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Abnormal cochlea linked to deafness in transgenic mice expressing human cytokeratin K8

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Summary. The cytokeratin intermediate filaments have a relevant role in the proliferation and differentiation processes of epithelial cells. To provide information about the role of K8 cytokeratin during the auditory receptor differentiation, two groups of adult mice were used: TGK8-4 transgenic and control animals. The TGK8-4 transgenic mice contained 12 kb of K8 human cytokeratin (HK8) locus (Casanova et al., 1995, 1999). The functional activity of the auditory receptor was analyzed by auditory thresholds. Morphological studies demonstrate that the auditory receptors of the TGK8-4 transgenic mice are highly immature. Immunocytochemical studies were made by using two monoclonal antibodies: CAM 5-2 (recognizing K8 human cytokeratin) and Troma-1 (recognizing both mouse and human K8 cytokeratin). These demonstrated significant differences between the auditory receptors of the transgenic mice and the control mice. These functional and morphological differences clearly suggest that K8 cytokeratin has a relevant role during the differentiation and tridimensional organization of the sensory and the supporting cells of the auditory receptor.

Key words: Cytokeratin, Cochlea, Transgenic mouse, Shambaugh cells, Tectorial membrane, Stria vascularis

Introduction

The cytokeratin intermediate filaments are a major constituent of the vertebrate epithelial cell cytoskeleton. They are encoded by a large multigene family and are classified as type I (acidic) and type II (basic) cytokeratins according to biochemical, immunological and sequence homology parameters (Moll et al., 1982; Steinert and Roop, 1988; Fuchs and Weber, 1994). Type I and type II cytokeratins are expressed as pairs that copolymerize into heteropolymer structures (Sun et al., 1984).

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Although the function of cytokeratins is not yet fully understood, they extend as an intracellular three-dimensional meshwork which contributes to the cell organization and orientation (Lazarides, 1982), providing mechanical strength to the cell (see reviews in: Fuchs, 1996; Pagan et al., 1996; Omary and Ku, 1997). In addition, the K8, K10, K16, K18 and K19 cytokeratins have been shown to be involved in the proliferation and differentiation processes (Takahashi et al., 1994; Casanova et al., 1999; Paramio et al., 1999). The pair K8/K18 are the first cytokeratins to be synthesized during the embryogenesis process of the single cell layer epithelia (Jackson et al., 1980; Oshima et al., 1983; Duprey et al., 1985). Hepatic lesions were observed in transgenic mice that expressed a K18 keratin disruption (Ku et al., 1995). Thus, target disruption of the mouse K8 gene causes a mid-gestational lethality (Klymkowsky et al., 1992; Baribault et al., 1993), even though embryonic lethality observed in K8-deficient mice seemed to be dependent on mouse strain analyzed (Baribault et al., 1994). The K8 cytokeratin disruption in the FVB/N mouse strain was associated with intestinal and hepatobiliary abnormalities (Baribault et al., 1994). Moreover, it has been reported that transgenic mice that over-expressed human K8 (HK8) cytokeratin showed exocrine pancreas alterations related to proliferation, differentiation and apoptosis processes (Casanova et al., 1999).

The auditory receptor exhibited the expression of several kinds of acidic or basic cytokeratins in the cochleae of the human being (Anniko et al., 1987, 1990, 1992; Bauwens et al., 1991; Anniko and Arnold, 1995), the guinea pig (Shi et al., 1990; Kuijpers et al., 1991a; Raphael and Altschuler, 1991), the rat (Kuijpers et al., 1991a,b, 1992), and the mouse (Wikström et al., 1988; Anniko et al., 1989; Berggren et al., 1990). The role of cytokeratins in the differentiation process of the auditory receptor still remains unclear.

The study of the auditory receptor of the TGK8-4 transgenic mice could provide relevant information to clarify some roles of cytokeratins during cochlear differentiation. In fact, an abnormal auditory receptor linked to deafness found in these transgenic animals

could be due to an aberrant development of this structure. Abnormalities in the auditory receptor, linked to auditory deafness, were also found in other congenital processes that involved an altered protein synthesis (i.e. hypothyroidism).

Material and methods

Animals

A total of 42 adult mice were analyzed. The animals were divided into two groups: control (27 animals) and TGK8-4 transgenic (C57BL10xBalb/c) (15 animals) mice. The control group was constituted of wild mice (6 C57BL10 mice and 6 Balb/c mice), and 15 non-transgenic (C57BL10xBalb/c) mice pertaining to the same litter of TGK8-4 transgenic mice.

