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Gene transfer into the mammalian inner ear using HSV-1 and vaccinia virus vectors

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Abstract

The introduction of foreign genes into cells has become an effective means of achieving intracellular expression of foreign proteins, both for therapeutic purposes and for experimental manipulation. Gene delivery to the nervous system has been extensively studied, primarily using viral vectors. However, to date less work has focused on gene delivery to the inner ear, and existing studies have primarily used adenovirus and adeno-associated virus. Using two recombinant viral vectors, herpes simplex type 1 (HSV-1), and vaccinia virus, bearing the *Escherichia coli lacZ* gene, we tested gene delivery to the guinea pig cochlea in vivo with β-galactosidase staining as an assay. The HSV-1 and vaccinia virus vectors were both found to infect and elicit transgene expression successfully in many cells in the guinea pig cochlea, including cells in the organ of Corti. These data demonstrate the feasibility of gene delivery to the inner ear using these two viral vectors. Such techniques may facilitate study of the auditory systems, and might be used to develop gene therapy strategies for some forms of hearing loss.

Key words: Gene transfer; Gene therapy; Viral vector; Herpes simplex virus; Vaccinia virus; Cochlea; Inner ear

1. Introduction

The efficiency of viral vectors in transferring foreign DNA into cells of the nervous system has provided a powerful method to manipulate cellular function. Although a great deal of effort has been directed at gene transfer to the central nervous system or to sensory ganglia, there has been less work done to date in gene delivery to the inner ear. Hearing loss resulting from the death of hair cells in the organ of Corti or neurons in the spiral ganglion could be prevented by the appropriate in vivo delivery of genes encoding neurotrophic factors. Gene replacement therapy could be used in some forms of inherited deafness. Eventually, factors promoting hair cell regeneration might be delivered as a means of restoring hearing loss.

Herpes simplex virus type 1 (HSV-1) vectors have become one of the most commonly used vectors to deliver foreign genes to post-mitotic cells in the mammalian nervous system and have been used successfully as gene therapy vehicles in several models of neurological dysfunction (for reviews see Suhr and Gage, 1993; Friedmann, 1994; Smith et al., 1995; Kelley, 1997). Vaccinia virus vectors have also emerged as an effective means of achieving transient expression of foreign proteins in both mammalian (Pettit et al., 1994, 1995) and amphibian (Wu et al., 1995) neurons.

The purpose of the present study was to assess the relative efficacy of gene delivery to the inner ear, including cell specificity and virus toxicity, using two viral vectors. High titer recombinant HSV-1 and vaccinia virus vectors bearing the *E. coli lacZ* gene were pressure injected into the guinea pig cochlea in vivo, and the

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tissues were later assayed for the presence of the *lacZ* gene product, β-galactosidase (β-gal). The HSV-1 and vaccinia virus vectors were found to infect and elicit transgene expression in many cell types. In the guinea pig cochlea, cells lining the scalae and cells in and around the organ of Corti expressed β-gal, suggesting that these vectors might be useful as gene therapy vehicles in the inner ear.

A preliminary report of this work was presented in abstract form (Derby et al., 1995).

2. Materials and methods

2.1. Viral vectors

All procedures involving viruses were performed in accordance with the Harvard Office of Biological Safety. The HSV-1 vector used in this study, designated *hr*R3, was obtained from Dr. S. Weller (University of Connecticut Medical School), and its construction has been described previously (Goldstein and Weller, 1988). This recombinant vector has the *E. coli lacZ* gene inserted in the ribonucleotide reductase (RR) gene locus, thereby disrupting RR expression. Expression of *lacZ* is driven by the ICP6 (RR) viral promoter. The *hr*R3 vector was passaged on African green monkey (Vero) cells, and plaque formation assays on Vero cell monolayers were used to monitor viral titers, which were approximately 1×10^{10} plaque forming units (pfu)/ml.

Recombinant vaccinia virus vSC56 was generated by Drs. S. Chakrabarti and B. Moss (National Institute of Allergy and Infectious Diseases) in HeLa cells. This vector has a thymidine kinase-negative/*lacZ*-positive ($TK^-/lacZ^+$) genotype and contains a strong synthetic early/late promoter regulating transcription of the *lacZ* gene (Chakrabarti et al., 1997). Virus stocks of 6.0×10^9 pfu/ml used for injections were prepared as described (Mackett et al., 1985; Earl and Moss, 1991).

