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Immunohistological analysis of neurturin and its receptors in human cochlea

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ABSTRACT

Objective: Difficulties in obtaining properly preserved human cochlea have been a major obstacle to in vitro study of this deeply located and hard bone-fortressed hearing organ. Our study aimed at investigating GDNF family ligands (GFLs) and their receptors in the human cochleae that were surgically obtained during a transcochlear approach dealing with life-threatening, intra-cranial meningiomas.
Methods: The specimens were properly fixed with 4% paraformaldehyde in the operating room. By using immunohistochemical techniques, distribution of GDNF, Neurturin (NTN, one member of GFLs), as well as cRet, GFRα-1 and GFRα-2 receptors in the human cochleae was investigated. Five cochleae from five adult patients were processed for the study. The patients had normal hearing threshold before operation.
Results: cRet receptor immunoreactivity was seen in the spiral ganglion neurons, mainly inside the cell bodies but rarely in the nerve fibers and not in the organ of Corti. Immunolabeling for GFRα-1 and GFRα-2 receptors was identified mainly in the cell bodies of the spiral neurons than in the nerve fibers. In the organ of Corti, GFRα-1 immunostaining could be demonstrated in the Deiters' cells, Hensen cells, inner pillar cells, and weakly in the inner hair cells but not in the outer hair cells; no structures in the organ of Corti were labeled with GFRα-2 receptor antibody. NTN immunostaining was found in the supporting cells of organ of Corti, including Deiters' cells, Hensen cells as well as Claudius' cells. In the spiral ganglia, NTN immunostaining was seen in both the cell bodies and the nerve fibers of neurons. GDNF immunoreactivity was not revealed in human cochlea.
Conclusion: Surgically obtained human cochleae were properly fixed and underwent immunohistochemical investigation of neurotrophic elements. NTN and its receptors discovered in current study can be responsible for the unique neuronal survival properties in human spiral ganglion (hSG); a prerequisite for the function of cochlear implants.

1. Introduction

The role of glial cell line-derived neurotrophic factor (GDNF) as survival factor for inner ear neurons has been well established [1–3]. In contrast, other members of GDNF family ligands (GFLs) have been less intensively studied. Neurturin (NTN or NTRN), one member of GFLs, was investigated only in a few animal studies concerning the auditory system. In other peripheral nervous systems (PNS) and central nervous system (CNS), NTN had been extensively studied since several years [4] with regard to its distribution and neurotrophic function. NTN supports survival and regulates differentiation of sympathetic, parasympathetic, sensory and enteric neurons [5]. NTN as well as GDNF has been shown to restore function in damaged dopaminergic neurons. Meanwhile, attempts to target these neurons with NTN gene therapy are in progress [6].

Among the receptors of GDNF and NTN, i.e. cRet, GFR-alpha-1 (GFRα-1) and GFR-alpha-2 (GFRα-2), only cRet receptor mediated hearing loss was identified when cRet knockin mice were used to produce the disease [7]. The hearing loss can be early-onset syndromic or late-onset non-syndromic depending on complete or partial monogenetic impairment of cRet phosphorylation [8].

So far the expression and function of GFLs and their receptors in human inner ear have not been reported although a few anatomical studies in animals suggest a potential protective role of NTN and its receptors in the inner ear [9,10]. Due to the rarity but obvious importance of the study about neurotrophic mechanism in human inner ear [11], we harvested human cochleae from operated patients suffering from life-threatening posterior cranial fossa meningiomas for immuno-localization of neurotrophic

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Table 1
Antibodies used in current study.

Antibody	Dilution	Poly or mono	Host	Clone	Catalog No.	Producer
cRet	1:100	Polyclonal	Rabbit	c-19	sc-167,	Santa Cruz Biotechnology, Inc.
GFRα-1	1:500	Polyclonal	Rabbit	N/A	AB5140	Millipore
GFRα-2	1:100	Polyclonal	Rabbit	H-89	sc-28953	santa cruz biotechnology, Inc.
NTN	1:100	Polyclonal	Rabbit	N/A	ab49203	Abcam
GDNF	1:50	Monoclonal	Mouse	B-8	sc-13147	Santa Cruz Biotechnology, Inc.
Tuj1	1:200	Monoclonal	Mouse	N/A	1637	Millipore

Poly, polyclonal antibody; mono, monoclonal antibody.

factors and receptors. These neurotrophic elements can be partly responsible for the unique survival properties of human spiral ganglion neurons (SGNs); a prerequisite for the function of cochlear implants.

In a previous analysis of several properly fixed adult human cochleae, tropomyosin kinase B (TrkB), the brain-derived nerve growth factor (BDNF) receptor, was found in SGNs. However, BDNF immunoreaction could not be verified in any structures in human cochleae including spiral ganglion and organ of Corti [11]. Questions thus arise where the neurotrophic substances are synthesized and located in human inner ear. We consequently extended our searching to GFLs and their receptors in human cochlea.

