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Spiral Ganglion Neurons Are Protected from Degeneration by GDNF Gene Therapy

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ABSTRACT

Perceptual benefits from the cochlear prosthesis are related to the quantity and quality of the patient's auditory nerve population. Multiple neurotrophic factors, such as glial cell line-derived neurotrophic factor (GDNF), have been shown to have important roles in the survival of inner ear auditory neurons, including protection of deafferented spiral ganglion cells (SGCs). In this study, GDNF gene therapy was tested for its ability to enhance survival of SGCs after aminoglycoside/diuretic-induced insult that eliminated the inner hair cells. The GDNF transgene was delivered by adenoviral vectors. Similar vectors with a reporter gene (*lacZ*) insert served as controls. Four or seven days after bilateral deafening, 5 μl of an adenoviral suspension (Ad-GDNF or Ad-lacZ) or an artificial perilymph was injected into the left scala tympani of guinea pigs. Animals were sacrificed 28 days after deafening and their inner ears prepared for SGC counts. Adenoviral-mediated GDNF transgene expression enhanced SGC survival in the left (viral-treated) deafened ears. This observation suggests that GDNF is one of the survival factors in the inner ear and may help maintain the auditory neurons after insult. Application of GDNF and other survival factors via gene therapy has great potential for inducing survival of auditory neurons following hair cell loss.

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INTRODUCTION

Cochlear implant technology has expanded rapidly over the last decade. The cochlear implant has provided great benefits to patients with profound hearing impairment. The implant sends sound to the speech processor and transforms the acoustic energy into an electrical signal that is passed to an electrode implanted within the cochlea. The functional state and the number of surviving spiral ganglion cells (SGCs) are among the factors that determine the success of the procedure. This article addresses the means of reducing SGC loss by adenoviral-mediated gene transfer leading to overexpression of glial cell line-derived neurotrophic factor (GDNF).

Following destruction of hair cells in the organ of Corti, spiral ganglion cells degenerate (Webster and Webster 1981; Sutton and Miller 1983; Jyung et al. 1989; Nadol 1990; Leake et al. 1991; Otte et al. 1978). There have been several reports of successful prevention or reduction of SGC degeneration, through the use of electrical stimulation, GM1 ganglioside, and, in particular, neurotrophins (Hartshorn et al. 1991; Lousteau 1987; Leake et al. 1991; Miller et al. 1997; Mitchell et al. 1997; Staecker et al. 1996; Ernfors et al. 1995, 1996; Schindler et al. 1995; Shah et al. 1995).

Neurotrophins and their receptors have been shown to be expressed in the developing and mature

cochlea and to have an important role in the development of the auditory system (Pirvola et al. 1992, 1994; Farinas et al. 1994; Jones et al. 1994; Ernfors et al. 1994, 1995; Despres and Romand 1995; Liu et al. 1995; Fritzsch et al. 1997, 1999). Studies have also begun to define the effects of providing neurotrophic factors on ear tissues and to determine the changes in the inner ear that occur in transgenic mice which do not express these survival factors. Some neurotrophins, such as BDNF,NT-3, and NGF, have been shown to protect SGCs and hair cells from trauma (Staecker et al. 1996; Miller et al. 1997; Ernfors et al. 1996; Schindler et al. 1995; Shah et al. 1995). By the late stages of embryogenesis, mice lacking the genes for production of both NT-3 and BDNF completely lack type I and type II spiral ganglion neurons, suggesting that these factors are essential for normal development and maintenance of spiral ganglion neurons (Ernfors et al. 1994, 1995; Farinas et al. 1994).

GDNF, the first member of a new family of trophic factors distantly related to the transforming growth factor-β superfamily, was initially isolated and cloned as a factor produced by B49 rat glial cells with pronounced trophic effects on cultured fetal rat midbrain dopamine neurons (Lin et al. 1993, 1994). Several studies from a number of laboratories have shown that GDNF is synthesized by many cell types throughout the body and affects the development and survival of a diverse set of neuronal and non-neuronal cells (Moore et al. 1996; Pichel et al. 1996; Henderson et al. 1994; Sanchez et al 1996; Buj–Bello et al. 1995; Trupp et al. 1995). The expression of mRNAs encoding GDNF is present in developing outer hair cells (OHCs) and inner hair cells (IHCs) and in the mature IHCs (Ylikoski et al. 1998). The GDNF family receptor α-1 (GFRα-1, formerly GDNFR-α) was detected in spiral ganglion neurons and in their non-neuronal sheath cells (Ylikoski et al. 1998; Stöver et al. 2000). GDNF has recently been shown to protect hair cells and SGCs of the cochlea against various insults (Ylikoski et al. 1998; Shoji et al. 2000; Keithley et al. 1998; Yagi et al. 1999).

Recombinant viral vectors have proved to be therapeutically successful in animal models; for example, in correcting genetic abnormalities, inhibiting inflammatory cascades to reduce brain injury, and providing trophic support for neuronal tissues. Studies investigating cochlear gene therapy have been initiated using a variety of viral vectors and rodents as the animal model (Geschwind et al. 1996; Lalwani et al. 1996, 1997, 1998; Raphael et al. 1996; Staecker et al., 1998; Weiss et al. 1997). The cochlea possesses several advantages as a target organ for gene transfer. Although it is encased in the temporal bone and is relatively isolated except through the endolymphatic duct and sac and the cochlear aqueduct, inoculation with vectors is technically feasible. The enclosed anatomy prevents the spread of viral particles to adjacent tissues. However, cochlear fluid spaces allow diffusible particles, such as viruses or proteins, to disperse rapidly throughout the cochlea. This is especially advantageous for restricting GDNF gene transfer to the inner ear since GDNF also functions in the kidneys and the enteric nervous system. Additionally, the blood–labyrinth barrier limits systemically administered GDNF concentration in the inner ear.

