Gene-Based Diagnostic and Treatment Methods for Tinnitus

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Abstract: The etiology of tinnitus combines hereditary and environmental factors. To help develop optimal therapies for tinnitus, it is necessary to characterize the genetic contributors to the pathophysiology and to design treatments at the level of the gene. Inner ear gene therapy involves delivery of genes into the vestibular or auditory portions of the inner ear for preventive or reparative therapies at the level of the sensory epithelium or the eighth nerve neurons. BDNF and GDNF are among the neurotrophic factors shown to be overexpressed with gene therapy and to protect the inner ear against trauma. Combined treatment with Ad.GDNF and electrical stimulation provided enhanced preservation of denervated spiral ganglion neurons. The use of viral vectors for gene therapy may involve side effects, including immune response to the viral proteins. Treatment with immunosuppressive medications can reduce the negative consequences of adenovirus-mediated gene therapy.

Key Words: adenovirus gene therapy; growth factors; hair cell; hereditary inner ear disease; spiral ganglion; tinnitus

Recent Progress in the Genetics of Tinnitus

Tinnitus is a multifactorial disorder that presents a challenge to affected patients, their families, and physicians. A combination of heredity and environment is likely to contribute to the pathophysiology of tinnitus. While the underlying cause of tinnitus is often difficult to determine, it can be associated with a wide variety of drugs and infectious, neurological, and vascular disorders (for review, see [1]). For the autosomal dominant genetic conditions of neurofibromatosis type II (NFII) and von Hippel-Lindau (VHL) disease, mutations in a single gene can cause multisystem effects, with tinnitus as a secondary phenomenon. In NFII, mutations in the gene schwannomin lead to acoustic neuromas with associated hearing loss and tinnitus that may be related to direct effects of the tumor or surgical repair of auditory structures. In VHL disease, mutations in the VHL tumor suppressor gene are associated with retinal, cerebellar, and spinal hemangioblastoma; renal cell carcinoma; pheochromocytoma; pancreatic tumors; and a rare tumor of the endolymphatic sac. Endolymphatic sac tumors in VHL patients often are associated with hearing loss and tinnitus.

In addition to these multisystem disorders, recent reports have demonstrated that single-gene mutations can result in isolated tinnitus and low-frequency sensorineural hearing loss (LFSNHL). Two genes implicated in the pathophysiology of tinnitus are WFSI (mutated in Wolfram syndrome) and COCH. Wolfram syndrome, a disorder that presents with diabetes insipidus, diabetes mellitus, optic atrophy, and deafness, is caused by mutations in the WFSI gene on chromosome 4p16. A recent report by Bespalova et al. [2] showed that WFSI mutations may be a relatively common cause of LFSNHL and tinnitus. In this study [2], five different missense mutations were identified in six families with LFSNHL and tinnitus, and one of these mutations was present in 1 of 336 control human DNA samples. A novel WFSI missense mutation was subsequently identified in a Japanese family with LFSNHL [3]. An earlier study by Ohata et al. [4] suggested that heterozygous carriers of WFSI mutations have an increased risk of hearing loss. Taken together, these studies indicate that the
population prevalence of individuals heterozygous for WFS1 mutations may be as high as 0.3–1.0%.

Tinnitus often presents in adults with Ménière’s syndrome, a condition of fluctuating hearing loss and tinnitus that can result in permanent hearing loss. Ménière’s syndrome is rarely hereditary; however, recently a large, 135-member Belgian family was identified as having 9 individuals with progressive vestibulocochlear dysfunction, tinnitus, and hearing loss who met the diagnostic criteria for definite Ménière’s syndrome proposed by the American Academy of Otolaryngology [5]. In their report, Verstreken et al. [5] described missense mutations in the COCH gene, also implicated in the nonsyndromic autosomal deafness locus DFNA9 [6].

