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Intermediate filaments in the inner ear of normal and experimentally damaged guinea pigs

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The hypothesis that proteins known to occur in glial cells in the central nervous system may be present in inner-ear supporting cells was investigated. Immunocytochemical techniques were used to look for the existence of two classes of intermediate filaments, vimentin and glial fibrillary acidic protein (GFAP), in cellular elements of the inner-ear epithelium in normal and experimentally damaged guinea-pig cochleas. Vimentin is present in two types of supporting cells in the normal organ of Corti: Deiters' cells and inner pillar cells. Differences in intensity and distribution of vimentin immunostaining are observed across the three rows of Deiters' cells. GFAP immunoreactivity was not detected in any supporting-cell type in the organ. Cochlear hair cells were not labeled for either GFAP or vimentin. Following hair-cell destruction by exposure to noise or the administration of aminoglycosides, GFAP and vimentin are not present in phalangeal scars replacing lost hair cells.

Inner-ear supporting cells; Cochlea; Intermediate filaments; Sound- and drug-damaged inner ear

Introduction

Little is currently known about the roles inner-ear supporting cells play in end-organ function. Supporting cells in the auditory receptor organ, as in the central nervous system (CNS), were originally thought to principally play a structural role in the maintenance of tissue architecture. Recent findings in the CNS and retina have radically changed perceptions of the roles glial cells play in both normal physiology and pathophysiology. Glial cells react to and regulate changes in their environment. For example, glial elements buffer extracellular spaces against fluctuations in potassium concentration (reviewed in Waltz and Hertz, 1983; Ripps and Witkovsky, 1985) and are involved in the uptake and catabolism of neurotransmitter discharged by axon terminals (Henn, 1982; for a review see Varon and Somjen, 1979). Recent electrophysiological data from inner-ear supporting cells (Furukawa, 1985; Oesterle and Dallos, 1985, 1986, 1990; Oesterle 1987) suggest that auditory supporting cells may share some roles played by their counterparts in the CNS and retina.

We investigated the hypothesis that intermediate filament proteins known to be present in CNS glia and retinal supporting cells, namely glial fibrillary acidic protein (GFAP) and vimentin, are present in inner-ear supporting cells. Intermediate filament proteins (IFs) are one of the three major fibrous protein systems comprising the eukaryotic cell cytoskeleton (for a review see Schliwa, 1986). Differences in antigenicity and number and size of component subunits have enabled a subdivision of the IFs into five major classes: the cytokeratins, vimentin, desmin, neurofilaments, and glial fibrillary acidic protein. Different cell types in mammalian tissue differ in the content and type of intermediate filament proteins expressed, and

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many recent studies have shown that the cell-type specific expression of the various IFs in normal and in transformed cells may be used to identify their embryonic origin, their developmental stage, and possibly cell function (Franke et al., 1979, 1982; Osborn and Weber, 1983; Virtanen et al., 1985; Erickson et al., 1987). In the case of neuroglial cells, specific polyclonal and monoclonal antibodies to GFAP and vimentin have brought increased resolving power, through immunofluorescence and immunoperoxidase labeling techniques, to the classification of CNS glial cell types. They have proved useful not only for glial identification in tissue actions and tissue culture (Bignami et al., 1972; reviewed in Bignami et al., 1980), but also as markers for 'reactive' glial cells after injury (Amaducci et al., 1981; Bignami and Dahl, 1976; Latov et al., 1979).

From ultrastructural studies, it is clear that IFs are not static structures but can undergo important cytoplasmic rearrangements during different cellular processes (e.g., cell division: Paulin-Levasseur and Brown, 1987) or alterations in expression in response to injury. For example, during astrogliosis astrocytes within and adjacent to the site of injury accumulate intermediate filaments (Nathaniel and Nathaniel, 1977), demonstrate increased immunostaining (Amaducci et al., 1981; Bignami and Dahl, 1976) and transcription of GFAP (Kretzschmar et al., 1986; Manuelidis et al., 1987) and increased content of vimentin (Dahl et al., 1981a,b 1982). Retinal Müller cells, the major type of non-neuronal cell in the vertebrate retina, normally do not express GFAP. However, after optic nerve crush, phototoxic insult to the retina, or photoreceptor degeneration the Müller cells begin to express GFAP (Bignami and Dahl, 1979; Eisenfeld et al., 1984). In general, during glial reactivity, when glial cells form scars in response to neural or sensory cell damage, dramatic increases in intermediate filament content are observed and increased GFAP and vimentin immunostaining are seen. The functional significance of dynamic rearrangements or expression of the IFs remains to be elucidated.

We used immunocytochemical techniques to look for the presence of GFAP and vimentin intermediate filament proteins in the various supporting-cell types present in the inner-ear epithelium of normal adolescent and adult guinea pigs. In view of demonstrations of increased expression of intermediate filament content during glial reactivity in the CNS and retina, it was also of interest to determine if the expression of GFAP or vimentin was modulated in the inner-ear epithelium in response to hair-cell degeneration. Therefore, GFAP and vimentin expression were examined in auditory end organs where hair-cell degeneration was induced by the administration of aminoglycosides or by exposure to noise.

Findings regarding the normal organ of Corti indicate that vimentin is present in two types of supporting cells: Deiters' cells and inner pillar cells. GFAP was not detected in any supporting-cell type in the normal organ of Corti. Neither GFAP nor vimentin appear to be present in mature phalangeal scars formed by supporting-cell processes replacing degenerating inner and outer hair cells. A preliminary report of portions of this investigation has appeared (Oesterle et al., 1988).

Materials and Methods

Subjects

Albino guinea pigs (Hartley strain; both sexes) weighing between 150 and 1000 grams served as experimental subjects. Normal animals and animals with experimentally induced hair-cell degeneration were studied. Hair-cell degeneration was induced by exposing animals to intense sounds or to ototoxic drugs. For the former, white noise, generated by a Grason Stadler Noise Generator (Model 901B), was band-pass filtered (Krohn Hite Model 3550; 800 to 2000 Hz or 400 to 800 Hz), attenuated (Hewlett-Packard 350D), and amplified (RAMSA WP-9055). Animals were individually confined under a JBL 2482 power horn in a sound attenuated box and exposed to noise at levels of 115 to 120 dB SPL (re 0.002 dynes/cm^2) for periods ranging from 2 to 18 hours. Sound-pressure level was monitored near the subject's head at the beginning, during, and at the completion of each exposure using a B and K 1 inch microphone (4145) and a Hewlett-Packard 3561A signal analyzer. After the completion of the sound exposure, animals were sacrificed at time intervals ranging from 5 hours to 14 days (5 h, 19 h, 3 days,

5 days, 6 days, 7 days, 12 days and 14 days; $N = 1, 1, 2, 1, 1, 2, 1$, and 1, respectively). Age-matched guinea pigs maintained in a quiet environment were used as controls. Hair-cell degeneration was also experimentally induced by administering single doses of amikacin (300 mg/kg) and amino-oxyacetic acid (AOAA; 15 or 20 mg/kg) as described by Bryant et al. (1987). Animals were sacrificed 4, 5, 7, and 14 days ($N = 4$, 1, 1, and 3, respectively) after the injections. Age-matched animals, not injected with the drug cocktail, were used as controls.