The care and use of animals in this study were in strict accordance with the animal welfare guidelines of the Declaration of Helsinky. All animals were sacrificed under deep general anesthesia.

Transgenic mice

The generation of a transgenic line that contains the unarranged cytokeratin HK8-12 has been previously reported (Casanova et al., 1995, 1999). TGK8-4 transgenic mice were produced by microinjecting a plasmid containing 12 Kb of the HK8 locus into fertilized oocytes (C57BL10x Balb/c) (Casanova et al., 1995, 1999).

Electrophysiological recordings

The electrophysiological recordings of the compound action potential (CAP) of the auditory nerve were carried out as previously described for the rat (Gil-Loyzaga et al., 1999; Carricondo et al., 2001). The CAP was evoked using filtered clicks (100 ms) at five frequencies, 22, 18, 16, 12, 8 and 4 KHz, with intensities from 100 to 30 dB (SPL) generated from a waveform synthesizer (Hewlett Packard HP8904 A), and recorded using an evoked potential compact unit (Mistral, Medelec Int.). The auditory thresholds of N1 wave were measured and plotted as a function of the stimulus intensity. One-way ANOVA was used to detect the significant differences between groups.

Histological procedure

After electrophysiological recordings the cochleae were rapidly removed and fixed using a solution containing 2.5 % glutaraldehyde in 0.1M phosphate buffer saline at pH 7.4 (PBS). The cochleae were decalcified in a saline solution containing 1% of ascorbic acid (Merchán-Pérez et al., 1999), postfixed in 1% aqueous OsO_4, deshydrated in ethanol (50% to 100%) and embedded in Spurr semi-thin sections, were then sectioned at 0.5 μm, stained with Richarson blue, then studied and photographed in a DMRB Leica photomicroscope.

Immunohistochemisty procedure

The cochleae submitted for immunohistochemistry were also rapidly removed, and quickly fixed in a 2% acetic acid in 98% ethanol solution, then postfixed in the same fixative solution for approximately 72 hours. The cochleae were decalcified in a saline solution containing 1% of ascorbic acid (Merchán-Pérez et al., 1999) for a short period (Tonnaer et al., 1990). Then they were dehydrated and embedded in paraffin. Samples were serially sectioned at 7-μm thickness from the mid-modiolar plane.

The immunocytochemical procedures were similar for the two different antibodies, CAM 5-2 and Troma-1, which were used in this study. The CAM 5.2 antibody (Becton Dickinson, California, USA) is a mouse monoclonal antibody that recognizes the HK8 cytokeratin (Casanova et al., 1995). The Troma-1 antibody is a rat monoclonal antibody that recognizes human and murine K8 cytokeratins (Brûlet et al., 1980). The sections were rinsed three times, in PBS, for 5 minutes each. Preincubation was carried out for 30 minutes in a PBS solution containing 30% of horse and rabbit normal serum, respectively. Alternate sections were then incubated overnight at 4 °C, in a solution containing 1/100 of one of the selected antibodies: CAM 5-2 or Troma-1. After being rinsed with PBS (three times, 5 minutes each), the sections were incubated in biotinylated anti-mouse and anti-rat IgG, respectively (Vectastin, Vector) at 1/200 in PBS, for 1 hour. Antigen-antibody immunoreaction was revealed using the avidin-biotin-peroxidase method (Vectastin, Vector), as has been widely reported (Merchán-Pérez et al., 1994; Gil-Loyzaga et al., 1997; Bartolomé et al., 1999).

Negative controls were obtained using the same procedure described above, but avoiding the primary antibodies.

Results

This transgenic TGK8-4 line contains 17 copies of transgene (Casanova et al., 1999). The expression of the transgenic HK8 was the parallel of the endogenous mouse K8, as demonstrated by northern and immunofluorescence analysis (Casanova et al., 1995). The transgenic animals, containing a low number of the transgene (1 to 12) copies, showed a normal phenotype, while those animals that carried an elevated number of the HK8 gene had a reduced growth, and homozygotic animals were not viable (Casanova et al., 1995, 1999). The expression of the transgenic HK8 in the TGK8-4 transgenic mice, which contained 17 copies of transgene, was parallel to the endogenous mouse K8 (Casanova et al., 1995). The different level of HK8 transgene expression is related to morphological alterations exhibited by adult TGK8-4 transgenic mice. These

Table 1. Summary of results obtained by immunocytochemical distribution of CAM 5-2 and Troma-1 immunoreactivity in different regions of the cochlea of control and TGK8-4 transgenic line. The control cochleae are constituted of the cochleae of the C57BL10, Balb/c mice, and the non-transgenic mice correspond to C57BL10 x Balb/c mice.

