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NEW ULTRASTRUCTURAL EVIDENCE FOR THE EXISTENCE OF A GLYCOCALYX ON HAIR CELLS OF THE ORGAN OF CORTI

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Abstract

The recently demonstrated presence of a glycocalyx with antennulae on the tallest stereocilia of the hair cells of the three outer rows (OHC-I, II and III), was confirmed. There was a decrease in the amount of glycocalyx from the apical to the basolaminar side at the tallest individual stereocilia and from OHC-III to OHC-I. Optimal visualization of the glycocalyx with antennulae in FE-SEM was only possible at low accelerating voltage (2-3 kV), after glutaraldehyde prefixation and tannic acid/arginine/OsO_4 postfixation (TAO method) or OsO_4/thiocarbohydrazide (TCH)/ OsO_4/THC/ OsO_4 postfixation (OTOTO method), respectively. The former is a non-coating method with tannic acid, the latter method uses TCH as ligand. The glycocalyx with antennulae could also be demonstrated in the transmission electron microscope (TEM) at 60-80 kV after each of these non-coating treatments. A similar type of glycocalyx has been demonstrated on cat duodenum microvilli after TAO or OTOTO postfixation in the field-emission scanning electron microscope (FE-SEM) at 2-3 kV and in TEM at standard accelerating voltage (60-80 kV). Cryofixation and observation of the same duodenum tissue sample at 5-10 kV in FE-SEM showed the same glycocalyx on the microvilli, demonstrating without any doubt the existence of the glycocalyx with a filamentous appearance (penicillium) even without chemical fixation.

Key Words: Organ of Corti, glycocalyx, antennulae, penicillium, low voltage field emission scanning electron microscopy, cryo field emission electron microscopy, transmission electron microscopy (TEM), non- coating techniques, thiocarbohydrazide, duodenum.

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Introduction

Recently, Jongebloed *et al*. (1996) demonstrated the presence of a glycocalyx with antennulae on the stereocilia of hair cells of the three outer rows of hair cells (OHC's) of the organ of Corti in the guinea pig at 2-3 kV in a field emission scanning electron microscope (FE-SEM). In that study, the tannic acid/arginine/osmium tetroxide (TAO) postfixation method was used, following a perfusion and immersion prefixation with glutaraldehyde. The presence of a glycocalyx could be demonstrated at the tallest stereocilia of each of the hair cells of the three outer rows. Demonstration of the glycocalyx with antennulae was also possible after osmium tetroxide/thiocarbohydrazide (TCH)/osmium tetroxide/TCH/osmium tetroxide (OTOTO) postfixation. However, this fixation procedure, with TCH as the active component, is less appropriate because it often results in brittle samples.

Earlier attempts to visualize the glycocalyx in a scanning electron microscope (SEM) failed, mainly due to the inferior preservation of the glycocalyx constituents and the relatively high accelerating voltage employed in a conventional SEM (with a W or LaB_6 cathode). Moreover, the high accelerating voltage applied (15-25 kV), necessary to obtain optimal resolution with a conventional SEM, required a rather thick conductive coating (4-6 nm), which often obscures surface details present.

Gold (Au) coatings are known for their decoration effects, which cause an artificial appearance of the surface, even at thin (2-3 nm) layers, particularly when Au is applied by means of diode- or ion sputtering techniques. With these techniques large grains or metal nuclei are formed on the specimen surface, and these grains can easily conglomerate to larger complexes. Thin coatings of Pt, W and in particular Cr, produced with so-called planar magnetron sputtering devices, give such small grains that almost no sub-structure is observable, not even in high resolution transmission electron microscopy (TEM) images (Apkarian, 1994; Stokroos *et al*., 1995).

The high accelerating voltages applied in the past resulted in a rather large electron penetration volume in the specimen. The backscattered electrons (BSE) emerging from that volume produce, after several collisions, a high yield of

type II secondary electrons (SE-II) at the specimen surface at some distance from the point of impact of the electron beam. In contrast, type I secondary electrons (SE-I) are generated at the point of impact of the beam. Lowering the accelerating voltage in a conventional SEM results in a poorer signal/noise ratio and attainable resolution, mainly because insufficient secondary electrons are generated at low kV. This explains why even the application of non coating techniques did not lead to visualization of the glycocalyx in a conventional SEM, neither at high nor at low accelerating voltage. At high kV the secondary electrons generated at the impact of the electron beam (SE-I), are drowned in the high yield of SE-II and SE-III generated by the emerging BSE, which do not give topographic information but rather contribute to the noise. At low kV, the low yield of SE is low, and an increase of the spot size results in poorer resolution. Only a high current density electron beam from a high brightness field emission cathode produces a very small spot with a high intensity on the specimen surface; this guarantees high resolution even at low kV.

Observation of a specimen prepared by the OTOTO or TAO method in the FE-SEM at 2-3 kV was successful for various reasons: [1] the resolution at low kV in FE-SEM is almost the same as that of a LaB_6-SEM at 15-25 kV, [2] backscattered electrons (BE), formed by primary electrons reflecting against the specimen surface, and secondary electrons (SE), formed by the impact of the primary electrons at the specimen surface, contribute to the final image, [3] thiocarbohydrazide or tannic acid, respectively, in combination with osmium tetroxide, can optimally preserve the mucopolysaccharide complexes in the glycocalyx.

In TEM the presence of a glycocalyx could be observed in an indirect way by means of tracers such as cationized ferritin and colloidal thorium, indicating the existence of anionic sites in the glycocalyx with antennulae (Slepecky and Chamberlain, 1986; Lim, 1986; Santi and Anderson, 1987; Osborne *et al*., 1988). Identification of these anionic sites was carried out by means of enzymatic digestion via treatment with neuraminidase and hyaluronidase, which made it impossible for the agents mentioned above to bind to those sites (Van Benthem *et al*., 1992).

The presence of filamentous material or antennulae has lead to speculations about its origin and whether it is a real entity or an artefact due to fixation. The aim of this investigation was therefore to prove whether the antennulae were real or a product of preparation/fixation, by posing and answering a number of relevant questions:

[a] Why have not others seen the antennulae before?

[b] Are all stereocilia covered by a glycocalyx with antennulae?

[c] Is there a relation between the tectorial membrane undersurface and the fine structure of the antennulae?

[d] Can the glycocalyx with antennulae be demonstrated in TEM images?

[e] Can a glycocalyx with filamentous material be demonstrated in other tissues, such as duodenum with these non coating methods?

[f] Is it possible to demonstrate glycocalyx in tissues with cryo-fixation?

Materials and Methods

Animal treatment and prefixation procedure

Cat duodenum microvillous glycocalyx. Young cats were anaesthetized with pentobarbital and given ketamine for muscle relaxation. The duodenum was first perfused for 2 min via the mesenteric artery with 0.1 M sodium cacodylate buffer, containing 2% polyvinylpyrrolidone (PVP) (molecular weight = 40000) and 75 mM $NaNO_2$ and subsequently prefixed by perfusion for 30 min with sodium cacodylate buffered glutaraldehyde (GA) (2%; pH 7.4). Approximately 10 cm of duodenum was removed and the contents of the lumen carefully washed out with 0.9% NaCl in distilled water. The tissue blocks were then fixed by immersion in the same fixative for 24 hours and then rinsed 6 times with buffer solution.

