Freeze-Fracture and Immunomorphological Analysis of Spiral Ganglion Cells

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KEY WORDS Spiral ganglion, Freeze-fracture, Intermediate filaments, Morphology, Cytoskeleton, Membrane, Labyrinth

ABSTRACT Freeze-fracture analysis of adult spiral ganglion cells of CBA/CBA mice revealed two types of membrane specializations. Most cells (type I) had a smooth surface and were surrounded by Schwann cells. Type II spiral ganglion cells showed numerous membrane specializations with well-delineated indentations similar to those previously found on hair cells adjacent to afferent and efferent nerve endings. Immunomorphological analysis (using well-defined monoclonal antibodies directed against different subclasses of intermediate filament proteins) revealed a unique co-expression of neurofilaments, vimentin and cytokeratins in spiral ganglion cells of 8- to 22-week human fetuses.

INTRODUCTION
Several cell types exist in the spiral ganglion, as in other sensory ganglia (Noden, 1980). The major criteria that facilitate their identification are similar in a number of mammalian species. The spiral ganglion is populated mainly by bipolar neurons whose processes are located on opposite poles of the cell body (Kellerhals et al., 1967). The perikaryon of these cells can be surrounded by a myelin sheath (Rosenbluth, 1962). In addition, multipolar cells also occur (Kimura et al., 1979). Sperdulik (1969) reported that the great majority of all ganglion cells in the cat are large and myelinated (type I), and only about 5% of all ganglion cells are devoid of the myelin sheath (type II). The proportions of type I vs. type II cells are similar in the human ganglion (Ota and Kimura, 1989; Pollak and Felix, 1985). In the present study, new information is presented regarding the membrane structure of spiral ganglion cells and their cytoskeletal organization.

The freeze fracture technique

Freeze-fracturing is a valuable technique applied in electron microscopy for visualization and analysis of the surface structures of biomembranes. During the fracturing process, the cleavage plane runs preferentially within the membrane, splitting the hydrophobic bonds between the bilayer phospholipids of the cell membrane, rather than along one side of the membrane (Branton, 1966). Thus, either the internal half of the membrane (the so-called protoplasmic leaflet or P-face) or the external half of the membrane (the so-called exoplasmic leaflet or E-face) is exposed. Generally, in most mammalian cell membranes the P-faces contain numerous particles, which are thought to represent the sites of membrane intercalated proteins. On the E-face there are fewer free particles, but there can be pits complementary to the particles on the P-face (Bretscher, 1974).

The cell cytoskeleton

The cytoskeleton of all eukaryotic cells consists of three types of filamentous structure: microfilaments (actin; 4-6 nm in diameter), intermediate filaments (8-10 nm in diameter) and microtubules (22-25 nm in diameter), each of which consists of polymers of protein subunits (Borisy et al., 1984; Wang et al., 1985). The morphological organization of the cytoskeleton has specific features in each cell type. The most common pattern of cytoskeletal organization has two characteristic features: preferential localization of actin structures at the cell periphery adjacent to plasma membrane, and an array of microtubules radiating from the perinuclear cell region toward the periphery. Intermediate filaments (IFs) form the network connecting central parts of the cell with the submembranous periphery. This general pattern is characteristic of most interphase cells of higher animals.

For microfilaments and microtubules, a dynamic equilibrium exists between subunits and polymers. Although little is known about the regulation of this equilibrium, many functions of these cytoskeletal components appear to depend on dynamic rearrangements. IFs include a large class of polymers having uniform structure and properties that can vary greatly in their subunit composition. It was long believed that there was neither a pool of unpolymerized IF proteins in the cells nor a dynamic equilibrium between their soluble and polymerized forms, such as there was for actin and tubulin. By contrast, Soeller et al. (1985) reported that a small amount of soluble vimentin was present in

Received November 4, 1988; accepted in revised form March 25, 1989.
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cytosol fractions. It was proposed that in the living cell a small pool of a distinct, soluble tetrameric form of vimentin may exist making exchange between this soluble pool and the one containing polymeric IP add vimentin possible.