	CONTROL CAM 5-2	CONTROL TROMA-1	TGK8-4 LINE CAM 5-2	TGK8-4 LINE TROMA-1
Reisner's membrane	-	+ Epithelial cells	++ Epithelial cells	++ Epithelial cells
Spiral limbus	-	+	++	++++
Epithelium innner-outer	-	+	+++	++++
spiral sulci		Cell surface	Cell surface	Cell surface
Corti's organ	-	+ Cell surface	+++ Cell surface	++++ Whole cell bodies
Tectorial membrane	-	-	++	++
Shambaugh's cells	-	-	+++	++++
Vascular stria	-	Layer I +	Layer I +++ Layer II +++ Layer III +++	Layer I ++++ Layer II ++++ Layer III ++++
Lateral wall matrix	-	-	+	+

Layer I: marginal cell layer; layer II and III: deep layers; control animals: C57BL10, Balb/c, and C57BL10xBalb/c.

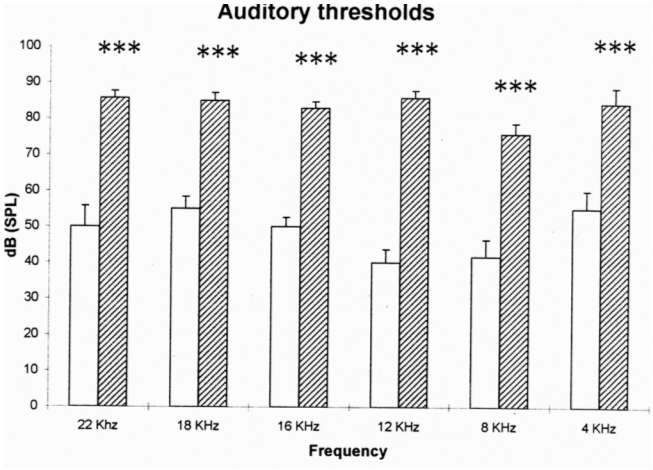

Fig. 1. Auditory threshold histogram. Means ± standard error of means (SEM) obtained from non-transgenic animals (grey bars) are plotted for each stimulation frequency. Statistical differences obtained by one-way ANOVA test between transgenic and non-transgenic mice are represented ***p<0.001.

TGK8-4 transgenic mice showed structural and functional alterations of acinar cells, leading to tissue destruction and adipose atrophy of the pancreas in aged animals (Casanova et al., 1999).

Electrophysiological results

The CAP auditory thresholds appeared to be significantly increased in TGK8-4 transgenic mice with respect to the non-transgenic mice (Fig. 1).

Morphological results

The non-transgenic mouse cochlea showed a completely mature auditory receptor (Fig. 2A,B). The Corti's tunnel opening and all the different cell types, in particular the inner hair cells (IHCs), the outer hair cells (OHCs) and the supporting cells, were easily identified (Fig. 2A). The tectorial membrane was placed upon sensory cells (Fig. 2A). The auditory nerve fibers crossed towards the osseous spiral lamina and reached the auditory receptor by habenula perforata (Fig. 2A). Moreover, the perikarya of the spiral ganglion neurons and myelinic axons were clearly observed going into the spiral ganglion (Fig. 2B).

The semi-thin cochlear sections from TGK8-4 transgenic mice (Figs. 2C, D) showed a total absence of the auditory receptor. The TGK8-4 transgenic mice showed dramatic differences with respect to the control mice (Fig. 2A,B). In the TGK8-4 transgenic mice, a single epithelial layer formed by non-structured epithelial cells was observed (Fig. 2C). The tunnel of Corti, the sensory and the supporting cells were not identified, and also the tectorial membrane was retracted (Fig. 2C). Scarce and isolated auditory nerve fibers were observed going into the osseous spiral lamina (Fig. 2C). Moreover, a few neuron perikarya were dispersed in the spiral ganglion (Fig. 2D).

Mouse K8 expression in the cochlea of control mice

A total absence of CAM 5-2 (which recognizes HK8) immunolabelling was observed in the cochlea of all strains (C57BL10, Balb/c mice and non-transgenic mice from C57BL10xBalb/c) of control animals (Table 1, and Fig. 3A). The positive Troma-1 (which recognizes

mouse and human K8) expression was observed in the spiral limbus area, the apical surface of the IHCs and OHCs, Hensen's cells, Reissner's membrane and the marginal cells of the stria vascularis (Table 1, and Fig. 3B), being negative on Shambaugh's epithelial cells (Fig. 3B).

