Intermediate Filament Proteins in the Embryonic Inner Ear of Mice under Normal Conditions and after Exposure to Ototoxic Drugs

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The expression of the intermediate filament subclasses cytokeratins (CKs), vimentin, and neurofilament triplet proteins (NFs) was analysed in the embryonic inner ear of the CBA/CBA mouse, in vivo and in vitro, using well-defined monoclonal antibodies. Some of the cultured inner ears were exposed to 1–10 μg/ml of the ototoxic drugs gentamicin, ethacrynic acid or cisplatin. There was no difference in the expression of IF pattern in the cultured inner ears as compared with the in vivo developed labyrinths. Exposure to the ototoxic drugs did not affect the expression of CKs, vimentin, or NFs. CKs 8 and 18 were identified in all inner ear epithelia. In the mature hair cells, only the apical surfaces showed immunoreactivity to CKs. In addition, CKs 7 and 19 were visualized in the epithelia involved in maintaining endolympth homeostasis. The ganglion cells showed co-expression of CKs, vimentin and NFs, each having a characteristic localization in the cells. Key words: intermediate filaments, cytoskeleton, inner ear, ototoxicity, in vitro.

INTRODUCTION

The cytoskeleton of higher eukaryotic cells consists of microtubules, intermediate filaments (IFs), microfilaments and interconnecting proteins. The IF protein composition of a cell may reflect both its embryonic origin and its function. IFs are grouped into five major classes: cytokeratins (CKs) which are identified in epithelia, desmin in muscle, vimentin in cells of mesenchymal origin, glial fibrillary acidic protein in astroglia, and neurofilaments (NFs) in neuronal cells (1).

In human epithelial cells there are at least 19 different CK sub-types, each of which probably has a corresponding group among other mammals (2). CKs are expressed in different combinations of polypeptides. A given epithelial cell can thus be characterized by the specific pattern of its CK components. IFs are important for the three-dimensional orientation of the cell. CKs, vimentin and desmin are closely related to the desmosomes (3, 4). Therefore IFs have a cytoskeletal function that may be of greater importance in tissue than in individual cells (3).

No drugs are known to specifically disrupt the IF network, but a variety of conditions exist that can alter the amount of IFs, or their internal organization. For example, viral infection of a cell can cause disturbances in the expression of vimentin (5) and CKs (6). Gamma irradiation during embryonic development of the inner ear induces the expression of NF in hair cells (HC) normally devoid of this subgroup of IFs (7). In cultured cells, changes in the expression of IFs can be induced by altering the growth conditions. A change in environmental conditions causing cell shape to turn from flat to spherical reduces the formation of vimentin, while reduced cell to cell contact results in a decrease
of CKs (5). In contrast to isolated cell lines, cell shape and cell to cell contacts are largely preserved when a whole organ, such as the inner ear, is cultured (8).

The purpose of our study was to compare the expression of IFs in various cells and tissues of the inner ear developed in vivo and in vitro and to ascertain whether the IF pattern changed when the embryonic inner ear was exposed to an ototoxic environment during morphogenesis and cytodifferentiation.

MATERIAL AND METHODS

Inner ears from CBA/CBA mouse fetuses were used. Gestational age was determined by the vaginal plug technique, considering day 1 as the day when the mucoid plug was observed. The in vivo specimens (N=5) were collected on the 20th (gestational day) GD. For organ cultures, pregnant mice were sacrificed by cervical dislocation on the 13th (otocyst stage), 15th or 16th GD. The inner ears were dissected in Hank's balanced salt solution and placed in an organ culture dish using Neuman and Tytell serumless medium supplemented with 15% fetal calf serum and 1% L-glutamine.

The inner ears were incubated at 35±0.2°C in atmospheric air supplemented with 5% CO2. The nutrient solution was renewed every second day. The explants were cultured for 5-8 days to an age corresponding to birth. The control material comprised six specimens from GD 13 and seven from GD 15. Twenty-six inner ears were exposed to ototoxic drugs in vitro (Table I). At the end of culturing, the specimens were shock-frozen in liquid isopentane cooled with liquid nitrogen, and serially cryosectioned at −30°C. Between 100 and 300 sections, with a thickness of approximately 4 μm, were obtained from each inner ear. Every 5th section was stained with haematoxylin-eosin to facilitate orientation. Based on the findings from these sections, adjacent sections were stained for immunomorphology using monoclonal antibodies (mAbs) against NFs, vimentin and various CKs (Table II).

