INTERMEDIATE FILAMENT PROTEINS IN THE HUMAN AUDIO-VESTIBULAR ORGAN

AN IMMUNOHISTOCHEMICAL STUDY

L.J.J.M. BAUWENS

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Studies on the distribution of cytoskeletal proteins are primarily motivated by the desire to further characterize the structure and organization of tissues. The cytoskeleton is largely made up of microtubules, microfilaments and intermediate-sized filaments. Intermediate filament proteins (IFPs) are ubiquitous constituents of virtually all eukaryotic cells and are present in the cytoplasm as filaments with a diameter of 7 to 12 nm, as well as at the periphery of the nucleus, forming the nuclear lamina [1]. In contrast to the other cytoskeletal proteins, most IFPs are characterized by a tissue-specific distribution [1]. However, co-expression of different types of IFPs is a common phenomenon, in particular in embryonic and malignant tissues.

In the past decade, it has become evident that IFPs can be subdivided into six distinct classes (see Table 1) [1, 2]. Types I and II comprise a family of 20 different cytokeratins, synthesized by different epithelia depending on their origin and state of differentiation. The type III IFPs include vimentin (present in cells of mesenchymal origin), desmin (expressed by different muscle cell types), glial fibrillary acidic protein (GFAP; found in astroglia) and peripherin (expressed by neuronal cells). Type IV IFPs are the neurofilament proteins, also expressed specifically by neuronal cells, while the nuclear lamins make up the class of type V IFPs. Type VI contains the most recent addition to the family, i.e. nestin [2]. This subdivision of IFPs is based on gene structure, nucleic acid and protein sequences, and immunohistochemical assays [1].

Table 1 Current classification and tissue-specific expression of intermediate filament proteins (IFPs)

	IFPs	*Cell type*
Type I	acidic cytokeratins (Cks 9-20)	epithelia
Type II	neutral to basic cytokeratins (Cks 1-8)	epithelia
Type III	vimentin	mesenchyme
	desmin	muscle
	GFAP	glia
	peripherin	neuronal cells
Type IV	neurofilament proteins	neuronal cells
Type V	nuclear lamin proteins	virtually all cells
Type VI	nestin	CNS stem cells

Immunohistochemical detection of IFPs with specific antibodies provides a method for a high degree of resolution of IFP-localization [1]. Since a given tissue can be characterized immunohistochemically by its expression of IFPs,

the detection of these cytoskeletal proteins has been used in the immunohistochemical identification of human tumors, but has also proven to be of particular value in morphological studies and studies on histogenesis [1]. However, although knowledge of the expression pattern of IFPs in various human tissues and organ systems has contributed substantially to the understanding of their composition and organization, only limited information has so far become available on structure-function relationships of this cytoskeletal network. Available data suggest a function in the organization of cytoplasmic space and involvement in the scaffold of the cytomatrix [1, 3]. Cytokeratins in particular are associated with a supracellular network since they link up with desmosome cell-to-cell attachment sites at cell boundaries [1]. However, since the exact functions of the different IFPs are not (yet) known, data on their tissue distribution are primarily of importance at the morphological level by providing a method to differentiate between different tissue types – among which different types of epithelia – and only seldom allow conclusions to be drawn concerning the functions of specific tissues or organ systems.

During the past decade, the intermediate filament composition of most (human) tissues has been extensively documented. However, the inner ear has long been neglected in this respect. This may be explained primarily by technical difficulties encountered in inner ear immunohistochemistry [4-6]. Differences in density between the membranous labyrinth of the inner ear on the interior (in many regions only a few cell layers thick), the bone of the surrounding otic capsule on the exterior, and the presence of large fluid-containing spaces in between, make it practically impossible to apply routine immunohistochemical techniques to the inner ear. This applies in particular to the human inner ear since in adults this structure lies deeply embedded in the temporal bone, while the surrounding otic capsule consists of the hardest bone encountered in the human body [4-8]. These characteristics not only interfere with tissue processing but, as a consequence of the time-consuming nature of the processing procedure, also necessitate chemical fixation in order to avoid autolysis and loss of antigenicity [4-7]. Since chemical fixation can only sufficiently preserve tissue morphology and antigenicity in the inner ear when performed within several hours after death (< 3 hours), certain requirements, such as an adequate internal hospital organization, have to be met. Nevertheless, for several reasons – such as the prescription in The Netherlands of legal regulations on the obligation of having an informed consent from either the patient before his or her death or the nearest relatives – the availability of adequately preserved specimens of the human inner ear remains very poor, whereas pathological specimens are extremely rare [8]. It is because of these organizational problems, together with the technical difficulties in tissue processing as described above, that data obtained with sophisticated immunohistochemical techniques on the adult human inner ear (temporal bone) are only now becoming available.

Objectives of this study

The human inner ear displays a complex anatomy, primarily due to its (functional) heterogeneity [8, 9]. Of the three primitive germinal layers, the ectoderm contributes to the formation of the membranous labyrinth and nerve supply of the neuroepithelium and the mesoderm to the formation of the periotic labyrinth and otic capsule [8]. Due to the complexity and the numerous developmental interactions during embryogenesis, the human inner ear comprises an interesting and challenging object for the study of the distribution of IFPs. The aim of this study was to investigate the expression patterns of these structural proteins in the human inner ear and thus make a contribution to a further understanding of its functional anatomy.

In Chapter 2 an improved tissue-processing technique for immunohistochemistry of the adult human inner ear is described. The technique avoids the immunocompromizing step of decalcification. The procedure allows reliable investigation of frozen sections of the chemically fixed, non-decalcified, adult human cochlea and vestibular labyrinth.

In Chapter 3 the general expression pattern of IFPs in the adult human cochlea is presented. In addition, some findings that may be of functional significance are discussed.

In Chapter 4 the various epithelia of the adult human cochlea are immunohistochemically characterized by their expression patterns of different cytokeratins.

In Chapter 5 the immunohistochemical expression patterns of IFPs, among which different subgroups of cytokeratins, in the adult human vestibular labyrinth are described.

In Chapter 6 a study on the immunohistochemical detection of IFPs in the surgically removed adult human endolymphatic duct and sac is presented. Furthermore, the epithelium of the duct and sac is immunohistochemically characterized by its expression pattern of different cytokeratins.

In Chapter 7 the results presented in the different foregoing Chapters are interrelated and summarized, and general conclusions are drawn.

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CHAPTER 2

AN IMPROVED TECHNIQUE FOR IMMUNOHISTOCHEMICAL INVESTIGATION OF THE ADULT HUMAN INNER EAR

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Introduction

Over the past years, immunohistochemistry has emerged as an outstanding tool for identifying cells and tissues in a functional way. This technique complements other morphological procedures and allows a further distinction of cells and tissues at the lightmicroscopical level. As a result, immunohistochemistry has been readily applied to the cochlea of several animal species. However, immunohistochemical studies of the human inner ear have remained scarce due to problems in applying routine immunohistochemical techniques to the human temporal bone.

Unfortunately, chemical fixation cannot be avoided. Moreover, since the human inner ear is surrounded by the bony labyrinth, prolonged decalcification prior to sectioning has always been considered to be inevitable. However, it is widely acknowledged that both these processing steps can alter the antigenicity of tissues and therefore may cause false-negative results [1-3].

We have developed a new procedure which essentially consists in removal of the bony labyrinth prior to sectioning, so as to be able to avoid the immunocompromizing step of decalcification. This processing technique enables the immunohistochemical investigation of frozen sections of the chemically fixed, non-decalcified, adult human inner ear.

The preservation of both morphology and immunoreactivity is illustrated by the immunohistochemical localization of the intermediate filament constituents cytokeratins and neurofilament proteins, since it is well established that detection of intermediate filaments in such frozen sections, whether or not preceded by fixation with protein-precipitating fixatives, yields reliable immunohistochemical results [4].

Materials and methods

Human temporal bones (N=12; age 32 to 73 years) were fixed within three hours after death by perilymphatic perfusion [5]. For detection of intermediate filament proteins (IFPs), acetone was used as a fixative. After removal at autopsy, temporal bones were immediately microdissected, essentially as described by Hawkins and Johnsson [6]. Osmium tetroxide (OsO_4) postfixation was omitted, because of its known deleterious effects on immuno-detectability of certain antigens. In order to maintain the tissue in optimal immunological condition and to prevent drying of the membranous labyrinth, the entire procedure was performed in cold PBS (4°C). After removal of the bony capsule, the membranous cochlea was detached from the modiolus by cutting through the osseous spiral lamina. Next, both the cochlear and vestibular parts of the membranous labyrinth were removed *in toto* from the remnants of the bony labyrinth (Fig. 1; membranous cochlea) and embedded in *Tissue-Tek® II.* Cryosections (4-5 μm) were incubated with monoclonal antibodies to cytokeratins (RCK 102,

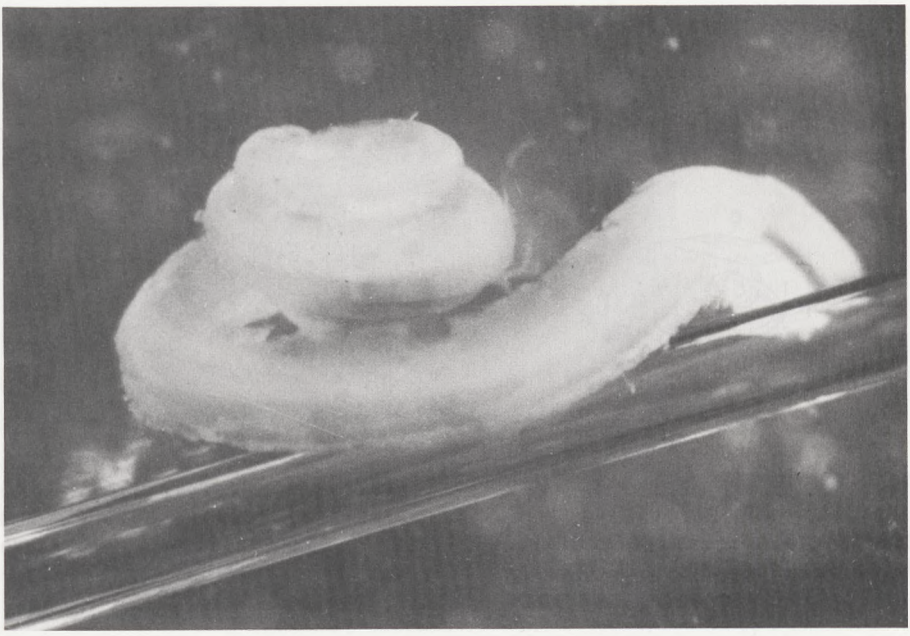

Fig. 1 The membranous cochlea, after microdissection and subsequent removal *in toto* from the remnants of the bony capsule.

dilutions 1:2.5 to 1:5, reacting with virtually all epithelial tissues) and to neurofilament proteins (MNF, dilutions 1:10 to 1:20) (both obtained from Euro-Diagnostics B.V., Apeldoorn, The Netherlands), using the peroxidase-antiperoxidase (PAP) technique and including appropriate positive control tissues (epidermis, liver, peripheral nerve) as well as negative control antisera. The entire procedure is summarized in Table 1 and schematically drawn in Fig. 2.

Table 1 Subsequent steps in tissue processing for immunohistochemistry of the chemically fixed, frozen, non-decalcified adult human inner ear.

1.	Tissue fixation by perilymphatic perfusion
2.	Removal of the temporal bone at autopsy
3.	Microdissection
4.	Removal of the membranous labyrinth from the remnants of the bony labyrinth
5.	Embedding in *Tissue-Tek® II*
6.	Cryosectioning (4-5 μm)
7.	Indirect immunoperoxidase staining technique

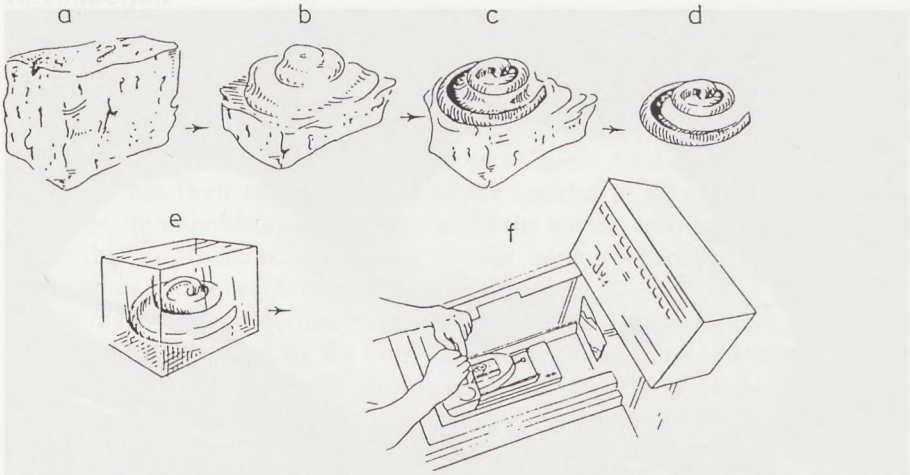

Fig. 2 Schematical representation of temporal bone processing procedure with emphasis on the cochlear part of the labyrinth. [a] Temporal bone after removal at autopsy. [b] Bony cochlea after the drilling procedure. [c] After removal of the otic capsule, the membranous labyrinth of the cochlea is visible. [d] Membranous cochlea removed *in toto*. [e] Embedding in *Tissue-Tek® II*. [f] Cryosectioning.

Results

Tissue morphology

The overall preservation of tissue morphology, although dependent on the patient's case history and on the time interval between death and fixation, was very satisfactory (Figs. 3-5).

Immunohistochemical staining results

All positive tissue controls yielded the expected staining reactions, thus proving the specificity of the antibodies.

In the cochlea, immunostaining with the broad-spectrum monoclonal antibody to cytokeratins occurred in all epithelial cells lining the cochlear duct, including most supporting cells of the organ of Corti (Fig. 3).

In the organ of Corti and macula utriculi, the monoclonal antibody to neurofilament proteins revealed immunostaining in the nerve fibres reaching the sensory epithelia and in their nerve endings at the sensory cells (Figs. 4 and 5).

Discussion

In tissue processing for immunohistochemistry, attention should be focussed

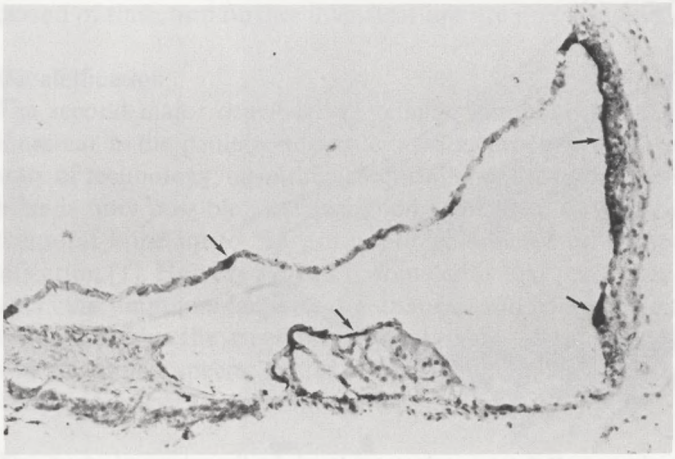

Fig. 3 Cochlear duct, immunostained with the broad-spectrum monoclonal antibody RCK 102 to cytokeratins.
Immunostaining (arrows) is seen in all cells lining the endolymphatic space. In the organ of Corti intense staining is present in the region of the basilar membrane and in the reticular lamina (x 40).

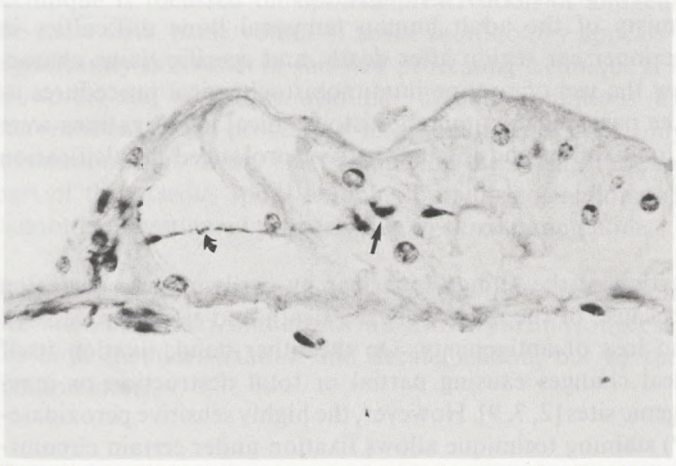

Fig. 4 Organ of Corti, immunostained with the monoclonal antibody MNF to neurofilament proteins.
Note immunostaining of the nerve endings at the inner and outer hair cells (straight arrow), and of the tunnel-crossing fibres (curved arrow) (x 100).

on the preservation of antigenicity as well as on tissue morphology [7]. Both are very much influenced by fixation and embedding techniques. It is widely acknowledged that optimal preservation of immunoreactivity is obtained by

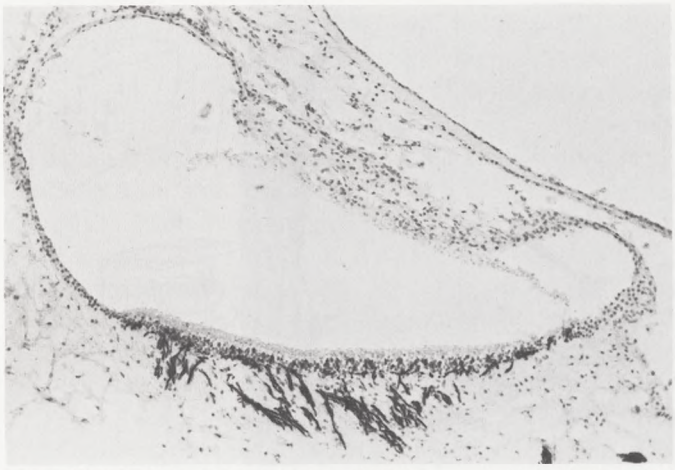

Fig. 5 Macula utriculi, immunostained with monoclonal antibody MNF to neurofilament proteins. Immunostaining of the neural bundles, and of their nerve endings at the sensory epithelium, is seen (x 20).

cryostat sectioning of fresh tissue [1, 2, 8]. On the other hand, tissue morphology is best preserved in chemically fixed, permanently embedded, tissue. Therefore, in all cases it will be necessary to reach a compromise.

In immunohistochemisty of the adult human temporal bone difficulties in gaining access to the inner ear region after death, and specific tissue characteristics, do not allow the use of routine immunohistochemical procedures as in other tissues. In the past, reliable immunohistochemical investigations were limited by the need for fixation and the necessity of prolonged decalcification prior to sectioning [1].

Fixation

Indeed, chemical fixation of the human inner ear by perilymphatic perfusion cannot be avoided because of the delay between death and tissue processing, causing autolysis and loss of antigenicity. On the other hand, fixation itself may produce chemical changes causing partial or total destruction or inaccessibility of the antigenic sites [2, 7, 9]. However, the highly sensitive peroxidase-antiperoxidase (PAP) staining technique allows fixation under certain circumstances [1, 10]. Recent investigations have shown that there are several fixatives which appear to be suitable for immunohistochemical investigation of the human inner ear, although some fixatives require further investigation [1, 11]. It is important to note that the choice which fixative to use depends on what kind of antigen is to be detected [7]. Furthermore, the duration of fixation is crucial. When fixation is too short antigens may diffuse away from the cell's cytoplasm, while over-fixation might cause irreversible denaturation of antigens [1]. Thus,

optimal fixation includes the use of the appropriate fixative during the right period of time, and further investigations are needed to determine these factors.