Among the different viral vector systems that exist, the adenoviral-mediated gene transfer system offers several important advantages as a tool for direct somatic cell gene delivery in the cochlea. The E1 and E3 genes are deleted, preventing viral DNA replication, and high titers of virus stocks are available. The adenovirus genome generally does not integrate into the host cell's chromosome, thus potentially limiting insertional mutagenesis. Finally, it is possible to infect quiescent as well as replicating cells. These advantages have enabled the development of adenoviral systems as effective drug delivery devices.

Here we report on our studies to test the influence of the viral-mediated GDNF transgene on SGC survival after aminoglycoside/diuretic-induced inner hair cell degeneration in guinea pigs.

MATERIALS AND METHODS

Thirty-two pigmented guinea pigs were used in these studies. Animals were outbred in the Murphy supplier colony. All animal experiments were approved by the University of Michigan Institutional Committee on the Use and Care of Animals and were performed using accepted veterinary standards.

Adenoviral vectors

We have used two replication-deficient recombinant adenoviruses. Both were based on the human adenovirus serotype 5 in which three transcriptional regions (E1A and E1B and a portion of the E3) have been deleted. The inserts in both vectors were driven by the cytomegalovirus (CMV) immediate promoter. The experimental vector contained the human GDNF gene and was designated Ad-GDNF. The control vector contained the *E. coli* β-galactosidase cDNA and was designated Ad-CMVntlacZ. Both vectors were prepared according to standard procedures (Lapchak et al. 1997; Akli et al. 1993). Viral suspensions in 10% glycerol were kept at -80°C until thawed for use.

Procedures to confirm deafening

Guinea pigs were deafened with a single dose of kanamycin (400 mg/kg SC). Two hours later, animals were prepared for cannulation of the jugular vein, as previously described (West et al. 1973), and infused with ethacrynic acid (40 mg/kg IV). Deafening was verified by auditory brainstem response (ABR) audiometry and visual assessment of hair cell damage in surface preparations. ABRs (4-, 12-, and 20-kHz pure tones) were performed twice before deafening (to determine baseline thresholds) and just before sacrifice. Animals were anesthetized with a mixture of xylazine (10 mg/kg IM) and ketamine (40 mg/kg IM), and needle electrodes were placed subcutaneously. The reference electrode was inserted beneath the pinna of the measured ear, the ground beneath the opposite ear, and the active electrode beneath the skin on the top of the head. The stimulus duration was 15 ms, the presentation rate 10/s, and the rise/fall time 1 ms. Around threshold, responses for 1024 sweeps were averaged at each intensity level, in 5-dB-SPL steps. Threshold was determined by visual inspection of the responses and defined as the lowest intensity level at which a clear waveform was visible in the evoked trace. Threshold shifts were calculated for individual animals by comparing thresholds immediately before sacrifice with the irbaseline thresholds.

Animals were deeply anesthetized and decapitated, and the ears were removed for histological evaluation just after ABRs. The inner ears were fixed in 4% paraformaldehyde for 2–3 hours. In all animals, the otic capsule, lateral wall, and tectorial membrane were removed, and the bony modiolus with the organ of Corti was carefully detached at the base of the cochlea. At this stage, tissues were permeabilized with 0.3% Triton X-100 in PBS for 10 min, then incubated for 30 min with rhodamine phalloidin (Molecular Probes, Eugene, OR) diluted 1:100 in PBS at room temperature. After washing, the organ of Corti turns were separated from the modiolus, mounted on microscope slides with Crystal/Mount (Biomeda Co., City, State), and examined for hair cell loss to confirm deafening. Tissue was photographed using a Leica DMRB epifluorescence microscope.

Deafening and viral administration

Bilateral deafening was performed in the 24 experimental animals as described above, 4 days or 1 week prior to viral injection. Animals were anesthetized with an intramuscularly administered solution of Rompun (xylazine, 10 mg/kg; Bayer, Shawnee Mission, KS) and Ketalar (ketamine HCL, 40 mg/kg; Parke Davis, Morris Plains, NJ). Chloramphenicol sodium succinate (30 mg/kg IM) was administered as prophylaxis, and 0.5 ml of 1% lidocaine HCl was injected subcutaneously for local anesthesia. The left temporal bone was exposed postauricularly and perforated to reveal the round window. Using a plastic cannula connected to the bent tip of a 30 gauge needle and a 25-μl Hamilton syringe, 5μl of an isotonic adenoviral suspension (approximate concentration of 10^{10} adenoviral particles per milliliter in sterile normal Ringer's solution) or artificial perilymph (AP) (sterile normal Ringer's solution) was injected into the scala tympani through the round window membrane. Animals inoculated with the Ad-GDNF were designated as the experimental group (inoculation 4 days after deafening, $N = 5$; 1 week after deafening, $N = 7$), animals inoculated with an Ad-lacZ vector served as the viral control (1 week after deafening, $N = 4$), and those receiving AP were the vehicle control (4 days after deafening, $N = 4$; 1 week after deafening, $N = 4$). After inoculation, the bulla was sealed with Durelon and the skin was closed with sutures. Subcutaneous postoperative administration of 5 ml of 0.9% saline provided rehydration. In all groups, the right ears were used as noninoculated controls.