Each section was incubated with one type of mAbs using the peroxidase-antiperoxidase technique for analyses of the initially unlabelled mAbs (11). The specimens were observed with a Zeiss Axiophot light microscope including phase contrast.

RESULTS

A similar principal pattern of IF expression occurred in vivo and in vitro developed inner ears as well as in drug-exposed labyrinths. The exposure to ototoxic drugs caused the same morphologic cell damage as earlier described for this in vitro model (12, 13). In all figures, the specimens had been analysed by light microscopy, using phase contrast.

Cytokeratins

Positivity to mAbs RPN 1164 and RPN 1166 (both detecting CK 8) and mAbs RPN 1160 and RGE 53 (both detecting CK 18) was found in all types of inner ear epithelia. In the mucolisa and the cribriform, the immunostaining of the sensory epithelium was restricted mainly to the basal parts and to the apical surface (Fig. 1). The primordial organ of Corti had a more general staining in the apical part of the cochlea than in the more differentiinated basal coil. In the undifferentiated cochlear epithelium all cell types stained with these mAbs (Fig. 2). The innervated and more differentiated parts of the cochlea showed a selective and distinct staining with a positive immunoreaction to these mAbs restricted to the stria vascularis, the cells of Deiter, Hensen and Claudius and to the apical surface of the hair cells (Fig. 3B). The supporting cells of the vestibular organs, the lining epithelia of the semicircular canals, the dark cells and the stria vascularis cells were consistently positive.
to CKs 8 and 18 with our set of mAbs, as was the endolymphatic duct and especially the sac, which displayed the strongest immunostaining.

The mAb RCK 102 (CKs 5 and 8) showed the same distribution as for CKs 8 and 18 (Fig. 4). In addition, an immunostaining of Reissner's membrane and outer sulcus cells (Fig. 5) and of ganglion cells was observed (Fig. 9). CK 7 (mAb RCK 105) and CK 19 (RPN 1165) were identified in the dark cells, the stria vascularis, Reissner's membrane (Fig. 3C), and the endolymphatic duct (ED) and sac (ES). In the stria vascularis the immunostaining was intense, whereas there was no staining with these antibodies in the dark cells of the in vivo inner ears, and only a weak and irregular staining of the dark cells in the in vitro cultured inner ears. The ED and ES showed strong positivity. Also parts of the epithelial lining of the semicircular canals showed a weak positivity to these mAbs. Positivity to the mAb RPN 1165 had the same distribution as for mAb RPN 1165 (CK 19). The mAbs RCK 106 and RPN 1161 did not stain inner ear epithelia.

A general finding was that well-differentiated epithelial cells had a more distinct and restricted positivity to cytokeratin antibodies than the less mature ones. For instance, the epithelia of the maculae stained less than that of the crista. This difference was most obvious in inner ear anlagen explanted early during development, such as the otocysts explanted on the 13th GD.

Table I. In vitro exposure of inner ears to ototoxic drugs

<table>
<thead>
<tr>
<th>Age at explantation (gestational day)</th>
<th>Drug</th>
<th>Concentration [µg/ml]</th>
<th>Duration of exposure</th>
<th>No. of explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>Gentamicin</td>
<td>10</td>
<td>8 d</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Ethacrynic acid</td>
<td>1</td>
<td>3 h³</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Cisplatin</td>
<td>1</td>
<td>8 d</td>
<td>4</td>
</tr>
<tr>
<td>16³</td>
<td>Cisplatin</td>
<td>1</td>
<td>3 h³</td>
<td>4</td>
</tr>
</tbody>
</table>

* During the first 3 h after explantation.
³ Specimens were divided into a cochlear and a vestibular part before explantation.