Decalcification

The second major drawback in reliable immunohistochemistry of the human inner ear is the prolonged decalcification procedure. Indeed, with the present state of technology, (semithin-) sectioning of the undecalcified human temporal bone is only possible after resin embedding [3]. Cryosectioning of the human temporal bone up to 3-5 μm is not possible at all without prolonged decalcification [1]. However, it has become clear that prolonged decalcification may alter the immunoreactivity of tissues, and these effects have been well-documented in the past [8, 12]. Only short decalcification, as is possible in several animal species but not in the human, has been shown not to affect immunoreactivity significantly [12-14].

The technique described in this study essentially consists in removal of the bony labyrinth prior to embedding and subsequent sectioning of the remaining membranous labyrinth, and thus excludes the immuno-compromizing step of decalcification in tissue processing for immunohistochemistry of the human inner ear. In addition, conventional paraffin embedding, which can cause denaturation of many cell surface antigens [15], is also avoided. Thus, the technique is focussed on optimal preservation of antigenicity (Figs. 3-5). At the same time, preservation of tissue morphology appears not to be disturbed significantly as a result of this new processing technique (Figs. 3-5).
However, the technique also has certain limitations as compared to the conventional tissue-processing techniques, since it is only applicable to the membranous labyrinth. For example, in the cochlea, the modiolus and central part of the osseous spiral lamina, containing ganglion cells and nerve fibres, cannot be investigated properly due to surrounding bone.

With this improved tissue-processing technique for immunohistochemistry of the human inner ear, limitations in sensitivity are no longer set by the requirement for both chemical fixation and decalcification, but by the need for chemical fixation alone.

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CHAPTER 3

EXPRESSION OF INTERMEDIATE FILAMENT PROTEINS IN THE ADULT HUMAN COCHLEA

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Authors: L.J.J.M. Bauwens, J.E. Veldman, H. Bouman, F.C.S. Ramaekers and E.H. Huizing

Introduction

Intermediate filament proteins (IFPs) are part of the cytoskeleton of eukaryotic cells and form a class of highly insoluble cytoplasmic filaments. These filaments are intermediate in size between the other two main constituents of the cytoskeleton, i.e. the microtubules and microfilaments. Biochemically, the main cytoplasmic IFPs can be grouped into the following subtypes: desmin (55 kD), which is expressed by muscle cells; vimentin (57 kD) by mesenchymally derived cells; cytokeratins (40-68 kD) by epithelial cells; neurofilament proteins (68, 150, 200 kD) by cells of neuronal differentiation; and glial fibrillary acidic protein (GFAP) (52 kD) by astroglia and related cells.

This cell type specificity of IFPs is of particular value in studies on histogenesis, as a given tissue can be characterized by its distribution of IFPs [1, 2]. However, it has become clear that there are exceptions to this general classification system. A few epitheloid tissues have been found not to contain cytokeratins but to express vimentin, and it has also been shown that some specialized cell types can co-express up to three different IFPs under certain circumstances [3]. Furthermore, it has become clear that certain tissue types in developing embryos may contain IFPs different from those present in their adult counterparts [4].

In the past decade virtually all kinds of human tissues have been investigated for their expression of IFPs. The cochlea, however, had not been studied in this respect until Flock et al. [5] first described the presence of prekeratin (= cytokeratin) in the supporting cells of the guinea pig organ of Corti. Since then, only few studies have been published on the distribution of one or several IFPs in the cochlea [6-14]. Moreover, until recently only animal species were investigated and data on the adult human cochlea were lacking.

Shi and Juhn [6] localized keratin in the cochlear duct of the guinea pig by means of an indirect immunoperoxidase technique on aldehyde-fixed, resin-embedded tissue. Anniko et al. [7] found vimentin and neurofilament proteins, but no cytokeratins, desmin, or GFAP in the newborn inner ear of the mouse using immunofluorescence on unfixed, frozen sections. In contrast, using an indirect immunoperoxidase technique on unfixed, frozen tissue, Anniko et al. [8, 9] did find cytokeratins in the cochlear duct of 14-18 week old human fetuses, in addition to vimentin and neurofilament proteins. This was confirmed by other studies, one on human fetal cochleas [10], and others on the mouse inner ear [11, 12], using immunofluorescence and indirect immunoperoxidase staining of frozen sections. Kasper et al. [13] described the expression of vimentin and cytokeratins in the stria vascularis of unfixed, decalcified, cryosectioned guinea pig cochleas. Finally, Schrott et al. [14] studied the expression of IFPs in aldehyde-fixed, decalcified, paraffin-embedded sections of cochleas of normal and mutant mice. However, desmin and GFAP reactivity could not be defined

because only the polyclonal antibodies to cytokeratins and vimentin, and the monoclonal antibody to neurofilament proteins appeared to be reactive on the paraffin sections. Cytokeratin and vimentin staining was found in the cochlear duct, whereas neurofilament staining was confined to a few scattered cells in the spiral ganglion [14].

The small number of studies on IFP expression in the cochlea, the partly conflicting data, and the absence of data on the adult human cochlea may all be explained by technical difficulties encountered in inner ear immunohistochemistry. Because of the delicate structure of the membranous labyrinth and the surrounding bone of the otic capsule, the immunoreactivity-compromizing steps of both decalcification and paraffin or resin embedding prior to sectioning are generally regarded to be inevitable. Immunohistochemistry of the adult human cochlea offers additional problems because chemical fixation cannot be avoided and the heavily calcified temporal bone necessitates prolonged decalcification [15, 16]. However, it has been well established that in immunohistochemical localization of IFPs, conventional formaldehyde fixation combined with paraffin embedding may lead to weaker, or even false-negative, staining results. Most reliable results are obtained by using frozen sections and, when fixation is necessary, by using protein-precipitating fixatives [1, 17, 18].

Recently, we developed a new procedure which enabled us to investigate frozen sections of chemically fixed, non-decalcified, adult human cochleas, thus preserving optimal antigenicity [16].
The aim of the present study was to analyze the expression patterns of IFPs in the adult human cochlea, using this new procedure.

Materials and methods

Tissue processing

Temporal bones (N=12) of six human adults (age: 32-73 years) without any known history of hearing impairment were fixed within three hours after death by perilymphatic perfusion with acetone (100%) for 10 min, followed by perfusion with phosphate-buffered saline (PBS). After removal at autopsy, temporal bones were immediately microdissected, essentially as described by Hawkins and Johnsson [19]. Osmium tetroxide (OsO_4) postfixation was omitted because of its known deleterious effects on immuno-detectability of certain antigens. In order to maintain the tissue in optimal condition and to prevent drying of the membranous labyrinth, the entire procedure was performed in cold PBS (4°C). After removal of the otic capsule, the membranous labyrinth was detached from the modiolus by cutting through the osseous spiral lamina. Next, the membranous labyrinth was removed *in toto* from the remnants of

the bony capsule, followed by embedding in *Tissue-Tek® II* and cryosectioning (4-5 μm).

Antisera

Sections were incubated with polyclonal rabbit and monoclonal mouse antibodies to five classes of IFPs, i.e. cytokeratins, vimentin, neurofilament proteins, desmin and glial fibrillary acidic protein (GFAP), most of which were obtained from Euro-Diagnostics BV, Apeldoorn, The Netherlands. Cytokeratins were detected with the affinity-purified polyclonal rabbit antibody PKE (dilutions 1:25 to 1:100) and with the broad-spectrum monoclonal antibody RCK 102 (dilutions 1:2.5 to 1:5; reacting with virtually all epithelial tissues). Vimentin was detected with the affinity-purified polyclonal rabbit antibody PVI (dilutions 1:700 to 1:1400) and with the monoclonal antibody RV 202 (dilutions 1:200 to 1:600) [20]. For detection of the neurofilament proteins three monoclonal antibodies were used, i.e. MNF (dilutions 1:10 to 1:20 – directed to the 68/200 kD subunits), RNF 401 and RNF 402 (dilutions 1:1 to 1:5 and 1:5 to 1:20 – directed to the 200 kD subunit) [21]. For detection of desmin and GFAP, polyclonal and monoclonal antibodies to desmin (PDE – dilutions 1:150 to 1:300 ; MDE – dilutions 1:1 to 1:2.5) and GFAP (PGF – dilutions 1:400 to 1:800 ; MGF – dilutions 1:1 to 1:1.5) were used.

Immunolabeling

Frozen sections (4-5 μm thick) were air dried at room temperature (15 min), fixed in 100% acetone (3 min), and air dried once more. After washing in PBS the specimens were incubated with 10% normal goat serum (NGS) (X 902, DAKOPATTS Corp., Glostrup, Denmark) in PBS for 20 min in order to diminish non-specific binding. The sections were then incubated with the primary antibodies at the appropriate dilutions in 10% NGS for 30 min. After washing in PBS, the sections were incubated for another 30 min with the secondary antibodies, i.e. rabbit anti-mouse IgG conjugated to peroxidase (P 260 DAKOPATTS Corp., Glostrup, Denmark) for detection of monoclonal antibodies or swine anti-rabbit IgG conjugated to peroxidase (P 217 DAKOPATTS Corp., Denmark) for detection of polyclonal antibodies, diluted in 10% NGS. 3-Amino-9-ethylcarbazole served as a chromogen for the peroxidase reaction. Finally, sections were counterstained with Mayer's hemalum, mounted in Kaiser's glycerol gelatin, and examined by light microscopy.

Appropriate human positive control tissues (including brain, peripheral nerve, muscle, liver, epidermis, tongue, esophagus etc.) were included to prove the specificity of the antibodies used. Negative controls were obtained by replacement of the primary antibody by either PBS, tissue culture medium (RPMI 1640, 10% FCS), or an irrelevant antiserum or antibody.

Results

All positive controls showed specific staining, thus proving the reactivity of the antibodies.

Immunostaining for the different antisera yielded similar results in all temporal bones, differing only in the degree of staining.

The overall staining pattern throughout the cochlear duct is depicted in Fig. 1 and summarized in Table 1.

CYTOKERATINS

Immunostaining with the polyclonal and monoclonal antibodies to cytokeratins occurred in all epithelial cells lining the endolymphatic space of the membranous labyrinth. This includes the Claudius' cells, the outer sulcus cells, the spiral prominence (especially those cells adjacent to the stria vascularis), the marginal

Table 1 Staining pattern for five types of intermediate filament proteins throughout the cochlear duct.

	Cytokeratins	*Vimentin*	*Neurofilament Proteins*	*GFAP*	*Desmin*
Reissner's membrane	+	+	-	-	-
Basilar membrane					
-mesothelial lining	-	+	-	-	-
-amorphous part	-	-	-	-	-
Nerve fibres	-	-	+	+/-[1]	-
Interdental cells	+	-	-	-	-
Spiral limbus	-	+	-	-	-
Inner sulcus cells	+	-	-	-	-
Organ of Corti					
-Border cells	+	-	-	-	-
-Deiter's cells	+	+	-	-	-
-Hair cells (IHC/ OHC)	-	-	-	-	-
-Pillar cells	+	+	-	-	-
-Hensen's cells	+	-	-	-	-
-Tectorial membrane	-	-	-	-	-
Claudius' cells	+	-	-	-	-
Outer sulcus cells	+	+	-	-	-
Spiral prominence					
-epithelial cells	+	+	-	-	-
-stroma	-	+	-	+/-[1]	-
Stria vascularis					
-marginal cells	+	-?	-	-	-
-intermediate- and basal cells	-	+	-	-	-
Spiral ligament	-	+	-	-	-

[1] questionable GFAP-like reactivity only with the polyclonal antiserum.

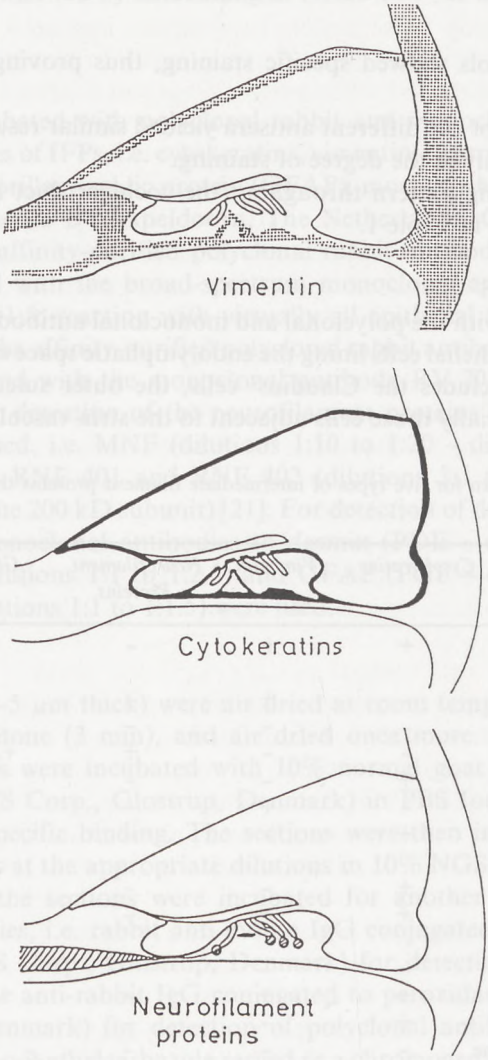


Fig. 1 Schematic representation of immunostaining for vimentin, cytokeratins and neurofilament proteins in the cochlear duct.

cells of the stria vascularis, Reissner's membrane (probably only the epithelial cells), the interdental cells, the inner sulcus cells, and the organ of Corti (Figs. 2a, 3 and 4).

In the organ of Corti staining was present in the border cells, the inner and outer pillar cells, Deiters' cells and Hensen's cells. Intense staining occurred at the appositions of these cells to the basilar membrane and along the surface

of the organ of Corti to which region these cells also contribute (i.e. reticular lamina). No staining, however, occurred in the hair cells (Figs. 2b and 5).

VIMENTIN

The polyclonal and monoclonal antibodies gave identical results. Immunostaining occurred in most supporting structures of the membranous labyrinth (Fig. 2c). The spiral ligament stained positive for vimentin. In the stria vascularis both intermediate and basal cells showed vimentin staining, while staining of the marginal cells seemed negative. Both the epithelial lining and the stroma of the spiral prominence showed positive immunostaining, as did Reissner's membrane (probably only the mesothelial cells). In the organ of Corti staining was limited to the inner and outer pillar cells and Deiters' cells (Fig. 2d). Other cells of the organ of Corti showed no immunoreactivity. Also, the Claudius' cells, the inner sulcus cells, the region of the interdental cells and the region of the neural bundles lacked reactivity for vimentin (Fig. 2c).

NEUROFILAMENT PROTEINS

All antibodies to the neurofilament proteins revealed identical staining results. Immunostaining occurred in the neural bundles reaching the organ of Corti (Fig. 2e). In the organ of Corti, nerve fibres and nerve endings at inner and outer hair cells stained intensely (Fig. 2f).

Because the preparation technique excludes the modiolus, only few ganglion cells could be detected, all apparently showing a variable staining with the neurofilament antibodies (Figs. 6 and 7).

DESMIN AND GFAP

No distinct immunostaining reaction was observed with our polyclonal and monoclonal antibodies to desmin and GFAP. Only questionable GFAP-like reactivity was seen at the nerve endings and adjacent nerve fibres of the organ of Corti and in the stroma of the spiral prominence, but only with the polyclonal antibody PGF.

Discussion

In this study we have demonstrated the presence of the IFPs cytokeratins, vimentin, and neurofilament proteins in the adult human cochlea. With regard to the absence of the other IFPs it should be stressed, however, that negative immunohistochemical staining results, without support of direct biochemical analysis, do not definitely prove the absence of the IFP concerned and are therefore to be interpreted with caution.

In contrast to their absence in the hair cells, cytokeratins were found in all other cells lining the endolymphatic space of the membranous labyrinth. This

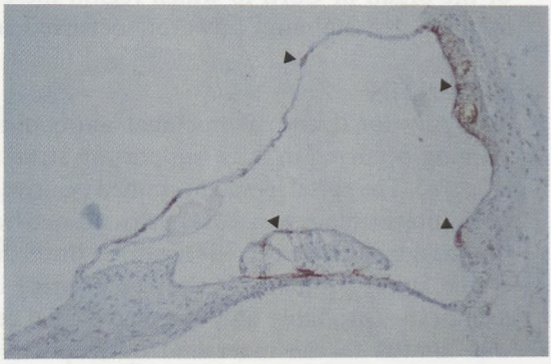


Fig. 2a

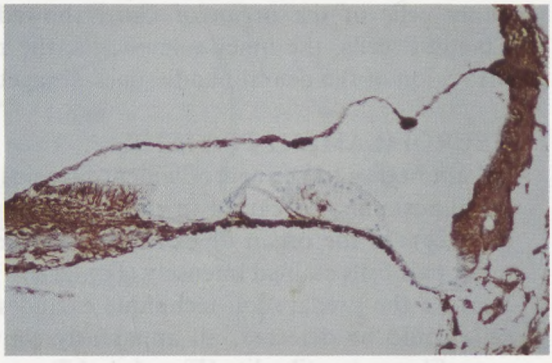


Fig. 2c

Fig. 2e.

Fig. 2 (a-f) Immunostaining of the cochlear duct with details of the organ of Corti.
[a] Cochlear duct, immunostained with broad-spectrum monoclonal antibody RCK 102 to cytokeratins. Immunostaining of all epithelial cells lining the cochlear duct is seen. Note intense staining of the spiral prominence, the marginal cells of the stria vascularis, Reissner's membrane and of certain regions in the organ of Corti (arrowheads) (x 30). [b] Organ of Corti, immunostained with broad-spectrum monoclonal antibody RCK 102 to cytokeratins. Immunostaining of the border cells (asterisk), the inner and outer pillar cells (double arrow), Deiters' cells (filled arrow) and Hensen's cells (open arrow). Intense staining at their appositions to the basilar membrane and at the surface of the organ of Corti to which region these cells also contribute. Note absence of immunostaining in the hair cells (x 80). [c] Cochlear duct, immunostained with monoclonal

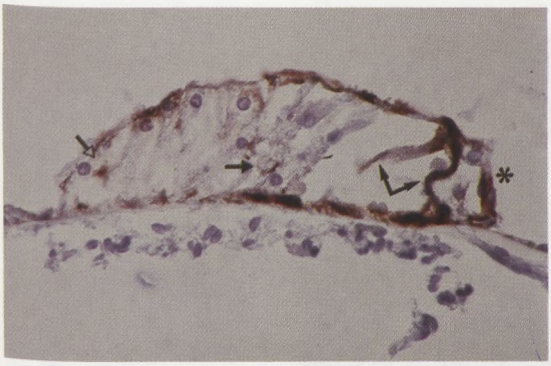


Fig. 2b

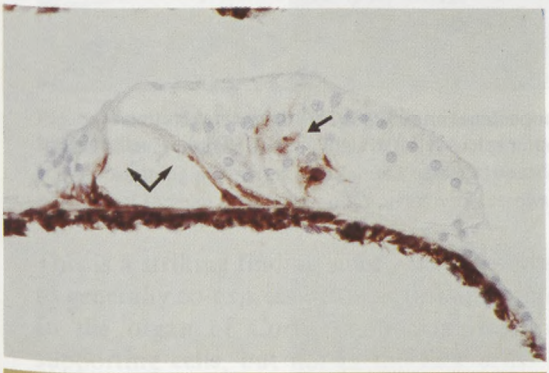


Fig. 2d

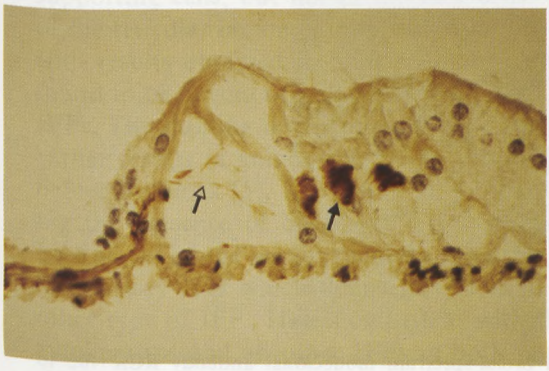


Fig. 2f

antibody RV 202 to vimentin. Note the absence of immunostaining in Claudius' cells, the inner sulcus cells, the interdental cells, in the region of the nerve bundles, and in most parts of the organ of Corti (x 30). [d] Detail from Fig. 2C, organ of Corti. Immunostaining is limited to the inner and outer pillar cells (double arrow) and Deiters' cells (single arrow). Note the staining of the mesothelial lining of the basilar membrane, in contrast to the absence of staining in the Claudius' cells (x 70). [e] Cochlear duct, immunostained with monoclonal antibody MNF to neurofilament proteins. Immunostaining of the neural bundles in the osseous spiral lamina (arrow) and of their nerve endings at inner and outer hair cells (x 30). [f] Detail from Fig. 2E, organ of Corti. Immunostaining of the nerve fibres reaching the organ of Corti, of their nerve endings at inner and outer hair cells (filled arrow), and of tunnel-crossing fibres (open arrow) (x 90).