Tissue preparation

In some experiments animals were anesthetized with urethane (1600 mg/kg; injected intraperitoneally) and the inner ear fixed in situ with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5). These subjects were decapitated after cardiac perfusion (with the same fixative), and the auditory bullae and positive control tissues (tissues known to contain the antigen, e.g., retina for vimentin staining and cerebellum for GFAP immunostaining) were dissected free and immersed in the fixative at room temperature for periods ranging from 3 to 5.5 hours. In other experiments a different protocol was used: the animal was anesthetized, decapitated, the bullae removed rapidly and opened widely, small openings were made in the basal and apical turns of the cochlea, and the ears were immersed in fixative for periods ranging from 3 to 5.5 hours. Paraformaldehyde (4%) was generally used for fixation. Some tissue was fixed with methanol Carnoy's fixative or periodate-lysine-paraformaldehyde (PLP; pH 7.4; McLean and Nakane, 1974).

After rinses in phosphate-buffered saline (PBS; 0.01 M; pH 7.5), the bony capsule and stria vascularis were removed and the cochlear tissue prepared in one of the following manners for immunocytochemical processing: a) cryostat sections, b) surface preparations, or c) whole-ear preparations. More specifically, to obtain cryostat sections of the organ of Corti, segments of the organ were dissected from the cochlea and immersed in 30% sucrose in PBS overnight. The cochlear half turns were placed in O.C.T compound (Miles laboratories), frozen rapidly with liquid nitrogen, and cut into 20 μm sections with a cryostat. Sections were picked up on poly-L-lysine coated slides. After air-drying overnight, immunocytochemistry was performed, as discussed later, using the ABC (avidin-biotin-peroxidase complex) staining procedure (Hsu et al., 1981) or indirect immunofluorescence techniques. Sections were dehydrated and cleared prior to coverslipping with D.P.X. mountant. To obtain surface preparations, segments of the organ (half turns) were carefully dissected from the cochlea and immunocytochemical procedures run on free-floating segments of the organ. The half turns were mounted in glycerol, coverslipped, and viewed as a whole mount (surface preparation). For whole-ear tissue, immunocytochemical procedures were run on the entire cochlea (minus the otic capsule and stria vascularis). The cochleas were osmicated (1% osmium tetroxide in PBS) for one hour, embedded in Spurr resin, and sectioned (3 or 5 μm).

Immunohistochemical procedures

The basic immunocytochemical procedure employed consisted of: 1) Enzymatically treating the sections and/or tissue to unmask antigenic activity (0.01% pronase for 10 min for GFAP; this step was unnecessary for vimentin), 2) washes with buffer (phosphate buffered saline, PBS; 0.01 M at pH 7.5), 3) treatment with a blocking buffer (4% goat serum in 0.5% bovine serum albumin (BSA) for GFAP experiments; 1% horse serum in PBS with 1% BSA and 0.05% Triton X-100 for the vimentin experiments) to block non-specific binding sites, 4) incubation in primary antibody overnight (GFAP antibody diluted with 0.5% BSA; anti-vimentin antibody diluted with blocking buffer). With some tissue, the control sections, the blocking buffer or an irrelevant antibody (anti-opsin) was substituted for the primary antibody and the sections subsequently treated identically to those incubated with primary antibody, 5) washes with PBS, 6) incubation in secondary antibody (diluted with 0.5% BSA for GFAP experiments; diluted with blocking buffer for vimentin experiments), 7) washes with PBS, and 8) incubation with ABC reagent (a complex of peroxidase conjugated with biotin and avidin; Vectastain ABC kit, Vector Labs; diluted in 0.5% BSA for GFAP experiments and diluted with blocking buffer for vimentin experiments). After washes with PBS, the

peroxidase reaction was developed with diaminobenzidine (0.075%) and hydrogen peroxide (0.01%) in Tris buffer (0.05 M Tris-HCl, pH 7.6). The colored end product was viewed by light microscopy using conventional optics or Nomarski differential-interference-contrast optics.

Immunocytochemistry was also performed on cryostat sections using indirect immunofluorescence techniques. Briefly, the primary antibody incubation was followed by incubation in secondary antibody conjugated to fluorescein-isothiocyanate (Zymed). After washes with buffer, sections were coverslipped in 80% glycerol and examined with a microscope equipped for epifluorescence.

Antibodies

A GFAP antiserum, a polyclonal antibody raised in rabbit against cow GFAP, was purchased from Dako (Santa Barbara, CA) and used at dilutions ranging from 1 : 200 to 1 : 2400. To detect the presence of vimentin, a monoclonal antibody raised in mouse against vimentin from porcine eye lens was purchased from the Dako Corporation and used at dilutions ranging from 1 : 20 to 1 : 200. For control experiments, a monoclonal antibody raised in mouse against human opsin was used at dilutions of 1 : 1 and 1 : 50. Secondary antibodies (biotin conjugated horse anti-mouse IgG, biotin conjugated goat anti-rabbit IgG, and fluorescein isothiocyanate (FITC) labeled goat antibody to mouse IgG were purchased from Vector Laboratories (Burlingame, CA) and Zymed (San Francisco, CA).

Controls

Method and antibody specificity were checked by substituting nonimmune sera for the primary antibody, substituting a monoclonal anti-opsin antibody for the primary antibody (a substance expected to be absent from the end organ), using a series of dilutions of the primary antibody, and processing positive control tissues alongside the auditory tissue. Positive control sections demonstrated that staining for vimentin and GFAP was highly specific.

Results

The primary objectives of these experiments were to characterize the expression and distribution of vimentin and GFAP in the cellular elements of the normal organ of Corti and to examine the modulation of the expression in the damaged ear. To this end patterns of vimentin and GFAP expression were examined in 10 normal, 9 drug-damaged and 10 sound-damaged guinea pigs. Findings regarding vimentin expression in normal and damaged end organs will be presented first and followed by a discussion of GFAP expression.