Table II. Specificity of monoclonal antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen recognized</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPN 1160</td>
<td>CK 18²</td>
<td>Amersham International</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bucks, England</td>
</tr>
<tr>
<td>1161</td>
<td>Not defined</td>
<td>As above</td>
</tr>
<tr>
<td>1163</td>
<td>Not defined</td>
<td>As above</td>
</tr>
<tr>
<td>1164</td>
<td>CK 8</td>
<td>As above</td>
</tr>
<tr>
<td>1165</td>
<td>CK 19</td>
<td>As above</td>
</tr>
<tr>
<td>1166</td>
<td>CK 8</td>
<td>As above</td>
</tr>
<tr>
<td>RGE 53</td>
<td>CK 18</td>
<td>9</td>
</tr>
<tr>
<td>RCK 102</td>
<td>CK 5 and 8</td>
<td>9</td>
</tr>
<tr>
<td>105</td>
<td>CK 7</td>
<td>9</td>
</tr>
<tr>
<td>106</td>
<td>CK 18</td>
<td>9</td>
</tr>
<tr>
<td>FV 24 BAG</td>
<td>Vimentin</td>
<td>10</td>
</tr>
<tr>
<td>NF Dakopatis</td>
<td>Neurofilament triplet proteins</td>
<td>Dakopatis Copenhagen, Denmark</td>
</tr>
</tbody>
</table>

² In terms of the current numbering system for human cytokeratins (3).
**Fig. 1.** Inner ear explanted on 15th GD and cultured for 6 days. Immunostaining with mAb RPN 1164 (CK 8). In the macula utriculi (MU), staining is restricted to the basal part of the epithelium and the apical surface. The non-sensory epithelia lining the endolymphatic space display positivity in the cytoplasm of all cells, with a strong reaction along their lateral borders. The cartilago (C) and the perilymphatic space (PS) are not stained.

**Fig. 2.** Apical part of cochlea from a 20-day-old mouse fetus. Immunostaining with mAbs RGE 53 (CK 18). This cochlear section had not yet become innervated. All types of cells comprising the cochlear epithelium display positive staining. GER, greater epithelial ridge; LER, lesser epithelial ridge; SL, spiral limbus; TM, tectorial membrane.

**Fig. 3.** Four consecutive sections from basal turn of the cochlea from a 20 GD mouse fetus. Irrespective of type of mAbs used, a distinct staining of the tectorial membrane (TM) occurred. (A). Immunostaining for NFs. Innervation is present around both outer (OHC) and inner hair cells (IHC), but immunoreactivity is more extensive around the IHC. (B). Immunostaining with mAb RGE 53 (CK 18). The cells of Deiter (CD), Hensen (CH) and Claudius (CC) are strongly positive. In the stria vascularis (SV) the intense staining is restricted to the apical cell membrane and to the basal part of the epithelium towards the basal membrane. The hair cells (arrows) show immunoreactivity only at
their apical surfaces. (C). Immunostaining with mAb RPN 1165 (CK 19). There is a distinct staining of the stria vascularis (SV) and Reissner's membrane (RM), but not in other epithelia. (D). Immunostaining for vimentin. The mesenchymal cells of the perilymphatic space (PS) show positivity. In the epithelia lining the endolymphatic space (ES), only a few cells (asterisk) in the spiral limbus (SL) and the basal cells (arrows) in the stria vascularis were stained.
Fig. 4. Inner ear explanted on 15th GD and cultured for 6 days. Immunostaining with mAb RCK 102 (CKs 5 and 8). The endolymphatic duct (ED) is strongly immunoreactive, with intensely marked cellular borders, whereas the epithelium of the semicircular canal (SSC) displays a considerably weaker staining.

Fig. 5. Basal coil of cochlea from an inner ear explanted on 15th GD and cultured for 6 days. Immunostaining with mAb RCK 102 (CKs 5 and 8) yields strong positivity in the apical surfaces of the outer sulcus cells (OSC), the stria vascularis (SV) and Reissner's membrane (RM). In the SV, basal cells also show immunoreactivity. Arrows indicate melanin granules.

Vimentin

MAbs detecting vimentin showed strong positivity to cartilage and mesenchymal cells of the perilymphatic space (Fig. 3D). In the cochlea, some basal cells in the stria vascularis and some cells in the spiral limbus showed a weak positivity to vimentin (Fig. 3D). A few
Fig. 6. Oticyst explanted on 13th GD and cultured for 8 days under continuous exposure to ethucrynic acid, 1 μg/ml. Vestibular ganglion. Immunostaining for NFs. Most of the ganglion cells show a strong immunoreactivity, but a few cells are weakly stained (arrow). In general, one end of the neuron with its axon hillock and axon itself became more heavily stained (double arrows) than the other parts of the cell.

