Prenatal Low-dose Gamma Irradiation of the Inner Ear Induces Changes in the Expression of Intermediate Filaments

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The expression of intermediate filaments (IF) was analysed in the inner ear in normally developed adult CBA/CBA mice and in mice of the same age which had been gamma irradiated in utero with a low dose 1–2 Gy single exposure. Well characterized monoclonal antibodies (mAbs) against all classes of intermediate filament proteins (cytokeratins—Cks, vimentin, neurofilaments, desmin and glial fibrillar acidic protein) were used. With the exception of neurofilament proteins, the expression of intermediate filament proteins was the same in adult normal and irradiated inner ears, irrespective of gestational age at exposure. A complex Ck pattern occurred in the various cell types comprising the membranous labyrinth. In spite of the differences in cell shape and internal organization of organelles, epithelia actively involved in inner ear fluid homeostasis (stria vascularis, dark cell epithelium, endolympathic duct and sac) revealed, according to our mAbs, the same expression of Cks, except for the mouse counterpart of human Ck 7, which was found exclusively in the stria vascularis and the endolympathic duct and sac. The pattern of intermediate filament composition in the labyrinth was the same in the mouse as in man. Irradiation on gestational days 12 or 13 (the otocyst stage)—but not at more advanced embryonic age—induced immunoreactivity for neurofilament proteins in vestibular hair cells (HC) and to a minor extent also in cochlear HC. No such positivity was found in the control material. Key words: cytoskeleton, intermediate filaments, immunomorphology, inner ear, gamma irradiation.

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There are a number of ototraumatic agents that cause functional and/or morphological damage to the inner ear. At advanced age, a morphological dysdifferentiation of the sensory epithelia occurs, although the hair cells (HC) themselves survive for a long time thereafter (1). During prenatal development toxic effects on the developing inner ear may cause either teratogenic or ototoxic effects, varying according to the developmental stage at the time of exposure (2). A selective inhibition of the basal membrane formation at the otocyst stage results in dysmorphogenesis (3).

Virtually all cells contain a cytoplasmic fibrillary network, comprising the three main components of the cytoskeleton: microfilaments (5–7 nm fibrils), intermediate filaments (10 nm in diameter) and microtubuli (25 nm fibrils) (4). The intermediate filaments (IF) are heterogeneous with respect to their composition. Five biochemically and immunologically distinct types of IF proteins (cytokeratins—Cks, vimentin, desmin, glial fibrillar acidic protein, and neurofilament triplet proteins) have been defined. The different types of IF are ultrastructurally indistinguishable and have the remarkable property of being expressed in a stable, cell-type-specific and differentiation-dependent manner (5). Tissue-
type specificity of a single type of IF, or their subgroup, does not exist, but the tissue specificity is expressed by the combination of various types of IFs and their subgroups.

We have recently described the immunomorphology of IFs in the inner ear, both in the mouse (6) and in the human (7). A developmental stage-dependent expression of IF proteins occurs in the developing inner ear, from the otocyst stage throughout morphogenesis and cytodifferentiation (8). Generally speaking, the expression of IF proteins is stable, for instance during both fetal development and malignant transformation. Exposure of the developing inner ear at the otocyst stage or later to a number of ototoxic or teratogenic agents, such as aminoglycoside antibiotics, ethacrynic acid or cisplatinum, did not affect the expression of IF proteins (9). Low-dose prenatal gamma irradiation given at the otocyst stage can cause dysmorphogenesis of vestibular organs, but did not macroscopically affect cochlear development (10). Cytodifferentiation of hair cells remained unaffected. However, a functional impairment of the adult cochlea occurred, as measured by loss of auditory brainstem recording (ABR) thresholds. Furthermore, irradiation acted as a sensitizer and caused premature aging (11).

In the present study we analysed the expression of IFs in the prenatally irradiated inner ear and report, for the first time, exogenically induced changes of the cytoskeleton in inner ear epithelia.