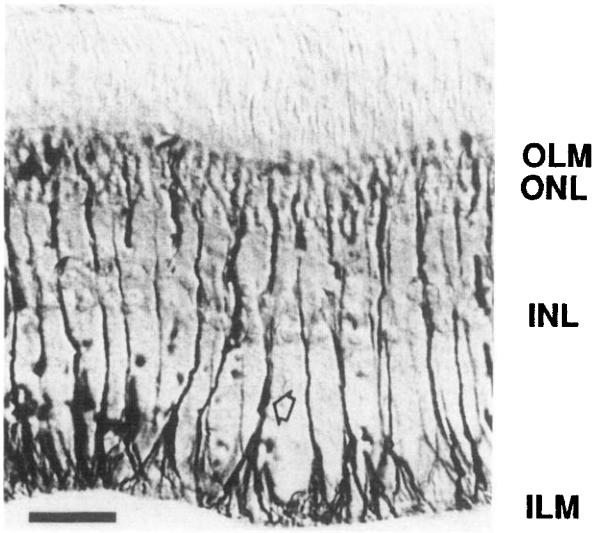

Fig. 1. Cryostat section (20 μm) of guinea-pig retina stained for vimentin (indirect peroxidase technique – ABC method) and viewed with Nomarski differential-interference contrast optics. Vimentin immunoreactivity is visualized with peroxidase-labeled secondary with a DAB chromagen, and immunostaining is manifested in the photomicrographs by a black color. Retinal Müller cells (arrow) are stained. Vimentin filaments are known to be present in Müller cells, non-neuronal cells in the retina (Drager, 1983; Schnitzer, 1985; Schnitzer et al., 1981; Shaw and Weber, 1983). Consequently, in the vimentin experiments retinal tissue was processed alongside the cochlear tissue (processed identically to the cochlear tissue). Normal staining patterns in the retinal tissue demonstrated the specificity of the Dako anti-vimentin antibody and indicated that the immunocytochemical procedures were performed correctly. Tissue in this and subsequent figures is fixed with 4% paraformaldehyde unless specified otherwise. Scale bar = 25 μm. OLM, outer limiting membrane; ONL, outer nuclear layer; INL, inner nuclear layer; ILM, inner limiting membrane.

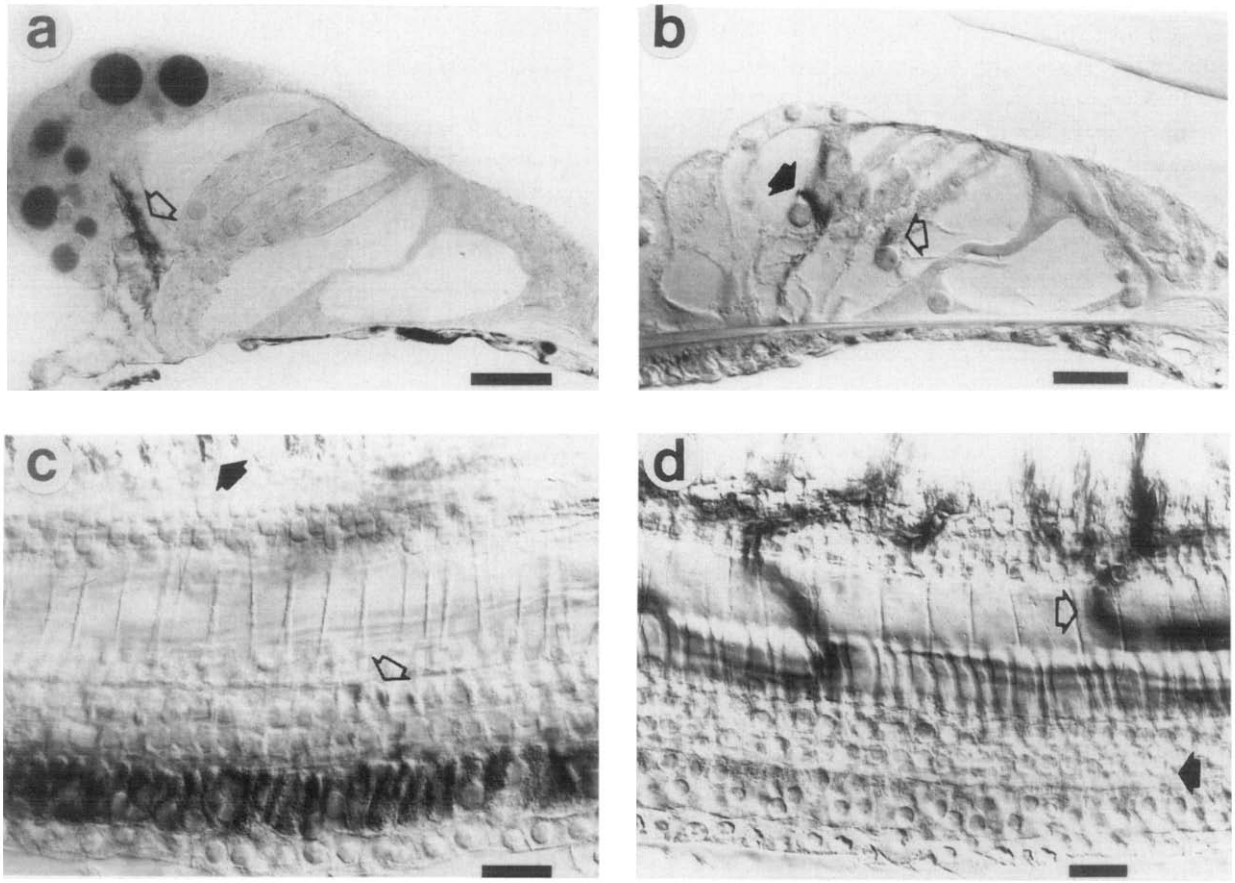

Fig. 2. Cochlear tissue from normal guinea pigs showing vimentin immunostaining (indirect peroxidase technique – ABC method). Parts (a)–(d) are viewed with Nomarski differential-interference contrast optics. (a) Spurr-embedded cross section (5 μm) through the apical-turn organ of Corti in an adult guinea-pig cochlea stained for vimentin filaments. Immunostained vimentin filaments are present in the peripheral-most Deiters' cell (open arrow). Immunoreactive label can also be seen in the tympanic border cells, cells lining the perilymphatic side of the basilar membrane. Inner pillar cells are negative for vimentin staining. As explained in the text, vimentin-positive inner pillar cells were observed in the majority of end organs examined, however, as illustrated here, and in 2b, they were not detected in some cochleas. Scale bar = 25 μm. (b) Spurr-embedded cross section (5 μm) through the basal-turn organ of Corti in an adolescent (260 g) guinea-pig cochlea stained for vimentin filaments. Vimentin immunolabel is visible in the peripheral-most Deiters' cell (arrow), extending from the supporting hillock towards the cell nucleus and in the lower portion of the stalk. Immunoreactive label is also visible in the first-row Deiters' cell (open arrow), adjacent to the cell nucleus. Labeled vimentin filaments are apparent in the tympanic border cells. Scale bar = 25 μm. (c) Surface preparation of the organ of Corti from the second turn of a normal guinea-pig cochlea stained for vimentin filaments. Hensen's cells are located near the bottom of the figure. Strong immunoreactive label can be observed in the peripheral-most row of Deiters' cells. Staining may also be observed near the nuclei of several first and second-row Deiters' cells (open arrow). Note the differences in the intensity and degree of immunostain across the three rows of Deiters' cells. Immunoreactive label is also visible in the habenular area (arrow). Scale bar = 25 μm. (d) Surface preparation of normal guinea-pig cochlea (turn 2) where nonimmune serum was substituted for the primary antibody. This substitution control serves as a specimen blank since any staining observed is due not to antibody localization of the antigen, but to nonspecific protein binding, endogenous peroxidase activity or nonspecific binding of the other antibody reagents. Observe the general lack of immunostaining in the end organ. Deiters' cells are unstained (arrow; the level of focus is near the Deiters' cell nuclei). Staining is observed in the vessel of the basilar membrane and vessel of the tympanic lip (open arrow). It is the result of endogenous peroxidase activity in red blood cells located within the vessels – it is not due to staining for the antigen but due to peroxidase activity already present in the tissue before staining. Scale bar = 25 μm.