Mouse and human K8 expression in the cochlea of TGK8-4 line mice

In the studies of immunocytochemical paraffin sections, the TGK8-4 transgenic adult mice cochleae appeared highly distorted (Fig. 4). The auditory receptor showed a degenerated cell mass in which the sensory and supporting cells could not be identified (Fig. 4A,B,D). Moreover, the tectorial membrane was distorted (Fig. 4A,B,D). In some slides, remaining cells of the Kölliker's organ, a transitory developmental structure of the auditory receptor, (see reviews in: Gil-Loyzaga et al., 1991; Remezal and Gil-Loyzaga, 1993; Gil-Loyzaga, 1997) were still present (Fig. 4A,B,D).

A positive CAM 5-2 (which recognizes HK8)

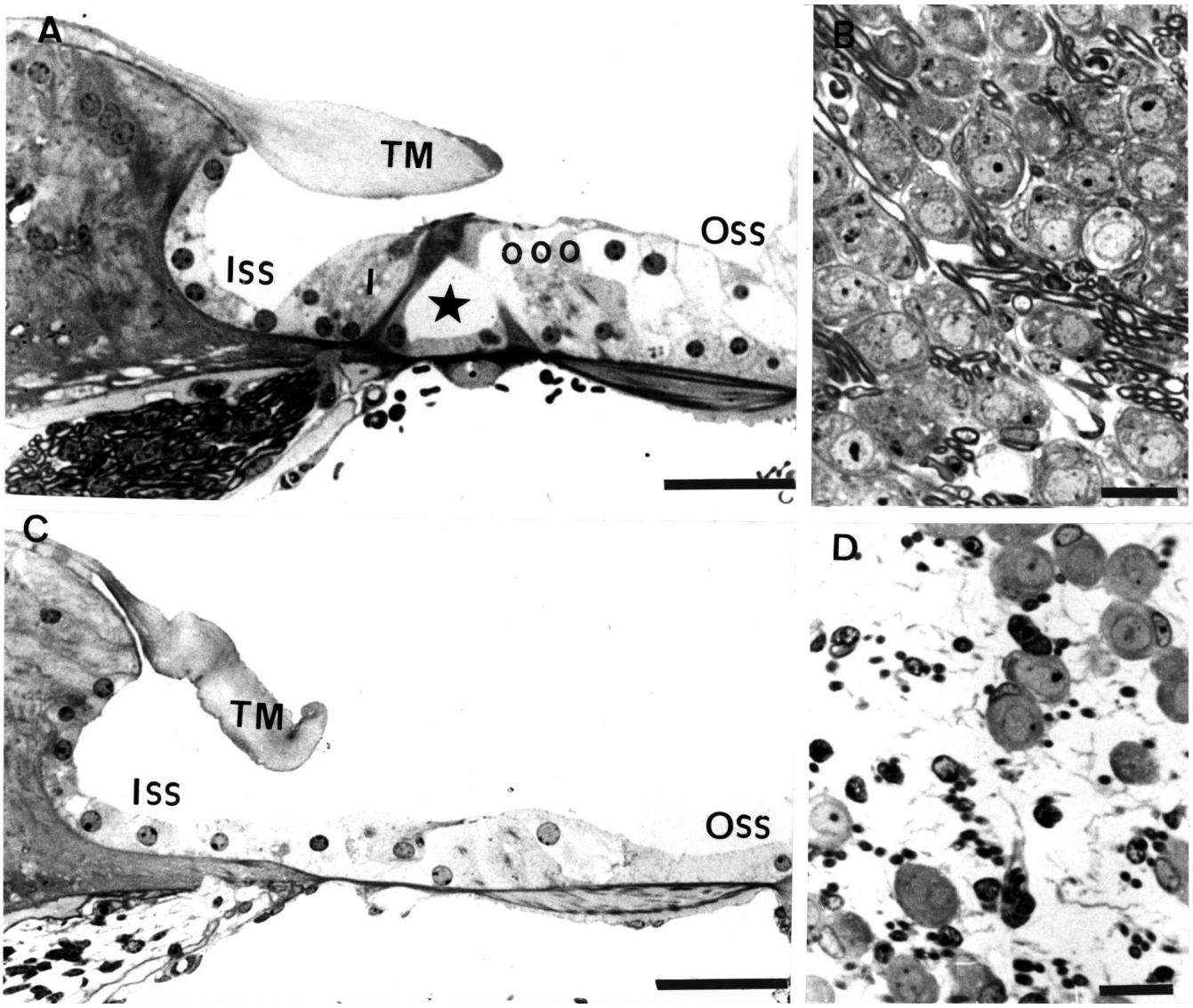

Fig. 2. The midmodiolar semi-thin cochlear sections **A.** The non-transgenic mice show a morphology of a completely mature auditory receptor. The OHCs (O), the IHC (I), the tectorial membrane (TM) and the tunnel of Corti (star) are clearly distinguished. The nerve auditory fibers crossing the osseous spiral lamina and reaching the auditory receptor. **B.** In the non-transgenic spiral ganglion the perikarya of neurons and myelinic axons are observed. **C.** The TGK8-4 transgenic cochlea shows a total absence of auditory receptor. A single epithelial layer is observed. The tunnel of Corti and OHCs (O), the IHC (I) and supporting cells are not able to be identified. The tectorial membrane appears retracted. Very few nerve auditory fibers are present in the osseous spiral lamina. **D.** In spiral ganglion, a few neuron perikarya are dispersed in the spiral ganglion. Scale bars: 50 μm: outer hair cells; (I) inner hair cells; (Iss) Inner spiral sulcus; (Oss) Outer spiral sulcus; (TM) Tectorial membrane; (star) tunel of corti.

