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The site of action of neuronal acidic fibroblast growth factor is the organ of Corti of the rat cochlea

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ABSTRACT **Here we show that the mature cochlear neurons are a rich source of acidic fibroblast growth factor (aFGF), which is expressed in the neuronal circuitry consisting of afferent and efferent innervation. The site of action of neuronal aFGF is likely to reside in the organ of Corti, where one of the four known FGF receptor (FGFR) tyrosine kinases—namely, FGFR-3 mRNA—is expressed. Following acoustic overstimulation, known to cause damage to the organ of Corti, a rapid up-regulation of FGFR-3 is evident in this sensory epithelium, at both mRNA and protein levels. The present results provide *in vivo* evidence for aFGF being a sensory neuron-derived, anterogradely transported factor that may exert trophic effects on a peripheral target tissue. In this sensory system, aFGF, rather than being a neurotrophic factor, seems to promote maintenance of the integrity of the organ of Corti. In addition, aFGF, released from the traumatized nerve endings, may be one of the first signals initiating protective recovery and repair processes following damaging auditory stimuli.**

The vertebrate auditory organ contains the sophisticated microarchitecture of elements aimed at transduction of mechanical sound energy to bioelectrical impulses in the auditory nerve. The organ of Corti, which consists of the hair cells (mechanoreceptor cells) and different types of supporting cells, is particularly vulnerable to loud noise and ototoxic drugs. It is the hair cells that degenerate first following insults, resulting in hearing loss. Degeneration of the innervating neurons occurs secondarily to the loss of hair cells. Because hair cells of the mature mammalian cochlea do not regenerate through proliferation, loss of each cell is irreversible. Thus, effective means of recovery and repair must be present in the cochlea to prevent progressive damage and preserve residual hearing. In the mammalian organ of Corti, the repair mechanism involves a scarring process, where supporting cells replace lost hair cells by a "phalangeal scar" (1). It is conceivable that growth factors regulate the cascade of cellular and molecular events associated with the repair, and, in order to act focally, their specific receptors should be present within the organ of Corti. A rationale for our focus on fibroblast growth factors (FGFs) has been earlier studies demonstrating expression of acidic FGF (aFGF) mRNA in the cochlear ganglion (2) and FGF receptor 3 (FGFR-3) mRNA in the developing auditory sensory epithelium (3). However, it is not known whether FGFRs are present in the mature organ of Corti.

FGFs form a family of at least nine heparin-binding polypeptide growth factors (reviewed by ref. 4) that have pleiotropic effects on various cell types during development and adulthood. In the nervous system, the prototypic members of this family, aFGF (FGF-1) and basic FGF (bFGF or FGF-2), are abundantly expressed, mostly in nonoverlapping cellular populations, and these factors have been shown, *in vitro*, to promote a wide variety of biological effects (4). Specifically, aFGF mRNA has been localized to distinct neurons of the central and peripheral nervous systems (2, 5–7).

Cellular responses to FGFs are mediated through transmembrane tyrosine kinase receptors. So far, genes encoding four high-affinity FGFRs have been isolated, designated FGFR-1 through -4. FGFRs interact with several ligands. Complexity is further increased, since splicing variants that have altered ligand and tissue specificity have been demonstrated for FGFR-1 to -3 (8, 9). In addition to the high-affinity binding sites for FGFs, low-affinity sites, represented by the heparan sulfate chains of proteoglycans (HSPGs), have been characterized on cell surfaces, extracellular matrix, and basement membranes. Besides serving as an extracellular reservoir for FGFs (10), recent evidence suggests that HSPGs are obligate partners in binding of the ligand to the tyrosine kinase receptors and in producing a biological response (11).

