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Regular Article

Transgenic BDNF induces nerve fiber regrowth into the auditory epithelium in deaf cochleae ☆

Seiji B. Shibata [a,b], Sarah R. Cortez [a], Lisa A. Beyer [a], James A. Wiler [a], Adriana Di Polo [c], Bryan E. Pfingst [a], Yehoash Raphael [a,*]

[a] Kresge Hearing Research Institute, Department of Otolaryngology, The University of Michigan, 1150 W. Med. Cntr. Dr., Ann Arbor, MI 48109-5648, USA
[b] Department of Otolaryngology, Kansai Medical University, Moriguchi, Osaka, 570-8506, Japan
[c] Department of Pathology and Cell Biology, University of Montreal, Montreal, PQ, Canada H3T 1J4

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ABSTRACT

Sensory organs typically use receptor cells and afferent neurons to transduce environmental signals and transmit them to the CNS. When sensory cells are lost, nerves often regress from the sensory area. Therapeutic and regenerative approaches would benefit from the presence of nerve fibers in the tissue. In the hearing system, retraction of afferent innervation may accompany the degeneration of auditory hair cells that is associated with permanent hearing loss. The only therapy currently available for cases with severe or complete loss of hair cells is the cochlear implant auditory prosthesis. To enhance the therapeutic benefits of a cochlear implant, it is necessary to attract nerve fibers back into the cochlear epithelium. Here we show that forced expression of the neurotrophin gene BDNF in epithelial or mesothelial cells that remain in the deaf ear induces robust regrowth of nerve fibers towards the cells that secrete the neurotrophin, and results in re-innervation of the sensory area. The process of neurotrophin-induced neuronal regeneration is accompanied by significant preservation of the spiral ganglion cells. The ability to regrow nerve fibers into the basilar membrane area and protect the auditory nerve will enhance performance of cochlear implants and augment future cell replacement therapies such as stem cell implantation or induced transdifferentiation. This model also provides a general experimental stage for drawing nerve fibers into a tissue devoid of neurons, and studying the interaction between the nerve fibers and the tissue.

Introduction

Hair cells in the cochlea transduce acoustic auditory stimulation to activate the auditory nerve. In normal ears, the afferent auditory neurons, spiral ganglion neurons (SGNs), maintain peripheral nerve fibers that synapse with the hair cells (Rusznak and Szucs, 2009, Spoendlin, 1985, Spoendlin and Schrott, 1988), but the loss of hair cells may lead to degeneration of nerve fibers from the sensory epithelium, and eventually to degeneration of the SGNs (Bichler et al., 1983, Jyung et al., 1989, Koitchev et al., 1982, Webster and Webster, 1981). Because lost mammalian auditory hair cells and neurons do not spontaneously regenerate, the hearing loss associated with their degeneration is permanent (Hawkins, 1973, Spoendlin, 1975).

Loss of inner hair cells is implicated in SGN degeneration, but the causal relationship between the two is complex. The extent of SGN degeneration differs between laboratory animals, where it is common and severe (Dodson and Mohuiddin, 2000, Jyung et al., 1989, Leake and Hradek, 1988, Sugawara et al., 2005), and human ears, where it is much less common (Linthicum and Fayad, 2009, Nadol and Eddington, 2006, Spoendlin and Schrott, 1990). Moreover, the correlation between the outcome of cochlear implant prosthesis therapy and the survival of the SGNs was not found to be strong in most cases (Blamey, 1997, Linthicum and Fayad, 2009). However, the loss of peripheral nerve fibers, the dendrites of the SGNs, is strongly correlated with the loss of inner hair cells and probably also with the supporting cells in both animal and human ears (Linthicum and Fayad, 2009, Sugawara et al., 2005). It is likely that the outcome of cochlear implant therapy is related to the condition of the remaining SGNs and the survival of peripheral dendrites.