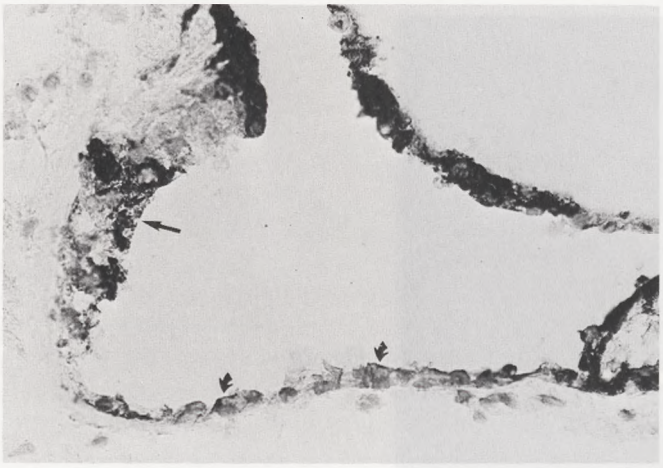


Fig. 3 Outer sulcus, immunostained with monoclonal antibody RCK 102 to cytokeratins.
Immunostaining of the spiral prominence, outer sulcus cells (straight arrow), Claudius' cells (curved arrows), Hensen's cells and Reissner's membrane (x 300).

Fig. 4 Inner sulcus, immunostained with broad-spectrum monoclonal antibody RCK 102 to cytokeratins.
Immunostaining of border cells, inner sulcus cells (open arrows), interdental cells (filled arrows), and Reissner's membrane (x 300).

is in accordance with the accepted epithelial origin of these cells and confirms earlier data on cytokeratin expression in the guinea pig and mouse cochlea [6, 10, 11, 14] as well as in the embryonic human inner ear [8, 9, 10]. Interestingly, the different mesothelial structures in the cochlea did not express cytokeratins.

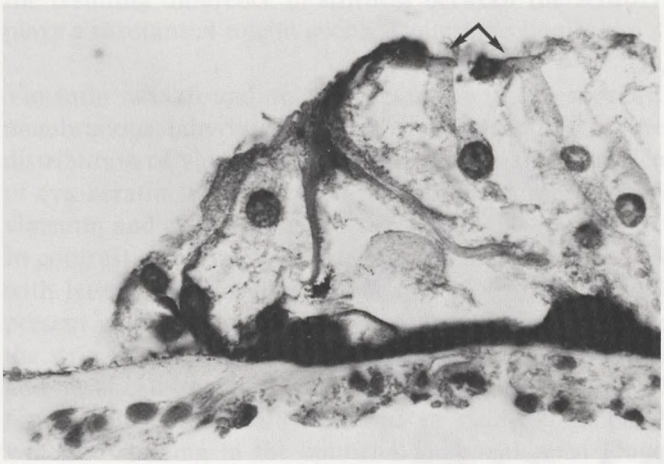
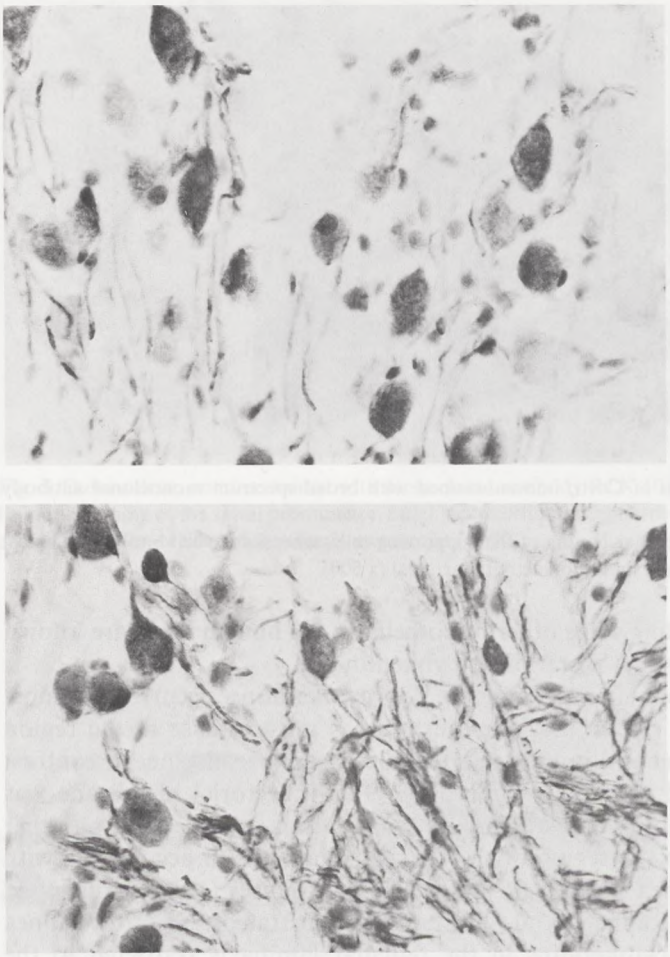


Fig. 5 Detail of the organ of Corti, immunostained with broad-spectrum monoclonal antibody RCK 102 to cytokeratins.
Immunostaining of the reticular lamina of the supporting cells, whereas the cell bodies and apical portions of the hair cells lack immunoreactivity (arrows) (x 500).

This is a striking finding since other mesothelia in the human body are known to generally co-express cytokeratins and vimentin [3].
In the organ of Corti immunostaining for cytokeratins occurred in most supporting cells, but not in the hair cells. It was most intense in the region of the reticular lamina and in the region of the basilar membrane. In contrast with results obtained in human embryos [8, 9], the tectorial membrane was found to be devoid of immunostaining for cytokeratins as well as for all other IFPs. The staining in the region of the reticular lamina is in accordance with earlier results in guinea pigs [5, 6, 10] and human fetuses [10]. As suggested before by Flock [5], Raphael et al. [10] have demonstrated that in the guinea pig the presence of cytokeratins in the reticular lamina is restricted to the headplates of the supporting-cell processes, enclosing the apical portions of the sensory cells which themselves seem to be devoid of cytokeratins or any other type of IFP. This is consistent with the absence of immunostaining for cytokeratins and all other IFPs in both inner and outer hair cells as seen in this study. The absence of IFP expression in hair cells is an unusual phenomenon and is not yet understood. It has been postulated that it may reflect the unique mechanical properties of the cells, their polarity, their specialized subcellular structural organization or the special junctional complexes between hair cells and supporting cells [10].
In the organ of Corti, the positive immunostaining results for cytokeratins at the supporting cells' appositions to the basilar membrane have not been reported earlier.
The "shell"-like distribution of cytokeratins in the supporting cells of the organ



Figs. 6 and 7 Spiral ganglion, immunostained with monoclonal antibody MNF to neurofilament proteins.
Immunostaining of the neural bundles and a variable staining of the ganglion cells (x 400).

of Corti, which "shell" encloses the non-cytokeratin-containing hair cells, suggests a role in determining and maintaining the mechanical structure of the organ. This is in good agreement with the suggested function of cytokeratins, namely maintenance of cell shape and tissue structure by intercellular attachment through desmosomes [22]. It has been postulated that the many cytoskeletal and contractile proteins in the reticular lamina are involved in the anchoring mechanism of the stereociliairy rootlets and thus could possibly regulate stereociliary stiffness [23]. The presence of cytokeratins in the reticular lamina of the supporting cells, together with their absence in the enclosed apical portions of the hair cells, seems of particular importance since it may be assumed that

the resulting difference in stiffness between the sensory and supporting cells plays a substantial role in cochlear micromechanics.

Vimentin was found to be present in most supporting structures of the membranous labyrinth. Apart from the region of the neural bundles, the distribution of vimentin appeared to be roughly complementary to the regions of cytokeratin staining, but there were certain cells showing co-expression for vimentin and cytokeratins.
In contrast to initial data obtained in human embryos [8, 9], but in agreement with later results in guinea pigs and mice [12-14], vimentin was found to be present in the stria vascularis and in the region of the spiral prominence. In the stria vascularis vimentin staining seemed to be limited to the intermediate and basal cells, as has been described by Schrott et al. [14] in mice. We could not confirm other data obtained in guinea pigs [13], reporting additional vimentin staining in the epithelial marginal cells. However, in the region of the spiral prominence, both mesenchymal and epithelial cells showed vimentin staining, and vimentin was also found to be present in the epithelial cells of the outer sulcus. Moreover, staining for vimentin occurred in certain supporting cells of the organ of Corti. This pattern of expression in the organ of Corti differs from earlier conflicting data on vimentin expression in human embryos which either showed staining for vimentin in the outer hair cells and a variable staining in the supporting cells [8, 9], or claimed the complete absence of vimentin in all epithelial cells of the cochlear duct, including the supporting cells of the organ of Corti [10].
Our results on vimentin staining of certain epithelial cells, i.e. the region of the spiral prominence and outer sulcus as well as the inner and outer pillar cells and Deiters' cells in the organ of Corti, demonstrate co-expression of vimentin and cytokeratins since all these epithelial cells were also found to express cytokeratins. However, although resolution was not sufficient to distinguish between the different cell layers of Reissner's membrane, the positive staining for both vimentin and cytokeratins in Reissner's membrane does very likely not reflect co-expression, but is thought to represent vimentin staining of the mesothelial cells and cytokeratin staining of the epithelial cells.
Although determination of co-expression of IFPs allows a further characterization of cells and tissues, its meaning is not yet understood. Co-expression of vimentin and cytokeratins has been found in human neuroepithelial tissues, but has also been reported in other epithelia and mesothelial cells [13, 21]. Furthermore, expression of vimentin in addition to another IFP has been described in many tissues during embryonic development [4], and this may account for the conflicting earlier data on vimentin expression in the organ of Corti of human embryos [8, 9].

Neurofilament proteins were found in the neural bundles and at the nerve endings at the sensory cells, which themselves showed no immunoreactivity

at all. In good agreement with Anniko et al. [8, 9], we found only few nerve fibres ending near the outer hair cells, while the majority was found adjacent to the inner hair cell region. Although neurofilament expression does not allow differentiation between efferent and afferent nerve fibres, one can speculate whether this difference nevertheless in some way does reflect the known difference in innervation between the inner (95% afferent innervation) and outer hair cells (majority of efferent innervation).
We could not confirm earlier results on co-expression of vimentin and neurofilament proteins in the neural bundles of human embryos [9], since in our material these structures lacked additional staining for vimentin. This difference may be accounted for by the transient expression of vimentin, in addition to another IFP, in many tissues during embryonic development.
Because of limitations set by our tissue-processing technique, our data on neurofilament expression by spiral ganglion cells are too limited to allow conclusions as to the proposed existence of two different populations of spiral ganglion cells, based on differences in amount of staining for neurofilament proteins [9].

No distinct immunostaining was obtained with our antibodies to GFAP. However, the polyclonal antibody PGF showed reactivity at the nerve endings and adjacent nerve fibres of the organ of Corti, without staining the further neural bundles as seen with neurofilament antibodies. In addition, some reactivity occurred in the stroma of the spiral prominence, a region where neurofilament proteins have never been detected. Unfortunately, these results could not be confirmed by immunostaining with our monoclonal antibody MGF.
The questionable GFAP-like reactivity in the spiral prominence seems an interesting finding since this region is thought to be involved in fluid homeostasis and in the past a possible regulatory function has been suggested, based on the finding of somatostatin-like immunoreactivity in this region [24]. Whether our staining results reflect artifacts due to cross-reactivity or reflect the true presence of glia-related cells in the spiral prominence remains uncertain. Further studies with other antibodies and perhaps with a more sensitive (submicroscopical) technique are required to confirm these preliminary results.

In conclusion, the expression pattern of IFPs in the adult human cochlea as described in this study largely confirms earlier data obtained in several animal species, although some notable differences occurred. Yet, when compared with the results on IFP expression in human embryos, there are numerous differences. These differences could be due to the fact that certain tissue types in developing embryos may contain IFPs different from those present in their adult counterparts.

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CHAPTER 4

CYTOKERATIN EXPRESSION IN THE EPITHELIA OF THE ADULT HUMAN COCHLEA

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Introduction

Cytokeratins (Cks) are intermediate filament proteins (IFPs) which are part of the cytoskeleton and form a class of highly insoluble cytoplasmic filaments. These are intermediate in size between the other two main constituents of the cytoskeleton, the microtubules and microfilaments. Biochemically, IFPs can be grouped into several major classes including desmin (55 kD) which is expressed by muscle cells, vimentin (57 kD) found in mesenchymally derived cells, neurofilament proteins (68, 150, 200 kD) synthesized by cells of neuronal differentiation, and glial fibrillary acidic protein (GFAP) (52 kD) detected in astroglia and related cells. A complex group of at least 20 different Cks (40-68 kD) is expressed by human epithelial cells [1-3]. Although it has become clear that there are exceptions to this rule, and that some specialized cell types can co-express up to three different IFPs under certain circumstances [4], the cell type specificity of IFPs is of particular value in studies on histogenesis, as a given tissue can be characterized by its expression of IFPs [1-4].
Due to an improved understanding of IFP biochemistry in recent years, it has become clear that Cks represent a family of polypeptides which are characterized by a remarkable biochemical diversity. Up to now, 20 different Ck subunits have been distinguished, two or more members of which are co-expressed in human epithelia [5, 6]. Moll et al. [6] have published the cataloge of human Cks and designated them 1 to 20, Ck 1 being the polypeptide with the highest molecular weight and isoelectric pH, and Ck 20 being the polypeptide with the lowest molecular weight and a low isoelectric pH. These Ck subunits occur in cell-type-specific combinations and their expression varies with the state of epithelial differentiation [5-7]. Recent data indicate that the human Cks can be divided into a neutral to basic (type II; nos. 1-8) and an acidic (type I; nos. 9-20) subfamily, and that most basic Cks form a pair with a specific acidic Ck counterpart [7]. Since different Ck pairs have been demonstrated in different kinds of epithelia, monoclonal antibodies, specific for a certain Ck polypeptide, allow the immunohistochemical classification of different epithelia [5-8]. So far, such monospecific antibodies have been described and prepared for Cks 3, 4, 5, 7, 8, 10, 13, 14, 16, 17, 18, and 19 [7, 8]. Representatives of most of these antibodies were used in this study.

Recently, we have reported on the expression of IFPs in the adult human cochlea using a new procedure allowing optimal preservation of antigenicity [9, 10]. All epithelial cells lining the cochlear duct were found to express Cks, thus confirming their epithelial origin [10].
The aim of the present study was to characterize the epithelia of the adult human cochlea by investigating the expression of different Ck subunits, in addition to our earlier results on the general expression of IFPs [10]. Of particular interest was the question whether the variety in differentiation and function of the epithelia concerned relates to differences in their Ck expression pattern.

Materials and methods

Tissue preparation

Temporal bones (N=12) of six human adults (age: 32-73 years) without a known history of hearing impairment were fixed within three hours after death by perilymphatic perfusion with acetone (100%) for 10 min, followed by perfusion with phosphate-buffered saline (PBS). After removal at autopsy, the temporal bones were immediately microdissected, essentially as described by Hawkins and Johnsson [11]. Osmium tetroxide (OsO_4) postfixation was omitted because of its known deleterious effects on immunoreactivity of certain antigens. In order to maintain the tissue in optimal condition and to prevent drying of the membranous labyrinth, the entire procedure was performed in cold PBS (4°C). After removal of the otic capsule, the membranous labyrinth was detached from the modiolus by cutting through the osseous spiral lamina. Next, the membranous labyrinth was removed *in toto* from the remnants of the bony capsule, followed by embedding in *Tissue-Tek® II* and cryosectioning (4-5 μm).

Antisera

Frozen sections were incubated with monoclonal antibodies to different Ck subunits. Most of the antibodies have been described elsewhere [8]. The Ck subunit classification as described in this paper is according to Moll et al. [6]. The following antibodies were used:

6B10 (dilution 1:1 – directed to Ck 4), RCK 102 (dilutions 1:2.5 to 1:5 – directed to Cks 5 and 8), RCK 105 (dilutions 1:1 to 1:5 – directed to Ck 7), M20 (dilutions 1:5 to 1:20 – directed to Ck 8), RKSE 60 (dilution 1:1 – directed to Ck 10) and 1C7 (dilution 1:1 – directed to Ck 13), which were all obtained from Euro-Diagnostics BV, Apeldoorn, The Netherlands.

Cam 5.2 (dilutions 1:80 to 1:160 – directed to Ck 8) was obtained from Becton & Dickinson, Mountain View, California, U.S.A., while RPN 1162 (dilutions 1:1 to 1:2.5 – directed to Ck 7) and RPN 1165 (dilutions 1:5 to 1:20 – directed to Ck 19) were obtained from Amersham International plc, Amersham, Bucks, England. Finally, the recently described monoclonal antibodies RCK 107 (dilution 1:1 – directed to Ck 14) [12] and RCK 106 (dilutions 1:1 to 1:2.5 – directed to Ck 18) [8] were applied.

Immunolabeling

Frozen sections (4-5 μm) were air dried at room temperature (15 min), fixed in 100% acetone (3 min), and air dried once again. After washing in PBS the specimens were incubated with 10% normal goat serum (NGS) (X 902, DAKOPATTS Corp., Glostrup, Denmark) in PBS for 20 min in order to diminish non-specific binding. The sections were then incubated with the primary antibodies at the appropriate dilutions in 10% NGS for 30 min. After washing in PBS, the sections were incubated with the secondary, peroxidase-conjugated, rabbit anti-mouse antibody (P 260, DAKOPATTS Corp., Glostrup, Denmark)

diluted in 10% NGS for another 30 min. 3-Amino-9-ethylcarbazole served as a chromogen for the peroxidase reaction. Finally, sections were counterstained with Mayer's hemalum, mounted in Kaiser's glycerol gelatin, and examined by light microscopy.

Appropriate positive human control tissues were included to prove the specificity of the antibodies (see Chapter 3). Negative controls were obtained by replacement of the primary antibody by tissue culture medium (RPMI 1640, 10% FCS).

Results

All positive controls showed the expected staining reactions, thus proving the specificity of the antibodies.

Immunostaining for the different antisera yielded similar results in all temporal bones, differing only in the degree of staining.