The bioavailability of aFGF *in vivo* is restricted by its inefficient secretion into the extracellular space due to the lack of N-terminal signal peptide (12–14). Based on this feature, an injury-dependent mode of release of aFGF and, consequently, its involvement in repair after tissue damage have been suggested (15). The cochlea appears to be an ideal *in vivo* model system for clarifying possible injury-related roles of aFGF and FGFRs, since it comprises well-defined cellular populations that are sensitive to specific ototoxic agents, the effects of which can be accurately monitored and evaluated. The present data provide evidence for the existence of a nerve-derived cortitrophic factor in the adult mammalian auditory organ that might be involved in maintenance of the organ of Corti as well as in protective and repair processes following trauma.

MATERIALS AND METHODS

Animals and Tissues. Twenty-five adult Wistar and 25 Sprague–Dawley rats (150–200 g) were used as control (nontreated) subjects. In addition, 40 adult Wistar rats were exposed in a free field to a narrow band of white noise with a center frequency of 4 or 8 kHz and sound pressure levels (SPLs) between 105 and 120 decibels (dB) for 5 or 10 hr. The animals were sacrificed within 12 hr following the exposures. For immunohistochemistry and *in situ* hybridization, cochleas were perilymphatically perfused and processed for 5-μm-thick paraffin-embedded sections as described (16). For immunoblots, the cochlear ganglion and its nerve and the organ of Corti were homogenized by sonication and the protein content was quantified as described (5).

Antibodies. The rabbit antiserum against human recombinant aFGF has previously been characterized (5, 13). A

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Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic FGF; FGFR, fibroblast growth factor receptor; SPL, sound pressure level; IHC, inner hair cell; OHC, outer hair cell; dB, decibels.

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polyclonal aFGF antibody from Sigma gave identical results. The polyclonal FGFR-3 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunohistochemistry. To detect the primary antibodies, we used rabbit ABC Elite kit (Vector Laboratories). The peroxidase reaction was visualized with 3,3′ diaminobenzidine and hydrogen peroxide. Sections were mounted without counterstaining and examined under bright-field or Nomarski optics using a Nikon Microphot-FXA microscope.

Immunoblotting. For Western blots, 25 μg of total protein extract was incubated with heparin-Sepharose (Pharmacia) overnight at 4°C. The pellet was washed twice, boiled in SDS sample buffer containing 100 mM dithiothreitol, and alkylated, and proteins were separated by 12% SDS/PAGE. Human recombinant aFGF (13) and bFGF (Boehringer Mannheim) were coelectrophoresed in separate lanes. Proteins were electrophoretically transferred to a nitrocellulose membrane that was blocked with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20. Following incubation with either of the aFGF antibody, signals were visualized by a horseradish peroxidase-conjugated secondary antibody and the chemiluminescence method (ECL; Amersham). For immunoprecipitation/Western blots, 25 μg of total protein was incubated in 10 mM Tris·HCl, pH 7.5/0.15 M NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/1 mM Na_2VO_3 and a cocktail of protease inhibitors overnight at 4°C with the FGFR-3 antibody, followed by addition of protein A-Sepharose (Pharmacia) and incubation for 4 hr. Precipitates were washed three times, processed as decribed above, and analyzed by 7.5% SDS/PAGE. After incubation with the FGFR-3 antibody, detection was performed by ECL.

cRNA Probes and *in Situ* Hybridization. The aFGF-specific cRNA was synthesized from a 465-bp cDNA fragment, in pGEM-4Z (Promega), encoding the open reading frame (13). FGFR-1 and FGFR-2 riboprobes were prepared from the 220- and 281-bp-long cDNA fragments, respectively, from the 5′ end of the corresponding murine cDNA, including the hydrophobic leader sequence. The cDNA fragments, cloned in pBluescript KS+ (Stratagene; ref. 17), were donated by Peter Lonai (The Weizmann Institute of Science, Rehovot, Israel). The FGFR-3-specific riboprobe was generated from a cDNA fragment, in pBluescript KS+, corresponding to the transmembrane and juxtamembrane portions of FGFR-3 cDNA (3). The FGFR-3 plasmid was provided by David Ornitz (Washington University Medical School, St. Louis). The 1000-bp FGFR-4 cDNA fragment, in pGEM-3Zf+ (18), encodes the immunoglobulin-like domains of the extracellular part of FGFR-4. It was a gift from Kari Alitalo (University of Helsinki, Finland). ^{35}S-labeled antisense and control sense cRNA probes were prepared, and *in situ* hybridization performed as described (16). Sections counterstained with hematoxylin were examined under a Zeiss Axiophot microscope. The sense probes, applied in parallel with the antisense probes, did not produce any signal above the backround level (data not shown).