Guinea pig hair cell stereociliar glycocalyx. Six weeks old animals were sacrificed by sublethal administration of sodium pentobarbital (60 mg/kg body weight). This was followed by a transcardial perfusion with a mixture of 0.1 M sodium cacodylate buffer, 2% PVP, 0.4% $NaNO_2$ and heparine (pH 7.4; 400 mOsmol; room temperature [RT], 5 min; flow rate 15 ml/min); subsequently a perfusion prefixation was carried out with 0.1 M cacodylate buffered glutaraldehyde (2%; pH 7.4; RT; 10 min; 450 mOsmol). Alternatively a similar perfusion and immersion prefixation was carried out with a so-called tri-aldehyde fixative consisting of a mixture of 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolein and 2.5 % dimethylsulfoxide (DMSO) in 0.08 M cacodylate buffer (pH 7.4; RT; 1500 mOsmol). Subsequently the bullae were removed and the round window and apex opened, for better exchange of various fixatives and endolymph. This was followed by an immersion prefixation for 24 hours with the same buffered fixative at 4°C, removal of the lateral bony wall and stria vascularis, and rinsing with buffer solution prior to the postfixation procedure.

Postfixation procedures for guinea pig and cat tissue

Postfixation was carried out according the conventional OsO_4 method, the TAO non-coating method or the OTOTO non-coating method respectively (Jongebloed *et al*., 1996).

Cryo fixation of cat duodenum tissue

Vibratome tissue slices of approximately 100 µm thickness were mounted on a TEM copper grid and the

excess buffer solution was removed with filter paper. Specimens were then slam frozen on a copper mirror cooled with liquid N_2 in a Reichert (Vienna, Austria) KF 80 apparatus. Then the frozen specimens were transferred to an Oxford Instruments (Oxford, UK) 1500 HT cryo-transfer unit, etched for approximately 60 sec, sputter coated with 1.5 nm Au/Pd and finally transferred to a FE-SEM.

Dehydration and drying or embedding and microscopical observation

The chemically fixed samples were dehydrated in an ethanol series up till 100% ethanol and then prepared for SEM and TEM respectively.

FE-SEM. The dehydrated samples were critical point dried (CPD) in liquid CO_2 and investigated without external conductive coating in a JEOL (Tokyo, Japan) FE-SEM, type 6301F, operated at 2-5 kV (cryo samples 5-10 kV); working distance: 6-15 mm and probe current 1.0-1.5 x 10^{-11} A.

TEM. The CPD SEM samples were coated with 1.5 nm Au/Pd, prior to embedding in epoxy resin (Serva, Heidelberg, Germany) under vacuum (1.3 x 10^4 Pa), sectioned according to standard methods and observed in an Akashi (Tokyo, Japan) TEM type 002A, operated at 60-80 kV.

Results

Why have not others seen the antennulae before?

There could be a number of reasons for this such as: inferior preservation of the glycocalyx by glutaraldehyde/osmium tetroxide (OsO_4), observation at a too high an accelerating voltage or a combination of both, as discussed in the Introduction. The presence of probable remnants of a glycocalyx has been acknowledged after conventional (GA/OsO_4) fixation and sputter-coating with 3-4 nm Au/Pd at 10 kV. The inferior preservation of the mucopolysaccharides in the glycocalyx by GA/OsO_4, requires the presence of a conductive coating of approximately 4-6 nm at 15-25 kV; the applied metal layer thoroughly obscured surface details. The accelerating voltage employed was in most cases too high to demonstrate these surface phenomena.

Are all stereocilia covered by glycocalyx with antennulae?

Three rows of outer hair cells (OHC-I, II and III) are shown in Fig. 1. From this image it is obvious that there is a gradient in thickness of the glycocalyx from OHC-III to OHC-I. The glycocalyx is thickest in OHC-III and decreases in thickness towards OHC-I, as can be seen in Fig. 2 (OHC-III), Fig. 3 (OHC-II) and Fig. 4 (OHC-I). Moreover, the glycocalyx is only present on the longest stereocilia of each hair cell, and decreases from the apical towards the basolamilar part. In the OHC-I the glycocalyx with antennulae is almost only present at the apical part of the longest stereocilia. The arrangement of the IHC stereocilia is not V- or W- shaped, but consists of rows of stereocilia positioned behind each other; no glycocalyx with antennulae is found here (Fig. 5). The stereociliar plasma membrane seems somewhat roughened, showing a sort of dense cap as observed already before by Dunnebier *et al*. (1995). In some of the micrographs Deiters' cells can be seen. The perfusion/immersion fixation of the samples shown in Figs. 1-5 was carried out with cacodylate-buffered glutaraldehyde. When a so-called tri-aldehyde prefixation was carried out with a mixture of glutaraldehyde, paraformaldehyde and acrolein (see Materials and Methods section), detachment of stereocilia from the cuticular plate occurred more often, as seen in Fig. 6. This mixture is a good fixative for TEM studies (De Groot, 1986), but our SEM studies show that the crosslinking between tectorial membrane and hair cell stereocilia increases, resulting in detachment of the stereocilia from the cuticular plate of the hair cells after CPD. Part of the stereocilia, sometimes complete rows of stereocilia of a hair cell, attach to the undersurface of the tectorial membrane, leaving only small stubs as remnants of the 3 rows of stereocilia present on one hair cell, as shown in more detail in Figs. 7 and 8.

Is there a relation between the tectorial membrane undersurface and the fine structure of the antennulae?

The tallest stereocilia of the hair cells of the three outer row (OHC-I, II and III) are connected to the undersurface of the tectorial membrane and form imprints in this membrane. In the sound state the tectorial membrane covers the outer and inner rows of hair cells and the phalangeal cells and the marginal end is connected to the base of the organ of Corti by means of a button-like structure. Observing the imprints of the longest stereocilia of the hair cells OHC-I, II and III (Fig. 9), it is obvious that around the imprints of OHC-I and OHC-II no remnants are found of the stereociliar glycocalyx with antennulae, in contrast with the area around the imprints of OHC-III. Fig. 10 shows part of the tectorial membrane undersurface with an imprint of the OHC-III stereocilia. The fine structure of the marginal end is different from that of the rest of the tectorial membrane, its undersurface shows a rather rough texture with a needle-like appearance, as can be seen in more detail in Fig. 11. The area around the imprints of the longest stereocilia of OHC-III is seen in more detail in Fig 12. Patches of the glycocalyx with antennulae are found close to the imprint. This is different from the area around the imprint of OHC-II stereocilia, which is seen in Fig. 13. Top and bottom side of the tectorial membrane have a completely different fine structure as shown in Fig. 14. The retracted tectorial membrane shows at the undersurface of the marginal zone (the part that originally was connected to the outer phalangeal cells) a needle-like structure, as can be seen in great detail in Fig. 11. At the top side it shows a reasonably smooth surface, even compared to the fibre-like structure of the main part of the tectorial membrane.

Can a glycocalyx with antennulae be demonstrated in TEM, analogous to SEM?