Tissue processing

At the completion of the experiment (28 days after deafening), the animals were deeply anesthetized, perfused intracardially with 2% paraformaldehyde and 2.5% glutaraldehyde, and decapitated, and the ears were removed for histological evaluation. The inner ears were postfixed in the same fixative for 2–3 hours. Cochleae were decalcified in 3% EDTA with 0.25% glutaraldehyde for 2 days. Tissues were then dehydrated in a series of ethanol and embedded in JB-4 (Electron Microscopy Scientific, Washington, PA). Sections were obtained at the midmodiolar area (3 μm thick) with every fifth section kept, stained with toluidine blue, and cover-slipped with Permount (Fisher Scientific, Pittsburgh, City, State).

An unbiased investigator inspected the collection of sections generated for each cochlea and selected the slides which had high-quality sections. Sections which had folds in the plastic, over- or understaining, or uneven thickness were eliminated. From the remaining high-quality sections, three sections from each cochlea were randomly chosen for counting.

In the guinea pig cochlea, midmodiolar sections include seven regions of Rosenthal's canal. We assessed the number of SGCs in three turns (basal, second, and third), always on the same side of the modiolus. The chosen slides were given a code name which was blind to the person who performed the counting. All neurons present in the section along with their nucleus were counted. SGC density (SGC number/10,000 μm^2) was calculated using Meta-Morph computerized

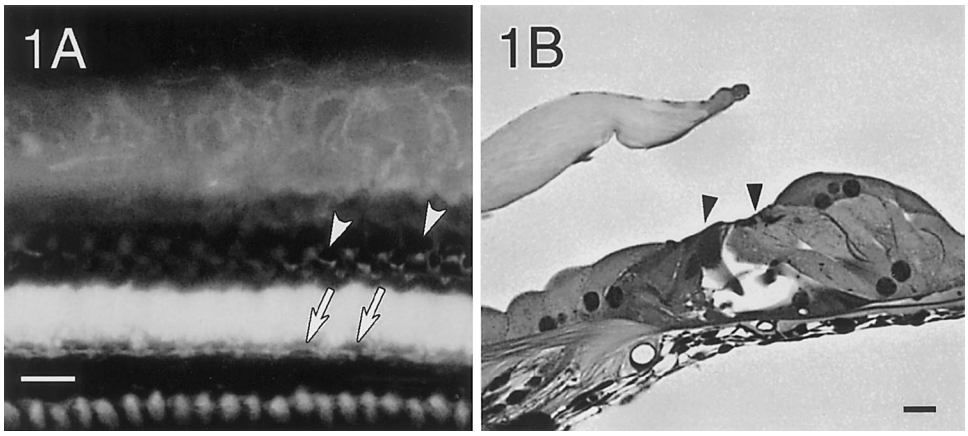

FIG. 1. A. A phalloidin-labeled organ of Corti specimen taken from the second turn of a cochlea 4 days after the deafening procedure. The focal plane is that of the inner hair cells, demonstrating a complete hair cell loss and typical scar formation (arrows). Each arrow points to the center of a scar, in two adjacent scars. Scars in the region of the outer hair cells can also be seen (arrow heads). **B**. Midmodiolar section of a guinea pig cochlea 28 days after deafening stained with toluidine blue. Both inner (arrowhead on left) and outer hair cells (arrowhead on right) are missing. Scale bars: (**A**) 20 μm, (**B**) 10 μm.

image analysis system (Miller et al. 1997; Komeda et al. 1999). The entire area of Rosenthal's canal in each counted section was circled for the purpose of area calculation (performed by the software). The boundaries of the canal were drawn at the interface between the bone and the soft tissue within Rosenthal's canal. After counting the three sections of each cochlea, the average value of the SGC density was calculated for each cochlea. Sigma Stat™ statistical software, (SPSS Science, Chicago, IL) was used for statistical analysis. Comparisons and statistical significance calculations of differences between inoculated and control (noninoculated) ears were made with two-sample *t* tests. Comparisons between several groups were accomplished by ANOVA. We also examined 4 nontreated cochleae with no deafening or inoculation and compared between experimental animals and nontreated (normal) cochleae (SGC percent survival = deafened ears/normal $\times$ 100).