Vimentin expression in normal guinea pigs

Fig. 1 shows a section of control tissue used to demonstrate the specificity of the Dako antibody to vimentin. Displayed is a cryostat section from guinea-pig retina processed for vimentin intermediate filaments. In this and all subsequent figures immunostaining is visualized by the presence of a dark precipitate (the reaction product of the peroxidase-labeled secondary and diaminobenzidine). In agreement with previously published results (mouse: Schnitzer et al., 1981; Drager, 1983; rat: Shaw and Weber, 1983; rabbit: Schnitzer, 1985), staining is confined to the Müller (glial) cells which are known to express vimentin. Photoreceptor cells and other retinal cells known not to contain vimentin are unstained.

In the cochlea unambiguous and discretely localized filamentous immunoreactivity for the anti-vimentin antibody is observed in the cytoplasm of Deiters' and inner pillar cells, two supporting-cell types in the organ of Corti. Vimentin immunostaining patterns in Deiters' cells of normal, adolescent and adult guinea pigs are illustrated in Fig. 2. Fig. 2a shows a cross section of normal organ of Corti treated with the anti-vimentin antibody. Immunolabeled vimentin filaments are evident in the cytoplasm of the third-row Deiters' cell, the peripheral-most Deiters' cell in the organ. Labeled filaments are visible in the vicinity of the cell nucleus and lower regions of the cell stalk. In general, labeled vimentin filaments are observed in the following regions of third-row Deiters' cells: running from the region of the supporting hillock towards the cell nucleus and on up towards the cup portion of the Deiters' cell beneath the outer hair cell (OHC) and in the lower part of the Deiters' cell stalk. The nuclei and phalangeal process are unstained.

As shown in Fig. 2a–c , vimentin immunostaining patterns in first and second-row Deiters' cells differ from third-row staining patterns. In Fig. 2a, for example, vimentin immunolabel is undetectable in the first- and second-row Deiters' cells. In general, vimentin label is either undetectable all together in first- and second-row Deiters' cells (e.g., Fig. 2a) or, when present, appears to be restricted to a specific region of the cell cytoplasm, the perinuclear region (Fig. 2b and c). Vimentin label in third-row Deiters' cells, as described above, is more extensive and is observed in other regions of the cell. Labeled vimentin filaments are seen in third-row Deiters' cells in all turns of the guinea-pig cochlea and in all normal cochleas examined. The observed differences in intensity, extent, and patterning of vimentin immunolabel across the three rows of Deiters' cells are apparent with all tissue processing methods employed in this study: cryostat sections, surface preparations of the organ, and whole-ear preparations.

To verify that the observed labeling differences across the various rows of Deiters' cells are not simply a result of the fixative employed, the inner ears of two normal subjects were fixed with paraformaldehyde, PLP, or methanol Carnoy's fixative and processed simultaneously for vimentin. A comparison of the material revealed identical patterns of vimentin staining. That is, the above reported differences in intensity and location of vimentin immunolabel across the various rows of Deiters' cells are observed with the three fixatives tested.

Vimentin immunoreactivity is also observed in a second end-organ cell type, the inner pillar cells. As illustrated in Fig. 3, vimentin immunostaining may be seen in the region of the inner pillar head. Most frequently, the immunolabel is restricted to a specific portion of the cytoplasm located under the inner pillar head inclusion (a dense body found in the heads of pillar cells in rodents) and adjacent to the tunnel of Corti fluids. Vimentin immunostain may also extend down into the upper regions of the inner pillar stalk. Vimentin-positive inner pillar cells were observed in the majority of end organs examined, however, as demonstrated in Fig. 2a and b, they were not detected in some cochleas.

Turning next to vimentin immunostaining in cells outside of the organ of Corti, vimentin immunoreactivity is seen in the following structures in normal guinea-pig cochleas: 1) fibroblasts in the spiral ligament (Fig. 4a), 2) external sulcus cells (Fig. 4a), 3) some Claudius' cells (Fig. 4a), 4) tympanic border cells (Fig. 2a and b; Fig. 3), 5) cells lining the perilymphatic surface of Reissner's membrane, 6) satellite-cell processes in the habenula perforata (Fig. 3), 7) Schwann-cell processes surrounding the cell bodies and periph-