Earlier attempts to demonstrate the glycocalyx in TEM failed, because the conventional GA/OsO_4 fixation failed to preserve the constituents of the glycocalyx, as discussed before. With thorium ions Van Benthem *et al.* (1992) could demonstrate the anionic sites at the glycocalyx; thorium could be seen in TEM as small dots. The TEM image of the organ of Corti (Fig. 15), shows successively an IHC, OHC-I, OHC-II and OHC-III hair cell. The glycocalyx with antennulae can barely be discerned on the stereocilia of OHC-III, the inset of this figure shows the glycocalyx more convincingly. As comparison to the TEM inset of Fig. 15, a FE-SEM image of an OHC-III hair cell with stereocilia is shown in Fig. 16. The glycocalyx with antennulae is clearly visible in particular at the apical side and decreases in thickness towards the basolaminar part (the part connected to the cuticular plate) of the stereocilia. When the OHC-II and OHC-III of Fig. 15 are shown in more detail, as seen in Fig. 17, it is obvious that the TAO non-coating technique can preserve the different cell organelles of the hair cells quite well. It is also obvious that the glycocalyx with antennulae is thicker in OHC-III compared to OHC-II. In the high magnification TEM image of the stereocilia with antennulae of an OHC-III hair cell it is not evident, whether the antennulae are connected to the stereociliar cytoplasm. At certain places it seems that the antennulae seem to end somewhere in the body of the stereocilium (Fig. 18). But the image is somewhat misleading, because due to the sectioning and the transmission image, a superposition of structures can occur. A close examination of the stereocilia shows the Au/Pd particles covering the stereocilia, but does not show very well the stereociliar "membrane". Possibly, as a result of the high contrast the phospholipid structure becomes blurred in the total contrast of the image. Fig. 19 shows the three stereocilia of an OHC-II in cross section with the antennulae mainly at the top. Fig. 20, however, shows two stereocilia of the inner row of hair cells. The surrounding membrane is much more evident as a dark line, although its contrast has increased as a result of the thin Au/Pd coating. This was necessary to improve the visibility of the glycocalyx with antennulae in the TEM image, as discussed before. The dense cap of the IHC stereocilia is evident as well; the microfilaments inside the stereocilium are obvious at certain places.

Can a glycocalyx with filamentous material be demonstrated in other tissues when post-fixed with the same non-coating methods?

Conventional (GA/OsO_4) fixation of the glycocalyx of the intestinal microvilli often leads to poor preservation and sometimes to conglomeration of the thin penicillium (antennulae). The duodenum of the cat is known to have a rather thick glycocalyx, which can be demonstrated in TEM quite well after glutaraldehyde prefixation and postfixation with OTOTO, TAO or a combination of osmium tetroxide/tannic acid/osmium tetroxide. Fig. 21 represents a TEM image of a cross section of the microvilli and penicillium (antennulae) of the cat duodenum after OTOTO treatment. When the microvilli are cut in a different direction (Fig. 22) the microvilli appear as small circles where the microfilaments within the microvilli can be seen as small dots and the glycocalyx as a granular mass at the top of the image. When an almost similar situation is observed with FE-SEM (Fig. 23) the glycocalyx can be observed on top of the microvilli and partly along the lateral sides of individual microvilli; the image is comparable with the TEM image of Fig. 21. When the glycocalyx is observed in top-view (Fig. 24), the typical net-like structure becomes evident which is

Figure 1. Three outer rows of hair cells (OHC-I, OHC-II and OHC-III) of the guinea pig organ of Corti, showing a decrease in glycocalyx covering from OHC-III to OHC-I. Note the inner pillar cells (IP) and Deiters' cell (DC) associated with microvilli (↗). Bar = 1 μm.

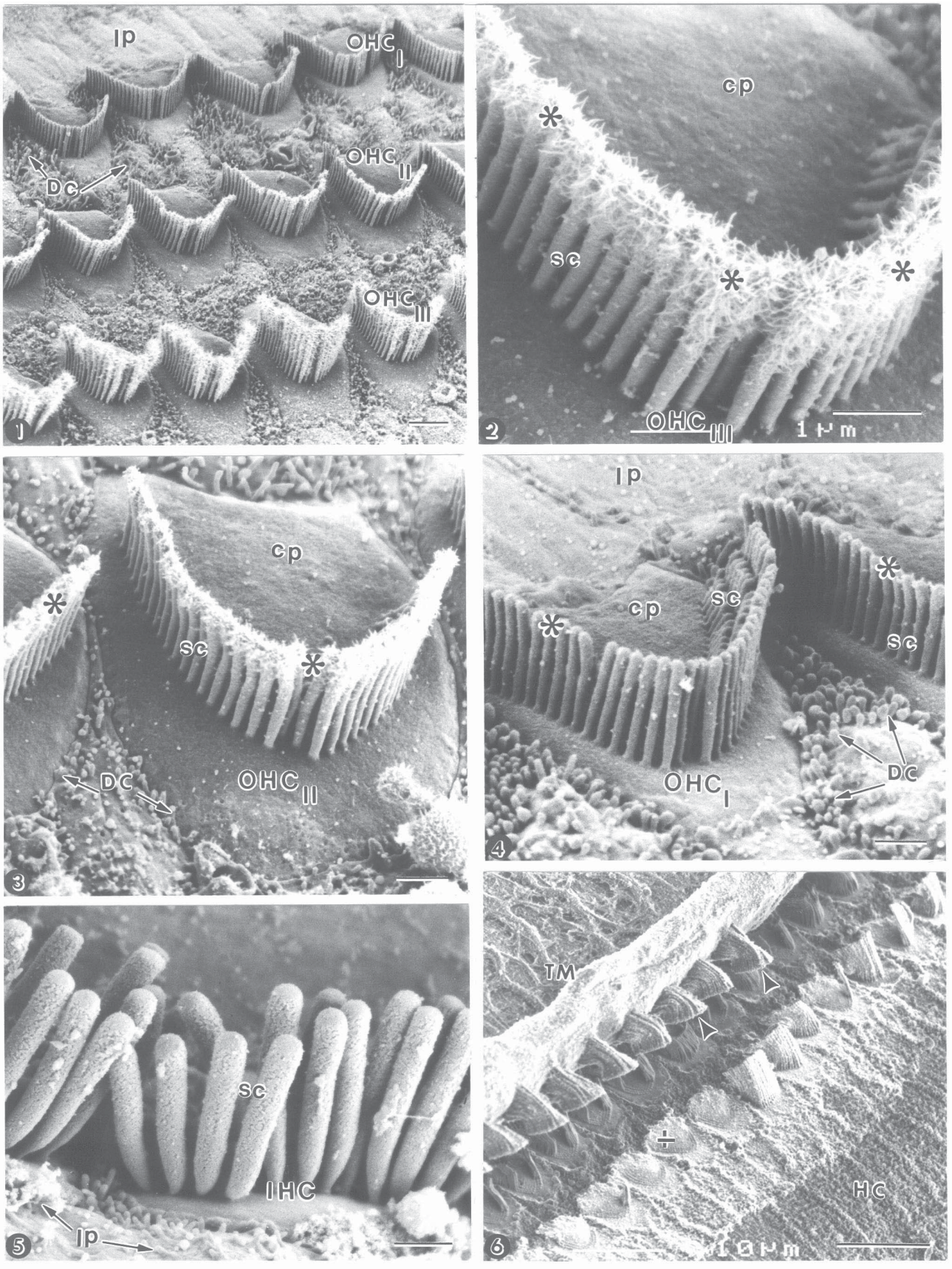
Figure 2. Part of OHC-III, showing glycocalyx with antennulae (*) particularly at the apical part of the longest stereocilia (sc), and cuticular plate (cp). Bar = 1 μm.