RESULTS

The specificity of the aFGF antibodies used has previously been confirmed (5). In Western blot analysis, both aFGF antibodies used specifically recognized human recombinant aFGF but not bFGF (Fig. 1). Recombinant aFGF and the cochlear samples yielded two or three bands with apparent molecular masses between 16 and 18 kDa, which have been shown to result from conformational heterogeneity and/or proteolytic processing at the N terminus of the aFGF polypeptide (13, 19). A prominent reaction was found in the neuronal compartment of cochleas of the Sprague–Dawley rats, whereas the intensity of reaction was clearly weaker in the Wistar rats

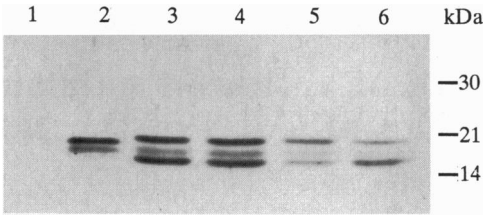

FIG. 1. aFGF protein in the neuronal compartment of the rat cochlea (cochlear ganglion plus nerve) as revealed by Western blot analysis. Molecular mass markers (kDa) are indicated. aFGF antibody shows specificity for two or three bands migrating with a molecular mass of 16–18 kDa. Recombinant bFGF (20 ng) and aFGF (3 ng) were electrophoresed in lanes 1 and 2, respectively. Sprague–Dawley (lanes 3 and 4) and Wistar (lanes 5 and 6) rats show clear differences in their aFGF content. Lanes 3–6 consist of different individuals and were loaded with the same amount of protein.

(Fig. 1). Exposure to noise did not significantly change the levels of cochlear aFGF (data not shown).

In the cochlea, aFGF protein and mRNA were localized by immunohistochemistry and *in situ* hybridization to the neuronal perikarya of cochlear neurons (Fig. 2). aFGF immunoreactivity was found in the nerve fibers reaching the organ of Corti and in the neurites projecting to the brainstem nuclei (Figs. 2 and 3 *a–d*). More than 90% of the neurons innervating the apical coil of the cochlea were immunostained, whereas a clearly larger number of neuronal somas of the middle coil and especially of the basal coil were unstained (Fig. 3 *e* and *f*). Nonneuronal cells of the cochlear ganglion and its nerve were negative. aFGF mRNA and protein (Fig. 2*a*) were also localized to the nonsensory epithelium of stria vascularis.

As in Western blotting, the difference in cochlear aFGF levels between the Sprague–Dawley and Wistar rats was evident by immunohistochemistry (Fig. 3 *a–d*) and, at the mRNA level, by *in situ* hybridization (data not shown). In both rat strains studied, striking immunostaining was localized to the neuritic segments situated immediately proximal to habenula perforata, the site where nerve fibers are compressed, because they emerge in densely packed bundles through narrow openings of the basilar membrane into the organ of Corti (Fig. 3 *a–d*). In the organ of Corti, aFGF immunoreactivity was localized to the region of nerve terminals beneath the IHCs (Fig. 3 *b* and *d*) and particularly to the large, apparently efferent nerve endings beneath the OHCs (Fig. 3*g*).

