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M. Ciges, A. Campos, Granada

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Preface

In recent years pathobiology has become an area which integrates findings from cell biology and pathology. It now seems clear that there is a continuum between normal structure and function and lesional states, at least in the initial phases of the pathological process. The study of lesions and their inception, together with their structural and functional bases, when approached from a biological and clinical standpoint, constitutes the basic objective of any pathobiological analysis.

The inner ear is an area in which the vulnerable, specialized structures of the cochlear and vestibular regions are subjected to a variety of physical and chemical agents, which are, at times, the consequence of medical treatment. The material in this volume was presented at the Inner Ear Pathobiology Symposium, held in Granada, September 18–20, 1989. Most of the articles deal with one of the following: (1) To elucidate the morphology, structure and development of specific areas of particular significance in certain pathological processes in the inner ear. (2) To characterize the initial development of lesions in the inner ear, not only from a morphostructural perspective but also in functional and, in some cases, clinical terms. These approaches are especially relevant to problems arising from ototoxicity. (3) To provide a solution, as far as current technology permits, to some of the methodological problems involved in the study of inner ear pathobiology. In this connection advanced techniques are described in this volume which provide us with a more direct access to the normal and damaged inner ear.

We are deeply indebted to the authors, who with their varied contributions not only bring us up to date on the biological and pathological aspects of the inner ear, but also point the way towards new avenues in our

understanding of how lesions arise, the most accurate methodology to study them and their clinical and diagnostic implications. We also express our appreciation to the organizations which supported the Symposium, particularly the Consejerias de Educacion y Ciencia and Salud y Servicios Sociales de la Junta de Andalucia and VR Medical Team. Their collaboration made it possible to bring some of the most highly qualified experts from around the world to Granada, where we were able to advance our current knowledge of inner ear pathobiology and strengthen ties of friendships and cooperation among researchers and clinicians interested in the inner ear. Finally, we would like to thank S. Karger publishers for their interest in making available to a wide readership the facts, ideas, and, of course, the applications of the recent findings and concepts brought to light in the Symposium.

Miguel Ciges
Antonio Campos

Contents

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Analytical Electron Microscopy and Monoclonal Antibody Techniques Applied to the Human Inner Ear

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Energy Dispersive X-Ray Microanalysis

Introduction

Analytical electron microscopy (EM) instrumentation has now made it possible to correlate the microscopic anatomy with its elemental chemical composition of the same tissue. This ability is a significant improvement over older, bulk methods of chemical analysis, which necessarily disrupt histomorphology. The quality and validity of the morphochemical correlations obtained by analytical electron microscopy depends closely, however, on the appropriate selection of specimen preparative techniques for the particular problem under consideration [1].

The route employed to bring any biological tissue from its in vivo state to in situ form in the microscope is potentially fraught with numerous pitfalls affecting both structural and chemical integrity. Often, a paradoxical trade-off exists between the two. Well-established EM preparative techniques that achieve excellent structural preservation (e.g. chemical fixation, osmification, staining) often sacrifice chemical integrity. Yet techniques suited for chemical preservation (e.g. freeze fixation) may disrupt the subcellular architecture or yield poor ultrastructural images.

[1] In cooperation with Lars-Eric Thornell[c], Ismo Virtanen[d], Torgny Stigbrand[e], Frans C. Ramaekers[f].

For many types of problems one needs to reach a compromise between the theoretical ideal of freeze fixation, and the excellent morphological data obtained with chemical fixation and/or conventional embedding. A useful general rule is that if one anticipates a future need for any sort of chemical or microanalytical study, it is always wise to freeze a portion of the tissue and to keep a corresponding portion of the tissue in an appropriate buffered formalin or glutaraldehyde solution.

XRMA (Microprobe) Techniques for Inner Ear Analysis

The use of X-ray microanalysis (XRMA) in inner ear research was originally reported by Flock [2, 3]. Thereafter, however, a rapid development in instrumentation and freezing techniques occurred. A number of specimen handling methods, freezing techniques and embedding methods for inner ear research have been developed by Anniko and Wróblewski as well as the application of XRMA in studies on the adult and embryonic inner ear both in experimental animals and in the human [4–6].

The methodological development has been performed on the labyrinth from rodent species, preferentially the guinea pig and the rat [5]. With the XRMA technique it is possible to perform microprobe measurements of extremely small volumes with the prerequisite that the analyzed volume should contain sufficiently high concentrations of any element present. Measurements of the total amounts of elements can detect concentrations as small as 10^{-18} to 10^{-19} g. Prior to cellular microanalysis, we have analyzed the general morphological state of each individual cell using the STEM mode. To ensure that freezing artifacts did not cause any significant changes in ion redistribution, only cells with a well-preserved structure were used for microprobe analysis.