IFs, named because of their size, may constitute the true cellular skeleton. They provide rigidity within cells, especially in areas of mechanical stress. IFs are cell and tissue specific, as shown by monoclonal antibodies (mAbs) directed against IFs. IFs are divided into five main classes: vimentin, glial fibrillary acidic protein (GFAP), cytokeratins (Cks), neurofilament triplet proteins (NFPs), and desmin. These classes cannot be visualized with routine morphological techniques, since all IFs have a similar ultrastructure.

The Cks display a marked heterogeneity, and 19 different polypeptides have been identified in the human epithelia. Individual Cks are rarely, if ever, organ specific. Tissue specificity lies in the combination of Cks that an epithelium expresses. Recently, a number of IF-associated proteins were identified (Clarke et al., 1986). Regarding microfilaments and microtubules, these associated proteins probably serve to regulate the distribution or the assembly of filaments. Some of these proteins are very large and may be involved in the crosslinking of IFs and other cytoskeletal elements.

Anniko and co-workers were the first to describe the immunomorphology of IFs in the inner ear, initially in the mouse (Anniko et al., 1986) and later in the human (Anniko et al., 1987a,b). A developmental stage-dependent expression of IF proteins occurs in the inner ear, from the otocyst stage to maturity (Wikström et al., 1988).

**MATERIALS AND METHODS**

**Material**

The material was composed of 19 2-month-old inner ears from CBA/CBA mice for freeze-fracturing and 17 human 14- to 22-week-gestation cochleas for immunomorphology.

**The freeze-fracture technique**

The inner ears were fixed by perilymphatic perfusion with 3% glutaraldehyde in 0.133 M sodium phosphate buffer through the round and oval windows. The specimens were immersed in fixative for 2 h, then microdissected in 0.15 M cacodylate buffer. The bony wall and the stria vascularis lateral to the organ of Corti were removed. The cochlea was divided in a mid-modiolar plane and each half was furthermore divided into two parts comprising respectively the upper and basal coils. The specimens were transferred to a solution of 30% glycerol buffer solution for a minimum of 2 h. Each coil was mounted on a gold specimen holder and fractured in a Balzer's 360M freeze-fracturing unit. The resulting replicas were mounted on copper mesh grids and examined ultrastructurally in a Jeol 1200 EX electron microscope.

**Immunomorphology**

The material was obtained from legal abortions and collected immediately. The inner ears were removed from the fetuses and shock-frozen in liquid isopenthan cooled by liquid nitrogen. In most cases, the cochlear and vestibular parts of the labyrinth were separated before freezing.

Each specimen was serially cryosectioned at -30°C (Anniko et al., 1988). Between 1,000 and 2,000 cryosections with a thickness of approximately 4 µm were obtained from each specimen. Every 10th section was stained with hematoxylin & eosin (H & E) to facilitate orientation. Based on the findings from these sections, adjacent sections were stained for immunohistochemistry with monoclonal antibodies (mAbs) against neurofilaments, vimentin and cytokeratins. All our material on spiral ganglion (SG) cells consisted of approximately 200 immunostained cryosections.

For immunohistochemistry, the following protocol was used. The sections were incubated with preimmune rabbit serum (diluted 1:20) in PBS for 20 min at room temperature (RT), and then with the different mAbs (at appropriate dilutions) for 90 min at RT in a moist chamber. After washing in PBS, rabbit antimouse Ig link antibody diluted 1:50 in PBS, with 0.1% bovine serum albumin (BSA), and 15 mM NaN₃, was added and incubated for 1 h at RT, followed by a PBS rinse. The mouse PAP complex, diluted 1:50 in PBS, was then incubated with 0.1% BSA and 15 mM NaN₃ for 1 h at RT. After washing again in PBS, the diaminobenzidine substrate (0.1% in 0.05 M Tris, pH 7.6) plus 0.03% H₂O₂ were added and left for 10 min. After a 5-min wash in water, the sections were dehydrated, cleaned, and mounted in DPX mountant (BDH Chemicals Ltd, Poole, England). The specimens were examined and photographed in a Zeiss Axiosphot photomicroscope. Each section was also analyzed by phase contrast.