Assay of vector activity

To test the ability of the Ad-GDNF to mediate production of GDNF in transduced cells, we performed an assay on cultured guinea pig fibroblasts. We used a fibroblast cell line (kindly provided by Dr. Thomas Carey) that had previously been described(Ptok et al. 1991). Cells were resuspended with trypsin–EDTA (Gibco-BRL, Grand Island, NY) and seeded on precleaned coverslips. The coverslips were placed in a six-well culture dish and maintained in DMEM (Gibco-BRL, Grand Island, NY) with 10% FBS at 37°C and 5% $C0_2$. Once cells had reached a near-confluence stage, the Ad-GDNF (1×10^{12} pfu/ml) solution was added into the culture. Four hours later, it was rinsed by replacing the culture media. Twenty-four hours later, the coverslips were removed from the incubator, briefly rinsed in PBS, and fixed in 4% paraformaldehyde in phosphate buffer for 1 hour. The specimens were then processed for GDNF-specific immunocytochemistry. The fixed cells were permeabilized with 0.3% Triton X-100 for 10 minutes, incubated in a polyclonal anti-GDNF antibody (Santa Cruz Biotechnology Inc. Santa Cruz, CA) for 60 minutes, rinsed in buffer and incubated with rhodamine conjugated goat–antirabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) for 30 minutes. Following a final rinse, cells were mounted on microscope slides with Crystal/Mount (Biomedia, Foster City, CA). The two negative controls were cells that were not exposed to the Ad-GDNF and cells that were treated with the vector, but the primary antibody was omitted from the reaction. Samples were analyzed and photographed with a Leica DMRB epifluorescence microscope.

RESULTS

Deafening

The deafening procedure used for these experiments was tested on a dedicated group of six animals that were killed four days ($N = 3$) and one week ($N = 3$) after deafening. The deafening procedure resulted in a complete or nearly complete loss of both IHCs and OHCs. In two animals from the four-day group, a few OHCs remained at the apex, and in the remaining animals, no remaining hair cells could be observed (Fig. 1A). Deafening was also confirmed in these animals by ABR testing. All animals ($n = 6$) had threshold shifts over 75 dB SPL across the test frequencies 1 week and 4 days after the deafening procedure.

In addition to the six animals that were used for deafening verification, the morphology of the organ of Corti could also be observed in the midmodiolar section used to count SGCs in the experimental groups. All cochlear sections from the experimental

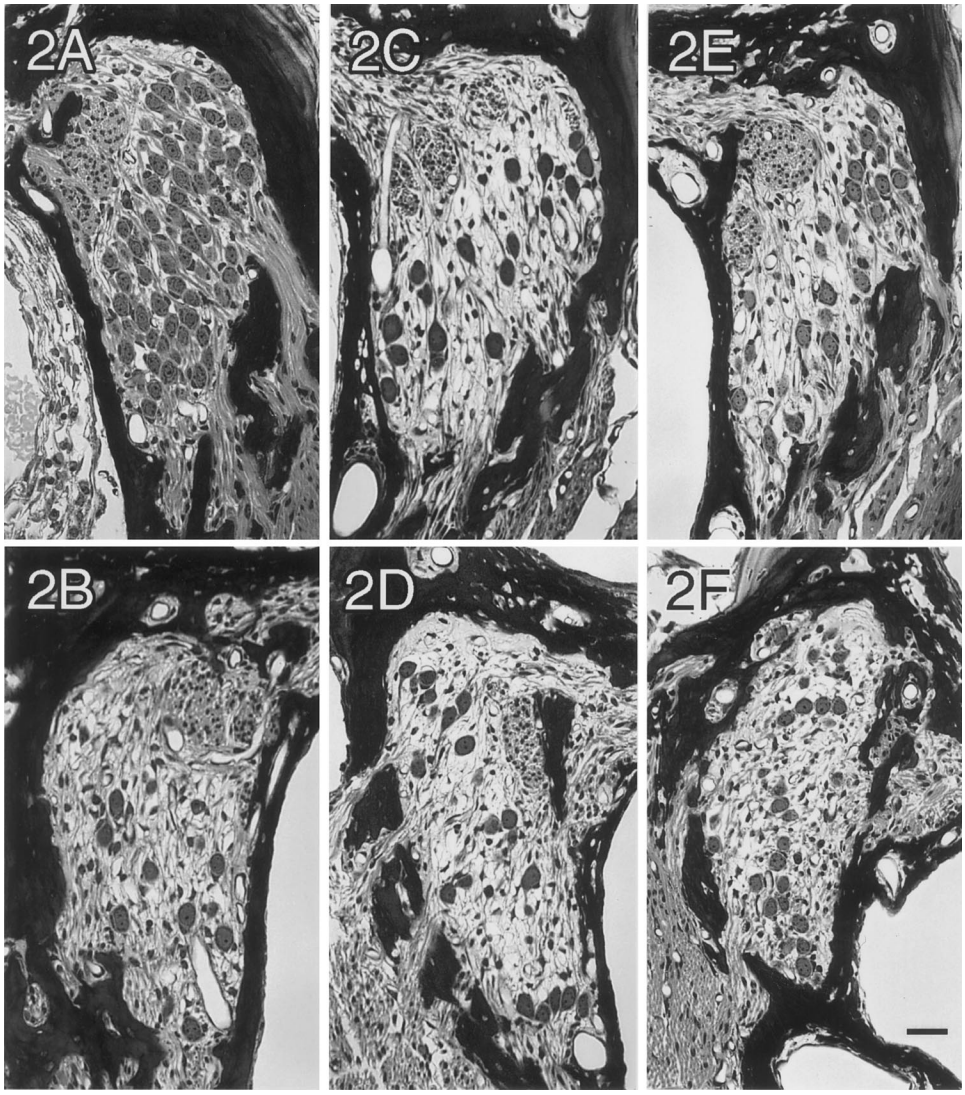

FIG. 2. Light micrographs of Rosenthal's canal at the basal turn of specimens taken from deafened animals (experimental groups). Midmodiolar sections of the basal part of the cochleae that received Ad-GDNF (**A**: inoculated ear, left: **B**: control ear, right), Ad-lacZ (**C**: inoculated ear, left: **D**: control ear, right), and AP (**E**: inoculated ear, left: **F**: control ear, right) stained with toluidine blue. The Ad-GDNF-inoculated ear (**A**) has better preservation of SGCs after aminoglycoside-induced trauma compared with the noninoculated right ear (**B**). In other treated ears (Ad-lacZ- and AP-inoculated ears, **C** and **E**) and noninoculated ears (right) (**B, D**, and **F**), there is a severe loss of spiral ganglion neurons. Scale bar-25 μm.

groups showed a complete loss of IHCs from base to apex (Fig. 1B).