To find out the target cells for the action of aFGF, expression of the tyrosine kinase FGFR mRNAs was studied by *in situ* hybridization. FGFR-1, -2 (Fig. 4 *a–c*) and -4 (data not shown) mRNAs were not found either in the organ of Corti or in the cochlear ganglion, whereas FGFR-3 mRNA was distinctly expressed in two types of supporting cells of the organ

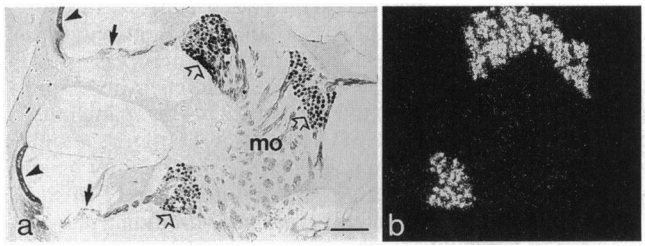

FIG. 2. Expression of aFGF protein and mRNA in the cochlea, as revealed by immunohistochemistry and *in situ* hybridization. (*a*) In the apical and middle coils, strong aFGF immunostaining is seen in neuronal somas (open arrows) and in peripherally and centrally projecting neurites. Immunoreactivity is also found in stria vascularis (arrowheads). Solid arrows point to the organ of Corti. mo, Modiolus. (*b*) Dark-field photomicrograph of emulsion autoradiogram showing strong aFGF mRNA expression in neuronal perikarya. (Bar = 110 μm.)

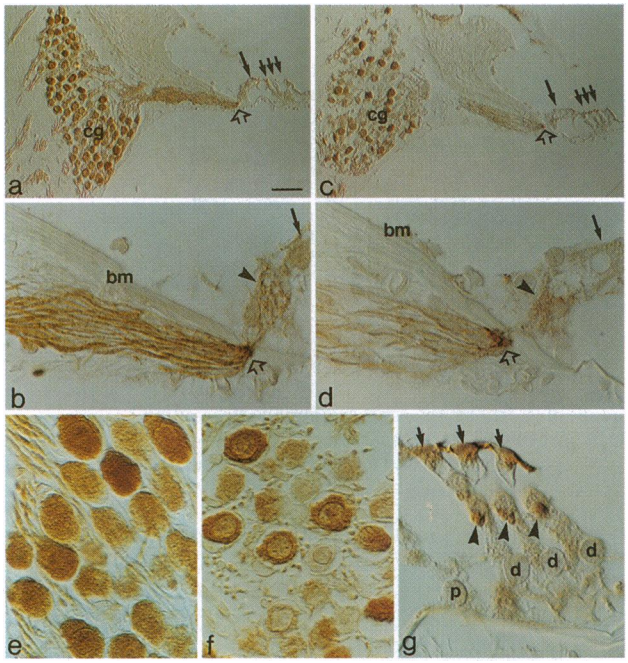

FIG. 3. aFGF immunoreactivity in cochleas of different strains of rats. Neurons of the upper middle coil show different levels of aFGF immunoreactivity in Sprague–Dawley (*a*) and Wistar (*c*) rats. At a higher magnification, accumulation of aFGF proximal to the habenula perforata and aFGF immunoreactivity in the region containing neural elements beneath the inner hair cells (IHC) are seen in Sprague–Dawley (*b*) and Wistar (*d*) rats. cg, Cochlear ganglion; bm, basilar membrane. A thick arrow points to the IHC and thinner arrows point to outer hair cells (OHCs). An open arrow marks the accumulation of aFGF and an arrowhead marks the plexus of nerve endings beneath the IHC. (*e*) Almost all neurons of the apical coil of Sprague–Dawley rats show strong aFGF immunoreactivity. (*f*) In the basal coil of the same cochlea, a neuronal subpopulation is lacking aFGF immunoreactivity. Most of the other neurons are only moderately immunostained. (*g*) In the organ of Corti, large efferent nerve terminals at the base of OHCs show aFGF immunoreactivity. Nonspecific staining is seen in the apical poles of OHCs and reticular lamina. p, Pillar cell; d, Deiters' cell. Arrows point to the OHCs. (Bar = 50 μm in *a* and *c*, 15 μm in *b, d, e,* and *f*, and 8 μm in *g*.)