Stocks of all vectors were stored at −80°C prior to their use.

2.2. In vivo experiments

2.2.1. Animal surgery and injections

All animal studies were performed in accordance with guidelines issued by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Viral inoculations and care of inoculated animals were performed in specially designated virus rooms. Albino-Hartley guinea pigs (150–200 mg) obtained from Elm Hill Breeding Labs (Chelmsford, MA, USA) were anesthetized with ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.) prior to surgery. The external auditory meatus and portions of the tympanic membrane were removed to expose the round window. Virus was injected with a glass micropipette (20 μm tip diameter) containing approximately 2 μl of viral suspension (*hr*R3, 2.0×10^7 pfu; vSC56, 1.2×10^7 pfu). The micropipette was inserted through the round window, and the complete volume of viral inoculum was gradually delivered (over a 2 min time span) using a micrometer-controlled hydraulic syringe. Control animals received an identical injection of approximately 2 μl of a 0.9% sodium chloride solution. Immediately after injection, the micropipette was removed, the wound was closed with surgical staples, and the animals were returned to their cages and allowed to recover.

2.2.2. Histology

At 2, 4, or 6 days, animals were reanesthetized with ketamine (120 mg/kg) and xylazine (13 mg/kg) and then killed by decapitation. Bullae containing injected cochleae were removed and excess bone and tissue were cut away to expose the cochleae. Cochleae were immediately placed in a petri dish containing 0.1 M phosphate-buffered saline, pH 7.4 (PBS). Under a dissection microscope, the round and oval windows were opened, and the scala tympani and scala vestibuli were perfused with approximately 10 μl of 10% formalin followed by 10 μl of a 2% solution of acetic acid injected separately through the round and oval windows with a Hamilton syringe. Following perfusion, the cochleae were placed in 10% formalin and postfixed at 4°C for 24 h. They were decalcified by treatment with 385 mM EGTA (pH 7.4) at 4°C for 48 h, with one change of EGTA at 24 h. The cochleae were then incubated in a freshly prepared solution of 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma Chemical Co.), as described (Boviatsis et al., 1994b), for a length of time sufficient to precipitate the β-gal reaction product (4–18 h). The whole stained cochleae were photographed and then dehydrated through a series of alcohols, cleared in xylene, embedded in paraffin wax, and sectioned at 5 μm. The sections were rehydrated, counterstained with eosin (0.5%), dehydrated, mounted, coverslipped, and photographed at low and high power magnification.

2.2.3. Analysis of targeted cells in vivo

The in vivo injections of *hr*R3 and vSC56 were each performed independently on four different animals. Multiple guinea pig cochlea sections were analyzed for each experiment. Results were similar for all animals. An estimation of general anatomical regions and specific cell types targeted by the different vectors was generated according to the localization and distribution of blue stained (β-gal-positive) cells. Areas of staining were assessed microscopically at low and high power and percent of field approximated.

3. Results

3.1. Guinea pig cochlea in vivo

β-gal-positive cells were observed in guinea pig cochleae injected with the HSV-1 and vaccinia virus vectors, *hr*R3 and vSC56, respectively. With both vectors, the highest number of β-gal-expressing cells was seen in the cochleae of animals which survived for 2 days following injection. However, both *hr*R3 and vSC56 did continue to produce β-gal-positive cells in cochleae: With *hr*R3, the number of positive cells at 4 days had dropped to about 20% of that at 2 days, but none were present at 6 days. With vSC56, there were about half as many positive at 6 days as at 2 days.

Fig. 1 shows a schematic overview (A), whole tissues (B, C, J), and various representative cross-sections (D–I, K–O) of cochleae that were injected with either saline (control), *hr*R3, or vSC56.

X-gal staining of *hr*R3- and vSC56-injected cochleae resulted in the labeling of numerous β-gal-positive cells that were categorized according to the general cochlear regions targeted by the two viruses. In general, the overall labeling reflected the distance from the round window. In each of the three and a half turns of the guinea pig cochlea, *hr*R3 was found to label cells in the spiral ligament (Fig. 1D, E), Reissner's membrane (Fig. 1E), and the lining of the scala vestibuli (Fig. 1H). Additionally, in the apical two turns, *hr*R3 labeled cells in the organ of Corti (Fig. 1D, F, G). Likewise, in all turns, vSC56 labeled cells in the spiral ligament (Fig. 1K, L), Reissner's membrane (Fig. 1L), and the lining of the scala vestibuli (Fig. 1N). In the basal two turns, vSC56 labeled cells in the organ of Corti (Fig. 1M) and in the lining of the scala tympani (ST) (Fig. 1O).