Figure 3. Hair cell of the middle row (OHC-II) with glycocalyx (*) mainly on the apical part of the stereocilia (sc), cuticular plate (cp) and microvilli (arrows) of Deiters' cells (DC). Note decrease of amount of glycocalyx in comparison with OHC-III. Bar = 1 μm.

Figure 4. Hair cell from the inner most row of outer hair cells (OHC-I) showing clearly three rows (short, medium and long) of stereocilia (sc), almost no glycocalyx (*) can be seen at the longest stereocilia (sc). Deiters cells (DC) with microvilli (arrows), Cuticular plate (cp) and inner pillar cells (IP) are shown. Bar = 1 μm.

Figure 5. Stereocilia (sc) of the inner row of hair cells (IHC), inner pillar cells (IP). Note the rather rough appearance of the outer stereociliar structure without antennulae and dense structure at the top. Bar = 1 μm.

Figure 6. Low magnification of the part of organ of Corti with completely broken-off (+) hair cells of the outer rows after tri-aldehyde prefixation (GA + PF + Acrolein). Complete hair cells (arrowheads) attached to the undersurface of the partly retracted tectorial membrane (TM). Note fibre-texture of TM upper surface and Hensens' cells (HC). Bar = 10 μm.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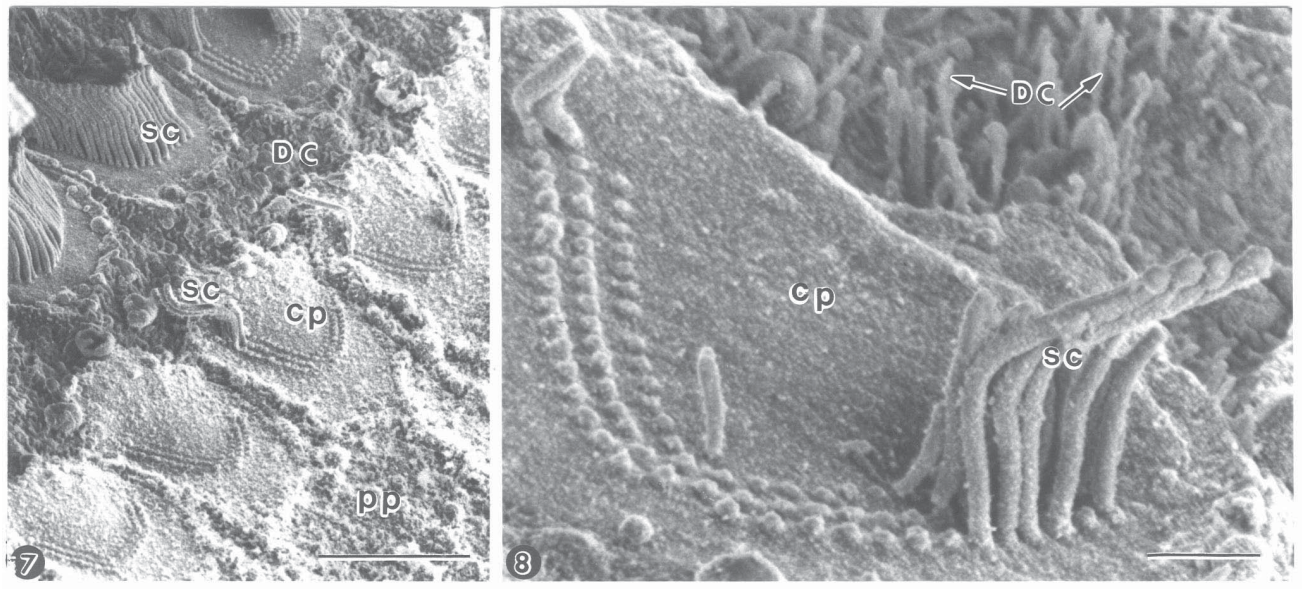

Figures 7-8. Detail of broken-off stereocilia (sc); note small stubs as remnants of stereocilia besides a few partly intact stereocilia from the rows with short, medium and longest stereocilia. Cuticular plate (cp), Deiters' cells (DC) with microvilli (arrows) and the phalangeal plate (pp). Bar = 5 μm (Fig. 7); Bar = 1 μm (Fig. 8).

(Figures on facing page)

Figure 9. V or W- shaped imprints of longest stereocilia of OHC-I, OHC-II and OHC-III in the undersurface of the tectorial membrane (TM). Note the different surface structure of the partly retracted (X) edge of the tectorial membrane. Bar = 5 μm.

Figure 10. Detail of the retracted edge of the tectorial membrane (TM) and the area around the OHC-III stereociliar imprint. Note the patches of glycocalyx material (*) mostly above the imprint. Bar = 1 μm.

Figure 11. Detail of the retracted edge of tectorial membrane with needle-like structure, note basic structure of tectorial membrane undersurface (Hardesty layer), as a groundwork of fibrils and small globular units (arrowheads), in particular in the top part of the image. Bar = 1 μm.

Figure 12. Higher magnification of the tectorial membrane around the OHC-III stereociliar imprint, note patches of glycocalyx material (*). Ground structure of the Hardesty layer consisting of fibrils and small globular units (arrowheads). Bar = 1 μm.

Figure 13. Detail of tectorial membrane undersurface with imprints of the tallest OHC-II stereocilia. Note the pattern of stereociliar indentations (*); no patches of glycocalyx are present. Bar = 1 μm.