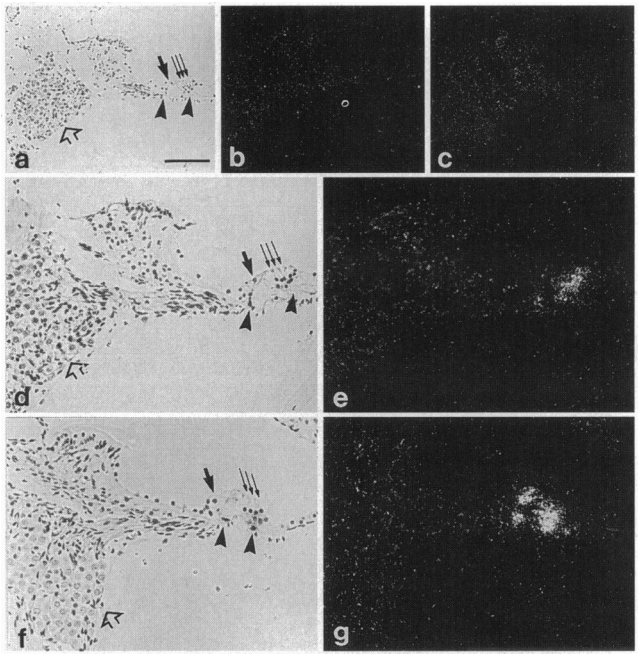

FIG. 4. Expression of FGFRs in the adult cochlea as revealed by *in situ* hybridization. (*a–c*) Bright- and dark-field emulsion autoradiograms of adjacent sections show that FGFR-1 (*a* and *b*) and FGFR-2 (*c*) mRNAs are not expressed in the ganglion or in the organ of Corti. (*d* and *e*) FGFR-3 mRNA is expressed in the supporting cells lying beneath the OHCs. (*f* and *g*) Following 10 hr of noise at 105 dB SPL, FGFR-3 message is up-regulated in the organ of Corti. An open arrow points to the cochlear ganglion. In the organ of Corti, a thick arrow marks the IHC, thin arrows mark OHCs, and arrowheads indicate the supporting cells. (Bar = 90 μm in *a–c* and 60 μm in *d–g*.)

of Corti, the Deiters' and pillar cells (Fig. 4 *d* and *e*). Low levels of FGFR-3 mRNA expression were also found in the limbus and lateral wall of the cochlea (data not shown).

Shortly after exposure to traumatizing, moderate levels of noise, expression of FGFR-3 mRNA was up-regulated in the organ of Corti, as deduced from *in situ* hybridization (Fig. 4 *f* and *g*). In the unlesioned adult cochlea, very few, if any, FGFR-3 transcripts were present in the OHCs and none in the IHCs (Fig. 5*a*). The noise-induced up-regulation of the receptor was seen in the region of the supporting cells and adjacent OHCs but not in the IHCs (Fig. 5*b*). This increased expression of FGFR-3 mRNA was reminiscent of its strong expression in the developing cochlea (Fig. 5*c*). By immunoprecipitation, noise-induced up-regulation of FGFR-3 in the organ of Corti was confirmed at the protein level (Fig. 6). The HEL cell line, which is known to express high levels of FGFR-3 mRNA, was used as a positive control to ensure that the precipitated band is of correct size (data not shown).

DISCUSSION

The present study demonstrates that aFGF mRNA and protein are prominently expressed in the neurons innervating the organ of Corti of the adult rat. aFGF is anterogradely transported to the auditory sensory epithelium, where its protein tyrosine kinase receptor, FGFR-3, is expressed in two types of supporting cells (Fig. 7). Our results suggest that this ligand–receptor interaction may have a role in maintenance of the integrity of the auditory organ. Additionally, based on the observed up-regulation of FGFR-3 after acoustic overstimulation, aFGF may have a role in protection and repair processes following damage to the organ of Corti.