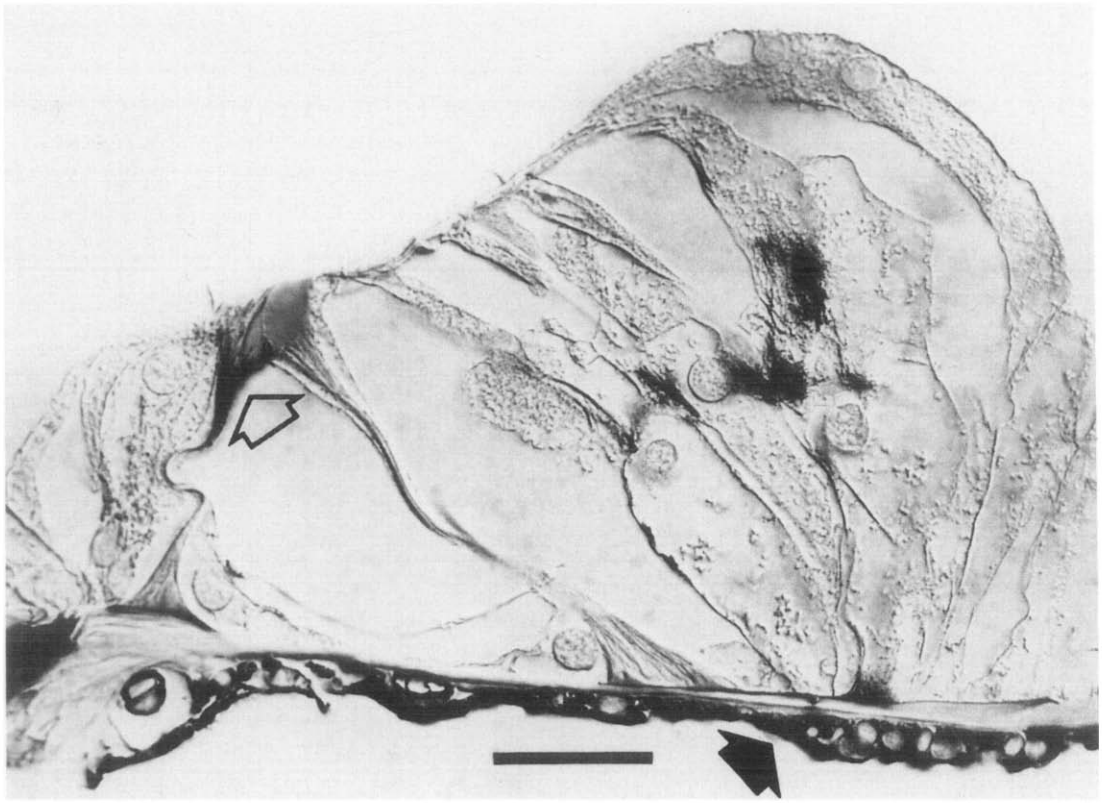

Fig. 3. Cross section (3 μm) of the organ of Corti from the second turn of a normal, adolescent (165 g), guinea-pig cochlea immunostained for vimentin (ABC method). Positive vimentin staining is visible in the head region of the inner pillar, near the inner pillar head inclusion, and extends into the upper regions of the pillar stalk (open arrow). Labeled vimentin filaments are also present in the Deiters' cells, tympanic border cells (arrow), and the habenular area. Tissue fixed with methanol Carnoy's fixative. Scale bar = 25 μm.

eral processes of spiral ganglion cells (Fig. 4b) and 8) areas of the spiral limbus (Fig. 4b). Vimentin immunoreactivity was not observed in other inner-ear structures including the Boettcher cells of the first cochlear turn.

Sections of the guinea-pig end organ treated with preimmune serum or anti-opsin antibody showed no detectable stain (e.g., Fig. 2d).

Vimentin expression in sound and drug-damaged guinea pigs

Vimentin expression was examined in end organs where hair-cell degeneration was induced by exposing the animal to intense sounds or to ototoxic drugs. The rationale was to determine if inner-ear supporting cells express vimentin in response to hair-cell degeneration. A variety of survival times (survival periods ranging from 5 h to 14 days) and exposure durations (ranging from 2 to 18 h) were employed. This enabled an examination of lesions of different severities and tissue at different stages of recovery. Extensive outer hair cell (OHC) loss was frequently observed in the sound- and drug-damaged animals, and marked inner hair cell (IHC) degeneration occurred in the lower turns of some drug-damaged animals. Regions of maximal damage and areas adjacent to the maximally damaged areas, where scattered loss of hair cells are observed, were carefully studied.

Findings regarding vimentin expression in experimentally-damaged end organs are illustrated in Fig. 5. Displayed in Fig. 5a is a surface view of the organ of Corti from a drug-damaged animal.

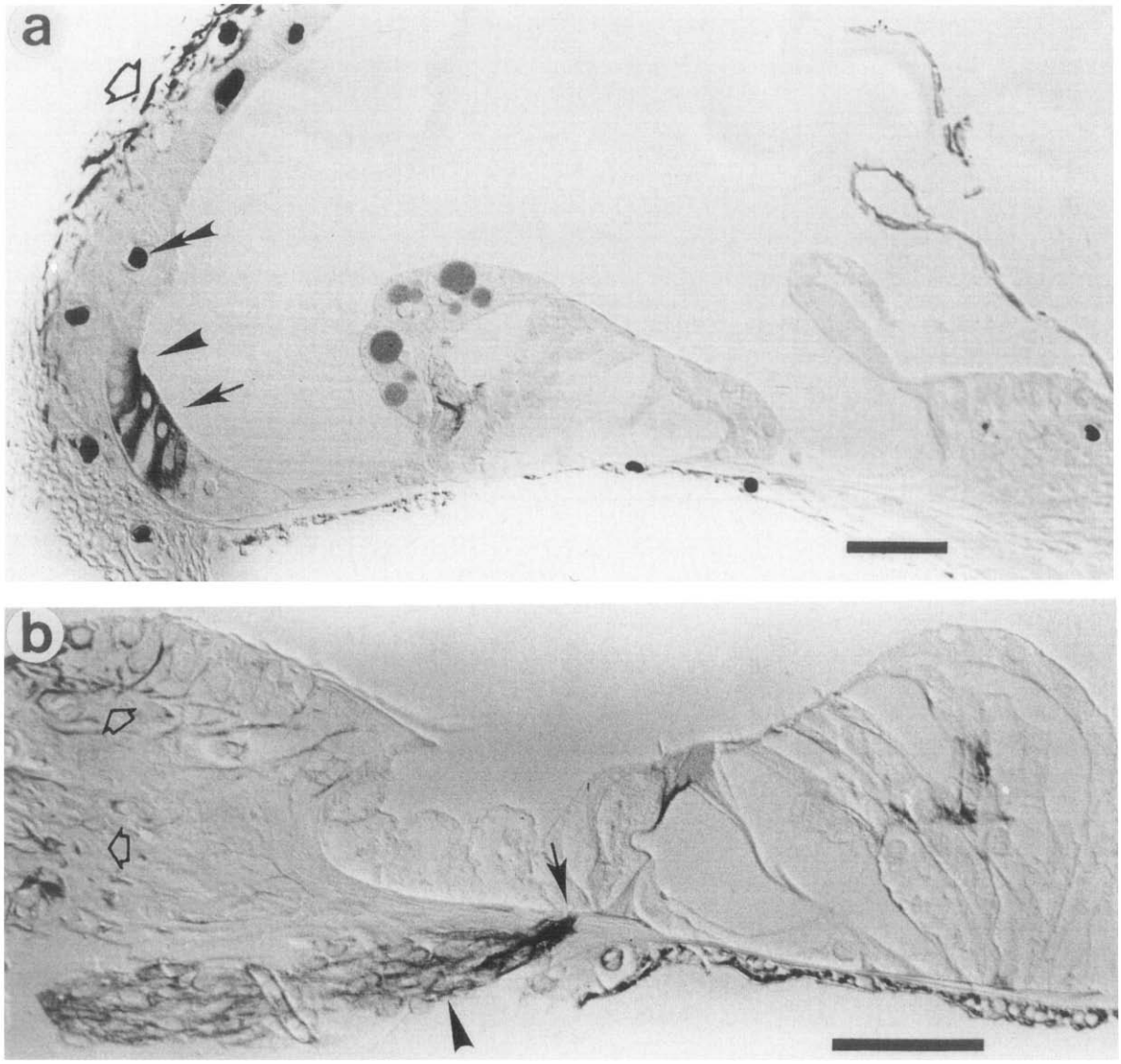

Fig. 4. Normal guinea-pig cochlea immunostained for vimentin (ABC method). The Spurr-embedded sections are viewed with Nomarski differential-interference contrast optics. (a) Cross section (5 μm) of an apical turn of an adult guinea-pig cochlea. Vimentin immunoreactivity is apparent in the following structures: fibroblasts in the spiral ligament (open arrow), external sulcus cells (single arrowhead), some peripheral Claudius' cells (arrow), tympanic border cells, peripheral-most Deiters' cells, and cells lining the perilymphatic surface of Reissners' membrane. Staining resulting from endogenous peroxidase activity in red blood cells (located within vessels) is indicated by the double arrowheads. Scale bar = 50 μm. (b) Cross section of the second turn of an adolescent guinea-pig cochlea (165 g). Tissue fixed with methanol Carnoy's fixative. Vimentin immunoreactivity is apparent in the following structures: areas of the spiral limbus (open arrows), Schwann-cell processes surrounding the cell bodies and peripheral processes of spiral ganglion cells (arrowhead), the habenula perforata (arrow), tympanic border cells, inner pillar cells and Deiters' cells. Scale bar = 50 μm.