Specific cell types infected within targeted regions are shown in Table 1 and summarized in Fig. 1A. In the spiral ligament, *hr*R3 was found to label fibrocytes of all types but type III, and with equal distribution of labeling throughout the entire region (Fig. 1D). vSC56, however, primarily labeled type II fibrocytes in the suprastrial zone, but also labeled some type I fibrocytes more centrally (Fig. 1K). In Reissner's membrane, both vectors labeled mesenchymal cells on the scala vestibuli (SV) side and epithelial cells on the scala media (SM) side (Fig. 1D, E, L). In the organ of Corti, *hr*R3 labeled Hensen's cells in the most apical turn (Fig. 1F) and Deiters' cells in the second most apical turn (Fig. 1G). In the most basal turn, vSC56 labeled mesothelial cells beneath the basilar membrane. It occasionally labeled only apical regions of inner and outer hair cells (Fig. 1M). The restricted distribution of label within individual hair cells is surprising. However, vaccinia virus initiates transcription in the cytoplasm rather than in the nucleus (Weisz and Machamer, 1994), and genes delivered by vaccinia may follow an expression gradient within the cell over time, with first expression appearing near the region of virus entry. Thus, it is possible that the hair cells expressing β-gal received vSC56 through their apical surfaces, and that at the time the animals were killed transcription in these cells was limited to the cytoplasmic region near the point of entry. Finally, *hr*R3 was found to label mesenchymal cells lining the scala vestibuli (Fig. 1H), and vSC56 was found to label mesenchymal cells lining both the scala vestibuli (Fig. 1N) and the scala tympani (basal two turns) (Fig. 1O). Animals were killed after 2 days. Cell rounding has often been implicated in vaccinia virus infection (Weisz and Machamer, 1994). In cochlea, infection with either *hr*R3 or vSC56 did not appear to affect the morphology of cells, although we would not have detected subtle morphological changes.

For each vector, the type and frequency of infected cell types was similar for two animals 2d post-infection and one animal 4d post-infection. The distribution was determined in one animal for each vector at 2d, by averaging estimated proportions of infected cells in five sections of all three turns. Fig. 2A summarizes the distribution of cells infected by *hr*R3 and vSC56, by showing the number of infected cells in each region as a proportion of total infected cells. Fig. 2B shows the estimated proportion of cells within each region that

Table 1
Specific cell types expressing β-gal in targeted regions in the guinea pig cochlea

	Spiral ligament	Reissner's membrane	Organ of Corti	Lining of scala vestibuli	Lining of scala tympani
*hr*R3	fibrocytes of: types I, II, and IV, type II (suprastrial zone)	mesenchymal cells, epithelial cells	Hensen's cells[a], Deiters' cells[b]	mesenchymal cells	
vSC56	fibrocytes of: type I, type II (suprastrial zone)	mesenchymal cells, epithelial cells	inner hair cells[c] (apical regions), outer hair cells[c] (apical regions), mesothelial cells[c] (beneath basilar membrane)	mesenchymal cells	mesenchymal cells[c]

All labeled cell types were observed in all cochlear turns unless noted otherwise. SV, scala vestibuli; ST, scala tympani.
[a]These cells were only labeled in the most apical cochlear turn.
[b]These cells were only labeled in the second most apical cochlear turn.
[c]These cells were only labeled in the two most basal cochlear turns.