The overall staining pattern throughout the cochlear duct is depicted in Fig. 1 and summarized in Table 1.

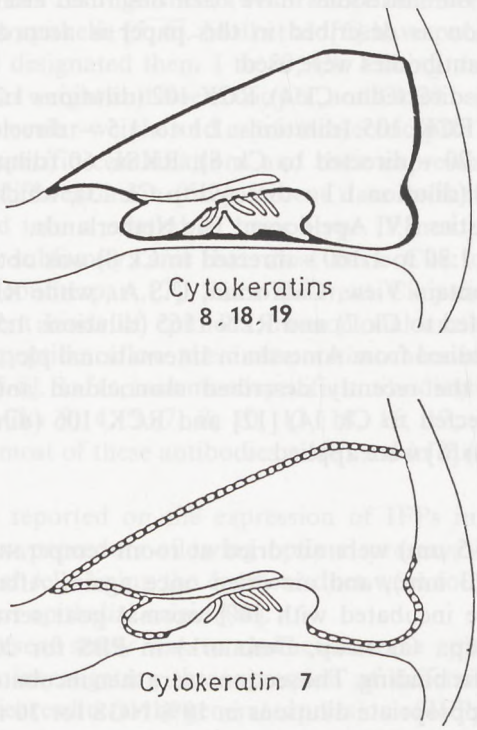

Fig. 1 Schematic representation of immunostaining results for cytokeratins 7, 8, 18 and 19 in the epithelia of the adult human cochlear duct.

Table 1 Cytokeratin distribution in cochlear epithelium as monitored with monoclonal antibodies on frozen sections of chemically fixed, non-decalcified, adult human cochleas.

	6B10 (Ck4)	RCK 102 (Cks5&8)	RCK 105 (Ck 7)	RPN 1162 (Ck7)	M20 (CK8)	Cam 5.2 (Ck8)	RKSE 60 (Ck 10)	IC 7 (Ck13)	RCK 107 (Ck14)	RCK 106 (Ck 18)	RPN 1165 (CK 19)
Reissner's membrane	–	+	+	+	+	+	–	–	–	+	+
Interdental cells	–	+	+	+	+	+	–	–	–	+?	+
Inner sulcus cells	–	+	+	+	+	+	–	–	–	+	+
Border cells	–	+	–	–	+	+	–	–	–	+	+
Deiters' cells	–	+	–	–	+	+	–	–	–	+	+
Hair cells (IHC/OHC)	–	–	–	–	–	–	–	–	–	–	–
Pillar cells	–	+	–	–	+	+	–	–	–	+	+
Hensen's cells	–	+	–	–	+	+	–	–	–	+	+
Tectorial membrane	–	–	–	–	–	–	–	–	–	–	–
Claudius' cells	–	+	+	+	+	+	–	–	–	+	+
Outer sulcus cells	–	+	+	+	+	+	–	–	–	+	+
Spiral prominence	–	+	+	+	+	+	–	–	–	+	+
Stria vascularis (marginal cells)	–	+	–	–	+	+	–	–	–	+	+

Immunostaining with antibodies RCK 102 (Cks 5 & 8), M20 (Ck 8), Cam 5.2 (Ck 8), RCK 106 (Ck 18) and RPN 1165 (Ck 19) occurred in all epithelial cells lining the endolymphatic space of the membranous labyrinth. These include the Claudius' cells, the outer sulcus cells, the spiral prominence (especially those cells adjacent to the stria vascularis), the marginal cells of the stria vascularis, Reissner's membrane (probably only the epithelial cells), the interdental cells, the inner sulcus cells and the organ of Corti (Fig. 2a, showing immunostaining with M20 to Ck 8). However, immunostaining of the interdental cells with antibody RCK 106 (Ck 18) was extremely weak or possibly even negative.

In the organ of Corti, staining for these antibodies was seen in the border cells, the inner and outer pillar cells, Deiters' cells and Hensen's cells. Intense staining occurred at the appositions of these cells to the basilar membrane and along the surface of the organ of Corti to which region these cells also

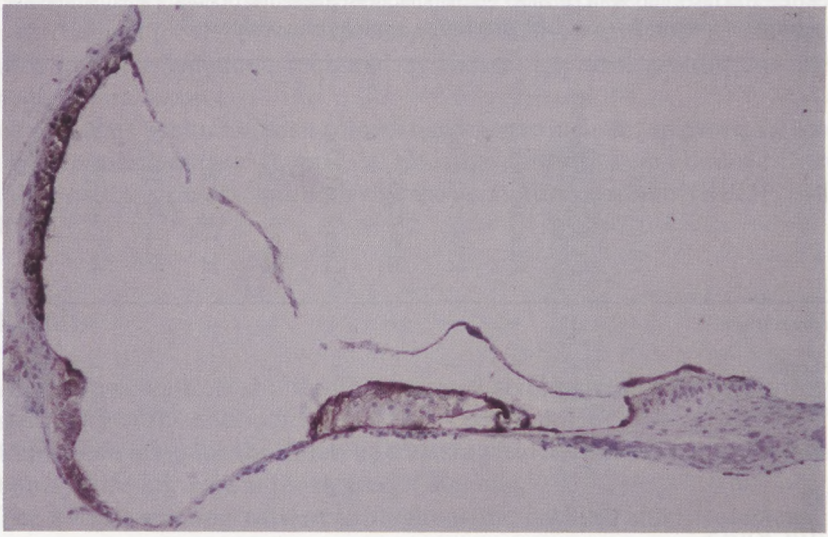

Fig. 2a

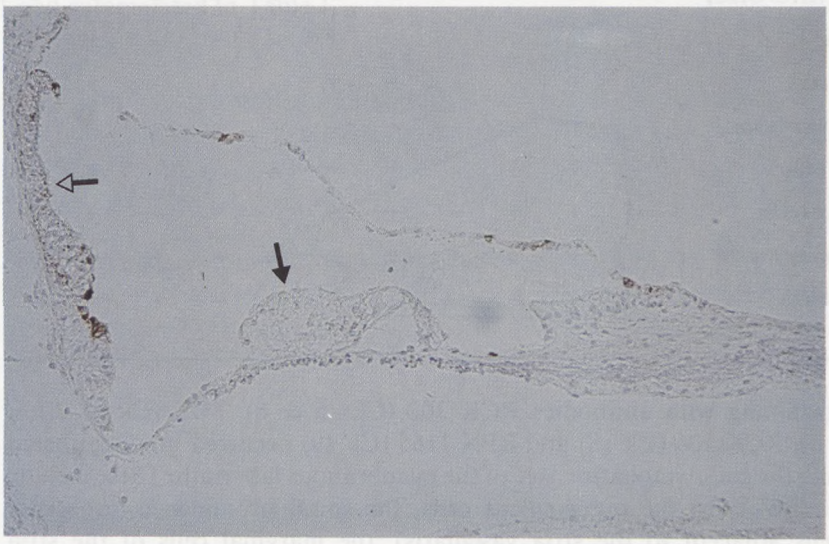

Fig. 2b

Fig. 2 (a-b) [a] Cochlear duct, immunostained with monoclonal antibody M20 to cytokeratin 8. Immunostaining of all epithelial cells lining the cochlear duct is seen, including the supporting cells in the organ of Corti (x 75). [b] Cochlear duct, immunostained with monoclonal antibody RCK 105 to cytokeratin 7. Note the absence of staining in the organ of Corti (filled arrow) and in the marginal cells of the stria vascularis (open arrow) (x 75).

contribute (Fig. 2a, showing immunostaining to Ck 8). No staining, however, occurred in the hair cells.

Immunostaining with antibodies to Ck 7 (RCK 105 and RPN 1162) was seen

in the region of the spiral prominence, the outer sulcus cells, the Claudius' cells, the inner sulcus cells, the interdental cells (not shown) and Reissner's membrane. In contrast to the expression pattern observed with the antibodies described above, no immunostaining was found with the antibodies to Ck 7 in the organ of Corti and in the marginal cells of the stria vascularis (Figs. 2b, 3a-c).
No immunostaining was observed with antibodies 6B10 (Ck 4), RKSE 60 (Ck 10), 1C7 (Ck 13) and RCK 107 (Ck 14).

Discussion

The general (not subunit-specific) expression pattern of cytokeratins (Cks) in adult human cochlear epithelia as described in this study is generally in agreement with earlier results in two previous studies [10, 13]. However, in contrast to Anniko et al. [13], we did find expression of Cks in inner and outer sulcus cells. Moreover, in our material, we were not able to confirm data indicating clear regional quantitative variations in Ck expression in the organ of Corti from apex to base [13], other than slight differences possibly related to the varying size of the Ck-positive Hensen's cells.

Since the expression of Ck subunits in different epithelia follows certain rules, a given epithelium or epithelial cell can be characterized by its specific Ck expression pattern [5, 6]. A clear correlation has been found between the complexity of an epithelium ("simple" epithelia vs. stratified epithelia vs. "complex" epithelia) and the type of the Ck subunits expressed [3, 5, 6, 7, 12]. "Simple" or single-layered epithelia express two to four of the group of Cks 7, 8, 18 and 19. Stratified epithelia express Cks 5 and 14/15, combined with one or more of the group of Cks 1, 2, 3, 4, 10, 12 and 13, depending on the differentiation programme (skin, corneal or esophageal type). Finally, "complex" epithelial structures such as glandular epithelia containing both basal and luminal cells, transitional epithelia and pseudostratified epithelia usually express a mixture of "simple" and "stratified" types of Ck polypeptides.

The first study on the expression of different Ck subunits in cochlear epithelia was published by Anniko et al. [14], who demonstrated the presence of Ck 18 in human fetal cochleas. Recently, Anniko et al. [15] published more detailed data which indicated the presence of Cks 7, 8 and 18 in human fetal cochlear epithelium. In another study on cryosectioned human fetal cochleas, Raphael et al. [16] concluded that the Ck expression of cochlear epithelia was characteristic of "simple" epithelia, although only two monospecific antibodies were used in this study. Hence, as in the aforementioned studies, the presence of many Ck subunits remained undetermined. Apart from our results on the general (not subunit-specific) expression of Cks in the adult human cochlea [10],

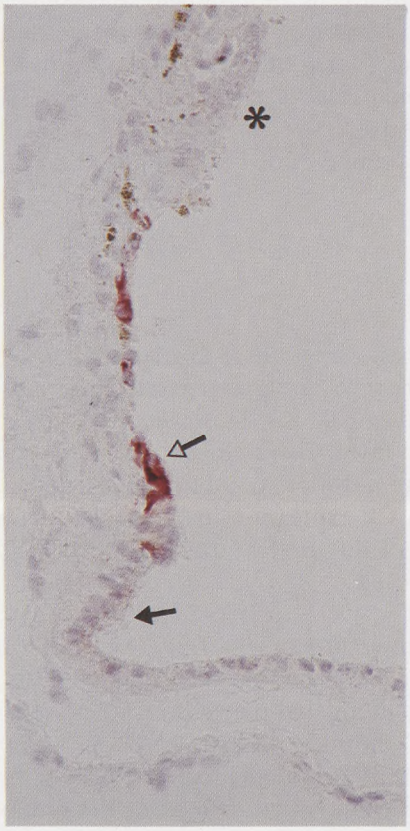

Fig. 3a

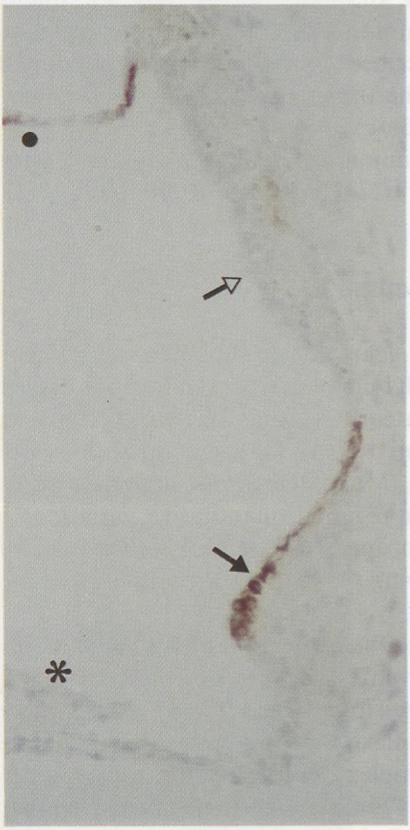

Fig. 3b

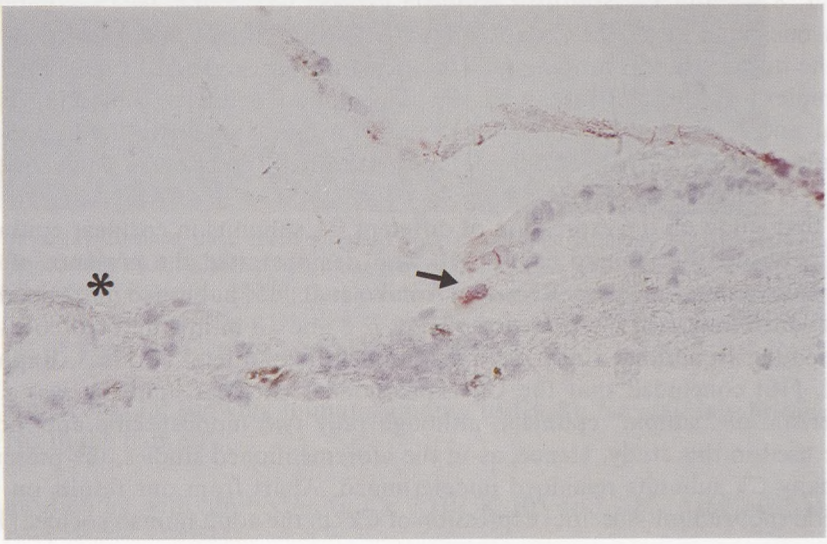

Fig. 3c

there exists only one other study on adult human cochlear epithelia [13] showing a similar general distribution of Cks with, however, regional variations. In that study only one monospecific antibody was used, thus allowing only detection of one Ck subunit, i.e. Ck 8. Thus, although several animal species have been investigated in this respect [17-20], until now no comprehensive data have become available on the expression of different Cks in adult human cochlear epithelia.

In this study we have used monospecific monoclonal antibodies to Cks 4, 7, 8, 10, 13, 14, 18 and 19, thus including markers for "simple", stratified (among which keratinizing) and "complex" epithelia as outlined above.
We have demonstrated reactivity for Cks 7, 8, 18 and 19 in the epithelia of the adult human cochlea, while Cks 4, 10, 13 and 14 could not be identified. However, since this immunohistochemical study was not combined with biochemical analyses, absence of staining does not totally exclude the presence of the Cks concerned and the results are therefore to be interpreted with caution.
Our immunohistochemical data show that the epithelia of the adult human cochlea express Cks which are characteristic of "simple" epithelia. Further characterization of "simple" epithelia by means of their Ck pattern is often not possible because similar Ck patterns are shared by several "simple" epithelia [7]. Thus, functionally heterogeneous "simple" epithelia such as the epithelia of the pancreatic duct, gall bladder, endocervix, endometrium, oviduct and lung alveoli all share the expression of Cks 7, 8, 18 and 19, as found in the cochlea [7].
However, within the cochlea two groups of "simple" epithelia could be distinguished on basis of their Ck patterns, i.e. their positive or negative immunostaining reaction for Ck 7. As revealed by staining with the two monospecific monoclonal antibodies to Ck 7, the organ of Corti and the marginal

Fig. 3 (a-c) [a] Detail of the outer sulcus, immunostained with monoclonal antibody RCK 105 to cytokeratin 7. Slight immunostaining of the outer sulcus cells (filled arrow) and Claudius' cells, intense immunostaining of the spiral prominence (open arrow). Note absence of immunostaining in the marginal cells of the stria vascularis (asterisk) (x 250). [b] Detail of the stria vascularis and spiral prominence, immunostained with monoclonal antibody RPN 1162 to cytokeratin 7. Note the absence of staining in the marginal cells of the stria vascularis (open arrow) while the adjacent cells of the spiral prominence (filled arrow) show strong immunoreactivity. Also note immunostaining of Reissner's membrane (dot) and absence of staining in the Hensen's cells of the organ of Corti (asterisk) (x 200). [c] Detail of the inner sulcus, immunostained with monoclonal antibody RCK 105 to cytokeratin 7. Immunostaining is seen in the inner sulcus cells (arrow). Note absence of immunostaining in the organ of Corti (asterisk) while Reissner's membrane shows clear positive staining (x 300).

cells of the stria vascularis were the only cochlear epithelial structures apparently devoid of this Ck subtype. Both antibodies, obtained from different fusions and likely to recognize different epitopes, gave identical results, thus reducing the likelihood of false-negative outcomes as a result of epitope masking. Although this immunohistochemical finding needs confirmation by direct biochemical analysis, it indicates that these two structures, in spite of their totally different functions, are the only epithelia in the cochlea that express Cks 8, 18 and 19 without co-expression of Ck 7.

Apparently, Ck 7 seems to be a discriminating Ck subunit in cochlear epithelia, as has also been shown in other human epithelia [21, 22]. Its presence in human tissues is restricted to a subgroup within the glandular types of normal human epithelia. It is present in various ductal and glandular epithelia but is absent in hepatocytes, prostatic acinar epithelium and in epithelia of the gastrointestinal tract [21, 22]. Indeed, the epithelia of the small intestine and colon represent one of the few examples of human epithelia which express the same set of Cks 8, 18 and 19 as we have found in the organ of Corti and stria vascularis [23]. However, similarities and differences in Ck pattern of different "simple" epithelia are not yet understood. Therefore, we cannot explain the deviant Ck pattern of the organ of Corti and stria vascularis, but it may well reflect their differences in functional state and/or in differentiation as compared to the other cochlear epithelia.

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CHAPTER 5

EXPRESSION OF INTERMEDIATE FILAMENT PROTEINS IN THE ADULT HUMAN VESTIBULAR LABYRINTH

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Introduction

Intermediate filament proteins (IFPs), together with microtubules and microfilaments, are prominent constituents of the cytoskeleton. An important feature of the main cytoplasmic subtypes of IFPs – cytokeratins (Cks), vimentin, desmin, glial fibrillary acidic protein (GFAP) and neurofilament proteins – is their tissue-specific distribution. In principle, in the mature vertebrate, a family of 20 different Cks is expressed by different types of epithelia, desmin is specifically expressed by muscle, vimentin by several cell types of mesenchymal origin, GFAP mainly by astroglia and neurofilament proteins by neuronal cells [1-3]. However, combinations of up to three of these different IFPs can occur in certain tissues, especially during embryogenesis [3]. Furthermore, different epithelia have been found to express different combinations of Cks depending on their nature and state of differentiation [4-6]. Thus, it has become clear that the IFP composition of a cell may reflect both its embryonic origin and its functional state.

Moll et al. [4] have recently published an updated version of their cataloge of human Cks and designated them 1 to 20, adding a newly detected cytokeratin to the former group of 19 constituents. They can be divided into two distinct families depending on their isoelectric pH and it has been shown that most basic Cks (type II; nos. 1-8) form a pair with a specific acidic Ck counterpart (type I; nos. 9-20) [4, 5]. Such pairs occur in cell-type-specific combinations while also a clear correlation has been found between the complexity of an epithelium ("simple" vs. stratified vs. "complex" epithelia) and the type of Cks expressed. "Simple" epithelia express two to four of the group of Cks 7, 8, 18 and 19. Stratified epithelia express Cks 5 and 14/15, combined with one or more of the group of Cks 1, 2, 3, 4, 10, 12 and 13, depending on the differentiation programme (skin, corneal or esophageal type). Finally, "complex" or "mixed" epithelia such as glandular epithelia containing both basal and luminal cells, transitional epithelia and pseudostratified epithelia usually express a mixture of "simple" and "stratified" types of Ck polypeptides [5, 6].