Fig. 7. Inner ear explanted on 15th GD and cultured for 6 days. Spiral ganglion cells. Immunostaining for NFs. A weak immunoreactivity occurs throughout the cytoplasm, but with one end of the cell more heavily stained (arrow).

Fig. 8. Vestibular ganglia from a 20 GD mouse fetus. Immunostaining for vimentin. Immunoreactivity is particularly strong in the periphery of the ganglion cells, probably indicating Schwann cells, and around outgrowing nerve fibres, whereas the ganglion cell body is only weakly stained.

Fig. 9. Section adjacent to that in Fig. 8. Immunostaining with mAb RCK 102 (CKs 5 and 8). The neuronal cytoplasm is intensely stained, but the Schwann cells lack positivity.
supporting cells in the vestibular parts of the epithelium lining the endolymphatic space also had an immunoreactivity to vimentin. A distinct immunoreactivity occurred in the vestibular (Fig. 8) and, although with less staining reaction, in the spiral ganglia. Schwann cells surrounding the ganglion cells and outgrowing nerve fibres had a strong immunoreaction to vimentin but there was also positivity within the neurons.

*Neurofilament proteins*

In the vestibular ganglia the majority of ganglion cells showed strong positivity to NF in the cytoplasm (Fig. 6). In contrast, few spiral ganglion cells were intensely stained (Fig. 7). Nerve fibres and nerve chalyses surrounding vestibular type I HCs were strongly positive. In the labyrinths cultured from the 15th or 16th GD and in the in vivo preparations, nerve chalyses were regularly stained also in basal parts of the cochlea (Fig. 3 A).

All ganglion cells showed immunoreactivity to NF (varying extent), vimentin and RCK 102 (CKs 5 and 8). The different types of IFs all had a characteristic distribution in the neurons. NF positivity could be found throughout the cytoplasm, but one end of the cell was invariably more intensely stained (Fig. 6). In some neurons only the axon hillock was positive to NF. Vimentin was found in the mesenchymal cells of the ganglion, in the periphery of the nerve cells and had a weak staining reaction within the neurons (Fig. 8). MAb RCK 102 (CKs 5 and 8) gave a distinct speckled staining of the cytoplasm of all ganglion cells (Fig. 9).

**DISCUSSION**

Our findings regarding the IF pattern in the embryonic mouse inner ear are principally the same as described for the human fetus (11). Inner ears exposed to ototoxic substances in vitro preserved their normal IF expression, even when explanted at the otocyst stage and having to pass both morphogenesis and cytodifferentiation in the presence of an ototoxic environment.

The CKs exists in pairs comprising an acidic and a basic or neutral CK constituent (14). In our study the expression of at least two sets of pairs was found. CKs 8 and 18 were identified in all inner ear epithelia. In addition to these two CKs, positivity to CKs 7 and 19 also occurred in the epithelia involved in the maintenance of the endolymph homeostasis.

The reason why mature HCs lack IFs, except at their apical surface, may be that differentiated HC possess tight junctions at their lateral surfaces, but in contrast to supporting cells, do not have other intercellular junctions such as desmosomes and gap junctions (15). During early embryonic development, all cells of the inner ear epithelia are connected to each other with both gap and tight junctions. The uncoupling of HCs with regard to gap junctions occurs in both in vivo and in vitro developed labyrinths (8), so that the HCs reach a morphological basis for being electrochemically isolated.

The ganglion cells showed immunoreactivity to three different subclasses of IFs, co-expression of CKs, vimentin and NFs. Each subclass showed a specific distribution in the ganglion cells. Since no other of our mAbs against CK 8 showed positivity in ganglion cells it is tempting to conclude that the immunoreactivity to mAb RCK 102 (CKs 5 and 8) was due to the presence of CK 5. The co-expression of these different subclasses of IFs in neurons may be related to the dual origin of the statoacoustic ganglion, being composed of cells both from the wall of the otocyst and the neural crest.

Combinations of CK pairs became more selective and showed more distinct positivity to mAbs, the higher degree of specialization the various epithelia reached during embryonic development. Quantitative differences in CK expression were estimated not only between the maculae and the cristae (which at birth had reached different degrees of maturation)
but similarly also between the apical and basal turns of the cochlea. It was noticeable that the endolympathic duct — and in particular the sac — showed a rather unspecific staining pattern to CKs. Whether this represents immaturity, or a high potential for adaptation to environmental influences, remains to be further explored.

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