expression in TGK8-4 transgenic cochleae was observed on the cell surface (Table 1, and Fig. 4A), and the cover net of the tectorial membrane was also positive (Fig. 4A). The marginal cell layer of the stria vascularis was strongly CAM 5-2 immunolabelled (Fig. 4A). However, the middle and basal cell layers only showed CAM 5-2 expression around the blood capillary net (Fig. 4A). Surprisingly, a positive CAM 5-2 expression was noticed in Shambaugh's epithelial cells and on the layer which covers the osseous lamina (Fig. 4A). Shambaugh's epithelial cells, which exhibited a conspicuous net of cytopodes distributed under the stria vascularis and within the spiral ligament, have never been clearly identified by immunohistochemistry (Fig. 4A).

In TGK8-4 transgenic cochleae, a strong positive Troma-1 expression (which recognizes mouse and human K8 cytokeratin) was observed in the degenerated epithelium (Table 1 and Fig. 4B,C,D), and on the cover net region of the tectorial membrane (Fig. 4B,D). Also, a strong Troma-1 expression was observed in all cochlear structures involved in the fluid endolymph-perilymph homeostasis: stria vascularis and Reissner's membrane. The marginal, middle and basal epithelial cells of the stria vascularis, were intensely immunolabelled (Fig. 4C); Troma-1 expression was observed surrounding the blood capillary net localized between the middle and basal layers (Fig. 4C). Also, a strong positive Troma-1 expression was observed in spiral prominence and