MATERIAL AND METHODS

Tissue. Pregnant CBA/CBA mice (n=14) were irradiated with a Siemens Gammastron 3 emitting 60Co gamma rays at a distance of 100 cm. The litters were exposed in utero either on gestation day (GD) 12 with 1 Gy (n=4), on GD 13 with 2 Gy (n=4) or on GD 16 with 2 Gy (n=6). For technical details of the irradiation technique, see ref. 10. Inner ears from newborn irradiated (n=22) and non-irradiated (n=8) CBA/CBA mice were used.

The inner ears were shock-frozen in liquid isopentane cooled by liquid nitrogen. All temporal bones were serially sectioned at −30°C with a section thickness of 4 μm. Every 5th section was stained with haematoxylin-eosin to facilitate orientation. Appropriate sections were taken for immunomorphology using the peroxidase-antiperoxidase (PAP) technique to identify the different IF proteins with well-defined monoclonal antibodies (mAbs). For further technical details, see ref. 7. All sections were mounted in DPX mountant (BDH Chemicals Ltd, Poole, Dorset, England) and observed and photographed in a Zeiss Axiophot photomicroscope. Each section was also analysed in phase contrast.

Antibodies. Sixteen different well characterized mAbs were used to detect IF proteins (Table 1).

RESULTS

The expression of the different types of IF proteins in cells and tissues in the labyrinth is presented below and depicted in Figs. 1–14. The overall morphology in the tissue sections was very well preserved and, at the light microscopic level, lacked obvious freezing artifacts.

Vimentin. The immunostaining was similar both in controls and in irradiated inner ears, irrespective of gestational age at exposure. Vimentin was present in cells of mesenchymal origin but was lacking in the epithelial lining of the membranous labyrinth. In the cristae ampullares, vimentin Dako showed a marked immunoreactivity at the apical surfaces of both hair cells and supporting cells. A weak staining occurred in vestibular and spiral ganglion cells. Our present findings are in agreement with those in our previously published data on the human fetal labyrinth (7).
Cytokeratins (Cks). In principle, the same pattern for expression of the various Cks was seen in both controls and irradiated inner ears, with a complex pattern of immunoreactivity of Ck subgroups. The localization of the most commonly detected Cks, i.e. the mouse counterparts of human Cks nos. 8 and 18 as revealed with the mAbs RPN 1164 (Ck 8) and RPN 1160 (Cks 18), is shown in Table II.

Immunoreactivity for RPN 1164 occurred in the stria vascularis (SV), the epithelial lining of the semicircular canals (SCC), the epithelium in the ampullary widenings (but not in the cristae), and especially in the cells lining the endolymphatic duct (ED) and sac (ES) (Figs. 1–3). The localization of the Ck 8 counterpart was also identified using the mAbs TS-1 and TS-7. A slightly different immunoreactivity pattern was seen when comparing these two mAbs and, also when compared with the staining pattern for RPN 1164. Both TS 1 and TS 7 showed a strong positivity in the epithelium of the ED and ES. The epithelial cells of the SCC and the ampullae, except for the most apical part of the cristae, were poorly stained by TS-1. In contrast, TS-7 positivity was lacking in SCC but was evident in the ampullary widenings, including the sensory organs. In the cochlea, positivity for TS-1 was not detected. Immunoreactivity for TS-7 was found in the epithelial lining of the cochlea and was stronger in the basal than in the apical half. The staining was most intense in cells of the SV and the outer sulcus cells (OSC).

Immunostaining for RPN 1160 (detecting the Ck 18 counterpart) occurred in the epithelial lining of the cochlea, except in the developing Kölliker’s organ (Table II). Positivity for RGB 53 (Ck 18) occurred in the cochlea at the apical surface of the SV, along the cell borders of the OSC and in the vestibular part of the labyrinth at the apical surface of the sensory organs. Immunoreactivity for RCK 102 (Cks 5 and 8 in the human epithelia) was found in the epithelial lining of the vestibular labyrinth (Fig. 4) and especially in the cells lining the ES and ED (Fig. 5). Immunostaining for RCK 105 (Ck 7 in human epithelia) occurred only in the SV, the ES and ED (Figs. 6–7).