The animal was sacrificed four days after the administration of AOAA and amikacin and the tissue processed for vimentin intermediate filaments. Three rows of OHCs have degenerated and been replaced by phalangeal scars. The characteristic 'Y' and 'X' shapes of the phalangeal scars are

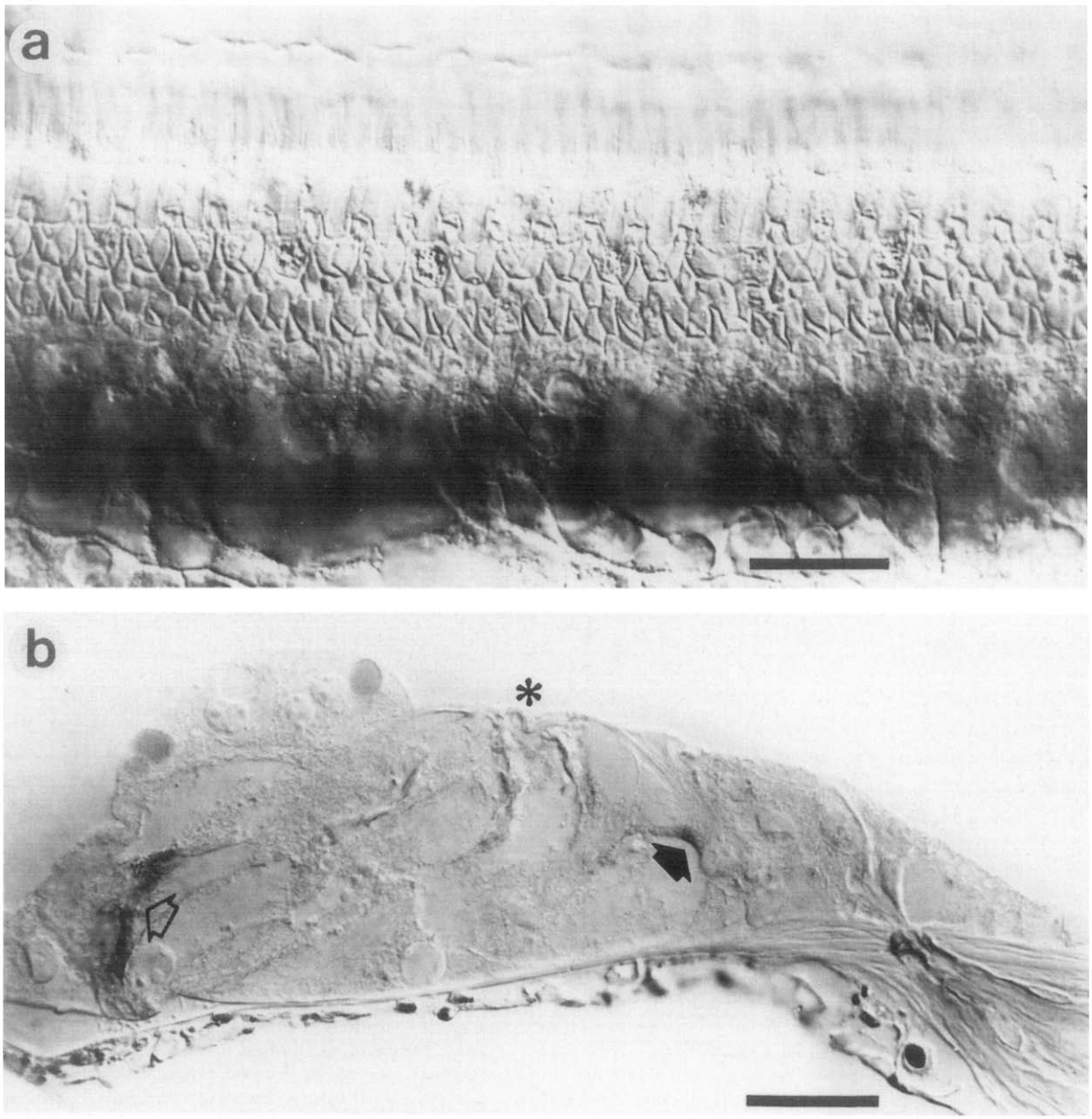

Fig. 5. Drug-damaged guinea-pig cochlea immunostained for vimentin (ABC method) and viewed with Nomarksi differential-interference contrast optics. (a) Glycerol-embedded whole mount preparation of the second turn of a drug-damaged cochlea from an adolescent guinea pig (300 g) sacrificed 4 days post-drug injection. The surface view of the reticular lamina shows the phalangeal scars replacing the three rows of missing OHCs. Immunostain is not detectable in the phalangeal scars. Cell bodies of the peripheral-most Deiters' cells are vimentin positive. Scale bar = 25 μm. (b) Cross section (Spurr embedded, 3 μm section) of the second turn of a drug-damaged organ of Corti from an adolescent guinea pig sacrificed 5 days post-injection. Vimentin immunostaining is not detectable in expanded supporting-cell processes replacing missing OHCs (asterisk). Positive vimentin staining is visible in the peripheral-most Deiters' cell (open arrow), the inner pillar cell (filled arrow), the habenula perforata, and tympanic border cells. Scale bar = 25 μm.

clearly evident in the photomicrograph. Inner hair cells are largely intact as evidenced by the presence of inner hair cell stereocilia. Noteworthy is the absence of vimentin immunoreactivity in the phalangeal scars replacing the missing outer hair cells. Strong vimentin label is present in the cell bodies of the peripheral-most Deiters' cells as seen by the dark band in the lower portion of the photomicrograph.

Cross sections of damaged end organs stained for vimentin (Fig. 5b) confirm these conclusions. Fig. 5b is from an animal sacrificed five days after drug injection. Marked OHC damage and the absence of detectable vimentin label in the reticular lamina may be observed. Note also that end-organ supporting cells not expressing vimentin normally (e.g., Hensen cells, outer pillar cells, inner phalangeal cells, and border cells) do not express vimentin filaments in response to the drug-induced end organ damage. Staining is evident in the peripheral-most Deiters' cell and inner pillar cell, cells expressing vimentin in their normal quiescent state.