In our present study, seven mAbs showed immunostaining of human spiral ganglion cells. The mAbs against NF triplet proteins were raised and characterized as described by Virtanen et al. (1985). In addition, commercially available mAbs of NF proteins (neurofilament Dako, Dakopatts, Copenhagen) were used. mAbs (RNF 402) detecting only the 200-kDa subgroup of NFs were also employed (Ramaekers, 1987, unpublished data). To detect vimentin, both mAbs K-17 (Ramaekers, 1987, unpublished) and commercially available vimentin (Dako) were used. The raising and the specificity of our mAbs RCK 106 (detecting Ck 18) and mAbs K-8.60 (detecting Cks 10 and 11) has been described recently (Ramaekers et al., 1987; Huszar et al., 1986).

Fig. 1. Electron micrograph (EM) of a freeze-fracture replica. A large number of SG cells with smooth cell surfaces are present. These are polygonal type I cells, according to the classification previously described by Spencllin (1969). The SG cells are enclosed by Schwann cells. (Reprinted from Anniko and Bagger-Sjöback, 1987.) ×1,300.

Fig. 2. Electron micrograph of freeze-fracture replica. One type II SG cell (see reference 4) shows a number of small, well-defined impressions on its surface. Two regions (arrows) contain an aggregation of particles larger than otherwise seen in most mammalian cell membranes (E-face), which represent sites of membrane intercalated proteins. An adjacent type I spiral ganglion cell is surrounded by several layers of myelin and is larger than the ganglion cell with impressions. a, myelinated axons; N, nucleus. (Reprinted from Anniko and Bagger- Sjöback, 1987.) ×4,000.
RESULTS

Freeze fracturing

The freeze-fracture features of SG cells were similar in the apical and basal parts of the cochlea. Two types could be identified. Most had a well defined surface and were surrounded by Schwann cells (Fig. 1). The nucleus in each of these SG cells is large, rounded and often eccentric. The cells often have bipolar appearance. No tight junctions or gap junctions were observed between the spiral ganglion cells and their satellite cells.

Intermingled among most SG cells were occasional ganglion cells whose surface displayed a large number of small, anatomically well-delineated impressions (Fig. 2). In several specimens, cells having more than 10 such impressions adjacent to each other were observed. Some of the impressions contained intracellular particles. A thin myelin layer, if occurring at all, surrounded these cells. Since the fracture surfaces were obtained coincidentally, concurrent morphometric analyses of the cells with membrane impressions could not be made. It was, however, possible to estimate that they constitute only a few percent of the total spiral ganglion cell population. The cells in both populations differed very little in size, although the cells with impressions were slightly smaller.

The average ratio of myelinated vs. unmyelinated SG cells was 24:1 (range, 20–31:1), i.e., approximately 4% (range, 3.8–4.4%) of the total ganglion cell population consisted of type II cells. Our calculations, based on sectioned material regarding the occurrence of type I and type II SG cells in the mouse, concur with similar studies in the guinea pig (Kellerhals et al., 1967), cat (Spoendlin, 1969) and human (Ylikoski et al., 1978). The studies showed that most SG cells are of type I, and are thus presumably associated with inner hair cells (i.e., are connected to the divergent afferent system).

Immunomorphology

Neurofilaments. Positivity for NF triplet proteins occurred in all nerve fibers irrespective of gestational age. In 14-week fetal or older inner ears only the axonal hillock of spiral ganglion cells stained (Fig. 3), in contrast to the vestibular ganglia, where many cells showed immunoreactivity throughout the cytoplasm (Fig. 4). Approximately 10–20% of all SG cells displayed positivity for NFs throughout the cytoplasm. The SG cells that differentiated earlier more often contained NFs in the entire perikaryon than was the case in younger (later developed) cells (Fig. 5).

Positivity for the mAb RNFL 402 (detecting only the heaviest 200-kDa triplet subunit) was evident in all nerve fibers, but with considerable differences in staining intensity at different locations of the labyrinth. Only 14–16-week-gestation specimens were analyzed. A fairly uniform immunoreactivity of axons and dendrites of SG cells was seen. However, in the vestibular nerve, several groups of fibers were more intensely stained than others (Fig. 6). Whether these differences in stainability reveal distinct groups of nerve fibers, or merely indicate different stages of morphological maturation, remains to be further analyzed in older temporal bones.