Immunostaining for PKK-1 (Cks 8, 18 and 19), PKK-2 (Cks 7, 17 and 19) and PKK-3 (Ck

Table I. Monoclonal antibodies used to identify intermediate filament proteins

The human Cks recognized are indicated by the catalogue numbers as introduced by Moll et al. (12)

<table>
<thead>
<tr>
<th>mAbs</th>
<th>Intermediate filament protein specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV24BA6</td>
<td>Vimentin</td>
<td>13 Dakopatts, Copenhagen, Denmark</td>
</tr>
<tr>
<td>Vim Deko</td>
<td>Vimentin</td>
<td>14</td>
</tr>
<tr>
<td>PKK-1</td>
<td>Cks 8, 18, 19</td>
<td>14</td>
</tr>
<tr>
<td>PKK-2</td>
<td>Cks 7, 17, 19</td>
<td>14</td>
</tr>
<tr>
<td>PKK-3</td>
<td>Ck 18</td>
<td>14</td>
</tr>
<tr>
<td>RCK 105</td>
<td>Ck 7</td>
<td>15</td>
</tr>
<tr>
<td>RCK 102</td>
<td>Cks 5 &amp; 8</td>
<td>15</td>
</tr>
<tr>
<td>RPN 1164</td>
<td>Ck 8</td>
<td>15 Amersham International plc, England</td>
</tr>
<tr>
<td>TS-1</td>
<td>Ck 8</td>
<td>15 Stigbrand, 1987</td>
</tr>
<tr>
<td>TS-7</td>
<td>Ck 8</td>
<td>15 Stigbrand, 1987</td>
</tr>
<tr>
<td>RPN 1160</td>
<td>Ck 18</td>
<td>15 Amersham International plc, England</td>
</tr>
<tr>
<td>RGE 53</td>
<td>Ck 18</td>
<td>15</td>
</tr>
<tr>
<td>Neurofilament</td>
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<td>13, 14 Dakopatts, Copenhagen, Denmark</td>
</tr>
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<td>13, 14 Dakopatts, Copenhagen, Denmark</td>
</tr>
<tr>
<td>Desmin</td>
<td>Desmin</td>
<td>13, 14</td>
</tr>
<tr>
<td>Desmin (polyclonal)</td>
<td>Desmin</td>
<td>16</td>
</tr>
</tbody>
</table>
18) occurred in the ED and the ES, but was lacking in most other inner ear epithelia. Groups of cells lining the SCC occasionally showed immunostaining for all three mAbs. In contrast, their immunoreactivity was very strong in the epithelial lining of the middle ear.

**Neurofilaments (NF).** Control ears and irradiated inner ears both revealed a distinct immunoreactivity for NF proteins in all nerve fibres.

There was a strong positivity in both types of vestibular HC, if irradiation had been performed on GD 12 or GD 13, but was lacking in specimens irradiated on GD 16 and in controls (Figs. 8–10). In the cochlea of the corresponding material, both outer (OHC) and

### Table II. Expression of cytokeratins in the inner ear of the normal CBA/CBA mouse and following prenatal low dose irradiation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>RPN 1160 (Ck 18)</th>
<th>RPN 1164 (Ck 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Irradiation GD</td>
<td>Irradiation GD</td>
</tr>
<tr>
<td></td>
<td>In vivo 12 13 16</td>
<td>In vivo 12 13 16</td>
</tr>
</tbody>
</table>