Our understanding of the genetic determinants of tinnitus is in its infancy. Clearly, single gene defects can cause tinnitus, either in association with multisystem disorders or in isolation. Although no diagnostic molecular testing is currently available to determine whether a given individual is at risk for developing tinnitus, such tests may become commonplace in those families with multiple affected members. Genetic testing can also be useful when individual genetic variants are found to determine responsiveness to pharmacological therapy. For tinnitus, gentamicin therapy is used to ablate inner ear hair cells, which may occasionally improve tinnitus in some patients at the expense of hearing ability [7]. Targeted therapies directed at the cause of tinnitus are sorely needed, both to circumvent the adverse side effects of hearing loss from gentamicin therapy and to treat the fluctuant symptoms. Several technologies for drug therapy have been used recently [7–10]. To help develop optimal therapies, it is necessary to better characterize the genetic contributions to the pathophysiology of tinnitus and to design innovative means for treatment at the level of the gene. Here we review recent progress in the use of biologically active genes for therapy in the inner ear.

INTRODUCTION TO INNER EAR GENE THERAPY

Inner ear gene therapy is a term used to describe the delivery of genes into the vestibular or auditory portions of the inner ear for preventive or reparative therapies. Potential applications for gene therapy in the inner ear include (1) replacing a defective (or missing) gene product that may be present owing to an inherited or acquired mutation; (2) delivering protective molecules that may reduce or eliminate the pathology caused by environmental stresses such as noise overstimulation or ototoxic drugs; (3) introducing genes that may induce regeneration of missing hair cells; and (4) delivering genes that counteract the destructive effects of autoimmune disease in the inner ear.

A regulated amount of gene delivery and transgene expression is desired for a specific duration, at a specific anatomical site, and with minimal side effects. These ideal requirements are not met by current delivery vectors [11–14]. Nevertheless, using available vectors, several genes have been tested for their ability to influence the response of the inner ear to insults. To date, all published data on in vivo gene therapy for the inner ear involve genes that encode secreted gene products. As such, it is not required that the viral vectors infect and transduce the ultimate target of the therapy. Rather, it is sufficient that cells derived from connective tissue, such as the mesothelial cells (lining the perilymphatic space), are transduced. These cells, in turn, secrete the gene product into inner ear fluids. In the future, as gene transfer technology improves, transduction of specific target cells will most likely become possible and allow us to expand the application of gene therapy to nonsecreted gene products.

Auditory Hair Cell Protection

Hair cell degeneration can occur owing to presbycusis, ototoxic drugs, overstimulation, or hereditary disease, and results in deafness, tinnitus, and vestibular pathology. Protection against environmental etiologies can be provided by several families of molecules, including antioxidants, antiapoptotic agents, and neurotrophic factors. Antioxidants and antiapoptotic agents are important candidates for gene therapy, but their use for gene transfer into the inner ear has not yet been reported. However, neurotrophic factors have been used in gene therapy experiments in the inner ear [15–19].

Neurotrophic factors influence neuronal development, growth, and survival [20–23]. Among the known neurotrophic factors is the family of neurotrophins, which includes NGF, BDNF, NT-3, and NT-4/5 and other important factors such as IGF-1, IGF-2, TGF-B1, CNTF, and the family of GDNF-related molecules. Many of the genes that encode neurotrophic factors (as well as their receptors and binding proteins) have been cloned and sequenced, providing insight into the function of each of these genes, from the level of the regulation of gene expression to their specific function in development, physiology, and pathology [24,25]. Studies of the various neurotrophic factors present in the inner ear suggest that overexpression of neurotrophic factors can provide protection against degeneration and enhance repair in the inner ear epithelium [26–31]. Delivery of growth factors into the inner ear via gene therapy technology has been reported for NT-3 [19,32], BDNF [15], and GDNF.

GDNF is a member of the TGF-β family of growth factor–encoding genes [33]. The GDNF family also
includes neurturin, artemin, and persephin [34,35]. GDNF binds to a dimeric receptor complex [36,37]. GDNFR-α is an extracellular cell-surface coreceptor, also known as α1 [38–40]. The transmembrane component is a tyrosine kinase receptor named RET [41]. Both GDNF and its receptors have been detected in inner ear tissues by reverse transcriptase–polymerase chain reaction [42].

To determine whether adenovirus-mediated transgene expression of GDNF protects sensory cells in the inner ear against ototoxic drug–induced degeneration, replication-deficient adenoviral vector encoding human GDNF (Ad.GDNF) was injected into the scala tympani of the left ear of guinea pigs prior to the systemic administration of ototoxic drugs [16]. Physiological testing (auditory brainstem responses [ABRs]) and hair cell counts were performed before the procedures and upon sacrificing the animals. Ad.GDNF significantly enhanced the survival of cochlear hair cells and preserved hearing in the left ears as compared to the contralateral (right) ears [16].