Various polyclonal and monoclonal antibodies to different IFPs have been developed over the past years, thus enabeling the immunohistochemical characterization of tissues with respect to these cytoskeletal constituents. In addition, monoclonal antibodies which recognize epitopes unique to single Cks allow the immunohistochemical classification of different epithelia.

During the last decade, virtually all types of human tissues have been investigated for their expression of IFPs. The inner ear, however, was initially only investigated in animal species [7-12]. Due to technical difficulties encountered in human inner ear immunohistochemistry [13], data on the adult human vestibular labyrinth are still lacking. IFP data on the adult human cochlea have only just become available [14-16]. Recently, studies have been published

on IFP expression in the embryonic human inner ear, including the vestibular labyrinth [17, 18]. These results do, however, only allow limited conclusions with respect to its adult counterpart since it is well-known that tissues in developing embryos may express different combinations of IFPs as compared to mature tissues [3]. In fact, our earlier findings demonstrated major differences between IFP data in the adult human cochlea [15] as compared to previous results in human embryos [17].

The aim of the present study was to immunohistochemically characterize the various tissues in the adult human vestibular labyrinth by investigating their IFP expression pattern. For this purpose, we used a previously described tissue processing technique that allows reliable immunohistochemical detection of these structural proteins on frozen sections of the chemically fixed, non-decalcified, adult human inner ear [13].

Materials and methods

Tissue preparation

Temporal bones (N=10) of five human adults (age: 59-76 years) without known history of inner ear disorders were fixed within three hours after death by perilymphatic perfusion with acetone (100%) for 10 min, followed by perfusion with phosphate-buffered saline (PBS). After removal of the temporal bones at autopsy, the bony vestibule and semicircular canals were exposed by dissection of the surrounding bone. Subsequently, these inner ear structures were micro-dissected, essentially as described by Hawkins and Johnsson [19]. Osmium tetroxide (OsO_4) postfixation was omitted because of its known deleterious effects on immuno-detectability of certain antigens. In order to maintain the tissues in optimal condition and to prevent drying of the membranous labyrinth, the entire procedure was performed in cold PBS (4°C). After removal of the bony capsule, the vestibular part of the membranous labyrinth was removed *in toto* from the remnants of the bony labyrinth. After this, the specimens were embedded in *Tissue-Tek® II* and cryosectioned (4-5 μm) [13].

Antisera

Sections were incubated with polyclonal rabbit and monoclonal mouse antibodies to five classes of intermediate filament proteins, i.e. vimentin, neurofilament proteins, desmin, glial fibrillary acidic protein (GFAP), and different cytokeratin subunits. Unless indicated otherwise, antibodies were obtained from Euro-Diagnostics BV, Apeldoorn, The Netherlands. The cytokeratin subunit classification used in this paper is according to Moll et al. [4].

CYTOKERATINS

Monoclonal antibodies 6B10 (dilution 1:1 – directed to Ck 4), RCK 102 (dilutions

1:2.5 to 1:5 – directed to Cks 5 and 8), RCK 105 (dilutions 1:1 to 1:5 – directed to Ck 7), M20 (dilutions 1:5 to 1:20 – directed to Ck 8), RKSE 60 (dilution 1:1 – directed to Ck 10) and 1C7 (dilution 1:1 – directed to Ck 13) were used. RPN 1162 (dilutions 1:1 to 1:2.5 – directed to Ck 7) and RPN 1165 (dilutions 1:5 to 1:20 – directed to Ck 19) were obtained from Amersham International plc, Amersham, Bucks, England. Finally, the recently described monoclonal antibodies RCK 107 (dilution 1:1 – directed to Ck 14) [20] and RCK 106 (dilutions 1:1 to 1:2.5 – directed to Ck 18) [21] were used.

VIMENTIN

This polypeptide was detected with the monoclonal antibody RV 202 (dilutions 1:200 to 1:600) [22].

DESMIN, GFAP AND NEUROFILAMENT PROTEINS

Desmin and GFAP were detected with polyclonal and monoclonal antibodies to desmin (PDE – dilutions 1:150 to 1:300 ; MDE – dilutions 1:1 to 1:2.5) and GFAP (PGF – dilutions 1:400 to 1:800 ; MGF – dilutions 1:1 to 1:1.5). For detection of the neurofilament proteins three monoclonal antibodies were used, i.e. MNF (dilutions 1:10 to 1:20 – directed to the 68/200 kD subunits), RNF 402 (dilutions 1:5 to 1:20 – directed to the 200 kD subunit) and RNF 403 (dilutions 1:1 to 1:5 – directed to the 160 kD subunit).

Immunolabeling

Cryosections (4-5 μm) were air dried at room temperature (15 min), fixed in 100% acetone (3 min), and air dried once more. After washing in PBS the specimens were incubated with 10% normal goat serum (NGS) (X 902, DAKOPATTS Corp., Glostrup, Denmark) in PBS for 20 min in order to diminish non-specific binding. The sections were then incubated with the primary antibodies at the appropriate dilutions in 10% NGS for 30 min. After washing in PBS, the sections were incubated for another 30 min with the secondary antibodies, i.e. rabbit anti-mouse IgG conjugated to peroxidase (P 260 DAKO-PATTS Corp., Glostrup, Denmark) for detection of monoclonal antibodies or swine anti-rabbit IgG conjugated to peroxidase (P 217 DAKOPATTS Corp., Glostrup, Denmark) for detection of polyclonal antibodies, diluted in 10% NGS. 3-Amino-9-ethylcarbazole served as a chromogen for the peroxidase reaction. Finally, sections were counterstained with Mayer's hemalum, mounted in Kaiser's glycerol gelatin, and examined by light microscopy.

Appropriate human positive control tissues were included to prove the specificity of the antibodies used (see Chapter 3). Negative controls were obtained by replacement of the primary antibody by either PBS or tissue culture medium (RPMI 1640, 10% FCS).

Results

Tissue morphology was well preserved in all specimens (Figs. 1-6). Most epithelial cell types, i.e. the sensory epithelia of both cristae and maculae, the cuboidal cells or so-called "transitional cells" adjacent to these sensory epithelia, the dark cell epithelium and the vestibular wall cells could easily be distinguished. However, cells of the planum semilunatum could not be clearly discerned, possibly due to the angle of sectioning. The ganglion cells of the vestibular (Scarpa's) ganglion were absent since the tissue-processing technique did not include their site in the internal auditory canal.

All positive controls demonstrated specific immunostaining, thus proving the reactivity of the antibodies.

In all specimens immunostaining for the different antisera yielded similar results, differing only slightly in the degree of immunostaining. The staining patterns for the different antisera throughout the vestibular labyrinth is summarized in Table 1 and depicted in Figs. 1-6.

CYTOKERATINS

Cks were detected in all distinguishable epithelial cell types lining the endolymphatic space of the vestibular labyrinth. Positive immunostaining with Ck antibodies was found in the following epithelial cell types: the cells of the sensory epithelia of the maculae and cristae which demonstrated intense immunostaining, the cuboidal or "transitional" cells adjacent to these sensory epithelia, the dark cell epithelium and the vestibular wall cells of both the vestibulum and the semicircular canals (Figs. 1a and 1b). However, all reactive antibodies demonstrated substantial differences in the degree of immunostaining (ranging from positive to negative) between adjacent, morphologically otherwise identical epithelial cells, especially in the nonsensory regions.

No immunostaining was observed with antibodies 6B10 (Ck 4), RKSE 60 (Ck 10), IC7 (Ck 13), and RCK 107 (Ck 14). Immunostaining for Cks 8, 18 and 19 in the sensory epithelia of the maculae and cristae was most intense in the basal region and at the apical surface of the epithelium (Figs. 2a, 2c and 6). However, it could not be discerned clearly at the lightmicroscopical level whether immunostaining was confined to either the sensory cells or the sensory supporting cells, or was present in both these cell types. The "transitional" epithelium and dark cell epithelium were also positive for Cks 8, 18 and 19 (Figs. 1a and 6). The vestibular wall cells of the vestibulum showed positivity for Cks 7, 8 and 19 (Fig. 1a), but strikingly not for Ck 18 as detected with antibody RCK 106. Even more striking was the finding that, in our hands, in the vestibular wall cells of the semicircular canals only Ck 19 could be detected (Fig. 1b). Both monoclonal antibodies to Ck 7 (i.e. RCK 105 and RPN 1162) revealed only very weak staining, limited to certain epithelial cells adjacent to the dark cell epithelium which were interpreted as vestibular wall cells. Immunostaining for Cks was absent in all other (non-epithelial) regions

Table 1 The overall staining patterns for the different IFP antisera throughout the vestibular labyrinth

	sensory epithelium maculae	sensory epithelium cristae	transitional epithelium	dark cell epithelium	vestibular wall cells - vestibulum	vestibular wall cells - semicircular canals	subepithelial connective tissue	nerve fibres and nerve endings	mesothelial lining	extracellular structures (cupulae, statoconial layers)
CYTOKERATINS										
- 6B10 (Ck 4)	–	–	–	–	–	–	–	–	–	–
- RCK 102 (Cks 5 & 8)	+	+	+	+	+	–	–	–	–	–
- RCK 105 (Ck 7)	–	–	–	–	+[1]	–	–	–	–	–
- RPN 1162 (Ck 7)	–	–	–	–	+[1]	–	–	–	–	–
- M20 (Ck 8)	+	+	+	+	+	–	–	–	–	–
- RKSE 60 (Ck 10)	–	–	–	–	–	–	–	–	–	–
- IC7 (Ck 13)	–	–	–	–	–	–	–	–	–	–
- RCK 107 (Ck 14)	–	–	–	–	–	–	–	–	–	–
- RCK 106 (Ck 18)	+	+	+	+	–	–	–	–	–	–
- RPN 1165 (Ck 19)	+	+	+	+	+	+	–	–	–	–
VIMENTIN										
- RV 202	+	+	+	+	+	+	+	–	+	–
NEUROFILAMENTS										
- MNF	–	–	–	–	–	–	–	+	–	–
- RNF 402	–	–	–	–	–	–	–	+	–	–
- RNF 403	–	–	–	–	–	–	–	+	–	–
GFAP										
- PGF	–	–	–	–	–	–	–	–	–	–
- MGF	–	–	–	–	–	–	–	–	–	–
DESMIN										
- PDE	–	–	–	–	–	–	–	–	–	–
- MDE	–	–	–	–	–	–	–	–	–	–

1) weak immunostaining in sporadic cells

of the membranous labyrinth, including the different mesothelial structures and such extracellular components as the cupulae or the statoconial layers of the maculae.

VIMENTIN

Immunostaining for vimentin was present in the subepithelial connective tissue fibroblasts and mesothelial lining (on the perilymphatic side) of the membranous labyrinth (Figs. 1c, 2b and 2d). Staining also occurred in all epithelial cell types bordering the endolymphatic space, including the cells of the sensory epithelia of maculae and cristae (with insufficient resolution to differentiate between sensory supporting cells and sensory cells; Figs. 2b and 2d), the cuboidal ("transitional") cells bordering these sensory epithelia, the dark cell epithelium and the vestibular wall cells of both the vestibulum and the semicircular canals (Fig. 1c). However, again a considerable variation in immunostaining between individual, otherwise identical, epithelial cells occurred as described for Ck staining. Immunostaining was absent in the nerve fibres reaching the sensory epithelia and in the cupulae and statoconial layers of the sensory organs (Figs. 2b and 2d).

NEUROFILAMENT PROTEINS

All monoclonal antibodies to neurofilament proteins revealed identical staining results. Immunostaining occurred in the nerve fibres reaching the sensory epithelia of the maculae and cristae (Figs. 3-4). Fig. 5 shows neurofilament reactivity in the nerve endings at the sensory cells, including the nerve calyces surrounding the type I hair cells.

DESMIN AND GFAP

All antibodies to these IFPs lacked immunostaining.

Discussion

In this study we have demonstrated the presence of cytokeratins (Cks), vimentin and neurofilament proteins in the adult human vestibular labyrinth whereas desmin and GFAP were found to be absent. Using monoclonal antibodies to different Ck polypeptides, we demonstrated the presence of Cks 7, 8, 18 and 19 in the epithelia of the vestibular labyrinth, while Cks 4, 10, 13 and 14 could not be identified with our antisera.

However, since biochemical analysis was not performed in support of negative immunohistochemical findings, absence of staining (desmin, GFAP and specific Ck subunits) does not definitely exclude the presence of the IFPs concerned and the results are therefore to be interpreted with caution.

Our present immunohistochemical results on Ck expression confirm in general earlier, albeit more limited, data on the embryonic human inner ear [17, 18].

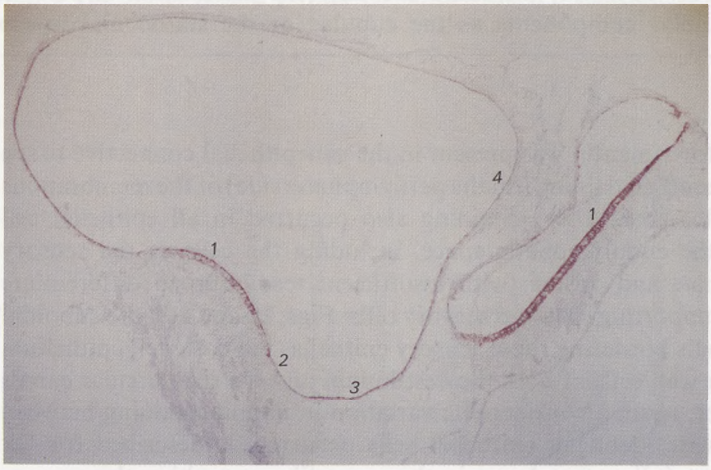

Fig. 1a

Fig. 1b

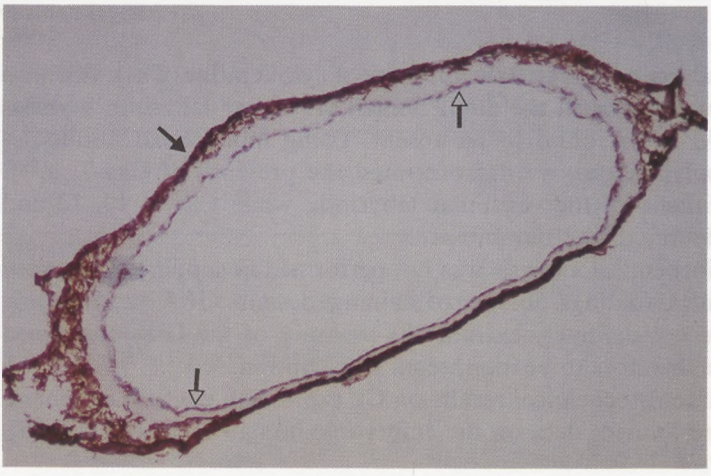

Fig. 1c

In addition, the Ck positivity of all cell types lining the endolymphatic space of the vestibular labyrinth supports the generally accepted epithelial origin of these cells. However,it is not clear whether the differences in immunostaining between adjacent, morphologically otherwise similar, individual cells reflect true differences in their Ck composition, since we are well aware of the fact that epitope masking may occur [21].
The absence of staining for cytokeratins in the different mesothelial structures in the vestibular labyrinth is striking since other mesothelia in the human body are known to generally co-express cytokeratins in addition to vimentin [3].
Ck staining of the different cell types in the sensory epithelia of the maculae and cristae, i.e. sensory supporting cells and sensory cells, remains controversial. In two subsequent studies on human embryos, Anniko and co-workers [17, 18] described immunoreactivity either in both cell types [18], or only in the supporting cells [17]. In the present study, insufficient resolution of our immunohistochemical technique in the different sensory epithelia did not allow definite conclusions on this matter. However, we demonstrated a particularly intense immunoreactivity for Cks along the surface and in the basal regions of the sensory epithelia of the maculae and cristae. This pattern highly resembles the staining pattern for Cks in the other neuroepithelial structure of the inner ear, the organ of Corti, in which staining was also most intense in the basal and apical regions. In this structure, Cks were demonstrated exclusively in the various supporting cells but not in the hair cells [15]. Because the basal regions of the vestibular sensory epithelia of maculae and cristae mainly contain the cell bodies of the sensory supporting cells, our finding of intense staining in this region suggests positive staining of at least this cell type. Immuno-electronmicroscopical studies are needed to elucidate the exact Ck distribution pattern in the vestibular sensory epithelia.
Since it has become generally accepted that Cks, at least partially, mediate tissue plasticity [23], our finding of intense immunostaining for Cks in the sensory epithelia of the maculae and cristae suggests that these sensory organs may be rigid structures. This cellular rigidity may then be considered to be

Fig. 1 (a-c) [a] Crista ampullaris and macula utriculi, immunostained with monoclonal antibody RPN 1165 to cytokeratin 19. Intense immunoreactivity of the sensory epithelia of crista and macula (1), moderate staining of the "transitional" epithelium (2), the region of the dark cell epithelium (3) and the vestibular wall cells (4) (x 20). [b] Cross-section through a semicircular canal, immunostained with monoclonal antibody RPN 1165 to cytokeratin 19. Immunoreactivity only of the vestibular wall cells lining the endolymphatic space (arrows)(x 70). [c] Cross-section through a semicircular canal, immunostained with monoclonal antibody RV 202 to vimentin. Intense immunostaining of the mesenchymal cells on the perilymphatic side (filled arrow), whereas the vestibular wall cells (lining the endolymphatic space) demonstrate only moderate immunoreactivity (open arrows) (x 70).

