxia or ethracrynic acid intoxication?

Authors: This type of experiment has not yet been performed. The X-ray microanalysis is sensitive enough and suitable to measure also minor changes in the elemental composition of the endolymph.

Additional Reference

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