The technical details of different XRMA methods for the inner ear have been published previously, including different temperatures for cryosectioning in the range −40 to −160 °C [5, 7]. Our present information is based on approximately 800 temporal bones from the mouse, about 60 each from the rat and the guinea pig and 22 from man. The advantages and risks of the different preparation methods can be summarized accordingly.

Bulk Specimens. If fractured or drilled (in liquid nitrogen), there is a risk that fluid may intermingle during the drilling procedure. The cochlea should preferably be fractured. The material for XRMA is freeze-dried or frozen hydrated. Soft tissue analysis is inaccurate because of poor resolution. If the specimens are microdissected prior to freezing there is a con-

siderable risk of fluid contamination. A large part of the microanalysis information is derived from the surface structures and upper parts of the cell volume analyzed. Microdissection of freeze-dried material is extremely time consuming and is not perfectly reliable because of the large number of cell types in the membranous labyrinth.

Cryosections (Sectioning at – 40 to – 80 °C). The material for XRMA is freeze-dried. Sections 16 µm thick are optimal for studying the elemental composition of fluid spaces and gross structures such as the cupula and the tectorial membrane. Fluid-filled spaces can be analyzed more accurately than when using thinner cryosections or plastic-embedded sections. Results obtained from soft tissues include the content of extracellular spaces.

A section thickness of 2–6 µm allows analysis of single cells. Nuclei and cytoplasm can be analyzed separately. Analysis of fluid-filled spaces is still possible. However, the distribution of fluids is less uniform than when using 16-µm-thick cryosections, because of crystallization during freezing.

Plastic-Embedded Material (Freeze-Dried Material Infiltrated with Plastics). Bone can be trimmed away prior to sectioning of the specimens. Sectioning is performed with a dry knife. Elemental analysis is performed at the subcellular level. By using Araldite (epoxy) embedding, different cell types in the inner ear can be analyzed with great accuracy. Both whole cochlea and parts fractured in liquid nitrogen can be processed. Some redistribution of intracellular ions may occur due to the water produced during polymerization. When using Lowicryl HM20, the tissue of interest has to be exposed (e.g. opening of the cochlea) prior to freezing. The possibility of analyzing inner ear fluids is thus lost. Only small pieces of tissue can be processed because of the limited penetration of ultraviolet light used for polymerization. No water is produced during polymerization of Lowicryl HM20. The specimens display a high contrast when analysed.

The Human Inner Ear. Soft tissue material must be obtained at surgery in order to preserve optimal conditions for microanalysis. The tissue has to be shock-frozen as soon as possible without prior orientation. This difficulty is overcome by advances in preparation technique described by Anniko et al. [8] using freeze-dried material infiltrated with epoxy resins at low temperature. After polymerization, the plastic-embedded inner ear

specimens can be handled at room temperature as ordinary specimens for transmission electron microscopy, but sectioned dry.

So far, from autopsy cases only otoconia are suitable to use for microanalysis [9] since autolysis of otoconia, as viewed with scanning electron microscopy, does not occur during the first 24–48 h after death [10].

Principal Information Obtained from Microanalysis

During the 1970s, XRMA of soft tissues was of particular interest, as the technique using monoclonal or polyclonal antibodies against well-defined proteins had not yet been particularly developed nor applied to the labyrinth. When studying the inner ear, however, the energy dispersive XRMA technique allowed analysis of morphologically defined structures at both cellular and subcellular levels. However, a significant limitation with this technique is that only measurements of the total amount of an element are possible, but not of only free or bound concentrations of the element.

The first results on differences in structural composition of cytoplasm between outer and inner hair cells (HCs) were obtained by the use of the microprobe technique [8]. Similarly, type I and type II vestibular HCs also showed slight mutual differences [11]. Electron probe microanalysis offers a sophisticated and specialized approach for localizing calcium in different types of cells in the inner ear. Recently, quantitative measurements of calcium using cryofixed, chemically untreated freeze-dried cochlear HCs demonstrated that the calcium content in basal part of HCs is at least twice as great as that of any other part in the HC [12] and thus a precondition for an isometric type of outer hair cell (OHC) contraction in situ [13].