In one subject, an animal sacrificed 14 days following exposure to intense sound (800–1700 Hz; 16 h at 120 dB), a portion of the second-turn end organ degenerated entirely and was replaced by a single, simple epithelial layer. Vimentin immunoreactivity was not detectable in this epithelial layer.

In general, vimentin immunoreactivity is undetectable in phalangeal scars (developing and mature *) replacing missing hair cells, OHCs or IHCs. No vimentin labeling was observed in phalangeal scars at all recovery times studied, times ranging from 5 hours to 14 days, in sound- and drug-damaged animals. Lesion size did not appear to affect immunolabeling patterns in that phalangeal scars in both small (several OHCs were missing) and large lesions (all three rows of OHCs absent and many IHCs missing) were unlabeled for vimentin filaments.

GFAP expression in normal guinea pigs

Glial fibrillary acidic protein, GFAP, is another class of intermediate filaments found in certain CNS glia **. GFAP distribution was examined in normal cochleas at the light microscope level using a polyclonal antibody directed against GFAP (Dako). GFAP immunoreactivity was not detected in any supporting cells, or hair cells, in the normal organ of Corti of adolescent or adult guinea pigs. The antibody, however, stained astrocytes and Bergmann glial fibers in guinea-pig cerebellar cortex (Fig. 6), cells known to express GFAP (Bignami and Dahl, 1973).

GFAP expression in sound and drug damaged guinea pigs

GFAP immunoreactivity was not detected in phalangeal scars replacing missing hair cells or in any supporting cells in the organ, suggesting that supporting cells in the end organ do not express GFAP normally or in response to hair-cell damage.

Discussion

Immunostaining in normal animals

To date, studies of vimentin and GFAP expression in the inner ear have predominantly dealt with embryonic or newborn tissues (Anniko et al.,

* As defined by Bohne (1976), 'mature' scars are characterized by the presence of a dense line of union between the adjacent phalangeal processes when studied by phase contrast microscopy. When phalangeal processes first come together 'immature' scars are formed. Immature scars can be distinguished from mature scars since only a faint line of union is visible between adjacent phalangeal processes. Immature scars lack electron-dense material in the processes adjacent to the plasma membrane that is present in mature scars (Bohne, 1976).

** Within the CNS it is now generally accepted that GFAP is a specific marker for astrocytes. The localization of GFAP in mouse tanycytes (Basco et al., 1981; de Vitry et al., 1981), mouse pituicytes (Suess and Pliska, 1981) and goldfish retinal Müller glia (Bignami, 1984) has been taken to indicate that these specialized neuroglial cell types are closely related to conventional astrocytes. Although GFAP has been localized in immature oligodendroglia both in vivo (Choi and Kim, 1984) and in vitro (Raff et al., 1983a,b), there is no evidence that GFAP is present in either microglia or mature oligodendrocytes. Several studies have shown that vimentin is found in glial cells of the CNS (Dahl et al., 1981b; Schnitzer, Franke, and Schachner, 1981; Yen and Fields, 1981). Vimentin is present at very early developmental stages in neuroepithelial cells and later becomes restricted to radial glial and astrocytes (Bignami et al., 1982). Vimentin has been reported to be present in retinal Müller cells (Bignami et al., 1982; Lemmon and Reiser, 1983).

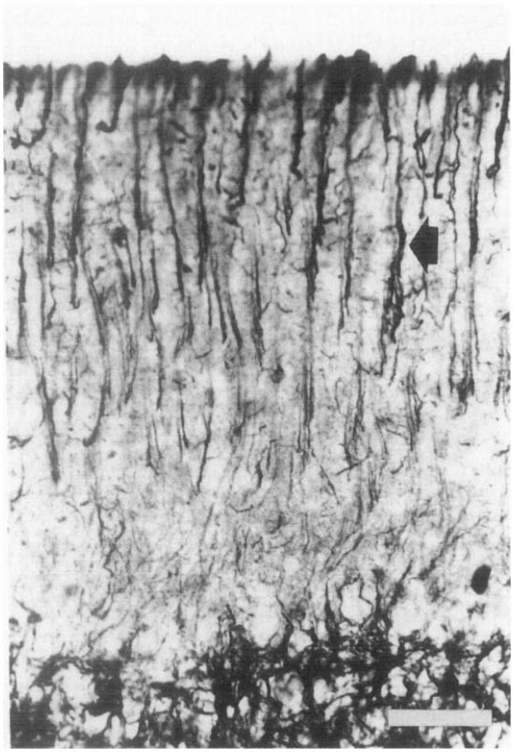

Fig. 6. Vibratome section (30 μm) of guinea-pig cerebellum stained for GFAP (indirect peroxidase technique -ABC method) and viewed with Nomarski differential-interference contrast optics. Stained Bergmann glial fibers (arrow) are visible traversing the molecular layer of the cerebellar cortex. Staining patterns in the cerebellar cortex demonstrated the specificity of the Dako anti-GFAP antibody and indicated that the immunocytochemical procedures were performed correctly. ML, Molecular layer; GL, granular layer. Scale bar = 50 μm.

1986, 1988; Kuijpers et al., 1988; Raphael et al., 1987) and little is known about the mature end organ. Our findings indicate an absence of detectable levels of GFAP in the cellular elements of the mature organ of Corti. Present results also establish that an immunohistochemically detectable vimentin protein is contained in Deiters' and inner pillar cells.

The apparent absence of GFAP in the mature guinea-pig organ of Corti is consistent with recent preliminary findings of Bauwens et al. (1988) reporting an absence of GFAP in the adult human organ of Corti. Vimentin findings described herein agree with preliminary work of Kuijpers et al. (1988) who reported the presence of vimentin in supporting cells of 5–15 day old rat organ of Corti. The identity of the supporting cells expressing vimentin filaments was not specified by Kuijpers and co-workers. Vimentin was not observed by Bauwen et al. (1988) in the adult human end organ, by Schrott et al. (1988) in mature mice, or by Raphael et al. (1987) in embryonic and newborn guinea-pig organ of Corti. Recent preliminary work of Raphael and Oesterle, (1989) confirms the existence of vimentin in isolated Deiters' cells from mature guinea-pig cochlea and supports the coexistence of vimentin and cytokeratin in these cells.

The present study provides evidence for a differential distribution of vimentin immunoreactivity among Deiters' cells in mature guinea-pig organ of Corti. Deiters' cells of the third row stain intensely for vimentin with the immunostaining occurring in vimentin fibers extending from the supporting hillock area up to the Deiters' nuclei, under the OHC base and into the lower stalk. In contrast, vimentin immunostaining in first- and second-row Deiters' cells, if present, is much less intense and confined to the perinuclear region. Thus, vimentin expression appears to depend on the radial position of Deiters' cells in the organ. The observed differences in vimentin immunoreactivity are intriguing and raise questions regarding the existence of subclasses of Deiters' cells and the idea of the existence of possible functional differences amongst the rows of Deiters' cells themselves. While previous studies have not reported radial differences in the distribution of cytoskeletal proteins in Deiters' cells, there is evidence that F-actin in OHCs in the guinea-pig organ of Corti is differentially distributed according to the radial and longitudinal location of the outer hair cells (Thorne et al., 1987).