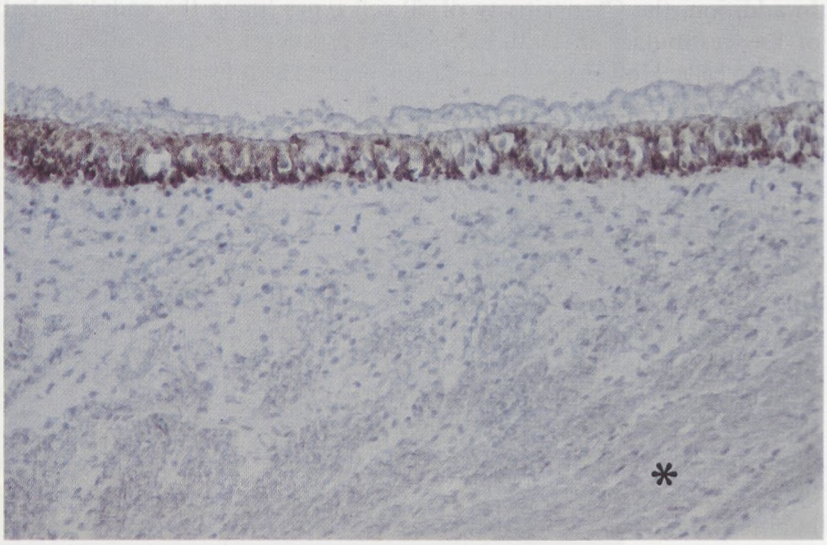

Fig. 2a

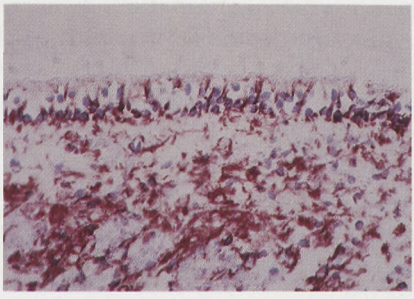

Fig. 2b

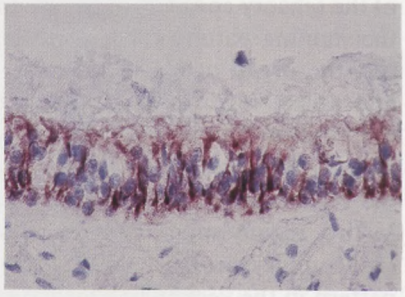

Fig. 2c

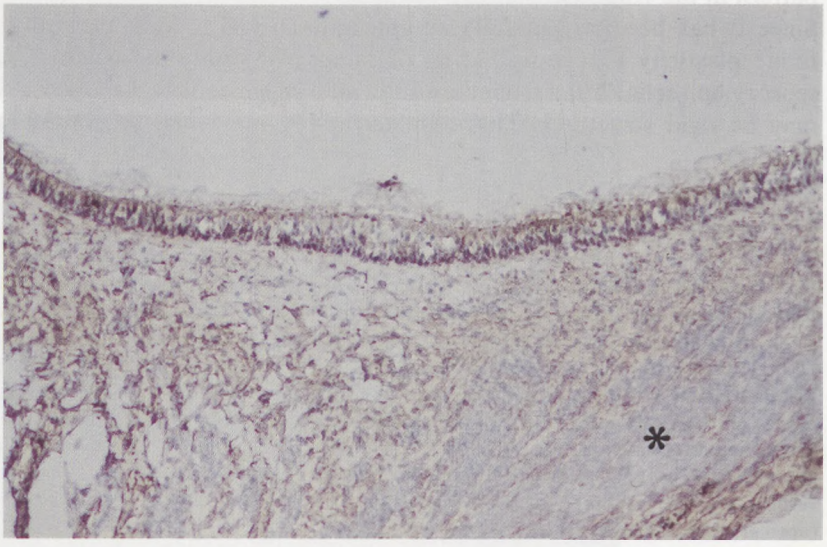

Fig. 2d

of importance in the mechano-electrical transduction process for the sense of equilibrium through a possible function in opposing the motion of the cupulae and statoconial layers.

The application of monoclonal antibodies to different Ck subunits enables the differentiation of epithelia by means of their CK expression pattern. In this study we used monoclonal antibodies which are monospecific for Cks 4, 7, 8, 10, 13, 14, 18 and 19, thus including markers for "simple", stratified (among which keratinizing) and "complex" epithelia as outlined in the Introduction. We have shown in this study that the vestibular epithelia contain Cks 7, 8, 18 and 19 which are characteristic of "simple" or single-layered epithelia. The same Ck subtypes have recently been demonstrated in the adult human cochlea [16]. However, in the adult human endolymphatic duct and sac, Cks typical of "complex" epithelia were found since in this part of the inner ear the additional presence of Ck 14 was demonstrated [24].

A further classification of "simple" epithelia on the basis of their Ck pattern is generally not possible since similar patterns are common within multiple, functionally heterogeneous, "simple" epithelia [16].

All Cks other than Ck 7 were found to have a rather uniform distribution in the different epithelial cell types of the vestibular labyrinth. However, the vestibular wall cells of both the vestibulum and semicircular canals demonstrated considerable differences in their immunoreactivity to different Ck antibodies. It is, however, uncertain whether these staining results represent true differences in their Ck composition or possibly reflect artifacts due to antigenic masking or due to differences in the affinity of the antibodies concerned.

The possibility of false-negative results due to epitope masking with our two different antibodies to Ck 7, however, is not conceivable, since these antibodies were obtained from different fusions and are therefore likely to recognize different epitopes. However, although results with both antibodies were identical, immunostaining was so weak that we are reluctant to draw any conclusions as to a possible differential expression of Ck 7 in the epithelium of the vestibular labyrinth.

All epithelial cell types (including the sensory epithelia) expressed vimentin

Fig. 2 (a-d) [a] Macula utriculi, immunostained with monoclonal antibody RCK 102 to cytokeratins 5 & 8. Intense staining of the sensory epithelium. Note absence of staining in the statoconial layer and in the subepithelial connective tissue, among which the neural bundles (asterisk) (x 120). [b] Macula utriculi, immunostained with monoclonal antibody RV 202 to vimentin. Intense immunostaining of the sensory epithelium. The subepithelial fibroblasts also demonstrate immunoreactivity, whereas the statoconial layer lacks immunostaining (x 175). [c] Detail of the macula utriculi, immunostained with monoclonal antibody RCK 102 to cytokeratins 5 & 8. See also Fig. 2a (x 300). [d] Macula utriculi, immunostained with monoclonal antibody RV 202 to vimentin. Absence of staining in the neural bundles (asterisk). See also Fig. 2b (x 100).

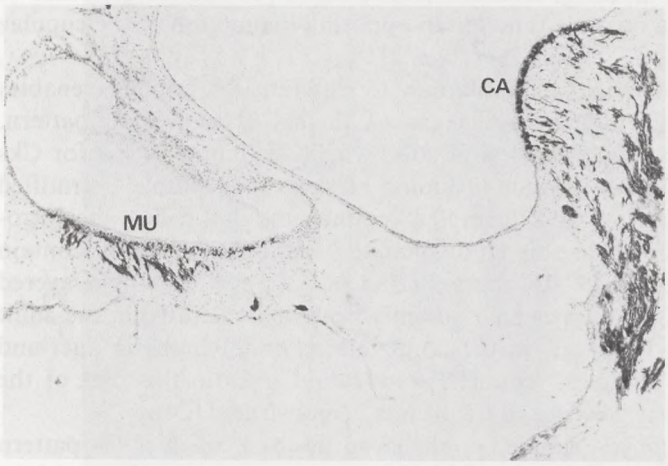

Fig. 3 Crista ampularis (CA) and macula utriculi (MU), immunostained with monoclonal antibody RNF 403 to neurofilament proteins. Immunostaining of the nerve fibres reaching the sensory epithelia (x 20).

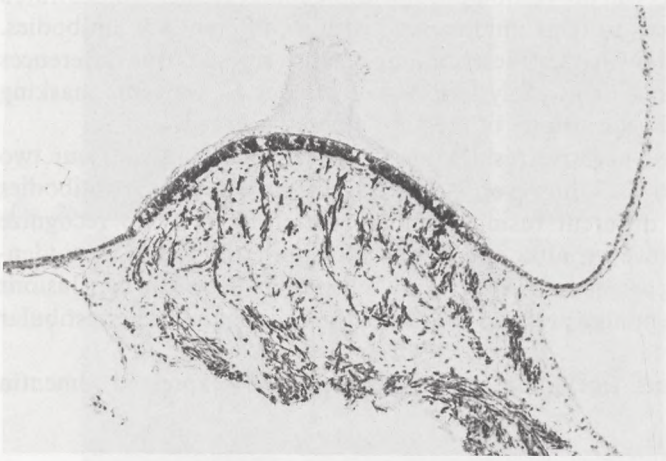

Fig. 4 Crista ampularis, immunostained with monoclonal antibody MNF to neurofilament proteins. Immunostaining of the nerve fibres reaching the sensory epithelium (x 45).

in addition to Cks, thus demonstrating co-expression of these IFPs. Interestingly, co-expression of Cks and vimentin has also been demonstrated in other parts of the adult human inner ear. In the cochlea, the pillar cells and Deiters' cells of the organ of Corti and the region of the spiral prominence and outer sulcus demonstrated co-expression of these IFPs [15], whereas in the endolymphatic duct and sac the entire epithelial lining demonstrated this phenomenon [24]. Although the finding of co-expression of Cks and vimentin enables a further

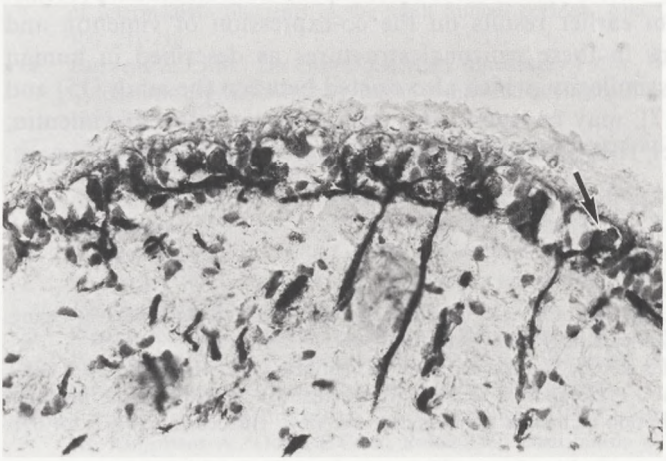

Fig. 5 Detail of the sensory epithelium of the crista ampularis, immunostained with monoclonal antibody RNF 403 to neurofilament proteins. Positively staining nerve fibres are seen penetrating into the epithelium after which they branch into smaller fibres. Staining of the nerve calyces surrounding the type I hair cells can be seen (arrow) (x 170).

Fig. 6 Crista ampularis, immunostained with monoclonal antibody RCK 102 to cytokeratins 5 & 8. Immunostaining is not only seen in the sensory epithelium, but also in the adjacent cuboidal or "transitional" cells (arrows) (x 45).

characterization of tissues, its meaning is not yet understood. Co-expression of these two IFPs has been demonstrated in several human neuroepithelial tissues [25], but has also been found in other epithelia and mesothelial cells [3].

All neuronal structures were found exclusively to express neurofilament proteins. We could not confirm earlier results on the co-expression of vimentin and neurofilament proteins in these neuronal structures as described in human embryos [17]. This dissimilarity, which also existed between the adult [15] and embryonic cochlea [17], may be due to the transient expression of vimentin, in addition to another IFP, in many tissues during embryonic development.

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CHAPTER 6

DIFFERENTIAL IMMUNOHISTOCHEMICAL DETECTION OF CYTOKERATINS AND VIMENTIN IN THE SURGICALLY REMOVED HUMAN ENDOLYMPHATIC DUCT AND SAC

This chapter has been submitted for publication in Eur Arch Otorhinolaryngol
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F. Linthicum, J.E. Veldman and E.H. Huizing

Introduction

Cytokeratins (Cks) are a family of polypeptides which belong to the class of intermediate filament proteins (IFPs) and are part of the cytoskeleton of epithelial cells. Vimentin, also an IFP, is in general synthesized by mesenchymally derived cells. Apart from this tissue-specific distribution, co-expression of Cks and vimentin has been described in several epithelia and mesothelial structures. Desmin, glial fibrillary acidic protein (GFAP) and neurofilament proteins constitute other IFPs, typically expressed immunohistochemically by muscle, astroglia and neuronal cells, respectively [1-3].

Up to now, 20 different Cks have been distinguished in human epithelia. According to Moll et al [4] they are numbered consecutively 1 to 20, representing differences in molecular weight and isoelectric pH. Furthermore, it has become clear that most basic Cks (type II; nos. 1-8) form a pair with a specific acidic Ck counterpart (type I; nos. 9-20) [5]. Such pairs of at least two or more Cks occur in cell-type-specific combinations reflecting both the cell's embryonic origin and its differential state [4-6]. A clear correlation has been found between the complexity of an epithelium ("simple" vs. stratified vs. "complex" epithelia) and the type of Cks expressed [4-8]. "Simple" or single-layered epithelia express two to four of the group of Cks 7, 8, 18 and 19. Stratified epithelia express Cks 5 and 14/15, combined with one or more of the group of Cks 1, 2, 3, 4, 10, 12 and 13, depending on the differentiation programme (skin, corneal or esophageal type). Finally, "complex" or "mixed" epithelia such as glandular epithelia containing both basal and luminal cells, transitional epithelia and pseudostratified epithelia usually express a mixture of "simple" and stratified types of Ck polypeptides. In addition, it has become clear that within such stratified and "complex" epithelia different cell types (i.e. basal, suprabasal or luminal) are distinguishable by their Ck expression pattern [8].

Over the past years, various polyclonal and monoclonal antibodies to different IFPs – and to different Ck subunits – have been developed, thus allowing the immunohistochemical characterization of tissues and the classification of different epithelia.

The mammalian endolymphatic duct (ED) and sac (ES) contain highly specialized epithelial cells of non-sensory origin. Since the human ED and ES is not clearly divided into areas with separate types of epithelia – a situation different from that in most lower animals – the nomenclature for subdivision of the human duct and sac as proposed by Bast and Anson [9] seems most appropriate. They divide the ED in the sinus and the isthmus portion while the ES is divided in the rugose and the smooth portion (i.e. endolymphatic sac proper). Since in man the rugose portion is restricted to the part of the

sac whithin the vestibular aqueduct (i.e. the intraosseous part), the smooth portion of the human sac may also be termed the extraosseous part.
Mainly based on animal experiments, various functions have been attributed to the different epithelial regions of the ED and ES, such as an active role in the turnover of inner ear fluids [10], a possible role in a local immunologic defense system [11], and a possible role in statoconial formation and degradation [12].
Over the past years there have been several reports describing the ultrastructure of the different epithelial cell types in both the human endolymphatic duct and sac [13-15]. However, apart from previous immunohistochemical studies on various animal species [16-18] and human embryos [19], the first report on the immunohistochemical characterization of the epithelium of the adult human ES has only recently been published [20]. Several Cks and vimentin were demonstrated in the epithelium. However, this study restricts itself to the extraosseous (smooth) portion of the human sac, thus excluding the highly differentiated epithelial cells of the intraosseous rugose portion and the ED. Moreover, only three monospecific antibodies to different Cks were used in this latter study [20], thus leaving the possible presence of many Ck subunits undetermined.

Recently, we have described the immunohistochemical detection of Cks in the epithelium of the adult human cochlea, demonstrating a Ck pattern typical of "simple" epithelia [21]. A similar Ck pattern was also found in the adult human vestibular labyrinth [22].
The aim of the present study was to characterize the epithelium of the adult human ED and ES by investigating its immunohistochemical expression of Cks and other IFPs in the various regions.

Materials and methods

Tissue preparation

The endolymphatic duct and sac of five patients (age: 21-69 years) were dissected and removed during surgery for acoustic neuroma through a translabyrinthine approach. Subsequently, the tissues were embedded in *Tissue-Tek® II* and cryosectioned (4-5 μm).

Antisera

Sections were incubated with polyclonal rabbit and monoclonal mouse antibodies to five classes of IFPs, i.e. vimentin, neurofilament proteins, desmin, glial fibrillary acidic protein (GFAP), and different Ck subunits. Unless indicated otherwise, antibodies were obtained from Euro-Diagnostics BV, Apeldoorn, The Netherlands. The Ck subunit classification as described in this paper is according to Moll et al. [4].

CYTOKERATINS

Monoclonal antibodies 6B10 (dilution 1:1 – directed to Ck 4), RCK 102 (dilutions 1:2.5 to 1:5 – directed to Cks 5 and 8), RCK 105 (dilutions 1:1 to 1:5 – directed to Ck 7), M20 (dilutions 1:5 to 1:20 – directed to Ck 8), RKSE 60 (dilution 1:1 – directed to Ck 10) and 1C7 (dilution 1:1 – directed to Ck 13) were used. RPN 1162 (dilutions 1:1 to 1:2.5 – directed to Ck 7) and RPN 1165 (dilutions 1:5 to 1:20 – directed to Ck 19) were obtained from Amersham International plc, Amersham, Bucks, England. Finally, the recently described monoclonal antibodies RCK 107 (dilution 1:1 – directed to Ck 14) [23], LL002 (dilution 1:1 – directed to Ck 14) [23], E 3 (dilution 1:1 – directed to Ck 17) [24] and RCK 106 (dilutions 1:1 to 1:2.5 – directed to Ck 18) [25] were used.

VIMENTIN

This polypeptide was detected with the monoclonal antibody RV 202 (dilutions 1:200 to 1:600) [26].

DESMIN, GFAP AND NEUROFILAMENT PROTEINS

Desmin and GFAP were detected with polyclonal and monoclonal antibodies to desmin (PDE – dilutions 1:150 to 1:300 ; MDE – dilutions 1:1 to 1:2.5) and GFAP (PGF – dilutions 1:400 to 1:800 ; MGF -dilutions 1:1 to 1:1.5). For detection of the neurofilament proteins three monoclonal antibodies were used, i.e. MNF (dilutions 1:10 to 1:20 – directed to the 68/200 kD subunits), RNF 402 (dilutions 1:5 to 1:20 – directed to the 200 kD subunit), and RNF 403 (dilutions 1:1 to 1:5 – directed to the 160 kD subunit).

Immunolabeling

Cryosections (4-5 μm) were air dried at room temperature (15 min), fixed in 100% acetone (3 min), and air dried once again. After washing in PBS the specimens were incubated with 10% normal goat serum (NGS) (X 902, DAKOPATTS Corp., Glostrup, Denmark) in PBS for 20 min in order to diminish non-specific binding. The sections were then incubated with the primary antibodies at the appropriate dilutions in 10% NGS for 30 min. After washing in PBS, the sections were incubated for another 30 min with the secondary antibody, i.e. rabbit anti-mouse IgG conjugated to peroxidase (P 260 DAKO-PATTS Corp., Glostrup, Denmark) for detection of monoclonal antibodies or swine anti-rabbit IgG conjugated to peroxidase (P 217 DAKOPATTS Corp., Glostrup, Denmark) for detection of polyclonal antibodies, diluted in 10% NGS. 3-Amino-9-ethylcarbazole served as a chromogen for the peroxidase reaction. Finally, sections were counterstained with Mayer's hemalum, mounted in Kaiser's glycerol gelatin, and examined by light microscopy.

Appropriate human positive control tissues were included to prove the specificity of the antibodies used (see Chapter 3). Negative controls were obtained by replacement of the primary antibody by either PBS or tissue culture medium (RPMI 1640, 10% FCS).

Results

In all specimens, the morphology was well preserved at the lightmicroscopical level (Figs. 1-3). In contrast to the ES which was easily distinguishable in all cases, the ED could only be discerned clearly in one specimen. However, since there is little or no morphological distinction between the end of the ED and the proximal part of the ES, there is a possibility that this structure actually represents the most proximal part of the ES. Although the majority of ES specimens comprised only the intraosseous or rugose portion of the ES, two specimens also contained a substantial part of the smooth or extraosseous portion (Fig. 1d).

All positive controls showed specific immunostaining, thus proving the reactivity of the antibodies.

Immunostaining for the different antisera yielded similar results in all five specimens, differing only slightly in the degree of staining.

CYTOKERATINS

The epithelium of both the ED and ES, which appeared single layered at the lightmicroscopical level, stained positively for RCK 102 (Cks 5 & 8), RCK 105 (Ck 7), RPN 1162 (Ck 7), M20 (Ck 8), RCK 107 (Ck 14), LL002 (Ck 14), E 3 (Ck 17), RCK 106 (Ck 18), and RPN 1165 (Ck 19) (Figs. 1-3, showing immunostaining for Cks 7, 8, 14, 18 & 19). However, although positive staining for all these antibodies was present in all regions of the ED and ES, substantial differences were seen in the degree of immunostaining (ranging from positive to negative) between morphologically otherwise similar individual cells. This especially occurred with antibodies RCK 107 (Ck 14), LL002 (Ck 14) and E 3 (Ck 17), which demonstrated immunostaining in roughly 40% of the epithelial cells throughout the ED and ES (with, however, large variations) (Fig. 1b). The cells which stained positively for these antibodies detecting Cks 14 and 17 were morphologically not distinguishable at the lightmicroscopical level from non-staining adjacent epithelial cells. The other immunoreactive Ck antibodies, i.e. RCK 102 (Cks 5&8), RCK 105 (Ck 7), RPN 1162 (Ck 7), M20 (Ck 8), RCK 106 (Ck 18) and RPN 1165 (Ck 19) stained the majority of epithelial cells throughout the ED and ES (Figs. 1a, 1c-e, 2a and 3).