Ionic shifts in individual cells of the stria vascularis can be measured in the same specimens as used for microprobe analysis of the endolymph [14]. In this way the active interaction between these two compartments can be analyzed with regard to the time of onset of ionic shifts and the degree of change, for instance following exogenically induced disturbances of inner ear fluid homeostasis due to ototoxic diuretics such as ethacrynic acid, furosemide, etc.

The most important contribution of the microprobe technique to inner ear research is the possibility of analyzing minute fluid compartments too small or too difficult to measure with micropipettes, for instance the determination of the maturation of endolymph and perilymph during embryonic development [15]. The mature fluid composition in the inner sulcus below the tectorial membrane and the tunnel of Corti has been

determined [8, 16]. In genetically induced deterioration of the ionic composition of endolymph, ionic shifts can be detected prior to morphological changes in the epithelial lining of the endolymphatic compartment [17].

The results of studies on the biochemical composition of the mature tectorial membrane (TM) have been rather contradictory. Histochemical studies have shown that the TM contains a large amount of glycoproteins. However, biochemical data show it to consist largely of protein [18]. Recently, Steel [19] reported that the TM consists largely of proteins distinct from any known cytoskeletal proteins. Several investigators have demonstrated that the bulk of the TM consists of type II collagen but that it also contains glycoconjugates, whose presence is consistent with our microprobe observations of high concentrations of sulphur, with considerable regional variations [20]. During the formation of the TM, organic material is secreted from surface cells of Kölliker's organ [21]. During this period, subclasses of the otherwise strictly intracellar intermediate filaments have been localized in the human embryonic (but not in the mature) TM [22]. The highly specialized extracellular structures such as the TM and the cupula show considerable differences in elemental composition [23]. The TM has large regional variations in elemental concentration, which appear already during embryonic development prior to the maturation of the ionic composition of endolymph, whereas the cupula seems very homogenous in all regions.

In otoconia from both rodent and human specimens, considerable changes in calcium concentration can be documented using the microprobe before any ultrastructural alterations can be visualized [9, 24]. During normal ageing, a phosphatization occurs as has also been found in certain types of genetically induced, premature inner ear degeneration [24].

During ossification of the otic capsule, the microprobe can localize calcification centers before any signs can be visualized in the microscope [25].

Future Prospects

The microprobe technique is very complex. Its limitations are more related to the preparation methods than to the instrumentation itself, which at present seems optimal for at least biological specimens. Microprobe analysis is still superior to any other technique regarding minute fluid compartments in biological material. Computerized analyses allow quantification using different standards, though they can be difficult to carry out in practice. Immunomorphological techniques using highly spe-

cific antibodies strictly define their target and provide more information than merely the elemental composition and are thus more suitable for analysis of cells and tissues. In a few cases, for instance in the search for certain elements such as calcium or magnesium, the microprobe is very useful. However, precautions must be taken to avoid redistribution of ions during the preparation of specimens. Further microprobe studies are called for regarding such highly specialized extracellular structures as the TM, the cupula and the otoconia.

Immunomorphology

Introduction

The cytoskeleton of higher eukaryotic cells comprises microtubules (22–25 nm in diameter), intermediate filaments (IFs; 8–11 nm in diameter), microfilaments (actin; 4–6 nm in diameter) and a large number of interconnecting proteins. Recently, interest has focused on the IFs because of their diversity and because various cell types can be characterized by their specific pattern of IF expression [26]. The IF composition of a certain cell may reflect both its embryonic origin and its function.

IFs, so named because of their size, may represent the true skeleton of the cell. They are generally insoluble in aqueous solutions containing salts or nonionic detergents and provide rigidity within cells, especially in areas of mechanical stress. The cytoskeleton not only gives structural support to individual cells, but contributes to the tensile strength of cells associated into tissues [27]. For example, in epithelial cell sheets, neighboring cells are held together mechanically by strong cell junctions, the spot desmosomes. Since another type of filament extends between the desmosomes, the IFs form a continuous network of cytoskeletal filaments extending throughout the epithelial cell sheet and thus serve to give the entire cell sheet its tensile strength.

The principles for the synthesis, expression and metabolic breakdown of IFs have been explored mainly in cell cultures. In clinical pathology, for instance, cytokeratins (Cks) in particular have been used as markers for the epithelial origin of tumor cells. Immunomorphological analysis of IFs in the inner ear was introduced by Anniko et al. [28] as recently as in 1986. We have since analyzed in detail the embryonic, fetal and adult human labyrinth with a number of newly developed/modified techniques using high-resolution light microscopy. Recently, we have postulated a hypothe-

sis for IF function in the human organ of Corti, introducing the concept of a possible isometric contraction of OHC on the perception of sound [29].