Based on earlier data, where aFGF and FGFRs have been found in different sets of neurons (and nonneuronal cells) of the nervous system (2, 4–7), a neurotrophic role for aFGF has been proposed. However, the present data suggest that the site of action of aFGF synthesized by the cochlear neurons is the organ of Corti, and not the ganglion. In contrast to the cochlear neurons, we have found distinct expression of aFGF as well as FGFR-1 and FGFR-2 transcripts in neurons of another sensory ganglion, the dorsal root ganglion (20), where an autocrine or paracrine mode of action is plausible.

The organ of Corti consists of the hair cells that are encircled by a supporting-cell framework and innervated by the aFGF immunoreactive neurites. Of the high-affinity FGF receptors, only FGFR-3 message was found in this sensory unit. In accordance, the protein product of the FGFR-3 gene binds and mediates a biological response preferentially through aFGF as compared to other members of the FGF family of growth factors (21). In the unlesioned organ of Corti, aFGF may act as a maintenance factor for the supporting cells, which, in turn, provide structural and perhaps also metabolic support for the adjacent hair cells. The observed accumulation of aFGF to a distinct site in the peripheral neuritic pathway gives further evidence for its anterograde transport and suggests that after traversing the basilar membrane, where the neurites lose their myelin sheath, aFGF might be released from the nerve endings into the extracellular space of the organ of Corti. It should be noted that, although aFGF lacks a signal sequence, increasing evidence suggests that there may exist a secretory mecha-

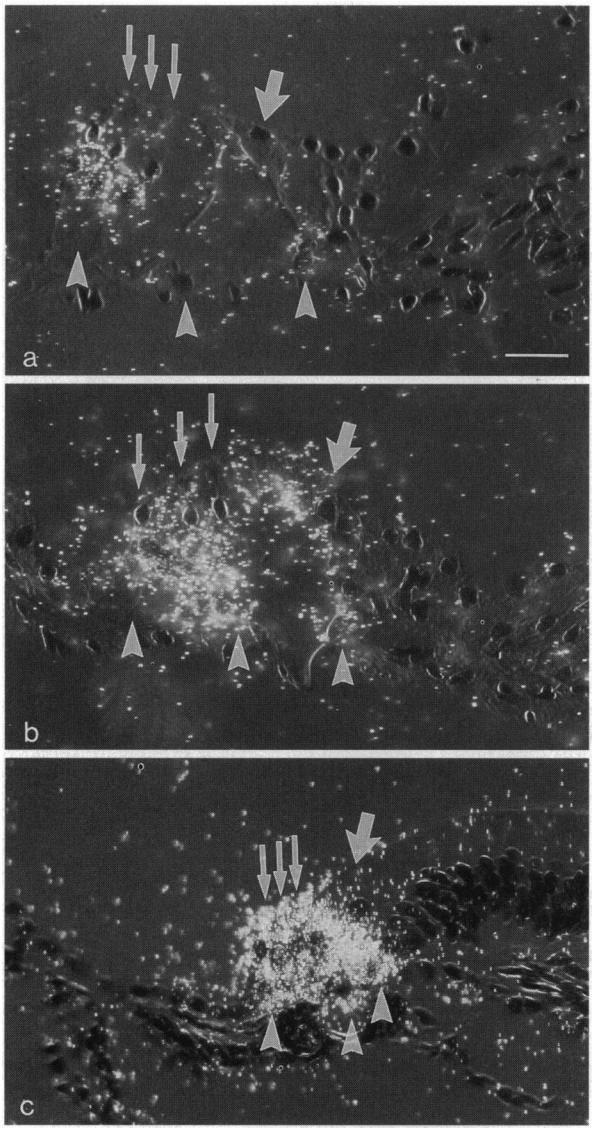

FIG. 5. Expression of FGFR-3 mRNA in the organ of Corti, as revealed by *in situ* hybridization and viewed under Nomarski optics. (*a*) In the nonexposed cochlea, expression of FGFR-3 mRNA is restricted to the inner and outer pillar cells as well as to the Deiters' cells. (*b*) An increased number of autoradiographic silver grains is found in the noise-exposed sensory epithelium. In addition to the supporting cells, basal parts of the OHCs appear to contain this message. (*c*) In the developing organ of Corti, at postnatal day 5, strong expression of FGFR-3 mRNA is seen in the differentiating supporting cells as well as in the OHCs. Thin arrows mark the OHCs, a thick arrow marks the IHC, and arrowheads point to the supporting cells. (Bar = 25 μm.)

nism(s) yet to be discovered (22). Under normal physiological conditions, these novel mechanism(s) of active export might provide sufficient amounts of aFGF available in the organ of Corti to perform the cortitrophic function.