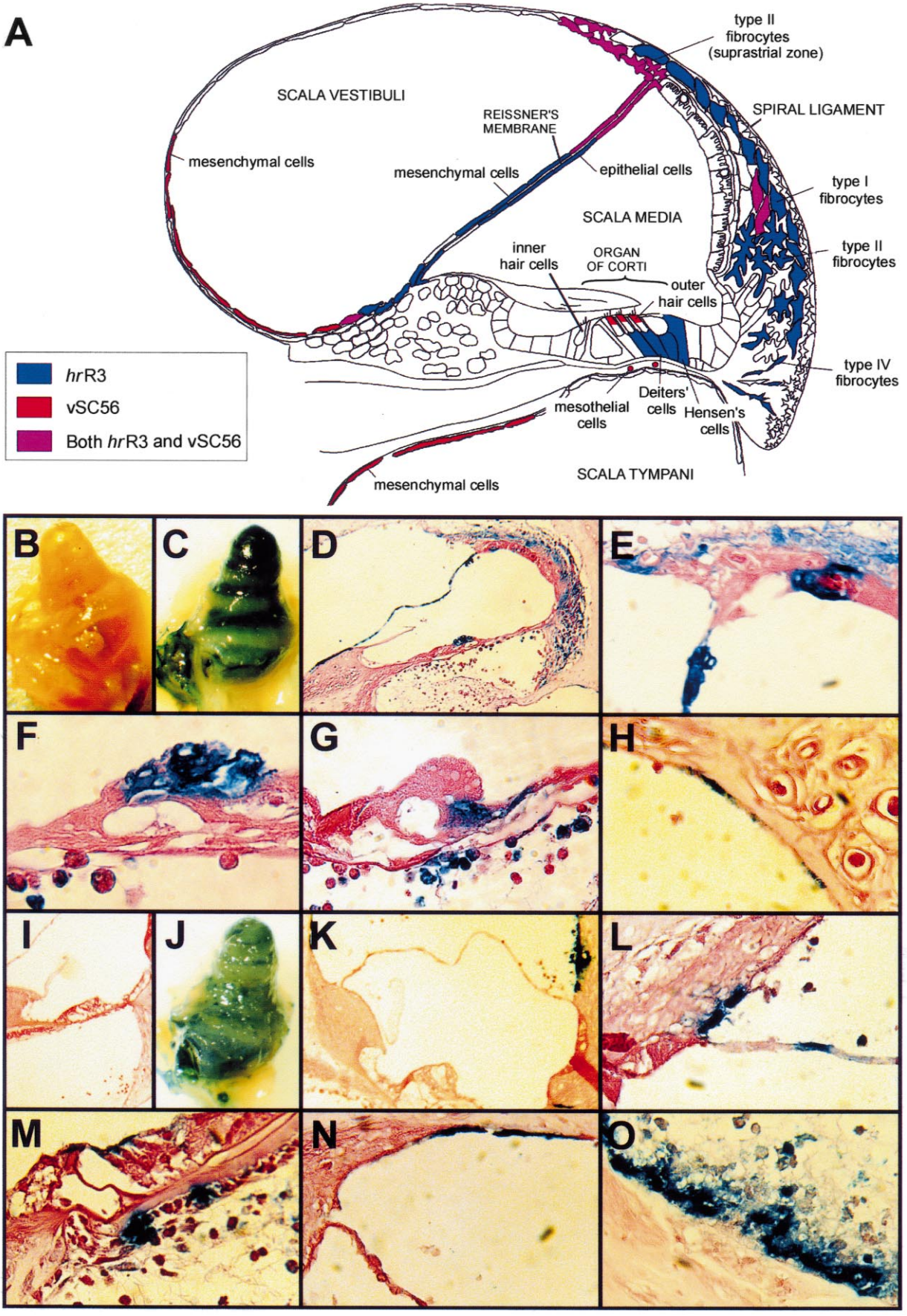
A
type II fibrocytes (suprastrial zone)
SCALA VESTIBULI
SPIRAL LIGAMENT
REISSNER'S MEMBRANE
mesenchymal cells
mesenchymal cells
epithelial cells
type I fibrocytes
SCALA MEDIA
inner hair cells
ORGAN OF CORTI
type II fibrocytes
outer hair cells
type IV fibrocytes
Deiters' cells
mesothelial cells
Hensen's cells
hrR3
vSC56
Both hrR3 and vSC56
mesenchymal cells
SCALA TYMPANI
B
C
D
E
F
G
H
I
J
K
L
M
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Fig. 1. A: Schematic overview of targeted regions and specific cell types infected with *hr*R3 and vSC56 in the guinea pig cochlea. Outline redrawn with permission from Kikuchi et al. (1995). B, C, J: Whole cochleae injected with saline (control) (B), *hr*R3 (C), and vSC56 (J). I: Cross-section of the second most basal turn in a saline-injected cochlea. Little inflammation was seen compared to cochleae injected with virus. D: Cross-section of the most apical turn in an *hr*R3-injected cochlea. Labeled cells are seen in the spiral ligament (fibrocytes of types I, II, and IV) (boxed area is magnified in E), Reissner's membrane, and the organ of Corti. E: *hr*R3-labeled cells in the spiral ligament (type II fibrocytes in the suprastrial zone) and in Reissner's membrane in the most apical turn. Both mesenchymal cells on the scala vestibuli side and epithelial cells on the scala media side of Reissner's membrane are labeled. F, G: *hr*R3-labeled cells in the organ of Corti. F shows labeled Hensen's cells in the most apical turn, and G shows