No immunostaining was observed with antibodies 6B10 (Ck 4), RKSE 60 (Ck 10) and IC7 (Ck 13).

VIMENTIN

Positive immunostaining occurred in the epithelium of the ED and ES, as well as in the subepithelial connective tissue fibroblasts (Figs. 2b and 2c).

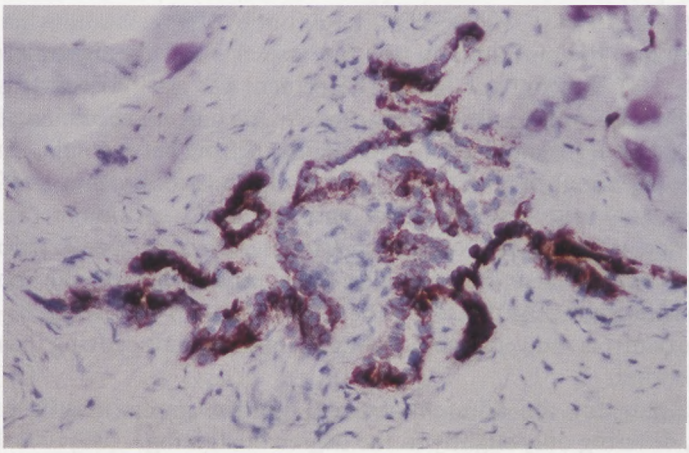

Fig. 1a

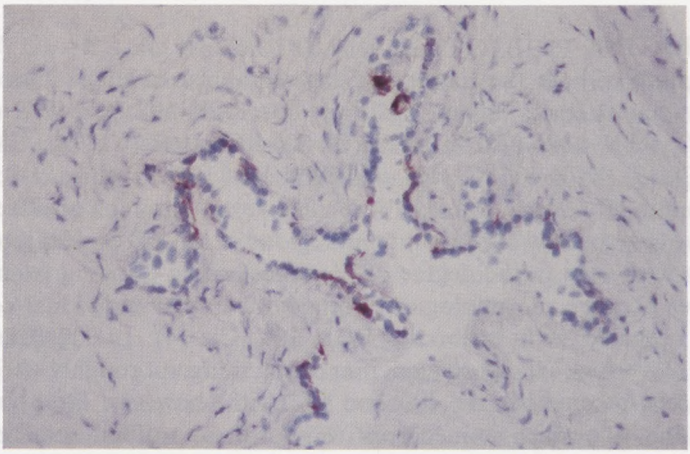

Fig. 1b

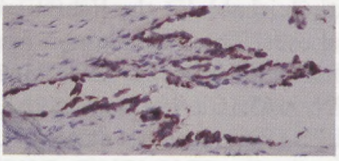

Fig. 1c

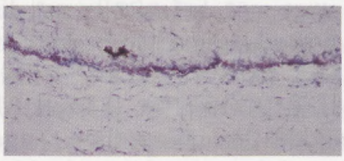

Fig. 1d

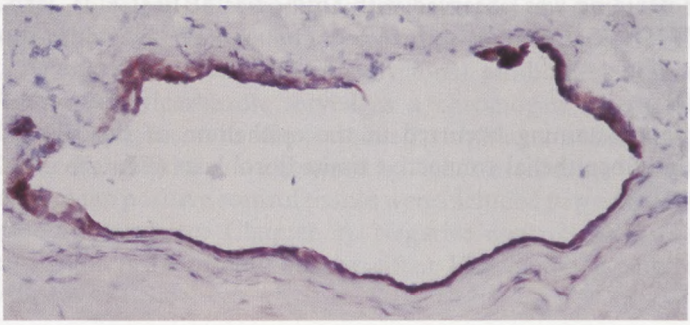

Fig. 1e

DESMIN, GFAP AND NEUROFILAMENT PROTEINS
All antibodies to these IFPs demonstrated absence of immunostaining.

Discussion

The procedure of surgical removal of the adult human endolymphatic duct (ED) and sac (ES) as performed in this study allows optimal preservation of immunoreactivity, since chemical fixation and decalcification are avoided. Thus, detection of antigens is performed on cryosections of freshly frozen tissue, offering the most reliable immunohistochemical results [27]. A disadvantage of the removal of the ED and ES during surgery, however, is that a substantial portion of the smooth or extraosseous part of the ES cannot be adequately dissected because of its close relation to the dura mater. Another disadvantage of our specimens could be the presence of an acoustic neuroma adjacent to the ES. However, we have found no reason to assume any influence on the immunohistochemical results.

The monospecific monoclonal antibodies to cytokeratins (Cks) 4, 7, 8, 10, 13, 14, 17, 18 and 19 as used in this study include markers for "simple" (single-layered), stratified (among which keratinizing) and "complex" or "mixed" epithelia as outlined in the Introduction.

We have demonstrated immunoreactivity for Cks 7, 8, 14, 17, 18 and 19 in the epithelium of the human ED and ES, while reactivity for Cks 4, 10 and 13 was absent. In addition, the epithelium of the adult human ED and ES demonstrated co-expression for Cks and vimentin. The remaining IFPs tested for, i.e. desmin, GFAP and neurofilament proteins, could not be detected immunohistochemically. Concerning the absence of these IFPs and of certain Ck subunits it must be stated, however, that absence of immunostaining –

Fig. 1 (a-e) [a] Intraosseous rugose portion of the endolymphatic sac, immunostained with monoclonal antibody RPN 1162 to cytokeratin 7. Intense immunostaining of the epithelium of the endolymphatic sac. However, note differences in the degree of staining between individual cells (x 120). [b] Intraosseous rugose portion of the endolymphatic sac, immunostained with monoclonal antibody RCK 107 to cytokeratin 14. Although the majority of epithelial cells lack immunoreactivity, other cells demonstrate clear immunostaining (x 110). [c] More distal portion of the endolymphatic sac with a villus protruding into the lumen; immunostained with monoclonal antibody RPN 1165 to cytokeratin 19. Clear immunostaining of the epithelial lining of the sac (x 60). [d] Extraosseous (or smooth) portion of the endolymphatic sac with its very narrow, slit-like lumen; immunostained with monoclonal antibody RPN 1165 to cytokeratin 19. Positive immunostaining of all epithelial cells (x 40). [e] Endolymphatic duct, immunostained with monoclonal antibody RCK 102 to cytokeratins 5 & 8. Intense immunostaining of the epithelial lining of the duct (x 80).

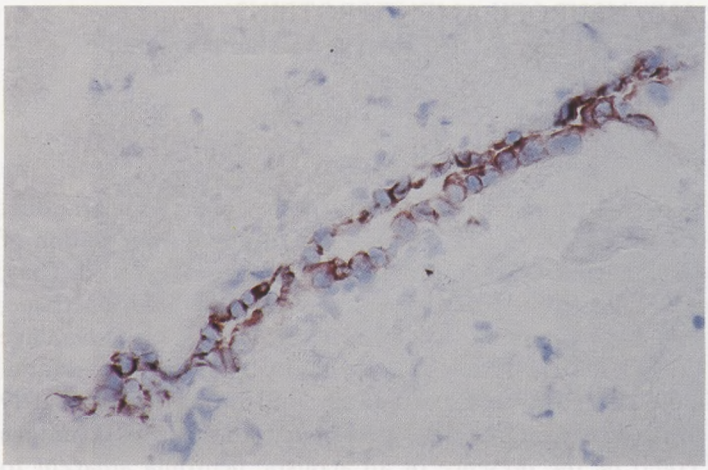

Fig. 2a

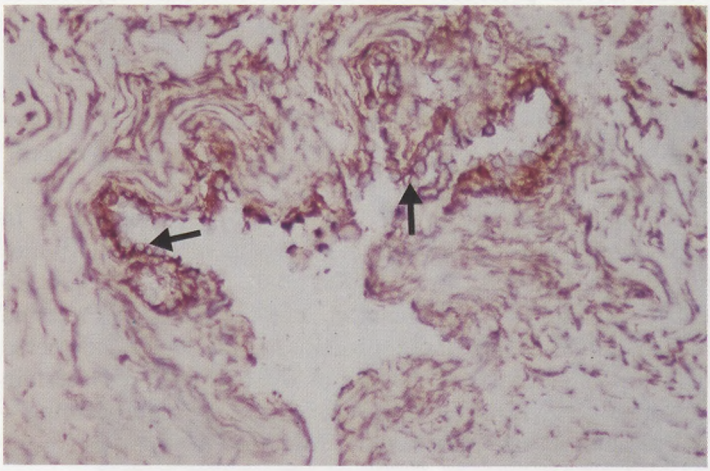

Fig. 2b

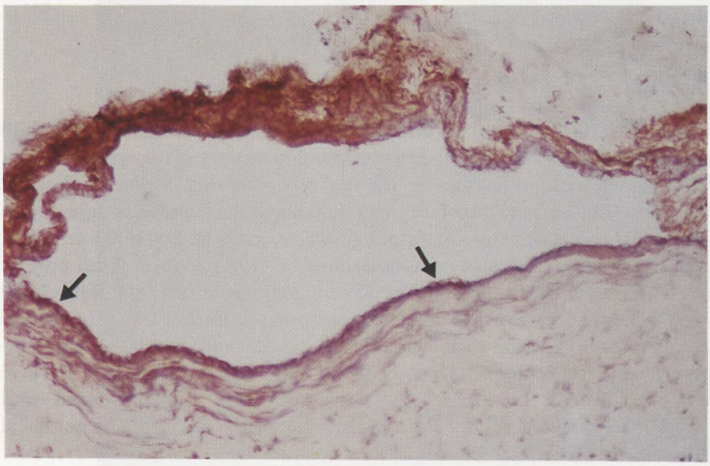

Fig. 2c

without support of biochemical analysis – does not definitely exclude the presence of the antigens concerned and therefore must be interpreted with caution.
Our results on the expression pattern of Cks in the adult human ED and ES are generally in agreement with earlier, albeit limited, data on mice [16-18]. These studies demonstrated the presence of Cks 7, 8, 18, and 19 in the ED and ES, without, however, testing for Cks 14 and 17, two subunits which we found to be present. However, when compared to a study by Anniko et al. [19] on human fetuses, the results of which may differ considerably from those obtained in the human adult due to transient expression of IFPs during embryonic development, there are notable differences. In this study, in which probes were used for Cks 7, 8, 10 and 18, the authors claimed the presence of Ck 10, apart from Cks 7, 8 and 18 [19]. We were not able to detect Ck 10 in our material, a polypeptide typical of keratinized epidermis of which the presence in the inner ear also seems very unlikely on theoretical grounds. Also, we were not able to confirm recently published data by Altermatt et al. [20] on the presence of Ck 13, besides Cks 18 and 19, in the extraosseous or smooth portion of the adult human ES, a subunit typical of non-keratinizing stratified epithelia as present in esophageal, tongue and cervical epithelia.

The Ck pattern in the epithelium of the adult human ED and ES as found in this study may be characterized as typical of a "complex" or "mixed" epithelium, since it constitutes a mixture of Cks typical of "simple" epithelia (Cks 7, 8, 18 & 19), a keratinocyte-specific Ck (Ck 14), and Ck 17 which is a Ck subunit that is frequently co-expressed with Ck 14 [24]. In addition, markers for both keratinizing (Ck 10) and non-keratinizing (Cks 4 & 13) stratified epithelia were absent.
As recently demonstrated by Purkis et al. [8], the 5/14 Ck-pair is expressed by all so-called basal cells (cells in contact with the basal lamina but out of contact with the epithelial surface) of each multilayered or "mixed" epithelium, whether it concerns glandular, ductal, secretory or stratified squamous epithelia. Furthermore, the 5/14 Ck-pair has been shown to be absent in all homogenous

Fig. 2 (a-c) [a] Intraosseous part of the endolymphatic sac, immunostained with monoclonal antibody RPN 1165 to cytokeratin 19. Immunostaining of all cells in the epithelial lining of the endolymphatic sac (x 200). [b] Intraosseous rugose portion of the endolymphatic sac, immunostained with monoclonal antibody RV 202 to vimentin. Immunoreactivity is seen in the epithelial lining of the endolymphatic sac (arrows) as well as in the subepithelial fibroblasts (x 150). [c] Endolymphatic duct, immunostained with monoclonal antibody RV 202 to vimentin. Immunostaining of both the epithelial lining of the endolymphatic duct (arrows) and of the underlying fibroblasts (x 70).

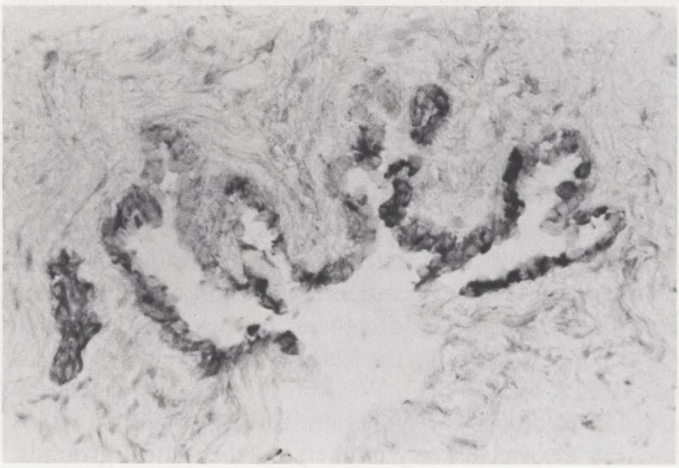

Fig. 3 Intraosseous rugose portion of the endolymphatic sac, immunostained with monoclonal antibody RCK 106 to cytokeratin 18. Clear immunostaining of practically all cells in the epithelial lining of the sac (x 120).

"simple" or single-layered epithelia, of which the cells border both the basal lamina and the epithelial surface [8]. Thus, our finding of Ck 14 expression in a subpopulation of cells throught the epithelial lining of the ED and ES, as detected with two different antibodies which are likely to recognize different epitopes, indicates the presence of basal cells and thus supports the classification of this epithelium as a "mixed" epithelium. Morphological data presented in two recent studies on the ultrastructure of the human ES [13, 15] and ED [14] demonstrated a highly differentiated epithelium in which an enormous variability exists in size and shape of the epithelial cells (either flattened, cuboidal or cylindrical). However, while Schindler [13] reported an occasionally pseudostratified appearance of the epithelium in the rugose folds and villi of the intraosseous portion of the sac, Bagger-Sjöbäck et al. [15] did not report such findings and described the epithelium to be purely single-layered. Our immunohistochemical data present new evidence of pseudostratification, since the expression of Ck 14 has, until now, never been found in homogeneous single-layered epithelia. The immunohistochemical classification of the epithelium of the human ED and ES as a "complex" or "mixed" epithelium differs strikingly from results by our group on the epithelia of the cochlea [21] and vestibular labyrinth [22] in which Cks were demonstrated typical of "simple" epithelia (i.e. Cks 7, 8, 18 & 19). Apparently, the epithelium of the ED and ES has a higher degree of complexity, and differs considerably in its immunohistochemical phenotype from the other epithelia in the adult human inner ear. Although differences in the ultrastructure of epithelial cells have been described between different regions of the ED and ES (e.g. differences in junctional characteristics between the duct and proximal part of the sac, and the rugose

and more distal part of the sac [28]), we were not able to detect clear regional differences in immunostaining for either cytokeratins or vimentin.

Similar to certain other epithelial structures in both the cochlea and vestibular labyrinth [22, 29], the entire epithelium of the ED and ES demonstrated co-expression of Cks and vimentin, as earlier described by Altermatt et al. [20] for the extraosseous or smooth portion of the adult human sac. However, the meaning of co-expression of Cks and vimentin is not yet understood and although it has been demonstrated in several neuroepithelial tissues [30], it has also been found in other epithelia and mesothelial cells [3].

Our immunohistochemical results indicate a high degree of cellular and functional differentiation of the ED and ES epithelium and give support to the view that the ED and ES are a functionally active part of the inner ear. Future studies will have to elucidate how certain functions of this structure correlate with its type of epithelial differentiation.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

Summary and conclusions

Although the principles of immunohistochemistry were outlined by Coons as early as 1941 and although immunohistochemisty had been fully adopted in various fields of research by the 1970s, the first immunohistochemical investigations of the mammalian inner ear were not reported until 1980. Since then, immunohistochemistry has greatly added to our knowledge of the structure-related function of the inner ear.
In the present study, the different tissues in the various parts of the adult human inner ear (i.e. cochlea, vestibular labyrinth and endolymphatic duct and sac) were immunohistochemically characterized by investigating their expression patterns of intermediate filament proteins (IFPs).

Tissue preparation

In Chapter 2 a new tissue-processing technique for immunohistochemistry of the adult human cochlea and vestibular labyrinth has been described.
The procedure can be summarized as follows: (1) Chemical fixation of the inner ear by perilymphatic perfusion within several (< 3) hours after death, (2) exposure of the bony cochlea, vestibule and semicircular canals by dissection of the surrounding bone, (3) microdissection of the bony labyrinth, (4) removal *in toto* of the vestibular and cochlear parts of the membranous labyrinth, (5) embedding in *Tissue-Tek® II*, and (6) cryosectioning (4-5 μm). This technique excludes the immuno-compromizing step of prolonged decalcification and thus results in optimal preservation of antigenicity. It enables reliable immunohistochemical detection of structural proteins such as IFPs.
All specimens of the adult human cochlea and vestibular labyrinth as described in this thesis were processed according to this technique. The specimens of the endolymphatic duct and sac were processed differently since microdissection of this part of the inner ear is extremely difficult because of the absence of a surrounding perilymphatic compartment. After having been removed during translabyrinthine surgery, they were embedded in *Tissue-Tek® II* and subsequently cryosectioned without prior chemical fixation or decalcification.

In Chapters 3 to 6 results have been presented on the immunohistochemical detection of several types of cytoplasmic IFPs in the human cochlea (Chapters 3 and 4), the human vestibular labyrinth (Chapter 5), and the human endolymphatic duct and sac (Chapter 6). Three classes of IFPs have been found to be present in the adult human inner ear, i.e., cytokeratins, vimentin and neurofilament proteins. Desmin and glial fibrillary acidic protein could not be detected.
Unlike the descriptions as presented in Chapters 3 to 6, the data on IFP-expression – and their possible functional implications – will be summarized in this section for each IFP separately, throughout the human inner ear.

Cytokeratins

Cytokeratins, in general only synthesized by epithelia, were detected in the entire cellular lining of the endolymphatic space of the inner ear, i.e. the cochlea, vestibular labyrinth and endolymphatic duct and sac. This finding is in accordance with the accepted epithelial origin of these cells. Interestingly, and in contrast to other mesothelia in the human body, the different mesothelial structures in the cochlea and vestibular labyrinth did not co-express cytokeratins in addition to vimentin.

Cochlea

While immunostaining for cytokeratins in the cochlea occurred in virtually all cells lining the cochlear duct and in most supporting cells of the organ of Corti, the hair cells lacked immunostaining for these as well as for all other IFPs.

Vestibular labyrinth

A similar staining pattern was seen in the vestibular sensory epithelia of the maculae and cristae. Staining was suggestive of cytokeratin positivity of only the supporting cells, although insufficient resolution of the lightmicroscopical technique did not allow us to definitely exclude staining in the vestibular sensory cells.