Cell damage may represent another mechanism providing a route out for small cytosolic proteins, which are not secreted via the exocytic pathway. In tissues such as the aortic endothelium and muscle, mechanical stress has been shown to give rise to transient disruptions of the plasma membrane (reviewed in ref. 15). bFGF has been demonstrated *in vitro* to be released through mechanically induced "wounds" (23, 24). Taking into account the prominent levels of aFGF in the cochlea, the target tissue of the potent mechanical force of noise, an attractive hypothesis would be that acoustic stress compromises the integrity of the neuritic plasma membrane and allows release of aFGF. Furthermore, the accumulation of

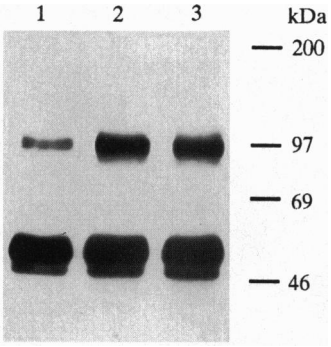

FIG. 6. Up-regulation of FGFR-3 protein in the noise-exposed organ of Corti as revealed by immunoprecipitation/Western blot analysis. Molecular mass markers (kDa) are shown. FGFR-3 antibody shows specificity for a band migrating with a molecular mass of 97 kDa. Lanes: 1, control; 2, 4 hr, 105 dB; 3, 10 hr, 105 dB.

aFGF distally in the peripheral neuritic pathway might allow rapid delivery of significant quantities of the polypeptide from the traumatized nerve endings. In fact, in response to intense noise, we have found pathological swellings and membrane ruptures in the afferent nerve endings of the rat cochlea (unpublished data), an observation that is in agreement with earlier studies performed with guinea pigs (25). However, we were unable to demonstrate elevated levels of aFGF in the noise-exposed auditory sensory epithelium. It is possible that aFGF is released into intracochlear fluids, which were not studied. Consistent with this hypothesis, injury-induced rapid release of aFGF has been demonstrated in the lesioned brain, where its levels in the Gelfoam filling the wound cavity are dramatically increased immediately following injury (26).

Previous *in vitro* data suggest that expression levels of protein tyrosine kinase receptors are regulated by the availability for their ligands, as shown, for example, for bFGF and FGFR-1 (27). The present results show noise-induced increased expression of FGFR-3 in the organ of Corti, at both mRNA and protein levels, and it is possible that this up-regulation is induced by release of aFGF from the cochlear neurons. The aFGF–FGFR-3 system might be involved in initiation of repair processes within the traumatized organ of Corti. The cellular localization of FGFR-3 message fits ideally with this suggestion, since the FGFR-3 mRNA-containing supporting cells are known to play a primary role in scarring of the damaged cochlear sensory epithelium (1). This response of the supporting cells is initiated within the first few hours of ototoxic damage (28). In agreement, we observed a rapid up-regulation of the receptor following excess noise. Synthesis of a trophic factor outside the site that is primarily affected by excess noise, as shown in the present study, might provide an efficient means of repair or recovery.

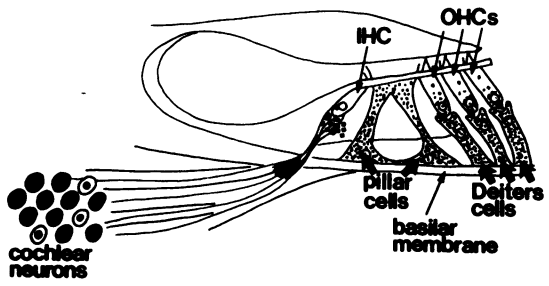

FIG. 7. Schematic representation of the distribution of aFGF in auditory neurons (filled cell bodies) and FGFR-3 in the organ of Corti of the cochlea (dots). An accumulation of aFGF is seen along the peripheral neuritic pathway.