labeled Deiters' cells in the second most apical turn. H: *hr*R3-labeled mesenchymal cells lining the scala vestibuli in the most apical turn. K: Cross-section of the second most apical turn in a vSC56-injected cochlea. Labeled cells are seen in the spiral ligament (type II fibrocytes in the suprastrial zone and type I fibrocytes centrally). L: vSC56-labeled cells in the spiral ligament (type II fibrocytes in the suprastrial zone) and in Reissner's membrane (both mesenchymal cells on the scala vestibuli side and epithelial cells on the scala media side) in the second most apical turn. M: Organ of Corti in the most basal turn of a vSC56-injected cochlea. Labeled cells include mesothelial cells beneath the basilar membrane and apical regions of inner and outer hair cells. N: vSC56-labeled mesenchymal cells lining the scala vestibuli in the most apical turn. O: vSC56-labeled mesenchymal cells lining the scala tympani in the most basal turn.
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expressed β-gal. For instance, about 15% of *hr*R3-infected cells were in Reissner's membrane, but nearly all Reissner's membrane cells were infected. The total number of β-gal-positive cells observed throughout *hr*R3-injected cochleae was about twice the total number of β-gal-positive cells observed throughout cochleae injected with vSC56. However, the titer of *hr*R3 that was used was two times higher than the titer of vSC56, suggesting that the ability of the two viruses to infect cells in the cochlea may be approximately the same.