SGC density

The control cochleae (Ad-lacZ, AP, or noninoculated ears) showed a severe loss of spiral ganglion neurons (Fig. 2) as previously demonstrated after similar trauma to the organ of Corti (Komeda et al. 1999; Miller et al. 1997).

Inoculation 4 days after deafening. In animals deafened 4 days before the inoculation, the extent of neuronal degeneration in the control cochleae (nine deafened, nontreated right cochleae; four left cochleae treated with AP) gradually became more severe toward the base. There was a statistically significant SGC survival for Ad-GDNF-inoculated ears at 28 days after deafening compared with the noninoculated ears and the AP-inoculated ears in the basal turn ($p < 0.05$) but not in the middle and upper turns (Figs. 3 and 4).

Inoculation 1 week after deafening. In animals deafened 1 week before the inoculation, the extent of neuronal degeneration in the control cochleae (11 deafened, nontreated; 4 treated with AP) was similar from base to apex (averaging 46.1%–60.3% SGC survival). Although there was some quantitative variability among the animals receiving Ad-GDNF, SGC density in all left ears receiving Ad-GDNF was higher in the basal and middle turns than in the opposite ears and the control ears (Figs. 5 and 6). In some left ears receiving the Ad-lacZ, the density of SGCs in the basal turn (averaging 26.8% survival) was less than in the opposite ears and the other control ears. In the middle and basal turns, but not in the upper turn, there was a statistically significant ($p < 0.05$) survival of SGCs for Ad-GDNF-inoculated ears at 28 days after deafening compared with the noninoculated ears (Fig. 5). There

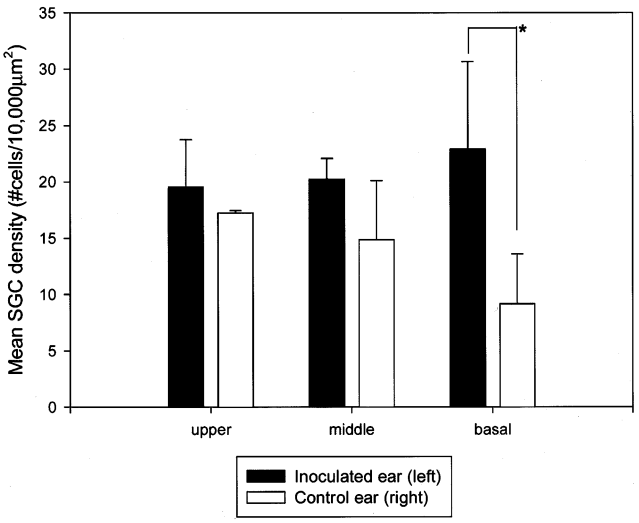

FIG. 3. The average spiral ganglion neuron density in the Ad-GDNF-treated group (4 days after deafening, $N = 5$). The inoculated (left) ears show statistically significant protection compared with noninoculated (right) ears ($N = 4$) in the basal turn ($p < 0.05$).

were also statistically significant differences between Ad-GDNF groups, and bothAd-lacZ and AP groups' ears in the middle and basal turns ($p < 0.05$) but not in the upper turn (Fig. 6). There was a trend toward lower SGC density in the Ad-lacZ-inoculated ears than in the AP-inoculated ears in the basal portion of the cochlea (Fig. 6).

The average SGC percent survival in the ears inoculated with Ad-GDNF 4 days after deafening (upper turn, 70.7%; middle turn, 61.5%; basal turn, 89.4%) is rather similar to that of the ears inoculated 1-week after deafening (upper turn, 65.4%; middle turn, 65.9%; basal turn, 88.6%). There is a trend for higher density of cells in the 4-day group. The data are summarized in Table 1.

Ad-GDNF tested in guinea pig fibroblast culture

Cells in the two negative controls did not show immunoreactivity with the GDNF-specific antibody (data not shown). In contrast, the cultures that were treated with the Ad-GDNF contained cells that stained positive for the antibody (Fig. 7).

DISCUSSION

This article describes a successful gene transfer procedure in the inner ear *in vivo* to protect SGCs after aminoglycoside/diuretic-induced cochlear insult. The kanamycin/ethacrynic acid deafness protocol yields a severe loss of hair cells in the entire cochlea and profound hearing loss. The loss of IHCs leads to a secondary degeneration of SGCs, as previously determined (Webster and Webster 1981; Komeda et al. 1999; Miller et al. 1997).