Functional implications

The finding of negative immunostaining for cytokeratins (and several other main types of cytoplasmic IFPs) in cochlear (and probably vestibular) hair cells is an unusual phenomenon and is as yet not understood. Interestingly, in contrast to the absence of cytokeratins in the cytoskeleton of these sensory cells, the surrounding supporting cells stained intensely for cytokeratin antibodies. Although very little is known about the exact functions of cytokeratins or any other IFP, it has become generally accepted that tissue plasticity is, at least partially, mediated by cytokeratins. They cross the cell's cytoplasm to link up with desmosome cell-to-cell attachment sites at cell boundaries, forming a supracellular network throughout the tissue. Taking this into consideration, the expression patterns for cytokeratins in the different sensory regions of the inner ear may well have functional significance.

In the organ of Corti, the presence of cytokeratins in the reticular lamina of the supporting cells, together with their absence in the enclosed apical portions of the hair cells, seems of particular importance since it may be assumed that the resulting difference in stiffness between the sensory and supporting cells plays a substantial role in cochlear micromechanics. Similarly, the intense staining for cytokeratins in the vestibular sensory epithelia (although the exact cytokeratin pattern in these epithelia has yet to be determined) suggests that the maculae and cristae are rigid structures of which the stiffness may be assumed to be of importance in the mechano-electrical transduction process for the sense of equilibrium. In this process, the motion of the cupulae and statoconial

layers may be thought to be opposed by the rigidity of these neuroepithelial structures.

Differential expression of cytokeratin subunits

In human epithelia 20 different cytokeratin subunits have been distinguished and various types of epithelial differentiation have been demonstrated to express different sets of cytokeratins. Thus, immunohistochemical detection of single cytokeratins enables the distinction of different types of epithelia.

In the epithelia of the cochlea and vestibular labyrinth the expression of certain cytokeratin subunits typical of "simple" or single-layered epithelia were found (Table 1). However, the epithelium of the endolymphatic duct and sac expressed a deviant set of cytokeratins (Table 1). In this part of the inner ear the same cytokeratins as found in the other parts were demonstrated, together with cytokeratin 14 (and cytokeratin 17) which are both subunits typical of the basal-cell compartment in multilayered or "mixed" epithelia. Since cytokeratin 14 has never been demonstrated in homogeneous single-layered epithelia, the expression of this subunit by a subpopulation of epithelial cells throughout the endolymphatic duct and sac presents new evidence of pseudostratification. This finding has been described earlier at the ultrastructural level in the human sac but has, nevertheless, remained controversial up to now.

In the cochlea, two groups of "simple" epithelia could be distinguished on the basis of their differential expression of cytokeratin 7, i.e., the epithelial structures of the organ of Corti and stria vascularis on the one hand, and all other cochlear epithelia on the other. However, it remains unclear why the organ of Corti and stria vascularis share the same cytokeratin phenotype (at least as far as the cytokeratins are concerned that we have investigated), in spite of their totally different structure and function.

Table 1 Expression of different cytokeratin subunits in the human inner ear

– cochlea	Cks 7, 8, 18 & 19	"simple" or single-layered epithelia
– vestibular labyrinth	Cks 7, 8, 18 & 19	"simple" or single-layered epithelia
– endolymphatic duct and sac	Cks 7, 8, 14, 17*, 18 & 19	multilayered ("complex") or "mixed" epithelia

*please note that the presence of cytokeratin 17 was not investigated in the cochlea and vestibular labyrinth.

Vimentin

This IFP type, primarily synthesized by cells of mesenchymal origin, was predominantly detected in the subepithelial fibroblasts of the membranous labyrinth, i.e. cochlea, vestibular labyrinth and endolymphatic duct and sac. However, vimentin also occurred in various epithelial structures where it

displayed co-expression with cytokeratins. In the cochlea, the pillar cells and Deiters' cells of the organ of Corti and the region of the spiral prominence and outer sulcus demonstrated co-expression of cytokeratins and vimentin. Also, the entire epithelial lining of the vestibular labyrinth and endolymphatic duct and sac demonstrated this phenomenon. Although the finding of cytokeratin and vimentin co-expression enables a further characterization of tissues, its meaning is not yet understood. It has been demonstrated in several neuroepithelial tissues but has also been found in other epithelia and mesothelial cells. Future investigations will hopefully clarify the meaning of this phenomenon.

Neurofilament proteins

This triplet of IFPs, synthesized by neuronal cells, was exclusively demonstrated in all neuronal structures of the cochlea and vestibular labyrinth. The endolymphatic duct and sac lacked this type of IFP since it only consists of non-sensory epithelia. In both the cochlea and vestibular labyrinth, staining occurred in the neural bundles reaching the sensory epithelia and in their nerve endings at the sensory cells. Limited data on the spiral ganglion cells in the cochlea revealed a variable staining pattern whereas the vestibular ganglion cells were not investigated in this study.

Postscript

Immunohistochemistry of the human temporal bone is gradually developing. It adds an outstanding tool to studies relating function and structure and complements morphological techniques in allowing a further distinction of cells at the molecular level.

However, most immunohistochemical studies on the inner ear, such as those investigating hair cell motility, have been performed on isolated cells from rodent species. Although they have certainly added considerably to our understanding of the fundamentals of hair cell function, they need continuation and confirmation in functional units such as the entire organ of Corti, the maculae sacculi and utriculi, the cristae ampullares and the endolymphatic duct and sac. They also need to be carried out on human material, as performed in this study on IFP analysis.

Immunohistological evaluations should therefore be added to routine temporal bone studies and fundamental inner ear research. Immediate post-mortem tissue preservation by means of intralabyrinthine perfusion with carefully selected fixatives, resin embedding with non-immuno-compromizing monomers, and specimen dissection enabeling immunohistochemistry on cryostat sections as described in this study, are all important steps forward in the process of applying newly developed immuno- and biotechnologies to temporal bone research.

SAMENVATTING

Samenvatting

Ofschoon de basisprincipes van de immunohistochemie reeds in 1941 door Coons zijn uiteengezet en hoewel in de zeventiger jaren immunohistochemische technieken al volledig waren ingeburgerd in de meeste wetenschappelijke disciplines, werd pas in 1980 de eerste immunohistochemische studie van het binnenoor gepubliceerd. Sedertdien heeft het gebruik van deze onderzoeksmodaliteit in grote mate bijgedragen aan onze verdere kennis van de structuur en functie van het binnenoor.
In deze lichtmicroscopische studie werden de verschillende weefseltypen waaruit het binnenoor van de mens is opgebouwd - cochlea, vestibulaire labyrint en ductus en saccus endolymphaticus - immunohistochemisch onderzocht. Daartoe werd het expressiepatroon van zogenoemde intermediaire filament (IF) eiwitten, cytoskeletaire componenten met een karakteristieke weefselspecificiteit, bepaald.

Techniek
In Hoofdstuk 2 is een nieuwe techniek voor immunohistochemisch onderzoek van het volwassen menselijke binnenoor (cochlea en vestibulaire labyrint) beschreven. In essentie bestaat deze techniek uit het volledig wegboren (of anderszins wegnemen) van alle botweefsel - waaronder de benige schil die het binnenoor omgeeft - vóórdat het membraneuze labyrint onder microscopische controle verwijderd wordt. Op deze wijze hoeft het binnenoorweefsel niet te worden ontkalkt. Deze procedure blijkt de weefsel-antigeniciteit vrijwel niet aan te tasten. Structurele weefselcomponenten, zoals de IF-eiwitten, kunnen betrouwbaar worden aangetoond.
De bewerkingsmethode kan in de navolgende stappen worden samengevat: (1) chemische fixatie van het binnenoorweefsel door intralabyrintaire perfusie met een geschikt fixatief binnen enkele (< 3) uren na de dood, (2) vrijleggen van de benige cochlea, vestibulum en halfcirkelvormige kanalen door het bot van het rotsbeen, waarin deze structuren liggen ingebed, onder microscopische controle weg te boren, (3) microdissectie (= verwijderen van de resterende benige schil) van de cochlea, vestibulum en halfcirkelvormige kanalen, (4) *in toto* uitnemen van zowel het cochleaire als vestibulaire deel van het membraneuze labyrint, (5) inbedden in *Tissue-Tek® II*, en (6) na invriezen tot -70 °C, snijden van 4-5 μm coupes met behulp van de cryostaat.
In de huidige studie werden alle preparaten van cochlea en vestibulaire labyrint op deze wijze verwerkt. De ductus en saccus endolymphaticus werden op een alternatieve wijze verworven en bewerkt. Microdissectie van dit onderdeel van het binnenoor is zeer moeilijk uitvoerbaar. Specimina werden peroperatief verwijderd bij patiënten die brughoekchirurgie ondergingen via de translabyrintaire benadering, waarna ze onmiddelijk werden ingebed in *Tissue-Tek® II* en cryostaat-gesneden. Chemische fixatie of ontkalking vond hierbij dus niet plaats.

In de Hoofdstukken 3 tot en met 6 zijn de resultaten beschreven van de immunohistochemische detectie en lokalisatie van de voornaamste cytoplasmatische IF-eiwitten in de humane cochlea (Hoofdstuk 3 en 4), het vestibulaire labyrint (Hoofdstuk 5), en de ductus en saccus endolymphaticus (Hoofdstuk 6). Drie van de vijf onderzochte typen IF-eiwitten bleken in het menselijk binnenoor aanwezig, te weten cytokeratines (specifiek voor epithelia), vimentine (specifiek voor mesenchymale weefsels) en neurofilament-eiwitten (specifiek voor neurale weefsels). Desmine en gliafilament-eiwitten (GFAP) konden niet worden aangetoond.
In tegenstelling tot de beschrijving en bespreking in Hoofdstukken 3 tot en met 6 van het expressiepatroon van IF-eiwitten voor elk afzonderlijk deel van het menselijk binnenoor, zullen nu de resultaten voor ieder IF-eiwit apart worden samengevat voor alle onderdelen van het binnenoor.

Cytokeratines
Cytokeratines, welke over het algemeen specifiek worden gesynthetiseerd door epithelia, werden gevonden in de volledige epitheliale bekleding van het endolymfatische compartiment van het binnenoor, te weten de cochlea, het vestibulaire labyrinth en de ductus en saccus endolymphaticus. Dit bevestigt de algemeen aanvaarde epitheliale oorsprong van deze cellen. In tegenstelling tot de meeste andere mesothelia in het menselijk lichaam, bleken de verschillende mesotheliale structuren in de cochlea en het vestibulaire labyrint geen cytokeratines, naast vimentine, te bevatten.

Cochlea
Alhoewel in de cochlea alle cellen die de scala media bekleden - inclusief de steuncellen in het orgaan van Corti - aankleurden voor cytokeratines, ontbrak aankleuring voor cytokeratines en voor alle verder onderzochte IF-eiwitten in de haarcellen van het orgaan van Corti.

Vestibulaire labyrint
Een vrijwel identiek patroon, suggestief voor aankleuring van alléén de steuncellen en niet van de haarcellen, werd eveneens gevonden in het zintuigepitheel van het vestibulaire labyrint. Wel dient opgemerkt te worden dat het oplossend vermogen van de lichtmicroscopische techniek niet afdoende is om aankleuring in de haarcellen in deze epithelia definitief te kunnen uitsluiten.

Functionele implicaties
Het ontbreken van aankleuring voor alle onderzochte IF-eiwitten in de haarcellen van het orgaan van Corti (en waarschijnlijk ook in de vestibulaire haarcellen) is een ongewone, en ook onbegrepen, bevinding. Daarnaast blijkt dat, terwijl kennelijk in het cytoskelet van de haarcellen cytokeratines ontbreken, deze in ruime mate aanwezig zijn in alle omringende steuncellen. Weinig is tot nu toe bekend over de specifieke functie(s) van IF-eiwitten. Wel is het

langzamerhand alom geaccepteerd dat de stijfheid of rigiditeit van weefsels mede bepaald wordt door de aanwezigheid van cytokeratines in het cytoskelet. Deze IF-eiwitten vormen een supracellulair netwerk in weefsels door hun intercellulair contact via desmosomen. Aldus zou het specifieke voorkomen van cytokeratines in het orgaan van Corti en in het vestibulaire zintuigepitheel van functionele betekenis kunnen zijn.
In het orgaan van Corti lijkt de aanwezigheid van cytokeratines in de lamina reticularis van de steuncellen, tezamen met hun afwezigheid in de omhulde apicale gedeelten van de haarcellen, van functioneel belang. Aangenomen mag worden dat de dientengevolge optredende verschillen in stijfheid tussen deze celtypen onderling een substantiële rol speelt in de micromechanica van het orgaan van Corti. Op gelijke wijze suggereert de sterke aankleuring voor cytokeratines in het vestibulaire zintuigepitheel dat de maculae en cristae rigide structuren zijn. Deze rigiditeit kan worden verondersteld van belang te zijn in het mechano-electrische transductieproces van het evenwichtsorgaan. De elasticiteit van de cupulae en statolietmembranen zou dan worden "tegengewerkt" door de stijfheid van deze neuro-epitheliale structuren.

Differentiële expressie van cytokeratine-subtypen
In menselijke epithelia kunnen momenteel 20 verschillende cytokeratine-subtypen worden onderscheiden. Het is gebleken dat verschillende typen epithelia verschillende combinaties van cytokeratines bevatten. Aldus kunnen immunohistochemisch, met monoclonale antilichamen die specifieke cytokeratine-subtypen herkennen, verschillende typen epitheel worden onderscheiden.
In de epithelia van de cochlea en het vestibulaire labyrint werden cytokeratines aangetroffen wier voorkomen typisch is voor "eenvoudige" of éénlagige epithelia (Tabel 1). Daarentegen werden in de ductus en saccus endolymphaticus cytokeratines aangetoond die typisch zijn voor "complexe" of "gemengde" epithelia (Tabel 1). Hier werden cytokeratine 14 (en cytokeratine 17), tezamen met dezelfde cytokeratines die ook in de overige delen van het binnenoor waren gevonden, aangetoond. Deze beide cytokeratines zijn specifiek voor de basale cellaag in een meerlagig of "gemengd" epitheel. Aangezien cytokeratine 14 nog nooit is vastgesteld in een homogeen éénlagig epitheel, vormt de

Tabel 1 Expressie van verschillende cytokeratine subtypen in het menselijke binnenoor

– cochlea	Cks 7, 8, 18 & 19	"eenvoudige" of éénlagige epithelia
– vestibulaire labyrint	Cks 7, 8, 18 & 19	"eenvoudige" of éénlagige epithelia
– ductus en saccus endolymphaticus	Cks 7, 8, 14, 17*, 18 & 19	meerlagige of "gemengde" epithelia

*NB de aanwezigheid van cytokeratine 17 is niet onderzocht in de cochlea of in het vestibulaire labyrint.

aanwezigheid van dit cytokeratine in een subpopulatie van epitheliale cellen in de ductus en saccus endolymphaticus een nieuw bewijs van pseudostratificatie. Dit fenomeen werd éénmaal eerder op elektronenmicroscopisch niveau in de humane saccus beschreven. Sedertdien is deze waarneming echter controversieel gebleven.
In de cochlea konden op basis van een differentieel expressiepatroon van cytokeratine 7 twee groepen "eenvoudige", d.w.z. éénlagige, epitheliale structuren worden onderscheiden, te weten het orgaan van Corti en de marginale cellen van de stria vascularis enerzijds, en alle overige epithelia anderzijds. Het is echter onduidelijk waarom het orgaan van Corti en de stria vascularis hetzelfde cytokeratine-fenotype delen (tenminste voor wat betreft de onderzochte cytokeratines), ondanks hun totaal verschillende structuur en functie.

Vimentine
Dit IF-eiwit, dat in het volwassen organisme over het algemeen wordt gesynthetiseerd door cellen van mesenchymale oorsprong, was primair aanwezig in de subepitheliale cellen (fibroblasten) van het membraneuze labyrint (cochlea, vestibulaire labyrint en ductus en saccus endolymphaticus). Echter, vimentine was ook plaatselijk aanwezig in verschillende epitheliale structuren. Hier was dan ook sprake van co-expressie met cytokeratines. In de cochlea toonden de spirale prominentia en sulcus externus, en de binnenste- en buitenste pilaarcellen en Deiterse cellen in het orgaan van Corti, co-expressie van cytokeratines en vimentine. De gehele epitheliale bekleding van het vestibulaire labyrint en de ductus en saccus endolymphaticus toonde ditzelfde fenomeen. Alhoewel de bevinding van co-expressie van cytokeratines en vimentine een verdere karakterisering van weefsels mogelijk maakt, is de betekenis ervan vooralsnog onduidelijk. Zij is tevens waargenomen in verschillende neuro-epitheliale structuren maar ook in andere epithelia en mesotheel. Verder onderzoek zal hopelijk in de toekomst de betekenis van dit fenomeen ophelderen.

Neurofilament-eiwitten
Deze door zenuwweefsel gesynthetiseerde IF-eiwitten werden uitsluitend aangetroffen in het neurale weefsel van de cochlea en het vestibulaire labyrint. Ze werden niet aangetroffen in de ductus en saccus endolymphaticus. Dit is niet uitzonderlijk aangezien dit deel van het binnenoor geen zintuigepitheel bevat. Zowel in de cochlea als in het vestibulaire labyrint waren deze IF-eiwitten aanwezig in de zenuwbundels van en naar de zintuigepithelia en ter plaatse van de zenuwuiteinden bij de haarcellen. Beperkte gegevens toonden een variabele aankleuring voor de neurofilament-eiwitten in de cellen van het ganglion spirale. De vestibulaire ganglioncellen werden in deze studie niet onderzocht.

Overwegingen t.a.v. toekomstig onderzoek

De toepassing van immunohistochemische technieken op het menselijke binnenoor (rotsbeen) is zich geleidelijk aan het ontwikkelen. Het is bij uitstek een fraaie modaliteit voor onderzoek naar de relatie tussen structuur en functie, waarin het een aanvulling betekent op morfologische onderzoekstechnieken, gezien het onderscheidend vermogen op moleculair niveau.

Tot op heden zijn echter de meeste immunohistochemische studies van het binnenoor, zoals bijvoorbeeld de onderzoekingen op het gebied van haarcel-contractiliteit, verricht op geïsoleerde haarcellen van diverse zoogdieren. Alhoewel deze studies van groot belang zijn en in hoge mate hebben bijgedragen aan ons begrip over de fundamentele principes van de werking van haarcellen, is er nu behoefte aan uitbreiding van vergelijkbaar onderzoek naar functionele eenheden zoals het orgaan van Corti, de maculae sacculi en utriculi, de cristae ampullares en de ductus en saccus endolymphaticus. Dergelijk onderzoek zou dan tevens op humaan materiaal moeten worden verricht, zoals in de huidige studie op het terrein van IF-analyse.

Daartoe moet immunohistochemisch onderzoek, naast de meer conventionele onderzoekstechnieken, een vaste plaats krijgen in het onderzoek van het menselijke rotsbeen. Intralabyrintaire perfusie met zorgvuldig geselecteerde fixatieven, het (voor inbeddingsdoeleinden) toepassen van speciale plastics die de antigeniciteit van weefsels ongemoeid laten, en het toepassen van dissectie-technieken – zoals in deze studie beschreven – die immunohistochemie op cryostaat-coupes mogelijk maken, zijn alle voorbeelden van belangrijke stappen voorwaarts in het proces van het toegankelijk maken van het menselijke rotsbeen voor immunohistochemisch onderzoek.

CURRICULUM VITAE

The author was born on 15 February, 1959 in Salisbury, Southern Rhodesia. After finishing secondary school in 1976, he studied medicine at the Medical Faculty of the University of Utrecht, The Netherlands, where he graduated in 1983 (MD). From 1983 to 1985 he worked as a resident in Internal Medicine (1983), Radiology (1983-1984), and Surgery (1984-1985).

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