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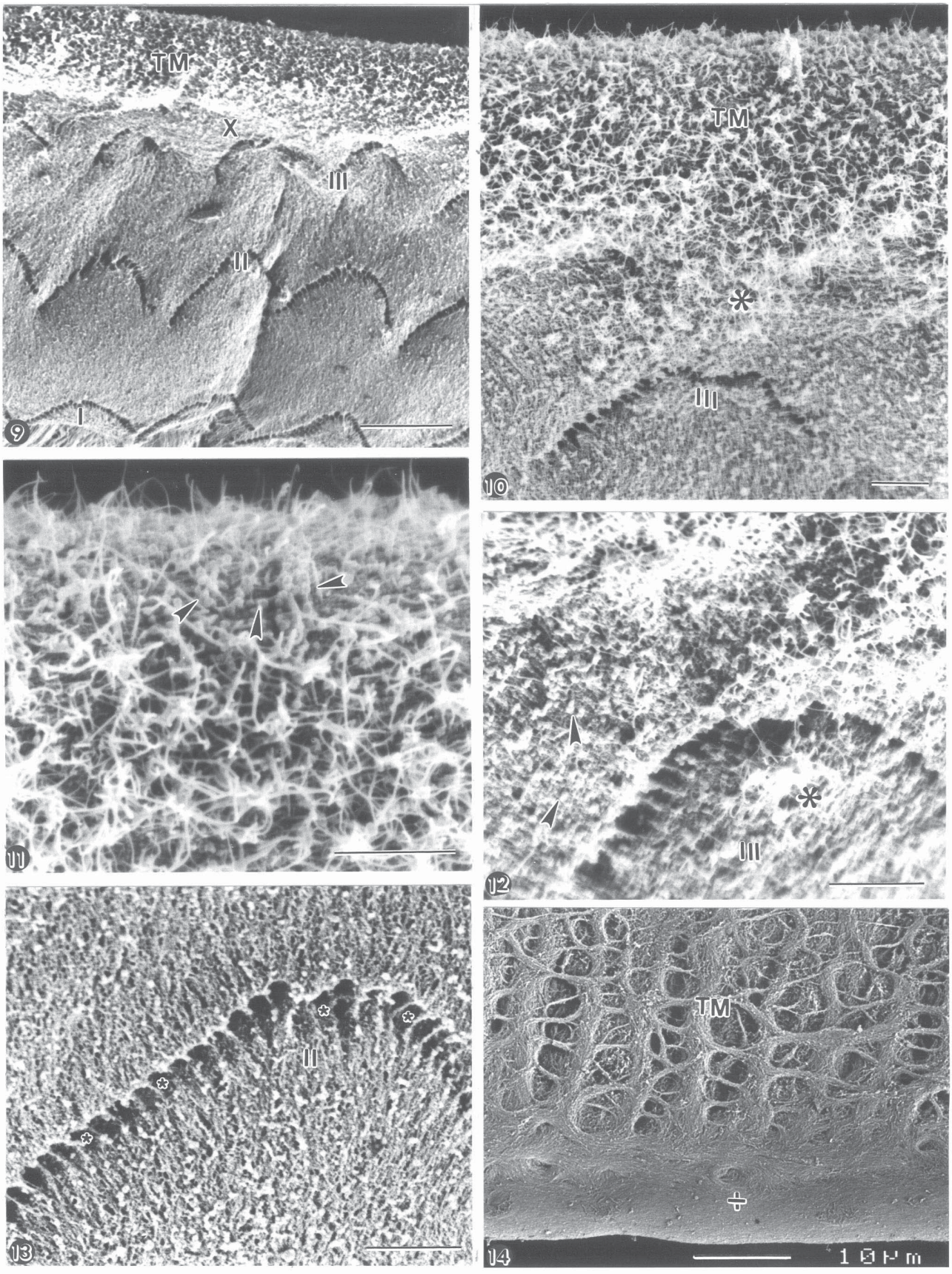
comparable with the structure shown in Fig. 21. At some places the glycocalyx seems slightly thinner than at other places, so that the apical surface of the microvilli becomes visible. Figs. 21 and 22 show that the non-coating treatment certainly can demonstrate the glycocalyx with filamentous material in other tissues as well.

Can a glycocalyx with filamentous material be demonstrated in tissues without chemical fixation?

To prove that a chemical treatment either as prefixation or postfixation procedure could not induce these antennulae, a preservation *without* chemicals was necessary. For technical reasons, it was decided to carry out cryofixation of the duodenum, while cryopreparation of segments of the organ of Corti is in progress. Because the vibratome slices are taken from a buffer solution (in this case sodium cacodylate), ice containing cacodylate salts will appear as well. The glycocalyx can be observed as a thin layer on top of the microvilli in a cross view, while at some distance from the glycocalyx the frozen buffer solution can be seen (Fig. 25). In a top view of a cryo-prepared sample cross fractures of microvilli are seen with the filamentous material of the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glycocalyx on top of and along the microvilli, proving that also without chemical fixation the glycocalyx with filamentous material can be observed. The cryo-specimen represents a fracture through a vibratome section of the intestine, hence different fracture faces will appear. The typical dotted structure inside each microvillus is due to the microfilaments (Fig. 26), and this is comparable with the dotted structure seen in Fig. 22. At some places the presence of glycocalyx both at the top of the microvilli as well as in between the microvilli is obvious. Sometimes the complete top of the microvilli including part of the interior was removed by fracturing, leaving a kind of hollow microvilli behind.

Discussion

Tannic acid is known for its strong binding to acid mucopolysaccharides (Murakami, 1974), which form the glycocalyx constituents. Therefore, visualization of antennulae after TAO postfixation method could be a result of strong adherence of the glycocalyx to the tectorial membrane (TM). The antennulae would be part of the TM undersurface, ripped off from that surface when the tectorial membrane and stereocilia separate by forces exerted during fixation and drying. The active group of tannic acid is galloyl glucose, consisting of three phenolic groups bound to glucose. This group can react with hydroxyl-, carboxyl-, and sulfonic groups of amino acids. It also can react with carbohydrates, such as hyaluronic acid and various glycoproteins by means of electrostatic binding. Moreover it can react with arginine, lysine and collagen by means of a non-electrostatic binding. Galloyl glucose is most effective with non-osmicated tissues; the immersion in a mixture of arginine-HCl, glycine, sucrose and sodium glutamate follows prefixation with glutaraldehyde (Murakami, 1974; Murphy, 1978; Chaplin, 1985).

Also with the OTOTO non-coating technique the glycocalyx can be demonstrated. The ligand thiocarbohydrazide (TCH) has three active groups: two NH_2 and one S=H group, which can react with tissue- bound osmium or additional OsO_4 (Kalicharan *et al.*, 1992; Jongebloed *et al.*, 1992).

FE-SEM images of conventionally fixed samples suggest the presence of a glycocalyx. But the image, even in FE-SEM does not come up to modern standards. Due to the poor preservation, the stability of the sample is insufficient leading to volume changes during SEM observation. The poor conductivity requires an external conductive layer to be applied which can obscure surface details and induce conglomeration of coating particles and fine filamentous material, reducing the quality of the image.

The prefixation plays an important role as well, particularly in the retraction of the tectorial membrane (TM).

(Figures 15-20 on facing page)

Figure 15. TEM overview of OHC-I, OHC-II and OHC-III hair cells with stereocilia, on the last one the antennulae are vaguely discernible; in the outermost left corner an inner hair cell with stereocilia (IHC) is observable. The inset shows a detail of a few stereocilia (sc) of an OHC-III hair cell with antennulae (*). Bar = 10 μm (main figure); Bar = 1 μm (inset).

Figure 16. Three dimensional FE-SEM image of similar stereocilia (sc) after TAO non- coating postfixation. Note the decrease in amount of the glycocalyx (*) with antennulae towards the basolaminar side of the stereocilia. Bar = 1 μm.

Figure 17. TEM micrograph of an ultrathin section of OHC-II and OHC-III hair cells and Deiters' cell (DC) with microvilli (arrows); note glycocalyx (*) with antennulae and various organelles in the hair cell cytoplasm (cy). Bar = 2.5 μm.