It remains unknown how the degeneration of deafferented spiral ganglion neurons occurs. It seems reasonable, however, that disruption of the trophic support and neuronal interaction between the hair cells and SGCs would be associated with the degeneration of SGCs. Neurotrophic factors are important for the development and maintenance of the nervous system, as well as the inner ear. These factors promote

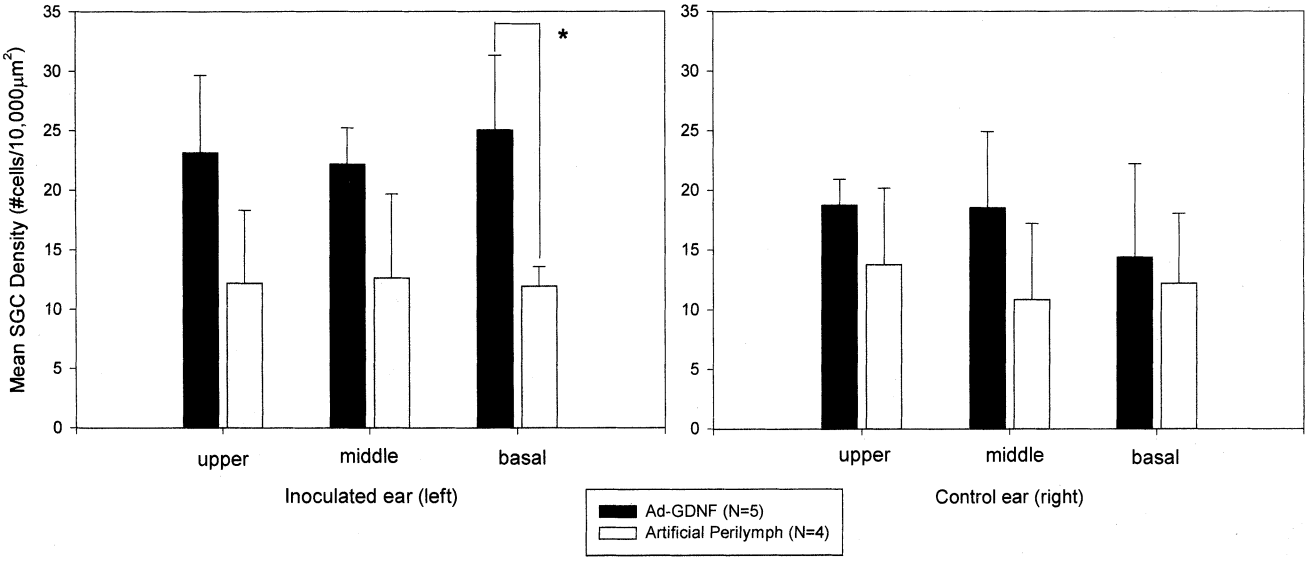

FIG. 4 Comparison between the Ad-GDNF and the AP treatment is demonstrated by presenting the mean spiral ganglion neuron density (and SD) among these groups in the 4-day interval animals. The SGC density in the Ad-GDNF-inoculated ears is significantly higher than the AP-inoculated ears in the basal turn ($p < 0.05$). The contralateral (right) ears have a tendency for higher neuronal density in the Ad-GDNF group compared with the AP group.

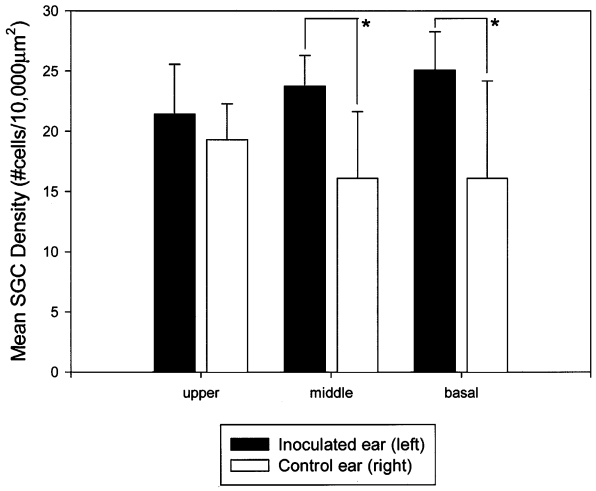

FIG. 5 The average spiral ganglion neuron density (number of cells/10,00 μm) and S.D. (error bar) in the Ad-GDNF-treated group (1 week after deafening, $N = 4$). A comparison between the SGC density in all inoculated (left) vs. noninoculated (right) ears demonstrates statistically significant protection in the middle and basal turns ($p < 0.05$).

neuronal survival and can prevent the neuronal degeneration associated with injury, toxin exposure, and neurodegenerative disease. Several studies using the osmotic pump infusion for chronic delivery of neurotrophic factors into the rodent cochlea have been reported. The chronic infusion of BDNF, NT-3, NGF, and GDNF can provide SGC survival after IHC insults (Ernfors et al. 1996; Staecker et al. 1996; Shah et al. 1995; Miller et al. 1997; Ylikoski et al. 1998). Viral-mediated gene transfer of BDNF using a replication-defective herpes simplex-1 vector has been shown to have a significant positive effect on the survival of denervated spiral ganglion neurons in the mouse (Staecker et al. 1998). Interaction between two or more neurotrophic factors is likely to influence the spiral ganglion. For instance, it has been shown that GDNF promotes survival of axotomized corticospinal neurons via BDNF (Giehl et al. 1998). It is therefore possible that GDNF may influence the spiral ganglion indirectly, via upregulating expression of BDNF or other neurotrophic factors.