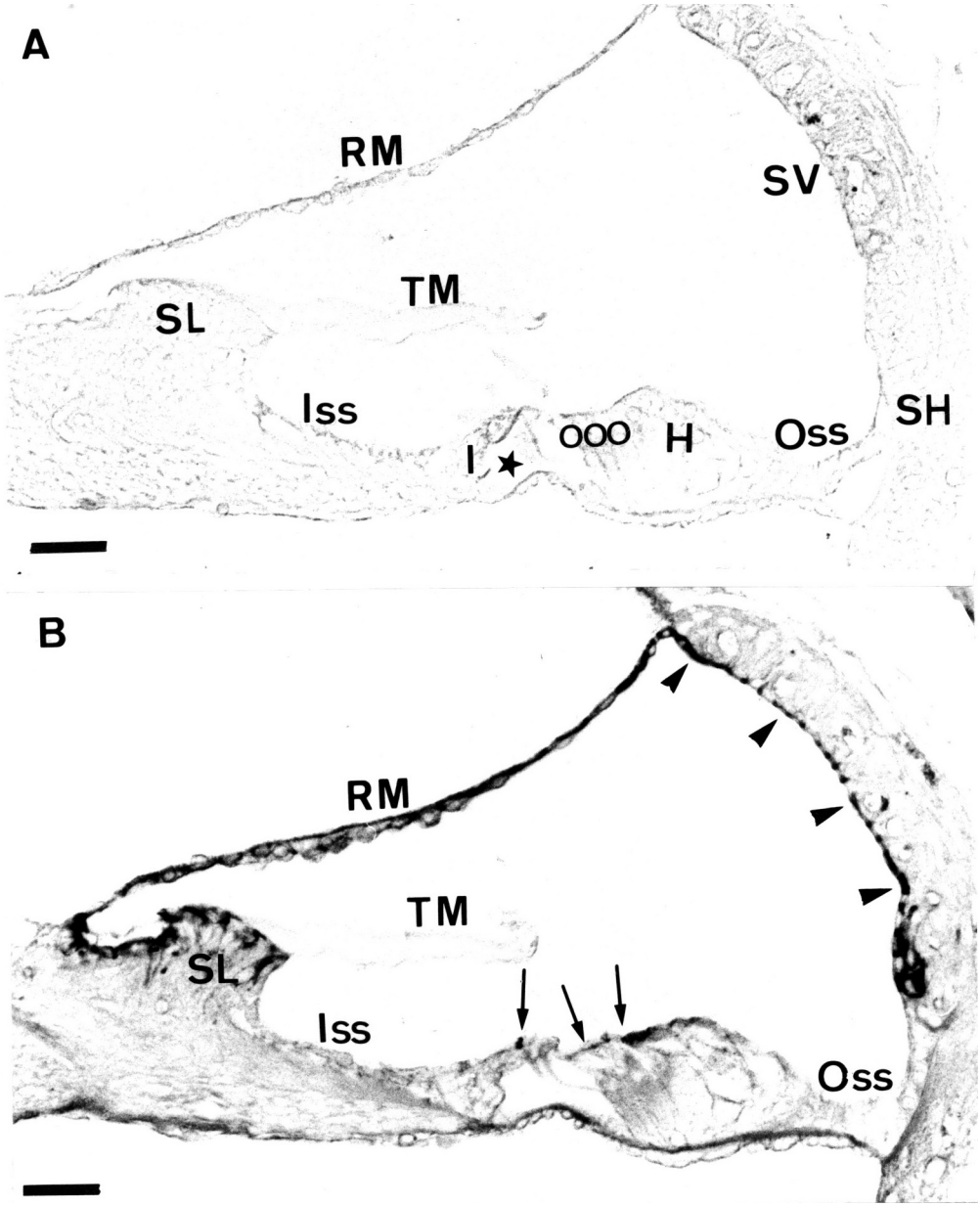

Fig. 3. Paraffin cochleae sections of control mice. **A.** A total absence of CAM 5-2 monoclonal antibody is observed. **B.** A positive Troma-1 expression is present in the spiral limbus (SL), apical region of organ of Corti (arrows), inner (Iss) and outer (Oss) sulcus, stria vascularis (arrowheads) and Reissner's membrane (RM). TM: tectorial membrane; (star) tunnel of Corti. Scale bars: 25 μm.