Although detailed studies have been performed on the distribution and function of actin on isolated OHC from several rodent species [30], knowledge is sparse regarding similar entities in the human labyrinth. Furthermore, experiments on isolated OHC do not reflect true in situ conditions, since in vivo OHC are firmly connected to the supporting cells and are exposed to the potassium-rich endolymph at the cuticular plate side and to the sodium-rich perilymph at their lateral and basal cell surfaces.

Materials and Methods

Tissue. The information published in the present paper is based on 42 temporal bones, 6- to 22-gestation weeks (GW) old, which were collected immediately at legal abortions. In addition, 14 adult (18–76 years old) temporal bones from healthy individuals who had died as a result of traffic accidents or heart attacks, were used.

Immunomorphology. The prenatal material was either lightly fixed in paraformaldehyde followed by cryofixation (6–14 GW old) or immediately cryofixed using liquid isopentane cooled with liquid nitrogen (14–22 GW old). In most cases, the cochlear and vestibular parts of the inner ear were separated prior to freezing. Each specimen was serially cryosectioned at –30 °C. Approximately 2,000 cryosections, about 4 µm thick, were obtained from 14-GW-old inner ears or older. Every 10th section was stained with hematoxylin-eosin to facilitate orientation. Based on the findings from these sections, adjacent sections were stained for immunocytochemistry (using the peroxidase-antiperoxidase (PAP) technique to identify initially unlabelled antibodies) with monoclonal antibodies (mAbs) against different subclasses of IFs, several neuropeptides, lecitin-binding glycoproteins, hormones, actin-associated proteins and fibronectin (table 1). In order that specific staining could be verified, it must have been found in specimens from at least three different temporal bones. For further technical details regarding methods for cellular and subcellular immunomorphological visualization in the prenatal human inner ear [37].

The adult temporal bones were perfused with paraformaldehyde within 6 h postmortem and processed according to our earlier published technique [38]. Following EDTA decalcification the temporal bones were embedded in Paraplast® at 54 °C and serially sectioned (section thickness approximately 5 µm). A grand total of more than 2,000 sections was obtained from each labyrinth. Every 10th section was stained with hematoxylin-eosin to facilitate orientation. Based on the findings, representative sections were taken for immunostaining. The sections were deparaffinized in xylol, trypsinized (0.5% trypsin, 37 °C, 15 min) and immunostained using the PAP technique (table 1).

All sections were viewed and photographed in a Zeiss Axiophot light microscope, using both bright-field and phase-contrast microscopy.

Sections from 11 prenatal temporal bones were taken for fluorescence visualization of F-actin, alpha-actinin, vinculin, alpha-spectrin, beta-spectrin and fibronectin. For F-

Table 1. Specificity of mAbs used in this study: the Cks are designated in terms of the current numbering system for human Cks [31]

Antibody	Antigen recognized	Reference
PKK-1	Cks 8, 18, 19	32
PKK-2	Cks 7, 17, 19	32
PKK-3	Ck 18	32
RCK 105	Ck 7	33
TS 1	Ck 8	34
TS 7	Ck 8	34
RPN 1164	Ck 8	Amersham International, UK
RCK 102	Cks 5 + 8	33
RGE 53	Ck 18	33
RPN 1160	Ck 18	Amersham International
RCK 106	Ck 18	33
Ck 18–2	Ck 18	33
RKSE 60	Ck 10	33
KA.5	Ck 10	35
K8.60	Cks 10 + 11	35
GFAP–BG	GFAP	Bio-Genex Laboratories, Dublin, Ireland
Vimentin-24	vimentin	32
Vimentin-BG	vimentin	Bio-Genex Laboratories
K-17	vimentin	Bio-Genex Laboratories
RV 202	vimentin	Bio-Genex Laboratories
RV 203	vimentin	Bio-Genex Laboratories
NF 13AA8	neurofilament triplet proteins	Virtanen, 1987, unpubl.
RNF 481	200 kDa NF	Ramaekers, 1987, unpubl.
RNF 402	200 kDa NF	Ramaekers, 1987, unpubl.
NF Dako	NF triplet proteins	Dakopatts, Copenhagen
AF 9	Thy-1	36
CC 8	glycoprotein 140, 100 kDa	36
CF 3	glycoprotein 120 kDa	36
SY 38	synaptophysin	ProGen Biotechnik, Heidelberg
HEA-125	glycoprotein Egp-34	ProGen Biotechnik
h-CDD/ANP	ANP	Milas, Malmö, Sweden
S-100	S-100 protein	Stigbrand, 1988, unpubl.
VIP	VIP	Amersham International
Neuropeptide Y	neuropeptide Y	Amersham International
Fibronectin	fibronectin	Dakopatts

actin, visualization was performed with rhodamine-phalloidin, whereas for actin-associated proteins and fibronectin, cryosections were incubated with rhodamine-marked antibodies.