β-gal expression resulting from *hr*R3 infection was concentrated in the spiral ligament, Reissner's membrane, and the organ of Corti (Fig. 2A), and was consistently greater than expression resulting from vSC56 infection in these regions (Fig. 2B). In contrast, β-gal expression induced by vSC56 was most prominent in the linings of the scala vestibuli and scala tympani, and was greater than that of *hr*R3 in these regions. In fact, *hr*R3 was not found to label any cells lining the scala tympani in any of the cochlear turns.

An inflammatory response resulting in apparent lymphocytic infiltration of the scala tympani was observed in all turns of the cochleae injected with *hr*R3 (Fig. 1D) and primarily in the basal two turns of vSC56-injected cochleae (Fig. 1O). Control cochleae injected with saline showed no signs of inflammation (Fig. 1I). Additional lymphocytic infiltration was observed around infected cells in the spiral ligament in all turns of cochleae injected with *hr*R3 (Fig. 1D), but little additional inflammation was seen around vSC56-infected cells outside of the basal two turns. While a minor inflammatory response in the scala tympani has been associated with the trauma of an intracochlear injection (Suzuki

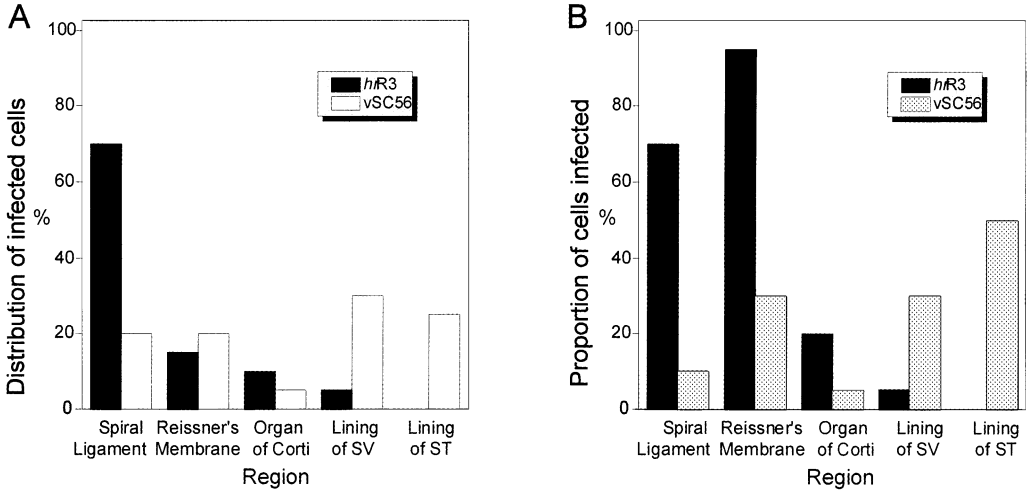

Fig. 2. Regional distribution of *hr*R3- and vSC56-infected cells. A: Infected cells in each region shown as an estimated proportion of the total number of infected cells in the guinea pig cochlea. B: Infected cells shown as a proportion of all cells in each region. *hr*R3 was concentrated in the spiral ligament and vSC56 was concentrated in the linings of the scala vestibuli and ST. *hr*R3 intensely labeled the spiral ligament and Reissner's membrane, but did not label any cells lining the scala tympani. vSC56 labeled most intensely Reissner's membrane and the linings of the scala vestibuli and scala tympani. Similar results were obtained in cochleae from four different animals for each vector.

and Harris, 1995), an acute inner ear immune response to the introduction of a foreign antigen into the cochlea has been well documented (Darrow et al., 1992; Stearns et al., 1993; Suzuki and Harris, 1995; Yeo et al., 1995). Furthermore, replication-defective herpes simplex viruses expressing *E. coli* β-galactosidase have been shown to elicit a strong humoral and cellular immune response against the enzyme (Brubaker et al., 1996).

4. Discussion

The data presented here show that an HSV-1 vector, *hr*R3, and a vaccinia virus vector, vSC56, can both be used to deliver the *E. coli lacZ* transgene to cells in the guinea pig cochlea in vivo. Both vectors induced β-gal expression in cells throughout the guinea pig cochlea, including cells in and around the organ of Corti. These results suggest that recombinant HSV-1 and vaccinia virus vectors may be effective in delivering genes for therapeutic proteins in some forms of hearing loss.

4.1. Assessment of viral infection and toxicity

The robust β-gal expression seen throughout guinea pig cochleae injected with *hr*R3 and vSC56 confirms successful infection and transgene delivery. The HSV-1 vector used, *hr*R3, is replication conditional, that is, it can only replicate in dividing cells (Goldstein and Weller, 1988); vSC56 is nominally replication competent, although its replication ability is likely to be attenuated in non-dividing cells due to low complementing levels of cellular tyrosine kinase (Buller et al., 1985). Most of the cells within the undamaged adult mammalian cochlea are non-dividing, though some evidence for slow cell division has been reported in the spiral ligament and in some other peripheral cochlear regions (Koburg, 1961; Ruben, 1967; Roberson and Rubel, 1994). Viral vectors, namely HSV and adenovirus vectors, have been shown to elicit strong immune responses against viral proteins and transgenes of foreign origin such as β-gal (Boviatsis et al., 1994a; Mineta et al., 1994; Brubaker et al., 1996; Wood et al., 1996). The propensities for *hr*R3 and vSC56 to replicate preferentially in dividing cells, then, make the spiral ligament and other peripheral cochlear regions in which slow cell division may be occurring particularly vulnerable to the cytopathic effects of these two vectors. Since a large portion of the spiral ligament was infected by *hr*R3, and only a small portion by vSC56, one would expect to see a significantly greater immune response associated with *hr*R3 infection than with vSC56 infection.

If HSV-1 or vaccinia virus are to be used to deliver genes for therapeutic purposes in the inner ear, the apparent immune response may be a concern, particularly that seen with *hr*R3. However, evidence suggests that hearing loss resulting from inflammation induced by a viral infection in the inner ear may be treated with immunosuppressants (Keithley et al., 1989; Darmstadt et al., 1990; Stearns et al., 1993). Furthermore, the use of viral vectors that are replication deficient, even in dividing cells, may prove to be less toxic to cells in the inner ear, and thus may be more useful for therapeutic applications. Newer recombinant HSV-1 vectors deleted in multiple immediate early genes have essentially no toxicity or expression of viral antigenic proteins, while retaining a large transgene capacity (>30 kb) and the ability to enter a stable latent state in neurons, which serves as a platform for long-term transgene expression (Glorioso et al., 1995; Samaniego et al., 1998). Also, plasmid DNA can be packaged in HSV-1 virions without helper virus or expression of viral genes (Fraefel et al., 1996).