Vimentin. All mesenchymal cells displayed vimentin staining (Fig. 7). Some SG cells showed a strong immunoreactivity, others a weak one (Fig. 8). Vimentin is present in SG cells irrespective of gestational age.

Cytokeratins. Spiral ganglion cells showed immunostaining for the mAbs K-8, 60 (detecting Cks 10 and 11) and the mAbs RCK 106 (detecting Ck 18). The former mAbs showed a fairly homogeneous positivity in the cell cytoplasm and to a minor extent in nerve fibers (Fig. 9), but the immunoreactivity for the mAbs RCK 106 varied between the individual SG cells (Fig. 10). Nerve fibers did not stain with the mAbs RCK 106.

DISCUSSION

When analyzing the fiber spectrum of the cochlear nerve in the cat, Arnesen and Kjelsberg-Osen (1978) found that unmyelinated exons could either be related to type II SG cells or could belong to aberrant efferent or postganglionic autonomic fibers. In a total population of approximately 10,000 fibers in the cochlear nerve of the adult mouse, Anniko and Arnesen (1988) showed that 2.6% (range, 2.2%–3.0%) were unmyelinated. Statistically, in our present study the number of unmyelinated nerve fibers corresponds to the number of type II SG cells. Any correlation, however, between these two observations remains speculative, since some of these unmyelinated fibers may belong to postganglionic autonomic fibers.

The type II SG cells show numerous membrane specializations with impressions that were morphologically similar to those previously found on hair cells adjacent to the afferent and efferent nerve endings (Anniko and Wróblewski, 1984; Romand and Romand, 1984). In freeze-fracture replicas, the postsynaptic membrane of the efferent nerve synapses lacks intermembrane particle specialization. We consider that many of the impressions on type II SG cells in the mouse are of efferent origin. Type II cells represent a convergent system of the outer hair cells and comprise about 5% of all afferent cochlear neurons (Spoendlin, 1972). Thus, synaptic contacts on the type II SG cells may exert a control over the transmission of information from a great number of outer hair cells to the central nervous system.

Synapses on SG cells have been previously described only in the human cochlea and monkey (Kimura and Ota, 1981; Kimura et al., 1987). Our findings in the adult mouse are in agreement with those described in man, but the mouse has more numerous nerve terminals. The findings of synaptic specialization of spiral ganglion cells in the human, monkey, and mouse suggest that the basic principles of structural organization apply to more than one species.

It is tempting to speculate that the two different populations of SG cells, differentiated by their NF immunoreactivity and freeze-fracture structure correspond to the two main cell types of spiral ganglion cells. The immunostained area of the axonal hillock in SG cells could represent the fibrillogenous zone of Held and could be compatible with the developmental pattern seen in the neurofilibrillar stain (Sobkowicz et al., 1973). Most of the toxic agents that alter the neuronal cyto-
Fig. 3. Cochlea (basal coil) from a 14-week human fetus. Immunostaining for neurofilaments (neurofilament Dako). The SG comprises cells with strong positivity only at one end of the perikaryon. Unfilled arrows point to the eccentric nuclei. x190.

Fig. 4. Inner ear from a 14-week human fetus. Immunostaining for neurofilaments (neurofilament Dako). The vestibular ganglion (VG) has many ganglion cells, which show strong positivity for neurofilaments throughout the cytoplasm (unfilled straight arrow). Nerve fibers show intense immunoreactivity (curved unfilled arrow). x190.
Fig. 5. Inner ear from a 8-week human fetus. Immunostaining for neurofilaments. Phase contrast microscopy. Many cells in the spiral ganglion (SG) show strong immunoreactivity throughout the cytoplasm (unfilled straight arrow), but SG cells with a very weak staining may also occur (unfilled curved arrows). ×180.

Fig. 6. Inner ear of a 16-week human fetus. Immunostaining with the mAbs RFN 402 (detecting only the 200-kDa component of the neurofilament triplet proteins). Cross section of the vestibular nerve (VN). There are considerable differences in positivity between different regions of the VN, although all nerve fibers show immunoreactivity. Intense staining is indicated with unfilled arrows. ×210.
Fig. 7. Inner ear of a 14-week human fetus. Immunostaining for vimentin (vimentin Dako). All cells in the area of the SG show some degree of positivity, including the SG cells (unfilled arrows). ×90.