**Sensory organs**

I. **Kölliker’s organ**

- GER: --
- LER: --
- TM: --
- IHC: --
- OHC-1: --
- OHC-2: --
- OHC-3: --

**Vestibular organs**

Hair cells

- Crista: +
- Utricle: +
- Saccule: +

Non-sensory cells

- Crista: +
- Utricle: +
- Saccule: +

**ED & ES**

ED & ES: +

**Epithelia involved in inner ear fluid homeostasis**

- Stria vascularis: +
- Spiral prominence: +
- Reissner’s membrane: -
- Epithelial cells: -
- Mesenchymal cells: -
- Dark cell epithelium: +

**Epithelial lining of the membranous labyrinth**

- Outer sulcus cells: --
- Spiral limbus cells: +
- SCC: +
- Vestibule: +

* Positivity only at cell surface.

- Individual variations between epithelial cells ranging from + to --.
Figs. 1–3. Specimens from inner ears irradiated with 2 Gy on gestation day (GD) 13, immunostained with mAbs RPN 1164 (Ck 8). Fig. 1 shows positivity in the cochlea only in the stria vascularis (arrow). The epithelial lining in the developing middle ear cavity was also stained. In Fig. 2, the dark cells (arrows) show distinct positivity whereas neither supporting nor sensory cells in the crista ampullaris (CA) became immunostained. Fig. 3 shows a strong positivity in all epithelial cells of the endolymphatic sac (ES). ×38 (Fig. 1); ×133 (Fig. 2); ×105 (Fig. 3).
inner hair cells (IHC) showed a weak but distinct immunoreactivity (Figs. 11–12). OHC which had not yet been reached by ingrowing nerve fibres lacked immunoreactivity.

The spiral and vestibular ganglia were both populated by groups of ganglion cells with a strong positivity for NF proteins. Although morphometrical methods were not used, it was estimated (in serially sectioned specimens) that the number of NF-positive ganglion cells was greater in inner ears irradiated on GD 12 and GD 13 than in those exposed on GD 16 or in controls.

Desmin. A distinct positivity occurred in a number of epithelial cells in the cristaæ and maculae but was otherwise lacking in the inner ear (Figs. 13–14).

Figs. 4–5. Inner ears irradiated on GD 12 with 1 Gy and immunostained with mAbs RCK 102 (Cks 5 and 8). Fig. 4 shows positivity in cells (mainly at their borders) lining the semicircular canals (asterisks). Fig. 5 shows strong positivity in the entire cytoplasm of cells lining the endolympathic sac (ES). ×105 (both figures).

Figs. 6–7. Inner ears irradiated on GD 13 with 2 Gy and immunostained with mAbs RCK 105 (Ck 7). Note positivity only at the cell borders in stria vascularis (SV; Fig. 6) and endolympathic sac (ES; Fig. 7). ×90 (Fig. 6); ×105 (Fig. 7).
Figs. 8-9. Inner ears irradiated on GD 12 with 1 Gy and immunostained with mAbs for NF. Note strong positivity in nerve fibres and adjacent HC both in macula utriculi (MU) (Fig. 8) and in crista ampullaris (CA) (Fig. 9). HC type I vs. type II are indicated with single vs. double arrows. ×315 (both figures).
Fig. 10. Crista ampullaris from inner ear irradiated with 2 Gy on GD 13 and immunostained with mAbs for neurofilament proteins. In several HC (arrows) a positivity occurred in the cytoplasm which stained less distinctly than adjacent nerve fibres. ×120.

Glia fibrillary acidic (GFA) protein. No positivity occurred in any of the inner ear specimens.

DISCUSSION

Differentiated mammalian cells both in vivo and in vitro can be reliably identified by their cell-type specific IF proteins (4, 5, 12). Since IFs are not isolated entities in the cytoplasm, their intimate association with other cellular organelles further characterizes the IF-associated proteins into two subcategories: those associating the IFs with surrounding structures, and those bridging the connections among the filaments themselves. The expression of IFs is regulated by evolutionarily related genes, of which some DNA sequences have been encoded.