2. Material and methods

We collected human surgical specimens from patients suffering from posterior cranial fossa meningioma. Surgery of these tumors is challenging due to location and risk for severing cranial nerves and blood vessels with postoperative sequelae. The trans-cochlear approach with re-routing of the facial nerve was developed since several years and gives a maximal exposure of the tumor avoiding cerebellar compression. Study on human materials was approved by the local ethics committee (no. 99398, 22/9 1999, cont., 2003, Dnr. 2013/190) and patient consent was obtained.

2.1. Fixation and sectioning of human cochlea

Five cochleae belonging to five adult patients (2 male, 3 females; ages 40–65 years) with normal pure tone thresholds for their age were dissected out as a whole piece during petroclival meningioma surgery. In the operating room, the cochleae were immediately placed in 4% paraformaldehyde diluted with 0.1 M phosphate buffered saline (PBS; pH 7.4). After a 24-h fixation, the fixative was replaced with 0.1 M PBS then with 10% EDTA solution at pH 7.2 for decalcification. After around four weeks, the thoroughly decalcified cochleae were rinsed with PBS. For frozen sections, the cochleae were embedded in Tissue-Tek (OCT Polysciences), rapidly frozen and sectioned at 8–10 μm using a Leica cryostat microtome. The frozen sections were collected onto gelatin/chrome-alum-coated slides and stored below–70 °C before immunohistochemistry.

2.2. Antibody and immunohistochemistry

The antibody against cRet was a polyclonal antibody from rabbit c-19 (catalog number sc-167, Santa Cruz Biotechnology, INC; dilution 1:100). The GFRα-1 antibody was a polyclonal antibody from rabbit (catalog AB5140, Millipore, Billerica, MA, USA; dilution 1:500). The GFRα-2 antibody (H-89) was a rabbit polyclonal antibody (catalog number sc-28953, Santa Cruz Biotechnology, INC, California, USA; dilution 1:100). NTN antibody was a rabbit polyclonal antibody (catalog ab49203, Abcam, Cambridge, UK; dilution 1:100). GDNF (B-8) antibody was a monoclonal antibody from mouse (catalog number sc-13147, Santa Cruz Biotechnology, INC; dilution 1:50). The antibody against neuron-specific class III beta-tubulin (Tuj1) was a monoclonal antibody from mouse (catalog number 1637, Millipore; dilution 1:200). The antibody data was summarized in Table 1 and more information can be found under discussion.

Immunohistochemistry (IHC) procedures on cochlear sections had been described in our previous publications [12,13]. Briefly, incubation of sections on slides with solution of the antibodies was carried out under humid atmosphere at 4 °C for 20 h. After rinsing with PBS (3 × 5 min), the sections were subsequently incubated with secondary antibodies conjugated to Alexa Fluor 488 and 555 (Molecular Probes, Carlsbad, CA, USA), counter-stained with a nuclear stain DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) for 5 min, rinsed with PBS (3 × 5 min) and mounted with a VECTA SHIELD (Vector Laboratories, Burlingame, CA, USA) mounting medium.

The sections used for antibody control were incubated with 2% bovine serum albumin (BSA) omitting the primary antibodies. As the result of the control experiment, there was no visible staining in any structure of the cochleae (Figs. 3C and 4D).

2.3. Imaging and photography

Stained sections were investigated with an inverted fluorescence microscope (Nikon TE2000, Nikon Co., Japan) equipped with a spot digital camera with three filters (for emission spectra maxima at 358, 461 and 555 nm). Both microscope and camera are connected to a computer system installed with image software (NIS Element BR-3.2, Nikon) including image merging and a fluorescence intensity analyzer. For laser confocal microscopy, the same microscope was used which is equipped with laser emission and detection system with three different channels. The optical scanning and image-processing tasks were run by the program Nikon EZ-C1 (ver. 3.80) including reconstruction of Z-stack images into projections or 3D images.

3. Results

Aiming to confirm the location of NTN and GDNF, members of GFLs, as well as cRet, GFRα-1 and GFRα-2 receptors for NTN and GDNF in human cochlea, five adult human cochleae were sectioned and immunostained as described under methods. The human cochlear specimens were surgically obtained from patients during a trans-cochlear approach for removal of life-threatening posterior cranial fossa meningiomas. In these cases, the inner ear tissue, the auditory nerves and the labyrinthine blood vessels were not contacted by the tumor; therefore the patients had nearly normal pure tone thresholds before the operation.

To have critical criteria for defining various cochlear cell types (e.g., hair cells and supporting cells in the organ of Corti, spiral ganglion neurons (SGNs) in the spiral ganglion), we used morphological, positional and bordering features of these structures observed under both dark- and bright-field conditions. That helped us to precisely identify the structures mentioned in the following description. No difference in immunostaining pattern