Principal Information Obtained from Immunomorphology

The expression of the IF subgroups Cks, vimentin and neurofilament triplet proteins (NFs) in both the fetal and the adult human inner ear is summarized in table 2. A further subclassification of Cks in the inner ear of the human fetus is presented in table 3.

Prenatal Development. A developmental stage-dependent pattern of inner ear expression of IF occurs [39]. The membranous labyrinth is the first sensory organ to form during embryonic development. At the otocyst stage, co-expression of vimentin and Cks was distinct throughout the epithelium. During inner ear development, from the otocyst stage to completed organogenesis, this co-expression was lost in most cells in the epithelial lining of the labyrinth. Thus, the period from the otocyst stage through organogenesis is characterized by not only a considerable morphological restructuring of epithelia with regard to cell shape and specialized cell structure, but also includes a change in gene expression of the cytoskeletal organization. This expression is very stable and even occurs when the inner ear anlage develops in the presence of ototoxic substances [40]. However, only when exposed to prenatal irradiation can a change in the cytoskeleton of HC be induced to express NFs, a very unique feature in cells which already have passed their terminal mitosis and display a normal ultrastructure [41].

The human inner ear has a more complex IF pattern than any inner ear so far known in animal species [39, 42]. *Epithelia involved in fluid regulation* (stria vascularis, Reissner's membrane and dark cell epithelium) revealed the same basic pattern of Ck immunoreactivity in spite of morphological differences in cell configuration [42].

The Adult Human Organ of Corti. The anatomic organization and gradation of the basilar membrane-TM-organ of Corti complex along the cochlear duct determines the frequency-specific area that is maximally stimulated by the travelling waves created by a stimulating frequency [44]. The unique feature of the mammalian auditory organ, including the basilar membrane, TM, and organ of Corti, is the differences in size and shape along its length [45]. The inner hair cells (IHC) are located just above the edge of the osseous spiral lamina, where the basilar membrane movement

Table 2. Expression of intermediate filament proteins in the fetal and adult human inner ear

Structure/cell type	Cytokeratins		Vimentin		Neurofilaments	
	fetal	adult	fetal	adult	fetal	adult
Inner ear ganglia						
Spiral ganglion cells	+	+	+ or −	−	+ or −	+ or −
Vestibular ganglion cells	+	+	+		+ or −	+ or −
Schwann cells	−	−	+	+	−	−
Organ of Corti						
IHC	+	−	+	−	−	−
OHC-1	+	−	+	−	−	−
OHC-2	+	−	+	−	−	−
OHC-3	+	−	+	−	−	−
Border cell	+	+	+	−		
Inner pillar cell	+	+	+	+	−	−
Outer pillar cell	+	+	+	+	−	−
Deiter's cell	+	(+)	+	−	−	−
Hensen's cell	+	+	−	−	−	−
Claudius' cell	+	+	−	−	−	−
Boettcher's cell	+	−?	−	−	−	−
Vestibular organs						
Hair cells (types I and II)						
Crista	+	−	+	−	−	−
Utricle	+	−	+	−	−	−
Saccule	+	−	+	−	−	−
Nonsensory cells						
Crista	+	+	+	−	−	−
Utricle	+	+	+	−	−	−
Saccule	+	+	+	−	−	−
Epithelia involved in inner ear fluid homeostasis						
Stria vascularis						
Marginal cells	+	+	−	−	−	−
Intermediate cells	−	−	+	+	−	−
Basal cells	−	−	+	+	−	−
Reissner's membrane						
Epithelial cells	+	+	+	+	−	−
Mesenchymal cells	−	−	+	+	−	−
Dark cells	+	+	−	−	−	−
Endolymphatic duct	− or +	− or +	− or +	− or +	−	−
Endolymphatic sac	− or +	− or +	− or +	− or +	−	−
Spiral prominence cells	+	+	(+)	+	−	−
Root cells	+	+	+	+	−	−