In the absence of hair cells, cochlear implant electrodes can stimulate SGN bodies and possibly their central axons, providing partial restoration of hearing to patients with severe or profound hearing loss (Wilson and Dorman, 2008). Inspired by the prevailing concept that better survival of SGNs would enhance outcomes of cochlear implant therapy, several approaches have been used to preserve neurons in deaf ears. Of these, increasing the levels of neurotrophins in the cochlear fluids has been the most successful

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* Corresponding author. Fax: +1 734 615 8111.
E-mail address: yoash@umich.edu (Y. Raphael).

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(Bowers et al., 2002, Fritzsch et al., 2004, Ylikoski et al., 1998). Neurotrophins were selected because studies had demonstrated that these growth factors have a role in development of afferent neurons in the organ of Corti (Altschuler et al., 1999, Fritzsch et al., 1999) and in protecting SGNs in ears where hair cells are lost (Aarnisalo et al., 2000, Duan et al., 2000, Nakaizumi et al., 2004, Staecker et al., 1996, Van De Water et al., 1996).

Several methods have been used to elevate neurotrophin levels for protecting neurons in deafened ears. Infusion of trophic factors such as neurotrophins via mini-osmotic pumps has been shown to significantly enhance the numbers of surviving neurons (Glueckert et al., 2008, Miller et al., 1997, Wise et al., 2005). Similar results were accomplished using forced expression of neurotrophins by introducing the genes for these growth factors into cells that line the perilymph (Bowers et al., 2002, Miller et al., 1997, Nakaizumi et al., 2004, Staecker et al., 1998). Short term secretion of a neurotrophin was also accomplished by implanting electrodes coated with a gel pre-soaked with the neurotrophin (Chikar et al., 2008, Hendricks et al., 2008).

Elevated levels of neurotrophins not only increased survival of SGNs, but also induced sprouting of peripheral nerve fibers (Glueckert et al., 2008, Wise et al., 2005). Growth or regeneration of nerve fibers, by itself, can further enhance SGN survival. To the extent that this new growth can be directed into the area of the auditory epithelium and the connective tissue beneath it (defined here as the basilar membrane area, BMA), and remain there for the long term, it could improve the function of the cochlear implant device by placing neurons closer to the electrodes and enhancing spatial selectivity of stimulation. Functional outcomes of novel restorative methods that might be developed in the future (such as cell replacement by stem cell therapy) would also benefit from the presence of nerve fibers in the tissue.

The severely traumatized guinea pig cochlea serves as a model for profound hearing loss in humans. Here, we tested the response of the auditory nerve in severely deafened ears to elevated levels of neurotrophins in the BMA and its vicinity. Specifically, neurotrophin over-expression was produced by viral vector transduction of cells in the BMA of deafened ears. To eliminate hair cells in the guinea pig cochlear epithelium and generate profound deafness, we used a perilymphatic injection of neomycin. This ototoxic regimen quickly eradicates hair cells and leaves behind a severely traumatized cochlear epithelium that also lacks the typical morphology of non-sensory cells (Duckert, 1983, Kim and Raphael, 2007). We refer to this severely lesioned tissue as the flat epithelium (FE). Presence of the FE has been observed after a long period of deafness following kanamycin treatment (Sugawara et al., 2005), after genetic hearing loss (Pawlowski et al., 2006) and other etiologies for animal deafness, and in human temporal bone studies of patients with congenital diseases, including some who also received a cochlear implant (Nadol, 1997, Nadol and Eddington, 2006, Teufert et al., 2006). Patients with profound deafness and FE are among those who receive cochlear implants, and may be the first candidate population for future stem cell therapies. In both therapies, presence of nerves in the BMA would be beneficial.

To deliver a neurotrophin gene to cells of the BMA, we used one of two viral vectors with the *BDNF* gene insert. In one group of animals, we used an adenovirus (Ad.*BDNF*) because of the high efficiency of gene transfer of this vector. In another group, we used an adeno-associated virus with the combined *BDNF* and *GFP* inserts (AAV.*BDNF-GFP)* as the AAV vector has long lasting transgene expression and no known side effects (Sapieha et al., 2006). We report that over-expression of BDNF in the BMA of deafened cochleae led to abundant regrowth of nerve fibers toward the cells that secreted the neurotrophin. The robust neurotrophin-induced neuronal regeneration into the BMA was accompanied by significant preservation of the SGNs.