GDNF is a distantly related member of the transforming growth factor-β superfamily, which acts as a potent survival factor for both the peripheral and the central nervous system. GDNF is a potent survival factor for a variety of nerve cells, such as dopaminergic neurons (Choi–Lundberg et al. 1997; Gash et al. 1996), motoneurons (Henderson et al. 1994), sensory or autonomic neurons (Buj–Bello et al. 1995), as well as corticospinal neurons (Giehl et al. 1998). GDNF is also an essential factor for the development of the enteric nervous system and the kidney during embryogenesis (Moore et al. 1996; Pichel et al. 1996;

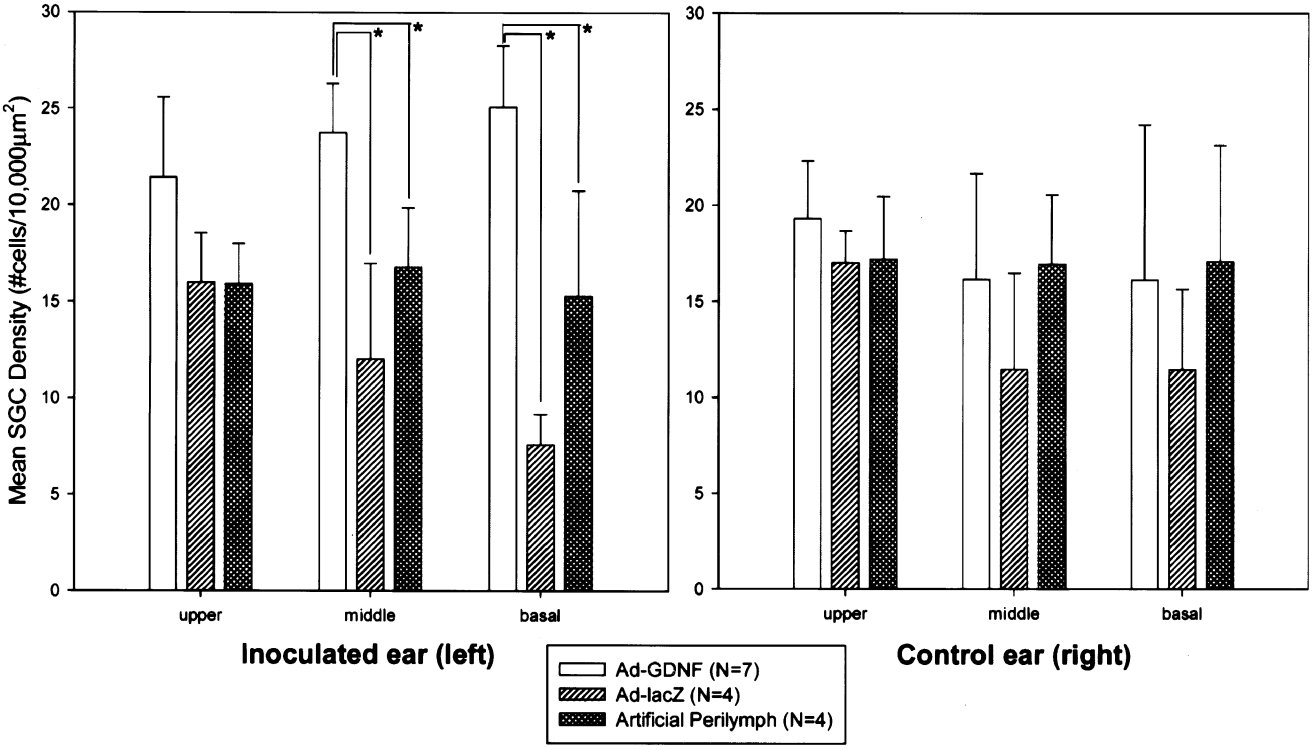

FIG. 6 The mean spiral ganglion neuron density among groups and S.D. (error bar) (1 week after deafening). The SGC density in the Ad-GDNF-inoculated ear is significantly higher than those of the Ad-lacZ and AP inoculated ears in the middle and basal turn ($p < 0.05$).

TABLE 1

Density of SGC in the experimental and control groups

	SG cell survival % (experimental ear/normal ear × 100)					
	Inoculated ear			*Noninoculated ear*		
	Upper	*Middle*	*Base*	*Upper*	*Middle*	*Base*
4 days after deafening						
Ad-GDNF	70.66	61.50	89.45	57.29	51.51	50.85
AP	37.23	35.08	42.09	42.19	30.14	43.10
7 days after deafening						
Ad-GDNF	65.44	65.95	88.55	58.99	44.77	56.91
Ad-Lac Z	48.87	33.36	26.82	51.98	31.80	40.42
AP	48.60	46.59	54.02	46.10	47.04	60.34

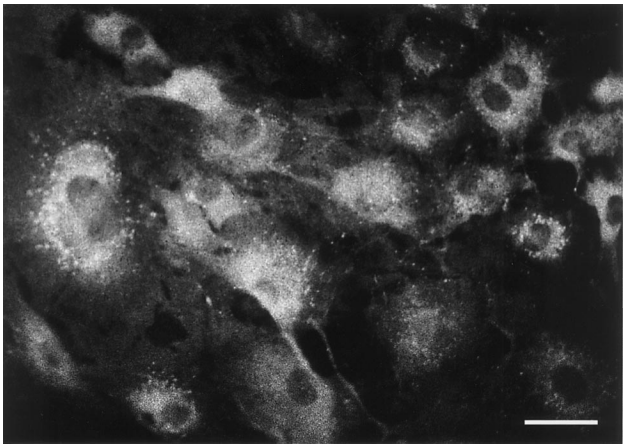

FIG. 7 Epifluorescence image of cultured guinea pig fibroblasts that were incubated with the Ad-GDNF vector and stained with a GDNF-specific antibody. The nuclei are negative whereas the cytoplasm stains positively with this antibody. Scale bar-10 μm.