Two other viral vectors have been used in recent studies to transfer the *lacZ* gene into guinea pig cochlea. A replication-deficient adenovirus injected through the guinea pig round window resulted in large numbers of *lacZ*-positive cells in the spiral ganglia, stria vascularus, spiral ligament and the lining of the fluid spaces (Raphael et al., 1996; Weiss et al., 1997). Supporting cells but not hair cells in the organ of Corti were transduced. In contrast, hair cells exposed to adenovirus in culture explants of either rat cochlea (Dazert et al., 1997) or mouse utricle (Holt et al., 1999) are efficiently transduced, suggesting that adenovirus requires access to the apical surface of hair cells to infect them. These different virus vectors differ in cellular tropism, transgene capacity and stability in host cells, and thus have complementary uses in gene delivery paradigms (Breakefield et al., 1997).

Adeno-associated virus delivered into the scala tympani by means of an osmotic mini-pump infected cells in organ of Corti, spiral ganglia, spiral limbus, and spiral ligament (Lalwani et al., 1996). Stria vascularis was not labeled. Both hair cells and supporting cells in vestibular epithelia were also infected with these scala tympani infusions (Lalwani et al., 1998a,b).

4.2. Implications for gene therapy

Cochlear implants provide a potentially effective means for treating some forms of hearing loss involving the death of hair cells in the organ of Corti. They function by electrically stimulating spiral ganglion neurons that no longer receive input from hair cells (Cohen et al., 1993), and their effectiveness relies critically upon a healthy population of spiral ganglion neurons (Hartshorn et al., 1991). However, spiral ganglion neurons apparently receive trophic support from hair cells, as they begin to die when the hair cells that they innervate

are lost. Thus, the implant effectiveness may be improved by protecting spiral ganglion neurons after hair cell death and prior to cochlear implantation.

Much evidence suggests that brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) are secreted by hair cells in the organ of Corti and are responsible for the maturation of spiral ganglion neurons, and for their maintenance and survival during injury (Pirvola et al., 1992; Ylikoski et al., 1993; Wheeler et al., 1994; Ernfors et al., 1995). Ciliary neurotrophic factor (CNTF) combined with NT-3 (Staecker et al., 1995b), and neurotrophin-4/5 (NT-4/5) (Zheng et al., 1995) have also been shown to promote the survival of cultured rat spiral ganglion neurons. Indeed, genetic delivery of BDNF to spiral ganglion neurons in vivo, using an HSV amplicon vector, has recently been shown to preserve these neurons, which otherwise tend to die when hair cells are killed with aminoglycoside treatment (Geschwind et al., 1996; Staecker et al., 1998). In preliminary experiments with *hr*R3, we have been able to deliver *lacZ* to spiral ganglion neurons in vivo by injecting virus directly into the guinea pig auditory nerve (data not shown). Thus, HSV-1 is a good candidate for delivering neurotrophic factors to spiral ganglion neurons.

While cochlear implants provide some restoration of hearing loss, the absence of hair cells still prevents the restoration of many aspects of normal hearing, including superior speech discrimination (Cohen et al., 1993). A more effective treatment would be the protection or replacement of hair cells themselves. Basic fibroblast growth factor (bFGF), transforming growth factor-α (TGF-α), and retinoic acid (RA) have all been shown to protect cultured rat hair cells from aminoglycoside poisoning (Low et al., 1995). The abilities of *hr*R3 and vSC56 to infect and induce β-gal expression in cells in and around the organ of Corti in vivo suggest that HSV-1 and vaccinia virus vectors might be used to deliver the genes for these factors to the organ of Corti in order to protect auditory hair cells from damage. Growth factors might eventually be delivered with viral vectors to accomplish mammalian auditory hair cell regeneration in vivo. These vectors would be particularly useful for this application since only acute expression of the factors would be needed to permit cellular regeneration. This situation would be unique in that it would circumvent the need for sustained expression in gene delivery, a persistent problem for most applications of viral vectors.

Finally, a growing number of defective genes implicated in inherited deafness have been discovered (for review see Steel and Brown, 1996), and in certain cases viral vectors bearing corrected versions of these genes could feasibly be used in the inner ear as a gene replacement therapy.

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