In mouse embryogenesis, the earliest IF proteins synthesized are Cks A and D, which are the murine polypeptide counterparts to the human Cks 8 and 18 (17). Later during development, i.e. after the onset of organogenesis, Ck patterns of fetal epithelial tissues are similar to, though not always identical with, those of corresponding adult tissues. Such a developmental stage-dependent pattern of IF expression was recently described in the mouse inner ear (6). Although a complex tissue-specific composition of Cks was observed in our study, an even greater degree of complexity has been found in the human inner ear (13). The principal distribution of Ck expression in the labyrinth, however, is similar in mouse and man.

Gamma irradiation given during critical periods of gestation induces malformation of litters. Low dose exposure early during inner ear development does not prevent organogenesis or cytodifferentiation, but causes ultrastructural and functional changes in vestibular...
Figs. 11–12. Cochlea from inner ears irradiated on GD 13 with 2 Gy and immunostained with mAbs for NF. Strong positivity occurred in nerve fibres directed toward hair cells. In the apical coil (Fig. 11), minor immunoreactivity was present only in the inner hair cells (arrow) which had an extensive innervation. In the basal coil of the same specimen (Fig. 12), NF positivity occurred in all three rows of outer hair cells (which also had been reached by ingrowing nerve fibres) (arrows) and the inner hair cell (double arrow), ×170 (both figures).

lar and cochlear HC and acts as a sensitizer to aging (10). The morphological changes were initially confined to the receptor cell surface with its highly specialized micromechanics of stereocilia and their insertion into the cuticular plate. A slowly occurring dysdifferentiation of HCs took place. Our finding of NF proteins in the irradiated HC cytoskeleton is unique. NFs are found principally in axons, are much less abundant in dendrites and preferentially concentrated in the axon as compared with the cell body (18). In many axons, NFs cannot be detected at all. A non-uniform distribution of NF epitopes is present in the CNS during development (19). In the human fetal inner ear, the heavy 200 kD NF protein is not present until rather late during cytodifferentiation of the labyrinth (Anniko & Thornell, 1987, unpublished).
Positivity for NF proteins was found only in inner ears irradiated on GD 12 or GD 13, i.e. the otocyst stage, but not if exposure was performed on GD 16 when organogenesis is largely completed and cytodifferentiation is in progress. We conclude that irradiation might disturb gene regulation of the cytoskeleton of the developing/differentiating HC, which is the most vulnerable cell type in the inner ear at this stage of development. In the adult inner ear too, the HC is the primary site of damage in a number of ototraumatic conditions.

Although several conditions are known to cause abrupt changes in IF protein expression, such changes have not been extensively described for NF proteins. Many—but not all—cells in culture start to express vimentin together with their celltype-specific IF protein (20). Cultured cells can also reversibly lose their specific IFs. Both during early embryonic development and in some cases of malignant transformation, epithelial cells can either acquire the expression of the vimentin-type of IF, or loose their Ck positivity. For instance, Ck-positive kidney tubular cells develop from vimentin-positive mesenchymal cells. Co-expression of NFs and vimentin occurs in several cell types, including those from the inner ear (7). Induction of NF expression in the inner ear has hitherto been unknown.

In spite of differences in cell shape and internal organization of organelles, epithelia actively involved in inner ear fluid homeostasis (SV, dark cells, ED, ES) revealed an identical Ck expression, except for Ck 7 which was found exclusively in the SV, the ED and the ES. A similar pattern has been described also in the human inner ear (7). The interspecies similarities in cytoskeletal organization of the two epithelia further emphasize their close interaction in the regulation of inner ear fluids and ions. Both in controls and in low-dose irradiated inner ears, the expression of vimentin and the various subtypes of CKs was identical. GFA protein was not detected in either irradiated or control specimens. Several of our mAbs detected the same subgroup of CKs, but expressed a difference in immunoreactivity between the various cell types of the inner ear. Such a difference may
represent differences in their epitopes recognized and can be used to further distinguish (classify) cells in a given type of epithelium.

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