Materials and methods

Animals and groups

Animal care and handling and all procedures described in this work were approved by the University of Michigan Institutional Committee on the Use of Care of Animals and performed using accepted veterinary standards. Male pigmented guinea pigs weighing 300–350 g were purchased from Elm Hill, Chelmsford, MA USA. Each animal was tested for normal Preyer's reflex before being included in the studies. Animal groups were Ad.*BDNF* (25), Ad.Empty (18), AAV.*GFP-BDNF* (13), and deafening only (7). The inner ears were assessed as whole-mounts by epifluorescence and/or confocal microscopy, or plastic cross-sections at the light or TEM levels.

Deafening and inoculation surgery

Animals were deafened unilaterally (left ear) by infusing 10 μl of 10% (w/v) neomycin sulphate solution (Neo-Rx, Pharma-Tek, in saline) into the scala tympani perilymph via the round window membrane. The surgical method of deafening is described in previous reports (Izumikawa et al., 2008, Kim and Raphael, 2007). This protocol resulted in a nearly complete degeneration of the organ of Corti in the basal and 2nd turn. Specimens with any residual differentiated supporting cells in the basal turn were excluded from the study. One week later, animals were inoculated with viral vectors into the scala media (Ishimoto et al., 2002) or scala tympani (Yagi et al., 1999).

Adenovirus and adeno-associated virus

Adenoviral vectors with mouse *BDNF* insert driven by a cytomegalovirus promoter have been described previously (Di Polo et al., 1998). This vector was given to the animal group labeled Ad.*BDNF*. We injected 5 μl of Ad.*BDNF* at a titer of 4×10^{12} adenoviral particle per ml. Control animals labeled as Ad.Empty received an adenoviral vector with no insert (a gift from GenVec, Inc., Gaithersburg, MD, USA). Adeno-associated virus vector with BDNF and GFP insert driven by a cytomegalovirus promoter (Sapieha et al., 2006), was given to the group designated AAV.*GFP-BDNF*. Viral suspension was preserved at −80°C and thawed on ice before use. We injected 5 μl of viral solution containing 1.68×10^{12} AAV particles per ml.

Immunohistochemistry analysis

Fourteen or 30 days after the inoculation, animals were decapitated under general anesthesia. Both temporal bones were extracted and the cochleae were perfused with 4% paraformaldehyde (PFA). After 2 h of fixation, cochleae were rinsed in phosphate buffered saline (PBS). Tissues were permeabilized with 0.3% Triton-X for 10 min, and then blocked against non-specific binding of secondary antibody by incubation in 5% normal goat serum for 30 min. Primary antibodies were monoclonal anti-neurofilament 200 kDa (Sigma) diluted 1:400 in PBS. Secondary antibodies were goat-anti mouse TRITC diluted to 1:200 in PBS. We counterstained the tissue for F-actin with Alexa Fluor 488 for 2 min, diluted to 1:300 in PBS. After the tissues were washed with PBS, whole-mounts of the BMA and surrounding tissue were obtained, mounted on glass slides and cover-slipped with Gel/Mount (Biomeda, Foster City, CA, USA). Whole-mounts were observed with a Leica DMRB epifluorescence microscope (Leica, Eaton, PA, USA) or a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany). Confocal images were acquired and processed with LSM image Browser (Carl Zeiss, Germany). Confocal images 2a-h, 5a, and 5d, are stacked sets of *z*-plane images. Stacked planes ranged from 4 to 28 images (total depth ranging from 3.3 to 19.9 μm).

For ears observed in plastic sections, the tissues were first stained with anti-neurofilament as primary antibody (as described above)