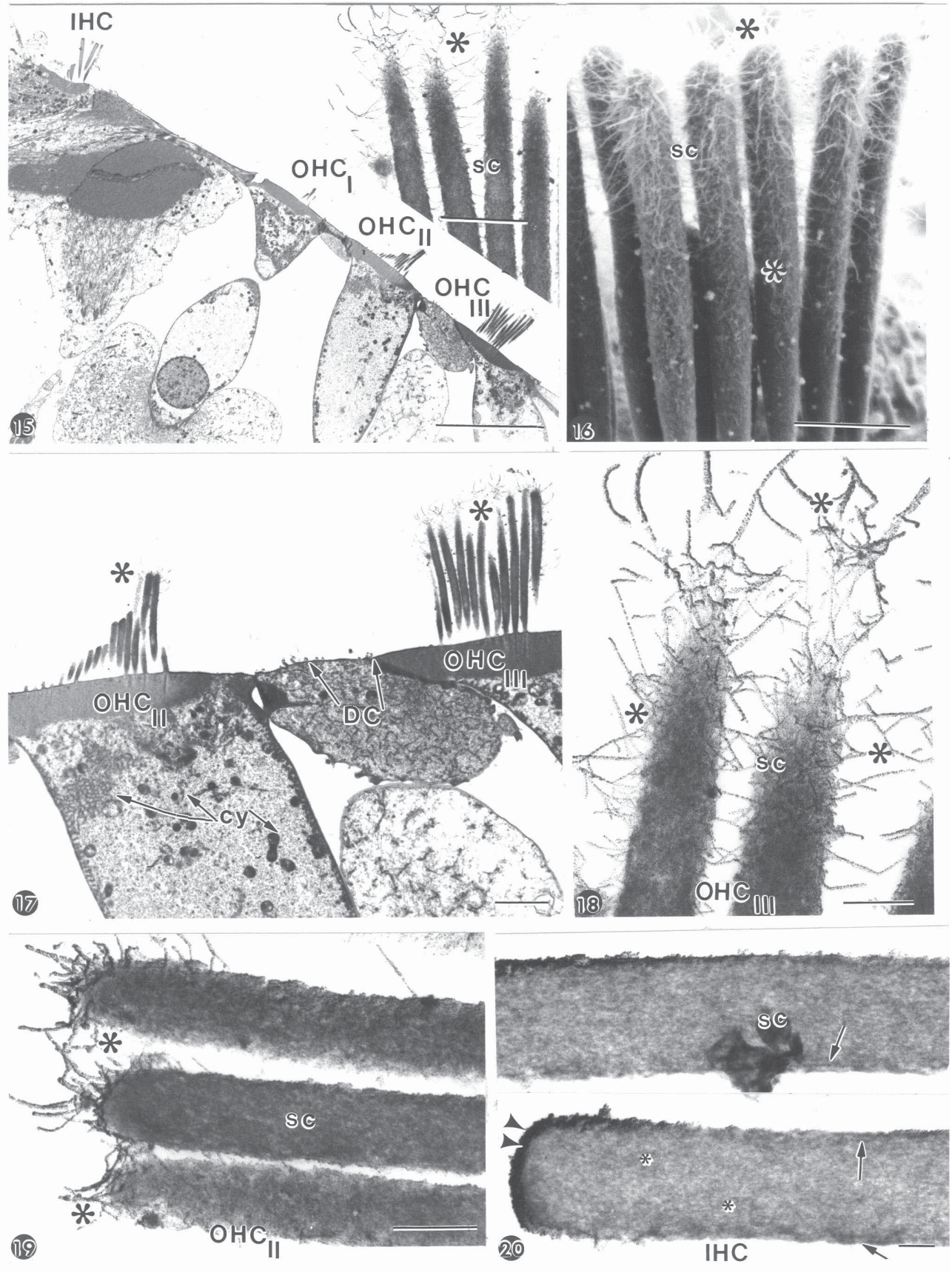
Figure 18. Detail of two stereocilia of an OHC-III cell after TAO non coating postfixation. The antennulae can be easily seen due to the (thin) Au sputter coating applied, prior to ultrathin sectioning. Bar = 250 nm.

Figure 19. Detail of OHC-II stereocilia (sc), showing glycocalyx (*) with antennulae. The high contrast of the TAO method masks the internal structure of the stereocilia. Bar = 250 nm.

Figure 20. Detail of IHC stereocilia (sc). Some arrangement in the cytoplasmic filaments can be seen at a few places (*), while at some places a highly contrasting inner and outer phospholipid layer is visible (arrows). The dense cap (arrowheads) on top of the stereocilium is evident. Bar = 100 nm.

One of the reasons for a certain indistinctness of the TM is the difficulty in obtaining a reproducible morphology. The TM is subject to marked distortion when it is fixed and dried or embedded for SEM and TEM examination, respectively. Werner (1937) reported about changes and discussed why the TM is rarely, if ever, chemically fixed in its natural position. He described how the TM can shrink in three directions: in height, in width and longitudinally (parallel to the axis of the cochlear duct).

Glutaraldehyde, used as perfusion and immersion prefixative, produces a fairly controllable retraction of the TM, in great contrast to a prefixation with a trialdehyde mixture of glutaraldehyde/paraformaldehyde/ acrolein. This fixative mixture produces such a strong cross-linking between the hair cell stereocilia and the tectorial membrane, so that complete stereocilia are ripped off from their cuticular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IHC
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plate (resulting from forces exerted during fixation) and attach to the undersurface of the TM. This is probably due to the high osmolarity of the mixture (> 1000 mOsmol) and/or the high reactivity of acrolein.

The TM can be divided in three zones: the limbal, the middle and the marginal zone, the latter ends externally in the marginal net (Lim, 1972). The middle zone contains a homogenous layer, which is continuous with the marginal band of the marginal zone. Several authors have reported on the protein and carbohydrate contents of the tectorial membrane, e.g., Tachibana *et al*. (1973), Ross (1974), and Steel (1980). Carbohydrates and glycocalyx may enable the TM to maintain a neutral electrical potential and thus have a polarizing effect between the negatively charged hair cells and the positively charged endolymph, or may allow displacement potentials to occur in the regions of the hair cell (Ross, 1974).

In the past several investigators have studied the relation between the stereocilia and the tectorial membrane (TM) with light microscopical techniques (De Vries, 1949; Hilding, 1952; Kimura, 1965). Kimura (1965) was the first to demonstrate in TEM, that outer sensory hair tips were inserted in the undersurface of the tectorial membrane; a finding which later was confirmed among others by Lim (1972). With SEM a more 3-dimensional image of the TM could be obtained (Kosaka *et al*., 1971; Lim 1977a,b; Soudijn, 1976; Hunter-Duvar, 1977; Saito and Hama, 1979; Kawabata and Nomura, 1981). But all these investigations were made with a conventional SEM, operated at relatively high accelerating voltage, and with specimens prepared with conventional fixation techniques requiring a relatively thick conductive coating. Lim (1977) found that the imprints of the outer hair cells can still be seen long after destruction of the organ of Corti by noise or intoxication, while Osborne and Comis (1990) studied the stereocilia of normal, post-mortem and drug-treated guinea pigs with high resolution SEM. Their findings could mean that, although the imprints are produced by the tips of the stereocilia, there might be an independent form of tissue organization in the tectorial membrane. One could argue that the tectorial membrane undersurface structure is equal to the antennulae structure. However, FE-SEM images of the TM undersurface show that its ground structure is entirely different from the filamentous structure of the antennulae. Except for the area around the imprints of the longest stereocilia of the OHC-III, no remnants of the antennulae could be found at the undersurface of the TM. Possibly part of the needle-like structure of the slightly curled marginal end of the TM adheres to the apical side of the antennulae of the OHC-III stereocilia.

The main body of the TM is formed by fine fibrils and an amorphous ground substance. Kronester-Frei (1978) found two types of protofibrils, type A and type B. Type A is mostly found in the basal layer and in the entire middle zone. Type B is located in the middle zone and marginal net. Kawabata and Nomura (1981) suggested that the amorphous ground structure could have a morphological differentiation as well. Kalicharan *et al*. (1996) demonstrated with FE-SEM and TEM different fibril orientations in the TM, which could be similar to both types of protofibrils.