Fig. 8. Inner ear of an 8-week human fetus. Immunostaining for vimentin (mAbs K-17). In the SG the nerve cells (unfilled arrows) show stronger immunoreactivity than do adjacent mesenchymal cells. ×110.
Fig. 9. Inner ear of an 8-week human fetus. Immunostaining with mAbs K-8.60 (detecting cytokeratins 10 and 11). Section through the acoustic ganglion. There is a distinct positivity in the SG cells (filled arrows), but none in outgrowing nerve fibers (asterisks). × 110.

Fig. 10. Inner ear of a 16-week human fetus. Immunostaining with mAbs RCK 106 (detecting cytokeratin 18). Phase contrast microscopy. In the SG several ganglion cells show distinct positivity of the entire cytoplasm (unfilled arrows). Nerve fibers lack immunoreactivity (asterisks). × 210.
skeleton affect NF distribution (Clarkson et al., 1986). Thus, theoretically, ototoxic substances would initially affect the cells that have a rich content of NFs. Even though most ototoxic substances primarily cause a morphological damage to hair cells, although early metabolic changes do occur in the SC cells (Darlstede and Bagger-Sjöbäck, 1977).

Neurofilaments are the only IFs known to contain side arms (non-alpha-helical terminal portions of the protein). These may be involved in lateral associations with other filaments or microtubules, via microtubule-associated proteins (MAPs). It has been suggested that the NF subunit triplet (approximately 68, 145, and 200 kDa) is a marker for slow axonal transport. Yet another possible role of NFs involves regulation of axonal caliber and shape. The proposed functions are based on studies of regenerating axons where NF content was determined by NF transport and correlated with axon caliber (Hoffman et al., 1984).

In principle, simultaneous expression of two classes of IF proteins, although frequently observed in cells in vitro, is rarely seen in vivo. But an expression of NFs, combined with transient expression of vimentin, has been reported in developing neuronal cells (Tappcott et al., 1981a,b). Moreover, in our material, a co-expression of NFs and vimentin occurred not only in the fetal human SG but also in the adult (Anniko and Arnold, 1988, unpublished data). Such co-expression has also been observed in mature spinal ganglion cells (Clarkson et al., 1986).

A transient co-expression of vimentin and Cks may occur in several organs during the development, e.g., in the embryonic kidney tubular epithelial cells of the collecting ducts. But in the adult kidney, only the cells of collecting ducts retain expression of Cks (Höthöfer et al., 1984). The simultaneous expression of Cks, NFs, and vimentin has not been previously described in human epithelium. We have recently found, however, such co-expression in the inner ear of the embryonic and the fetal mouse both in vivo and in vitro (Berggren et al., 1989). A developmental change in expression of IFs, from epithelial Ck-positive to neuronal NF-positive, has been reported in the early otic ganglion of the guinea pig (Raphael et al., 1987). Our results, however, indicate that, at least in the human, the co-expression of NFs, vimentin and Cks in the SG cells may persist into adulthood (Anniko and Arnold, 1988, unpublished data). The expression of Cks in the SG cells may reflect their potential epithelial origin (Politzer, 1956).

CONCLUDING REMARKS

The SG cells show high complexity with regard to both the synaptic membrane specializations in type II cells and to the internal organization of the cytoskeleton in type I and II cells. Our study provides information regarding both freeze-fracture images of adult SG cells in the CBA/CBA mouse, and the intracellular organization of the fetal inner ear cells in the human. The latter cells reach maturity between 18 and 22 weeks of gestation. To confirm the validity of our findings on a principal basis in mammals, further studies are needed on the freeze-fractured human adult SG cells and of their cytoskeletal structure.

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (M.A.: 12X-7305; L.-E.T.: 17X-3954), the Foundation Tysta Skolan (M.A.), the Ragnar and Torsten Söderberg Foundation (M.A.), the Ingrid Juelius Foundation (I.V.) the Academy of Finland (I.V.), the Dutch Cancer Foundation (R.W.F.), and the University of Umeå. The skilful technical assistance of Ms. Monika Andersson is gratefully acknowledged.

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