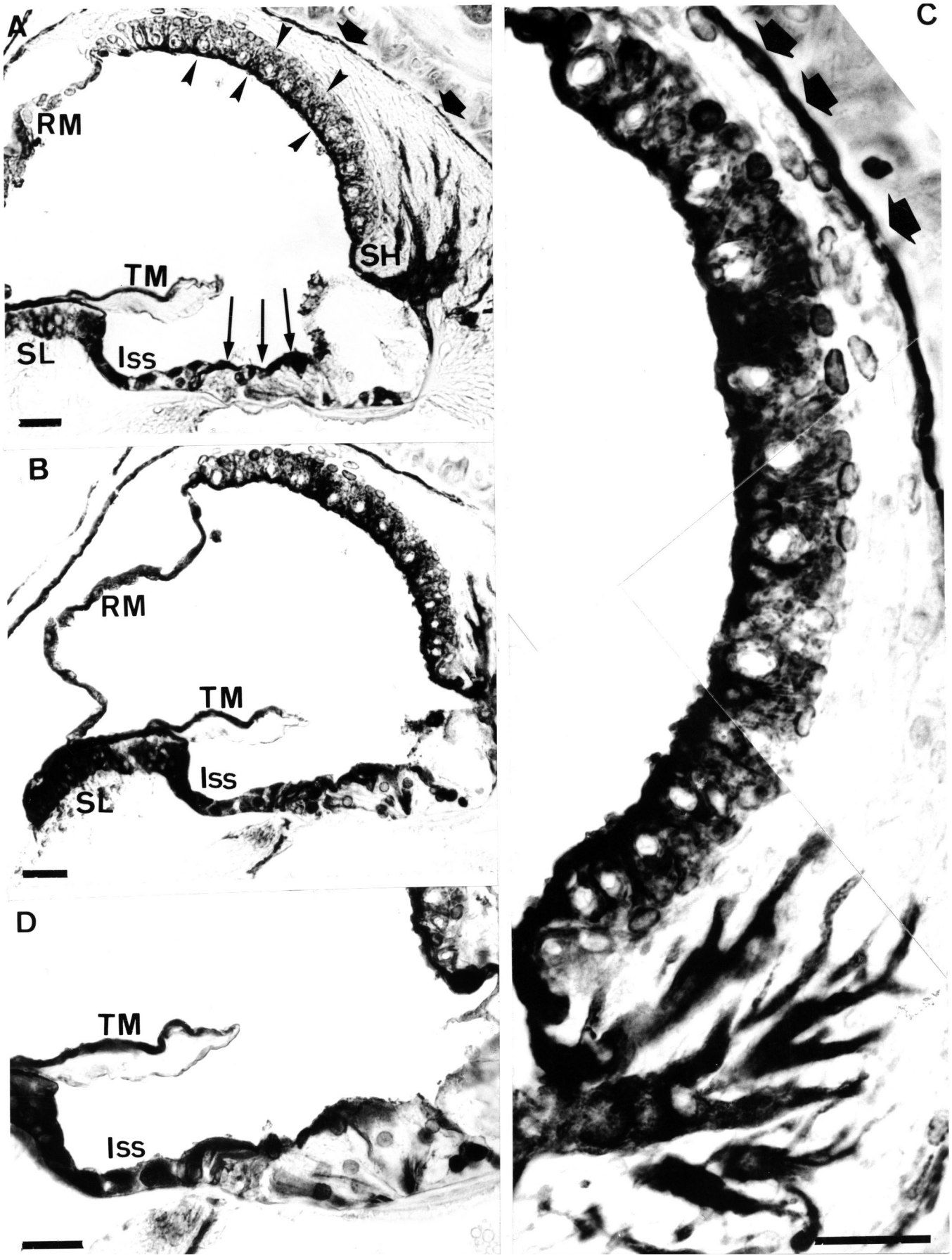
A
RM
SH
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Fig. 4. Paraffin cochleae sections of transgenic mice TGK8-4 line. **A.** A midmodiolar section through the cochlear duct. The CAM 5-2 immunolabelling is present in the spiral limbus (SL), the inner (Iss) and the outer spiral sulcus, the surface of the organ of Corti (thin arrows), and Shambaugh's cells (SH). The stria vascularis shows a strong immunolabelling in the marginal cells layer (arrowheads). The intermediate and basal layers show a diffuse but positive immunoreaction (arrowheads), Reissner's membrane (RM), the cover net of the tectorial membrane (TM), and the osseous lamina (thick arrows). **B-D.** A panoramic distribution of Troma-1 staining. **B.**The immunolabelling is observed in the spiral sulcus (SL), the cover net of the tectorial membrane (TM), the inner (Iss) and outer spiral sulcus, and Reissner's membrane (RM). **C.** A high magnification. Shambaugh's epithelial cells, the three layers of the stria vascularis, and the osseous lamina (thick arrows) show a high Troma-1 immunoreaction. **D.** Also, the surface of all the sensory and supporting cells are strongly labelled. Scale bars: A-C, 25μm; D, 50μm.

within Shambaugh's cells (Fig. 4B,C). Shambaugh's cells exhibited well developed cytopodes extending deep within the spiral ligament, constituting a net of pegs densely positive for the Troma-1 antibody (Fig. 4C). As was observed for CAM 5-2, a positive immunoreaction was noticed in the layer which covers the osseous lamina (Fig. 4A).

A total absence of immunoreactivity with both CAM 5-2 and Troma-1 antibodies was observed in negative controls obtained by the omission of the primary antibody.

Discussion

The auditory receptor of TGK8-4 transgenic mice shows dramatic functional and morphological alterations here described for the first time, which could be related to the abnormal expression of cytokeratins in these mice. The functional auditory nerve activity was analyzed by the usual CAP N1 wave electrophysiological recordings (Gil-Loyzaga et al., 1999; Carricondo et al., 2001). The TGK8-4 transgenic mice showed a significant threshold increase with respect to the control mice (non-transgenic mice). This abnormality in the physiological activity of the auditory nerve of adult TGK8-4 transgenic mice could indicate significant alterations in the auditory receptor. This assumption fits well with the reported immaturity of the auditory receptor and the scarce spiral ganglion neuron population. The low spiral ganglion neuron population reported in these animals exhibits some similarities with other degenerative genetic deafness pathologies.