The fact that the physiological function of the antennulae was unknown provided a basis for the idea that these filamentous structures probably were artificial. Lim (1986) and Slepecky and Chamberlain (1986) proposed the existence of a kind of micro-environment around the stereocilia, which could play a role in the K^+/Na^+ equilibrium around the stereocilium at sound transfer. The differential

(Figures 21-26 on facing page)

Figure 21. TEM image of an ultrathin section of cat duodenum with closely packed longitudinally arranged microvilli (mv). The epithelial cells show microfilaments (mf); the glycocalyx or penicillium (*) is found on the top and in between the microvilli after OTOTO postfixation. Bar = 600 nm.

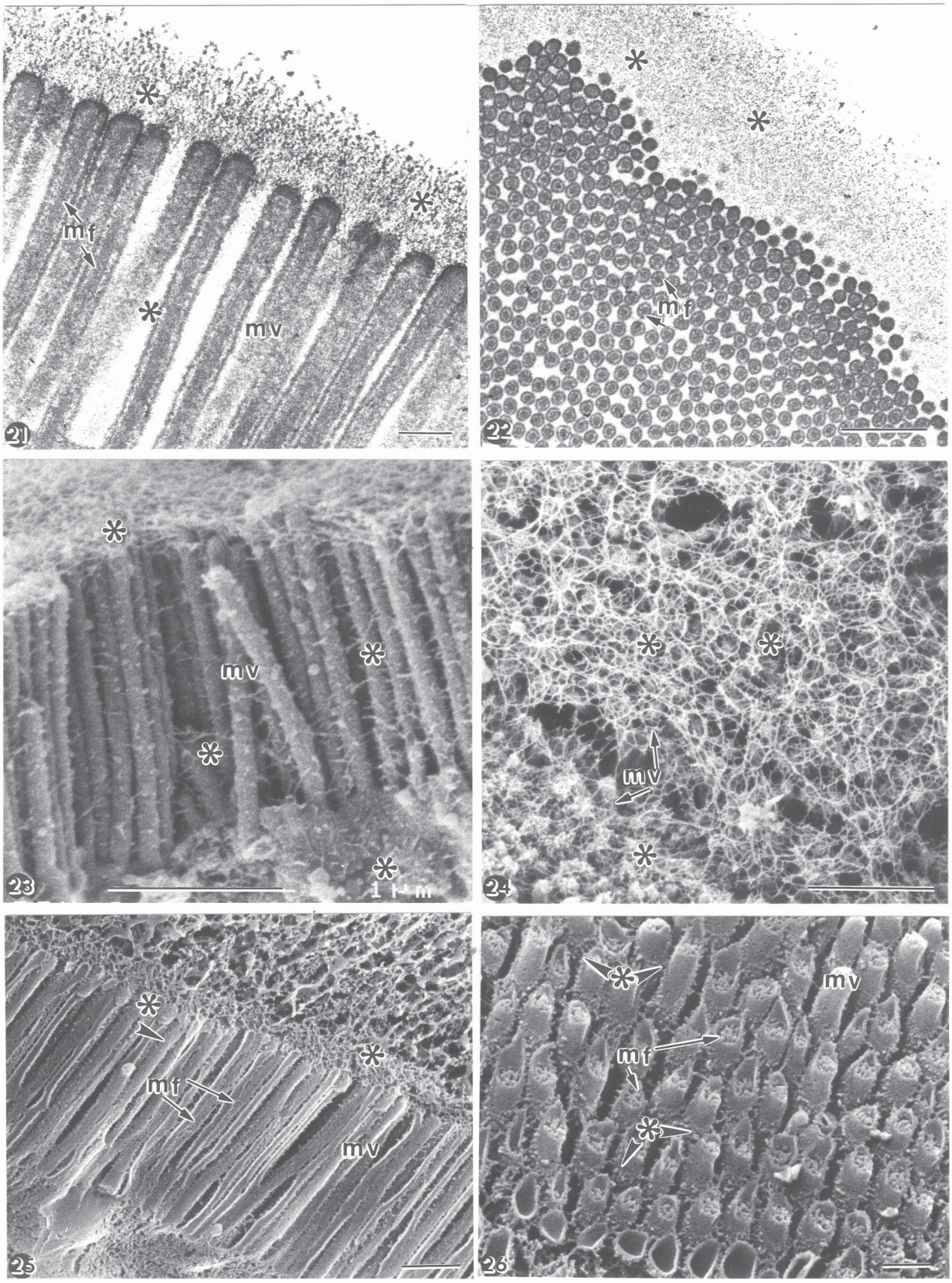
Figure 22. TEM image with transverse view of similar microvilli and associated glycocalyx (*). In the core of the microvilli, the microfilaments (mf) can be seen as dense areas, even at this low magnification. Bar = 1 μm.

Figure 23. FE-SEM image of an identical area as in Fig. 21, presenting a 3-dimensional view of the epithelium with associated microvilli (mv). The glycocalyx (*) is found on top of and in between the microvilli and is more abundant at the apical side. Bar = 1 μm.

Figure 24. FE-SEM image of the cat duodenum microvillous glycocalyx (*), seen in top view after TAO postfixation. The structure of the penicillium network is equally well preserved after both TAO and OTOTO (see Figs. 21-23) postfixation. Bar = 1 μm.

Figure 25. Cryo-FE-SEM image of similar duodenal epithelial microvilli (mv) with the glycocalyx (*) in particular observable at the top; note the plasma membrane bordering on the microfilaments (mf). Bar = 500 nm.

Figure 26. Cryo-FE-SEM image, showing fractured duodenal microvilli in more detail. The glycocalyx with a spark-like appearance can be seen along the microvilli (compare with Fig. 23). The microvillus core shows microfilaments in projection (compare with Fig. 22). Bar = 200 nm.


mf
mv
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distributions of the various glycosaminoglycans in the glycocalyx could account for the ability of adjacent calcified and uncalcified substances to coexist (Fermin *et al*., 1995).

The presence of a glycocalyx can be seen in TEM images after non-coating fixation. The stereociliar structure reacts quite easily with osmium and therefore the limiting membrane is sometimes difficult to distinguish from the dark stained cytoplasmic contents. A closer look shows the microfilament structure inside the stereocilia, but it cannot be seen whether the antennulae have connections to the cytoplasm of the hair cell.

Observation of TEM images of the hair cell body shows that the TAO method, used for (FE)-SEM, also gives a good preservation of the cell constituents and the cell membrane at the TEM level and a good contrast in the image, taking into account that no contrasting with uranyl acetate and/or lead citrate was carried out. However, a standard fixation with GA/OsO_4, followed by section contrasting with U/Pb is slightly better for TEM visualization of the cytoplasmic contents. On the other hand, non coating treated SEM specimens can be embedded for TEM observation without fixation problems, which is not possible with SEM samples fixed by standard methods.

Earlier attempts to visualize the morphology of glycocalyx with antennulae in TEM were not very successful, due to inferior preservation of the glycocalyx constituents, as discussed before. The antennulae themselves are very thin, so they only produce a minimum of electron scattering in the TEM, which makes them difficult to observe. By sputter coating the FE-SEM sample prior to embedding and ultrathin sectioning, the hair cells with stereocilia were covered with a 1-2 nm thin layer of Au/Pd. The presence of a very thin metal coating on top of the stereocilia with glycocalyx improved the visualization of the antennulae, because the metal particles clearly mark the shape of the antennulae in the TEM image.