Sanchez et al. 1996). In addition, GDNF has been shown to promote neurite outgrowth in the enteric nervous system (Schafer and Mestres 1999) and motoneurons (Zurn et al. 1994).

The mechanism of GDNF signaling involves two receptors. It first binds to the Ret receptor, then to a coreceptor, a tyrosine kinase and glycosylphosphatidylinositol (GPI)-linked receptor named GFRα (Jing et al. 1996; Treanor et al. 1996; Durbec et al. 1996). mRNAs encoding GDNF have been detected in the developing OHCs and IHCs and in the mature IHCs (Ylikoski et al. 1998). The GFRα-1 has been detected in the spiral ganglion neurons and in their non-neuronal sheath cells during development and in adult animals. Using RT-PCR, Stöver et al. (2000) demonstrated that GDNF, GFR-1, and c-ret mRNA were detected in the specific cochlear tissues, including the modiolus. The level of Ret expression in the normal inner ear was rather low (Stöver et al. 2000). However, to explain the protective effect of exogenous GDNF, it is possible that the trauma in the cochlea leads to upregulation of GDNF or its receptors. An alternative explanation is that GDNF might act on SGCs through an unidentified pathway or different receptors, e.g., GFRα-2, to which GDNF binds with lower affinity.

Once the protective pathway is activated, GDNF is likely to prevent apoptosis in the spiral ganglion. TUNEL positive cells were demonstrated in the SGCs in the aged gerbil cochlea (Zheng et al. 1998). Pai et al. (1998) showed apoptosis of cochlear spiral ganglion neurons after nitric oxide exposure by DNA fragmentation and the TUNEL method. These studies suggest that spiral ganglion neurons degenerate by undergoing active cell death. GDNF has been shown to suppress apoptosis of developing and postnatal substantia nigra dopamine neurons in vitro (Burke et al. 1998; Clarkson et al. 1997) and is likely to have similar capability in the spiral ganglion *in vivo*. More work will be necessary to understand the protective mechanism of GDNF on the denervated spiral ganglion.

At this stage, the transgenic expression of GDNF is unregulated. The data generated with the cultured guinea pig fibroblasts demonstrate the ability of the vector to transduce guinea pig fibroblasts and lead to synthesis of the gene product. Using adenovirus vectors with a reporter gene insert, we have previously shown that the transduced inner ear cells were mostly fibrocytes lining the perilymphatic space (Yagi et al. 1999). Based on these two sets of data we speculate that inoculating the inner ear with Ad-GDNF resulted in transduction of the connective tissue cells lining the perilymphatic space. These cells, in turn, secreted GDNF into the perilymph, making it accessible to the spiral ganglion.

In reporter gene experiments, the transduced cells were seen mostly in the basal turn and lower second turn. Although these cells secreted GDNF which diffused throughout the cochlea from base to apex, the protective effects were more robust toward the basal

turn. One of the possible explanations of this gradient is that the basal turn might contain the highest concentration of GDNF molecules. The reasons for the variability and gradient in the protective effect of GDNF need to be elucidated. Moreover, to design a clinically relevant application of this methodology, it will be necessary to use vectors that enable regulated gene expression of the inserted transgene.

The timing of gene transfer and the duration of transgene expression are important factors in the success of the procedure. Although most SGCs are present one week after deafening, the trophic support provided by a peripheral source and neuronal activity must already be decreased at that point. We speculate that earlier inoculation could provide better preservation of SGCs after deafening. The distribution and extent of the gene expression appeared stable at least three weeks after the inoculation (Raphael et al. 1996), but the level of GDNF expression may be decreased. In addition to the delivery technique, vector technology also needs to be improved to facilitate clinical feasibility. In addition to regulated gene expression, it should also be desirable to have vectors that are nonimmunogenic and nonpathogenic, and to achieve cell type-specific infection.

Electrical stimulation enhances the survival of denervated SGCs (17, 28). To assess the clinical applicability of gene therapy with neurotrophic factors, it is necessary to determine the cumulative effect exerted by electrical stimulation and GDNF transgene over expression on neuronal survival. Other combination therapies should also be examined, e.g., overexpression of GDNF with various neurotrophins and other agents. The studies reported here indicate that adenoviral-mediated overexpression of GDNF significantly enhances SGC survival in the high-frequency region of the cochlea after IHC elimination caused by the combination of kanamycin and ethacrynic acid. The data suggest that GDNF may be involved in the maintenance of denervated SGCs, directly or indirectly. Although gene transfer technique in the inner ear must be improved before gene therapy can become a realistic therapeutical strategy in humans, the application of GDNF and other growth factors via gene transfer may be useful for enhancing the survival of the auditory nerve following deafness.

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