Notably, up-regulation of FGFR-3 was observed following a moderate noise, when only a small number of hair cells was immediately wiped out. It was contributed, not only by the supporting cells surrounding the OHCs but apparently also by the OHCs themselves. However, the putative up-regulation of the receptor in the affected OHCs is still to be confirmed by methods other than radioactive *in situ* hybridization. This noise-induced increase in expression of FGFR-3 mRNA recapitulates the developing state: the embryonic (3) and early postnatal (this study) sensory epithelia, including the differentiating OHCs, contain high levels of FGFR-3 mRNA. The present data suggest a role, either direct or indirect, for the aFGF–FGFR-3 system in protection of OHCs against noise traumas. These sensory cells are known to be much more susceptible than the IHCs.

Protection of the organ of Corti from acoustic damage could be mediated partially through aFGF synthesized by the afferent neurons that terminate mainly beneath the IHCs and partially through the efferent olivo-cochlear system. The cochlear efferents contain aFGF in the neuronal perikarya located in the lateral and medial olive of the brainstem (7) as well as in the efferent nerve endings situated beneath the OHCs (this study). Thus, in the auditory organ, aFGF–FGFR-3 interaction might be involved in the neuronal circuitry consisting of the afferent and efferent innervation, and it could be speculated to be involved in protection of the organ of Corti from noise trauma in two ways. (*i*) The efferent system might modulate excessive oscillations of the basilar membrane through active motility of the OHC–Deiters' cell complex (29, 30), which has been suggested to be affected first during acoustic trauma (31). (*ii*) When OHC damage occurs, progression of damage is prevented by scar formation by Deiters' and pillar cells (1, 28).

Additional evidence for the involvement of aFGF and FGFR-3 in the traumatized cochlea is obtained from the spatial expression pattern of aFGF: the lowest numbers of aFGF-containing neurons are present in the lower middle and upper basal turns of the cochlea, corresponding to the region of the organ of Corti, which is known to be by far the most sensitive to ototraumatic hazards (32). Furthermore, recent research has shown that there is a toughening phenomenon in the mammalian cochlea: prior moderate noise exposures ("conditioning") can modulate the degree of decrease in hearing sensitivity following subsequent high-intensity exposures (33). It is tempting to speculate that the aFGF–FGFR-3 system is involved in this phenomenon. The significance of differences in aFGF content in the cochlear neurons between Wistar and Sprague–Dawley rats and its possible correlation to noise susceptibility remain to be established. Clinically, it would be of interest to investigate whether exogenous, pharmacological doses of aFGF would have a protective effect on the traumatized auditory organ.

Electrophysiological studies on the supporting cells of the organ of Corti suggest parallels in the functioning of these cells and the neuroglia of the central nervous system (CNS) (34). Also, scar formation by the auditory supporting cells is a counterpart to the deposition of a glial scar in the CNS. FGFR-3 mRNA is abundantly expressed in the glial cells of the brain (3), and aFGF is synthesized by the neurons (5, 7). Taken together with the present data, it is possible that this receptor–ligand interaction has a more general role in the recovery and repair processes. As pleiotropism in the function of aFGF is well-established, healing activated by the aFGF–FGFR-3 interaction might have differential manifestations: healing is associated with nonproliferative events in the mammalian auditory organ, whereas mitogenesis forms a prominent feature in the formation of the glial scar in the CNS. Finally, as the mature auditory sensory epithelium of non-mammalian species has the capacity of proliferative regeneration following insults, and knowing that it is the supporting cells that give rise to new hair cells through mitoses and subsequent differentiation (reviewed in ref. 35), it would be of interest to find out whether the aFGF–FGFR-3 system is involved in this process.

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