To test whether GDNF overexpression can protect cochlear hair cells and auditory function from permanent noise-induced hearing loss, guinea pigs were overstimulated with noise bilaterally 4 days after Ad.GDNF inoculation of the left ear. ABRs were measured at the onset of the experiment (baseline) and just prior to sacrifice. Animals were sacrificed 7 days after the inoculation, and their hair cells were counted. The extent of protection afforded by Ad.GDNF was determined by comparing the inoculated (left) ear to the noninoculated (right) ear. In all 9 animals, Ad.GDNF protected cochlear function and structure. In the right organ of Corti, there was a severe loss of hair cells, whereas in the left ear there was only a narrow lesion at the center of the area sensitive to the noise [43].

**Hair Cell Rescue After Trauma**

In cases in which inner ear trauma has occurred, rescue of the remaining hair cells and function becomes a clinical challenge in the cochlea and the vestibular epithelium. The timing of rescue procedures is critical, as delayed interventions may result in hair cell loss. It is necessary to determine which posttraumatic interventions may rescue hair cells and to define the time frame of effective rescue.

The ability of Ad.GDNF to rescue or repair guinea pig vestibular epithelium was tested by simultaneous inoculation of Ad.GDNF and administration of vestibulotoxic drugs. With this protocol, transgene overexpression is likely to yield significant GDNF secretion into the perilymph 12 hours after the insult. Significant rescue of hair cells was afforded by the Ad.GDNF as compared to controls [18].

The choice of molecules for hair cell rescue includes neurotrophic factors and other proteins. It is necessary to identify the best gene products that can rescue hair cells and hearing and to determine the time frame during which effective rescue can be accomplished. It is noteworthy that protein or drug therapy may be more appropriate for rescue of hair cells, as the action of such agents is immediate on administration. In contrast, gene therapy invariably involves (at least) several hours of delay between viral inoculation and secretion of the transgene.

**Vestibular Hair Cell Protection**

Balance impairment and vertigo are extremely debilitating. In older individuals, these problems often lead to devastating falls. As in deafness, loss of hair cells is thought to be the main reason for vestibular impairment. Hair cell regeneration has been shown to take place in the vestibular epithelium, unlike the organ of Corti. However, regeneration in the vestibular periphery is incomplete [44–48] and, therefore, vestibular function deteriorates with age and does not fully recover at any age after hair cells are lost owing to ototoxins. Consequently, it would be helpful to develop preventive and therapeutic approaches for disorders affecting the vestibular epithelium, including hereditary disease, ototoxins, and aging.

One approach for vestibular hair cell protection that has been pursued via gene therapy involves neurotrophic factors, specifically GDNF. To determine the effects of Ad.GDNF on vestibular hair cells exposed to gentamicin, guinea pigs received a gentamicin injection into the middle ear cavity of the left ear. This treatment leads to a severe degeneration of vestibular hair cells in the saccule and the utricle. Seven days later, Ad.GDNF was inoculated through a cochleostomy into the scala vestibuli of the same ear. Control groups included vehicle control (artificial perilymph) and vector control (Ad.LacZ). Upon sacrificing the animals, utricles were obtained, and missing hair cells (scars) were counted. The density of scars (scar number per square millimeter) was calculated for experimental and control groups. The results demonstrated a statistically significant decrease in utricular scarring owing to gentamicin in ears that were inoculated with Ad.GDNF as compared with ears in control groups [18].

**Spiral Ganglion Cell Survival**

The loss of mammalian cochlear hair cells is irreversible. Once a severe or complete depletion of the hair cell population occurs, a secondary response takes place in the spiral ganglion (SG) [49–53]. This response may
include physiological changes in the remaining neurons (qualitative changes); degeneration of neurons (quantitative changes, decrease in numbers); sublethal neuronal changes, such as degeneration of the peripheral axons; and changes in the surrounding tissues, including Schwann cells and connective tissue elements surrounding Rosenthal’s canal. In the complete absence of cochlear hair cells, the only way to restore hearing is with the use of a cochlear implant. Maximizing the number and health of remaining SG neurons can significantly enhance the success of the implant. As with hair cells, several attempts have been made to influence the survival of deafferented SG neurons with gene therapy.