It is conceivable that fixation effects or difficulties with antibody penetration might explain the heterogeneity of vimentin immunostaining among Deiters' cell rows. However, fixation effects (i.e., the antigen is not fixed in the cell and is lost during rinses and incubations, or the antigen is not accessible to the antibody due to the degree of cross linking - overfixing and losing antigenicity) seem unlikely considering that the vimentin staining variations were observed when a number of different fixatives and fixation times were em-

ployed (PLP, Carnoys and paraformaldehyde) and in all 3 tissue processing methods. Possible antibody or reagent penetration problems were also ruled out; staining patterns for vimentin filaments were similar in cryostat and whole-mount sections.

The present study also provides evidence for the absence of the glial-specific protein, glial fibrillary acidic protein (GFAP), in the mature guinea-pig organ of Corti. It is possible, however, that GFAP intermediate filaments are present in the end organ but that procedural difficulties prevented their detection — that the findings of this study are a false negative. The following observations argue against this possibility and suggest that GFAP intermediate filaments are not present in the mature guinea-pig organ of Corti: 1) GFAP intermediate filaments stained astrocytes and Bergmann glial in the cerebellum (a tissue known to contain GFAP which was processed alongside and identically to the cochlear tissue; e.g., Fig. 6). The presence of this GFAP staining demonstrates the viability of the antibody and immunocytochemical procedures utilized. 2) GFAP immunocytochemical procedures were run on cryostat and whole-mount sections of the end organ, and GFAP staining was not observed in any cellular elements in the organ of Corti in either type of tissue preparation. The absence of GFAP staining in the cryostat sections of the organ rules out possible antibody or reagent penetration problems, and 3) GFAP staining was not present in the cellular elements of the end organ even when very concentrated antibody solutions were utilized.

The function of intermediate filaments (IFs) and the significance of their cell-type-specificity are unknown. Consequently, it is difficult to speculate what the presence of vimentin filaments in Deiters' cells, the observed differences in vimentin immunoreactivity across the various rows of Deiters' cells, and the absence of GFAP from the organ may mean. Others studying IF expression in the CNS have proposed that the IFs might function primarily as a structural framework for the positioning of cellular organelles (Lehto et al., 1978; Summerhays et al., 1983) and possibly for the transmission of signals from cell surface to the nucleus (Metuzals and Mushynski, 1974; Jones et al., 1985; Geiger, 1987). Experiments attempting to elucidate intermediate filament function by microinjection of intermediate-filament specific antibodies are notable by their failure to disturb normal cell behavior (Eckert et al., 1981; Gawlitta et al., 1981; Klymkowsky, 1981; Lane and Klymkowsky, 1982; Lin and Feramisco, 1981). Experimental data demonstrating that intermediate filament subunit proteins have a high affinity for nucleic acids (Nelson and Traub, 1981; Traub and Nelson, 1982) has led to the proposal that intermediate filaments act in the regulation of gene expression (Traub, 1985).

Immunostaining in damaged ears

Loss of hair cells and their replacement by expansion of supporting-cell phalanges is well known to occur both after acoustic trauma (Hamernik et al., 1984; Hunter-Duvar et al., 1982; Lim and Melnick, 1971) and as a consequence of the effects of aminoglycosides (Dodson et al., 1982; Harpur and Bridges, 1979; McDowell, 1982; Wersäll, 1981; Wersäll et al., 1973). The non-neural elements of the end organ are activated at injury sites and extend cellular processes into spaces vacated by degenerating hair cells. Phalangeal scars are formed, presumably, in order to restore or prevent breaches in the limiting membrane, the reticular lamina, and the structural integrity of the barrier separating endolymphatic from perilymphatic fluids is maintained. Information is lacking about what triggers the supporting-cell reactivity, and the cellular dynamics associated with the formation of the phalangeal scars are not fully understood.

In the CNS, dramatic increases in GFAP and vimentin immunostaining are associated with the formation of glial scars. GFAP is expressed by reactive astrocytes, and following injury the intensity of anti-GFAP staining increases dramatically (Bignami and Dahl, 1976; Latov et al., 1979; Amaducci et al., 1981). Increased anti-vimentin immunostaining has been described in degenerated optic nerves (Dahl et al., 1981a,b). Other investigators noted intense anti-vimentin staining of reactive glia both in tissue culture and in cerebral cortical tissue following stab wounds (Fedoroff et al., 1984b). Increases in vimentin synthesis (Ben Ze'ev, 1985; Ferrari et al., 1986; Thomas and Thomas, 1986) appear to be associ-

ated with rapidly growing cells, presumably due to the extensive remodeling of cytoskeletal components required for mitosis, migration, and process outgrowth.

In the vertebrate retina, the expression of GFAP is associated with reactive Müller cells. Retinal Müller cells, the major type of non-neuronal cell type in the vertebrate retina, begin to express GFAP in response to optic nerve sectioning, penetrating wounds to the eye, phototoxic insult to the retina, or heritable degeneration of photoreceptors (Bignami and Dahl, 1979; Eisenfeld et al., 1984). Müller glia do not stain for GFAP in their normal quiescent state (Björklund et al., 1985; Björklund and Dahl, 1985; Dixon and Eng, 1981).

Findings of this study indicate an absence of detectable GFAP or vimentin immunoreactivity in phalangeal scars (developing and mature scars) replacing lost hair cells (IHCs and OHCs). Thus, in contrast to CNS glia and retinal Müller cells, GFAP and vimentin can not be used as markers for reactive organ of Corti supporting cells after injury. These findings suggest that support-cell scars in the inner ear form in the absence of GFAP and vimentin expression and that the expression of these IFs is not involved in scar formation universally.

Auditory supporting cells derive from placodal tissue whereas retinal supporting cells come from an outpouching of the neural tube and CNS glia derive from neural-tube ectoderm (Rubel, 1978). Albeit the auditory supporting cells have a different embryonic origin than these other supporting cells, they appear to share some roles played by their counterparts in the CNS and retina - the maintenance of a homeostatic environment for the sensory cells and neural endings (Furukawa, 1985; Oesterle and Dallos, 1990). The absence of GFAP and vimentin expression in reactive organ of Corti supporting cells, along with an absence of GFAP expression in normal organ of Corti supporting cells, indicates that auditory supporting cells have immunocytochemical characteristics which differ from those of CNS glia and Müller cells in situ (see footnote p. 10). They suggest that auditory supporting cells may not use the same cytoskeletal proteins as CNS glia. These data do not rule out, of course, other possible functional similarities between the auditory supporting cells and the neuroglia (e.g., potassium buffering, neurotransmitter uptake, etc.).

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