Cat duodenum is known for its rather thick microvillous glycocalyx, which also creates a micro-environment for ion transport and for protection of the intestinal cell. Conventional OsO_4 postfixation often gave poor results both in SEM and TEM, and only remnants of the glycocalyx with penicillium were visible, indicating that preservation was inferior and that the various rinses during the fixation and dehydration process extracted non-stabilized material. Neither postfixation with the combination thiocarbohydrazide/OsO_4 in TEM or SEM, respectively (Takahasi, 1978), nor the combination OsO_4/tannic acid/OsO_4 in TEM (Kalicharan *et al*., 1984) resulted in an improvement. There are two reasons for that failure: the employed accelerating voltages were too high (as discussed before) and tannic acid is less reactive in already osmicated tissues. Tannic acid is most reactive in combination with arginine, as is the case in the TAO method. But tannic acid can also be employed in combination with already osmicated tissue; this method is known as the (G)OTO method (Jongebloed and Kalicharan, 1992). GOTO stands for glutaraldehyde/ OsO_4/tannic acid/OsO_4, but it is not an appropriate method for the preservation of mucopolysaccharides. However in the (G)OTU non-coating method, tannic acid is employed with already osmicated tissue, but the final step in the postfixation procedure is carried out with uranyl acetate (Jongebloed and Kalicharan, 1992). The cytoplasmic contents are well preserved by this combination, but it does not preserve the glycocalyx constituents. The (G)OTU method is quite similar to the standard TEM fixation with GA and OsO_4 and post staining with uranyl acetate/lead citrate. Postfixation by means of the TAO method showed the intestine glycocalyx very well, in top view and in cross view in FE-SEM at low accelerating voltage (2-3 kV) and in TEM at standard accelerating voltage (60-80 kV), as discussed before.

To exclude the possibility that only a chemical postfixation of the glycocalyx could show the filamentous structure of the glycocalyx, intestine samples were cryo-fixed. In stead of incubation of the sample in a cryoprotectant, samples were fixed by perfusion for 10 minutes with glutaraldehyde prior to slam freezing. At the apical surface and in between the microvilli the glycocalyx could be observed, which proved that the glycocalyx with penicillium is a real structure, and not an artefact as a result of a special postfixation procedure.

Conclusions

[1] Optimal preservation of the delicate constituents of the glycocalyx is the first prerequisite for glycocalyx visualization.

[2] Low kV FE-SEM operation is a second prerequisite for glycocalyx visualization

[3] The visualization of antennulae/penicillium is not dependent of one particular kind of chemical fixation.

[4] The presence of a penicillium can also be demonstrated in Cryo-FE-SEM.

[5] The antennulae/penicillium is a real structure and not an artefact.

[6] The visualization of the antennulae in TEM after non-coating treatment is certainly improved by deposition of a thin metal coating prior to embedding and ultrathin sectioning.

[7] The fine structure of the tectorial membrane undersurface and that of the antennulae are different.

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Discussion with Reviewers

Reviewer I: What is the difference between antennulae and penicillium?
Authors: In fact they are the same, but in the older literature the glycocalyx present at the intestine epithelial cells microvilli is always referred to as penicillium.

Reviewer I: Alcian blue is a cation too, but not a tracer.
Authors: This is correct, but Alcian blue does not give sufficient contrast by itself in TEM images, so to make it visible in the TEM a heavy metal ion should be coupled to it; this is not the case with tracers.

Reviewer I: Does glycocalyx without antennulae exist?
Authors: As far as we know the antennulae are part of the glycocalyx.

Reviewer I: Is there a reason for coating the samples for TEM, as seen in Figs. 18 and 19 and mentioned in conclusion (6)? See antennulae after conventional fixation in TEM without any coating in Fawcett (1981), Figs. 17, 18, on page 39.
Authors: Several authors in the past have demonstrated the glycocalyx on microvilli of intestinal epithelial cells in TEM after conventional GA/OsO_4 fixation. However, the preservation was not always optimal, which caused either agglomeration of the penicillium or loss of part of that glycocalyx. Good results have been obtained with OsO_4-tannic acid-OsO_4 fixation by Takahishi (1978) both for SEM and TEM. The TEM images were made from ultrathin sections of OTOTO prepared samples for SEM and those sections were not contrasted with U/Pb as is common with TEM sections. The FE-SEM samples were sputter coated with 1.5 nm Au/Pd to improve conductivity in the FE-SEM; moreover, this improved visualization of the antennulae in TEM.

Reviewer I: No answer is given to the posed question [a] in the section "Results".
Authors: We do not agree with this. We have given four possible answers: [1] the preservation of the glycocalyx has not been sufficient, [2] the relatively thick metal coating applied, necessary for viewing the specimen at 15-25 kV, obscured the possible presence of a glycocalyx and, moreover, caused agglomeration of glycocalyx material, [3] the accelerating voltage applied was much to high to visualize surface phenomena as discussed in the text, or [4] a combination of the three previously given explanations.

Reviewer I: The stereocilia penetrate into the tectorial membrane. The imprints/remnants of the antennulae should occur inside the sterociliary indentations, and not above these on the underside of the tectorial membrane.
Authors: From the pictures it is obvious that the indentations are rather shallow, so only the apical part of the (longest) stereocilia is connected with the undersurface of the tectorial membrane. If the GA prefixation is used, contrary to the tri-aldehyde fixation, there is a nice separation between the stereocilia and the undersurface of the tectorial membrane (TM). We have not observed remnants of the glycocalyx including the antennulae at the inside of the imprint. Accidently we found some remnants in the area around the imprints of the OHC-III stereocilia, as shown in the image. In general the undersurface of the TM was free from remnants, except for the area close to the rim (which has a needle-like appearance) originally attached to outer phalangeal cells. The marginal end or rim probably is rather firmly attached, so when forces are exerted during fixation and drying, small patches of glycocalyx can become detached.

Reviewer I: Many authors show connections between the stereocilia (tip-links, side-links). Why can these not be seen in any of the pictures of the stereocilia and how does the glycocalyx appear in this region?
Authors: We should state first that we have not specifically looked for these tip- and side-links, we have seen them and can refer to Jongebloed *et al*. (1996), where we have shown those connections in stereo images. The visualization of those connections is strongly dependent of the viewing direction, they appear at the inside of the V or W shaped stereocilia arrangement. The connections are partly hidden by the glycocalyx, certainly those between the longest stereocilia. The connections between the shortest and the medium long stereocilia should be better visible; they occur in an area which is less covered by glycocalyx, due to the gradient in thickness.

Reviewer I: The dark layer seen in Fig. 20 is not a inner and outer membrane, but an inner and outer phospholipid layer of the plasma membrane. Is the dense cap the glycocalyx without antennulae ?
Authors: The first statement is correct. We have not found

glycocalyx on the inner hair cell stereocilia. We only can speculate that the dense cap is a thickening of the phospholipid layer, which is even more visible due to the metal sputter coating applied prior to the embedding.

Reviewer III: Can you speculate about the significance of the gradient in thickness of the glycocalyx from OHC-III to OHC-I, illustrated in your work, with respect to function?
Authors: This is a question not easily answered. It is assumed that the glycocalyx creates a kind of micro-environment mainly around the apical part of the stereocilia. On the other hand it is commonly thought that the different rows of hair cells play an essential but also different role in sound transfer. Whether the thickness of the glycocalyx plays a more or less important role in this is not known. Further research should be undertaken to establish this.

Additional Reference

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