**CYTOKINE GENE THERAPY**

Cytokines and their inhibitors play a cardinal role in promoting and modulating inflammatory responses in injured tissues. One of the best-studied cytokines, interleukin-1β (IL-1), is a primary inflammatory cytokine that acts as a local or systemic mediator of inflammation [54]. Cytokine inhibitors targeting IL-1 are of specific interest for antiinflammatory therapy. Blocking the production or action of IL-1 reduces inflammation and is therefore useful as an antiinflammatory treatment [55]. However, in addition to its immunomodulatory action, IL-1 has been shown to enhance production and secretion of growth factors such as NGF [56,57].

To determine the influence of adenoviral-mediated IL-1ra transgene expression on the survival of SG neurons after experimentally induced hair cell loss, the hair cell population of guinea pigs was systemically eliminated with ototoxic drugs [58]. The left ear then was inoculated with Ad.IL-1ra. SG counts performed 4 or 8 weeks later revealed that in the left (inoculated) ears, Ad.IL-1ra significantly accelerated cell death in the SG. In control animals that received Ad.IL-1ra inoculation without the deafening procedure, Ad.IL-1ra did not influence the normal population of SG neurons. These data demonstrate that the IL-1ra impact is evident only in denervated SG neurons [58], suggesting that IL-1-mediated signaling is important for preservation of denervated SG cells, probably by signaling overexpression of growth factors.

**BDNF**

BDNF influences development of the apical region of the cochlea, whereas NT-3 influences the base [27]. BDNF has protective effects on the mature SG throughout the cochlea. These protective effects can be attributed either to cross-reactivity with trk-C receptors or to direct influence via another (uncharacterized) receptor. In a gene therapy experiment to test the protective capacity of BDNF in the inner ear, a replication-defective herpesvirus vector was used in mouse ears [15]. This vector contained inserts for both BDNF and the β-gal reporter gene and was designated HSV.bdnflac. Cochlear hair cells were eliminated with ototoxic drugs, which resulted in a degeneration of approximately two-thirds of the SG neurons 4 weeks after the toxic insult. In ears injected with HSV.bdnflac, preservation of the neurons was nearly complete. This study demonstrated effective and significant BDNF gene therapy for enhancing SG neuron survival in mice. The study also demonstrated expression of the reporter gene in SG cells, suggesting that the mechanism of action of BDNF in these neurons is paracrine [15].

**GDNF**

In a set of experiments to determine the protective effects of GDNF gene therapy on denervated SG cells, hair cells in guinea pig inner ears were eliminated systemically with ototoxic drugs (kanamycin and ethacrynic acid). Several days later, when hair cell loss was complete, 5 µl of Ad.GDNF was inoculated into the left cochlea (scala tympani, via the round window). Control groups received Ad.lacZ or artificial perilymph. Animals were sacrificed 1 or 2 months after the onset of the experiment, and their inner ears were prepared for SG cell counts. These studies demonstrated that the Ad.GDNF enhanced SG cell survival in the left (virus-treated) deafened ears as compared to the untreated (contralateral, control) ears [17].

**Electrical Stimulation Combined with GDNF**

Electrical stimulation provided by electrodes in hair cell-depleted ears can enhance survival of SG neurons [59–65]. GDNF also increases survival of SG cells after inner hair cell loss (see preceding section). To determine the combined effects of the AD.GDNF and electrical stimulation on SG neurons in hair cell-depleted ears, we deafened guinea pigs and provided Ad.GDNF treatment to one group, electrical stimulation via a cochlear implant electrode to a second group, and combined therapy for a third group. The right (contralateral) ears served as untreated controls. Animals were sacrificed 43 days after deafening, and SG cells were counted. Treated ears exhibited significantly higher SG neuron survival than nontreated ears. The combined treatment with Ad.GDNF and electrical stimulation provided significantly better preservation of SG cell density than did either treatment alone. It is likely that the combined treatment with a neurotrophic factor (such as GDNF or BDNF) with electrical stimulation in
cochlear implant patients will enhance the benefits of cochlear implants [66].