The mouse K8 cytokeratin (Troma-1 positive expression) distribution in the cochleae of control mice is similar to that found in other studies previously reported dealing with the mammalian cochleae, including that of man (Anniko et al., 1989, 1990, 1992; Berggren et al., 1990, Shi et al., 1990; Achouche et al., 1991; Bauwens et al., 1991; Kuijpers et al., 1991a,b; 1992; Yeh et al., 1993; Anniko and Arnold, 1995; Leonova and Raphael, 1997). Therefore, K8 cytokeratin, and also K18 and K19 type, were found in the organ of Corti cells, spiral prominence and the marginal cells of the stria vascularis, the Reissner's membrane, and the spiral limbus. However, the present study has also shown K8 cytokeratin expression on the epithelium of the inner and outer spiral sulci. Minor differences could be due to the age of the animals, the mouse strains, the different monoclonal antibodies and methodology for cochlear decalcification (Merchán-Pérez et al., 1999).

The TGK8-4 transgenic mice showed a highly abnormal and immature auditory receptor. In fact, TGK8-4 adult cochleae still showed the absence of Corti's organ (absence or immaturity of sensory hair cells, supporting cells, and the tunnel of Corti) and distortion of the tectorial membrane clearly reminiscent of some of the main abnormalities found in the cochleae of hypothyroid animals (Deol, 1973, 1976; Uziel et al., 1981, 1983; Gil-Loyzaga et al., 1990, 1994; Prieto et al., 1990; Remezal and Gil-Loyzaga, 1993; Gil-Loyzaga, 1997). Also, the remnants of Kölliker's organ, a transitory epithelium which disappears during cochlear maturation, is a common feature between both pathologies (see Deol, 1973; Uziel et al., 1983; Gil-Loyzaga et al., 1991; Remezal and Gil-Loyzaga, 1993; Gil-Loyzaga, 1997).

The expression of HK8 in the TGK8-4 transgenic mice cochleae was found in some additional areas to those observed in the wild and non-transgenic mice. In the TGK8-4 transgenic mice, the K8 transgenic cytokeratin cochlear expression, identified by the positive CAM 5-2 and Troma-1 immunolabelling, was also observed in the inner and outer epithelial sulci cells, the cover net of the tectorial membrane, the stria vascularis and in Shambaugh's cells. The three layers of the stria vascularis expressed K8 cytokeratins, in contrast to that which was observed in control animals where only the marginal (first) layer exhibited these molecules (Achouche et al., 1991; Bauwens et al., 1991; Yeh et al., 1993). This over-expression of K8 cytokeratins in transgenic TGK8-4 mice is relevant because it is well known that only the marginal layer of the stria vascularis has an epithelial origin, while the deep layers are epithelioid cells modified from the connective tissue. Shambaugh's cells, of which little is known, are migrated elements in the spiral ligament (Shambaugh, 1908; Duvall, 1969). They could be involved in the fluid homeostasis of the endolymph (Duvall, 1969) in a similar manner to Reissner's membrane, spiral prominence (Yamamoto and Nakai, 1969; Takahashi and Kimura, 1970) or spiral limbus (Bauwens et al., 1991; Yeh et al., 1993). The over-expression in these cells parallels with the deep layers of the stria vascularis and could result in alterations of endolymph production. This requires further research.

Previously, it has been described that an increase in the amount of K8/K18 filaments in TGk8-4 mice modifies the maturity of the acinar cells of the exocrine

pancreas (Casanova et al., 1999). The HK8 expression is now also noted together with a modification in the cover net of the tectorial membrane of the adult animals. A similar finding in immature human fetuses (Anniko et al., 1987) indicated that this over-expression could be linked to cochlear immaturity. This immaturity could affect the matrix composition of the tectorial membrane that contains N-linked glycoconjugates and O-glycosidic linkages, which have been shown to be involved in the architecture of this membrane (Gil-Loyzaga, 1997). The K8 and K18 cytokeratins contain N-linked glycoconjugates and O-glycosidic linkages (Chou et al., 1992; Coulombe, 1993). The expression of HK8 in the cover-net region of the tectorial membrane could interfere with the maturation of this membrane. The K8 over-expression in TGK8-4 mice suggested that, at least, this cytokeratin could be involved in the development, organization and maturation of the different types of epithelial cells and membranes of the cochlea. Intermediate filaments (among others cytokeratin), microfilaments and microtubules are components of the tridimensional network which forms the cytoskeleton (Flock et al., 1982; Osborn, 1983). The correct synthesis and arrangement of microtubules has been largely involved in the normal maturation of the inner ear (Slepecky and Chamberlain, 1987; Tannebaum and Slepecky, 1997). The present report clearly demonstrates that the normal expression of cytokeratin, a major component of the organ of Corti cells, must be necessary for a complete and mature development for this receptor. Studies are in progress to further analyze the role of cytokeratins during maturation of the auditory receptor.

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