TECHNICAL ISSUES RELATED TO PROTECTION VIA GENE THERAPY

Several technical findings pose a need for special consideration in the design and interpretation of gene therapy in the inner ear. One issue is related to findings that the vectors may reach the contralateral ear, resulting in contralateral transgene expression [67,68]. The route of transmission is most likely through the cochlear aqueduct, and the extent of contralateral gene transfer depends on the volume of inoculated vector [68]. It should be considered that cells lining the cerebrospinal fluid-filled ventricles must also be transduced. In protection experiments that are based on left-right animal comparisons, the extent of protection in the ipsilateral ear may be an underestimate, because the contralateral (control) ear may also be protected, owing to the contralateral transgene expression.

The influence of the surgical procedure and the vehicle control are not negligible. Several experiments have demonstrated protective effects of reporter gene inoculation against trauma in the organ of Corti [16, 18]. Although the mechanism for this phenomenon is not clear, it most likely involves the protective effect of a mild immune response due to the surgery or presence of the virus. Mild immune responses may be protective; however, a severe inflammation may lead to detrimental influence on the tissue. Such inflammation may certainly result from repeated inoculation with adenovirus or herpes simplex virus vectors. Treatment with immunosuppressive medications can reduce the negative consequences of repeated adenovirus-mediated gene therapy in the inner ear [69].

FUTURE GOALS

Gene therapy is a relatively new tool with great promise for future use. However, much work is needed to optimize gene transfer technology in the inner ear. Attempts to use gene therapy in the clinic for treatment of terminal diseases have resulted in several disappointing results. For instance, adenovirus-mediated gene therapy via the respiratory epithelium aimed at treating cystic fibrosis has not proved very successful [70,71]. Gene therapy using GDNF for treating Parkinson’s disease also had very limited success [72–75]. An attempt to treat ornithine transcarbamylase deficiency with increased adenovirus titer resulted in the death of one patient. These initial clinical failures and the tragic loss of a patient underlined the need for much basic work, for improving vectors and gene therapy protocols. In the last 2 years, however, the fruit of the enormous effort on behalf of the gene therapy community may be approaching a degree of ripeness, as a growing number of animal studies and clinical trials with are reporting positive outcomes.

Of particular interest are the encouraging data on initial successes in treating hereditary diseases with gene therapy. One recent success involves the treatment of hemophilia, as evidenced by several clinical trials. One approach currently being tried is intrathecal infusion of adeno-associated virus (AAV) vector carrying factor IX (deficient in hemophilia B) [76]. Another successful clinical trial involves treatment of X-linked severe combined immunodeficiency, which is a lethal disease [77]. In this case, treatment with transplanted stem cells previously transfected with the wild-type gene (ex vivo gene therapy) corrected the immune deficiency of affected patients. A third exciting example is an animal study aimed at treating one of the most clinically severe forms of congenital blindness. The disease, Leber’s amaurosis, is caused by retinal photoreceptor degeneration. An AAV vector carrying the wild-type RPE65 gene was used to treat dogs with a model disease and resulted in restoration of visual function [78]. This example is particularly relevant to inner ear gene therapy, as it involves a sensory system and a disease mechanism in which the sensory cells interact with their surrounding cells (retinal pigment epithelium in the eye) for normal maintenance or degenerative processes [78]. Gene therapy for cancer and cardiac disease has also seen significant successes in recent years [77,79,80].

With regard to inner ear gene therapy, our expanding knowledge of genes of interest along with the constant improvements in vector technology will likely facilitate future clinical trials. Proof of the principal that insertion of the wild-type gene can rescue an inner ear phenotype in a mutant mouse has been presented using transgenic insertion of wild-type BAC into shaker-2 mice [81]. Transgenic technology is not a viable therapeutic avenue for human disease. However, using somatic-cell gene transfer technology, treatment of hereditary inner ear disease, including tinnitus, may become possible.

One important future application of inner ear gene therapy is to induce regeneration of hair cells lost due to environmental insults or presbycusis. Regarding treatment for tinnitus and other inner ear diseases caused by environmental factors, it is necessary to determine the usefulness of gene therapy with antioxidants or antiapoptotic agents. Advances in vector technology will facilitate inner ear gene therapy by allowing us to achieve cell-specific transfection (or transduction) with
regulated gene expression mediated by nontoxic and nonimmunogenic vectors. Along with the identification of genes that encode molecules with potential applications for protection, repair, and regeneration in the inner ear, the development of gene transfer technology will likely equip us with new and sophisticated tools for treating tinnitus and other inner ear diseases.

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