Objective: To demonstrate and compare polycationic-mediated cochlear gene transfer with linear polyethylenimine (PEI) via cochleostomy and osmotic pump infusion method.

Design: A dissociated cochlear culture was used to select the optimum nitrogen to phosphate ratio of PEI/DNA complexes to be used in vivo. The PEI–enhanced green fluorescent protein reporter gene DNA complex was introduced with single inoculation (cochleostomy) or with sustained delivery (osmotic pump method) into guinea pig cochleas and examined for transgene expression.

Subjects: Male Albino Hartley guinea pigs (250-350 g).

Results: The relatively low transfection efficiency of PEI limits its potential when compared with viral counterparts; however, sustained release of the vector solution was able to improve PEI's transfection efficiency. The PEI-infused cochleas maintained intact cellular and tissue architecture with absence of inflammation. Transfection confined to the perilymphatic space highlights the need to target the gene vector into the scala media if transfection is targeted at cells within the organ of Corti.

Conclusion: These findings indicate that PEI is able to transfect the cochlea in vivo with sustained delivery and present an alternative for nonviral cochlear gene therapy.


DURING THE PAST DECADE, numerous strategies have been developed for gene delivery to the inner ear as a potential clinical treatment. Gene therapy stands out as a new and promising approach in the treatment of hearing disorders, and the cochlea provides an ideal target for gene therapy for various reasons. The cochlea contains a small localized population of cells (in the organ of Corti) that is responsible for many common hearing disorders: hence, successful treatments targeted to this small cell population are likely to cure many hearing-related problems. Because the cochlea is a confined and isolated space of the inner ear, it limits the spread of gene transfer vectors to surrounding tissue, minimizing unwanted effects after introduction. The limited direct blood supply within the cochlear perilymphatic and endolymphatic spaces also reduces the risk of triggering undesired immune responses. The perilymphatic and endolymphatic fluid spaces allow the diffusion and spread of vectors into areas remote to the site of inoculation, while still confined within the cochlea's perimeter. To summarize, the cochlea is a low-volume fluid-filled space with a small population of specialized cell types that could be potential targets of gene therapy with a small number of vector particles. These advantages combine to make the cochlea an attractive site for gene therapy.

Gene therapy strategies have been shaped by the choice of agent, vector, and route of delivery. The choice of vectors for gene delivery can be broadly classified as viral and nonviral. In light of some viral vectors' immunogenicity and their oncogenic potential, the use of viral vectors to deliver genes without major adverse effects in the infected host remains a great challenge. The death of an 18-year-old gene therapy patient in 1999 is regarded as the greatest casualty of gene therapy. This tragedy marked the cessation of several viral gene therapy trials. Another major setback in gene therapy research in recent years has been the development of leukemialike conditions in some patients leading to subsequent death.

Nonviral gene delivery vectors have emerged as a promising alternative to their viral counterparts. However, in inner-ear research, fewer studies using nonviral vectors have been carried out. The greatest challenge in using nonviral vectors is improving their low transfection efficiency. Currently available nonviral vectors are routinely less efficient than viral vectors. How-
ever, nonviral vectors have their own advantages, such as the ability to transfer a gene cassette of unlimited size and type, absence of a viral component that may evoke immunogenic and inflammatory response, inability to replicate or recombine to form infectious agents, inability to integrate into a host genome (which minimizes the risk of insertional mutagenesis), and ease of manufacture. Hence, research has been aimed at altering nonviral vector formulations and/or delivery approaches to help compensate for their lower transfection efficiency. Polyethyleneimine (PEI), a cationic polymer well known as a superior nonviral vector for gene transfection, is often regarded as the criterion standard in nonviral gene delivery. It possesses both strong DNA compaction capacity and high endosomolytic competence where every third atom of PEI is a protonable amino nitrogen atom, which makes the polymeric network an effective “proton sponge” at virtually any pH. This aids in endosomal escape and translocation of the DNA to the nucleus without significant DNA degradation (see also Figure 1 for mechanism).

This study explored the use of PEI to achieve polycationic-mediated cochlear gene transfer. We assessed and compared the transfection efficiencies of PEI-mediated gene transfer via cochleostomy and the osmotic pump infusion method and demonstrated successful PEI-mediated cochlear gene transfer with the osmotic pump infusion method. This highlights the importance of selecting the most suitable route of delivery for nonviral vectors to achieve successful transfection and that sustained release of the vector solution compensates for the generally lower transfection efficiency of nonviral vectors in the cochlea.

**ANIMAL MODEL**

Male Albino Hartley guinea pigs (230–350 g) were used in all in vivo experiments. The guinea pig model was used because of its large cochlea size compared with those of mice and rats, hence allowing greater ease of surgical manipulation without causing significant injury. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Biological Resource Centre–Biopolis (Singapore).

**VECTOR**

The polycationic polymer used was the 22-kDa linear PEI (ExGen500, No. R0521; Fermentas, Burlington, Ontario, Canada) with a nitrogen concentration of 100mM in every 1 μL of PEI. DNA (plasmid DNA encoding the enhanced green fluorescent protein [eGFP] reporter gene) and PEI were first diluted in sterile 5% (wt/vol) glucose solution. The PEI was added to diluted DNA at a nitrogen to phosphate (N:P) ratio of 6 (N:P ratio is a measure of the ionic balance of the PEI-DNA complex) and vortexed vigorously for 10 seconds. The resultant PEI-DNA complex (PEI-eGFP) was incubated for at least 10 minutes at room temperature before use.

**COCHLEAR CELL CULTURE**

The cochleas of the guinea pigs (300–350 g) were harvested and dissected carefully in cold extracellular solution (142mM sodium chloride, 5.37mM potassium chloride, 1.47mM magnesium chloride, 2.5mM calcium chloride, and 10mM HEPES; 300 mOsm, pH 7.2). After the spiral ligament, stria vascularis tissues, and tectorial membrane were removed, the sensory epithelium was carefully dissected and collected for enzymatic disaggregation in a mixture of 0.125% trypsin and 0.123% collagenase for 5 to 10 minutes at 37°C with shaking. The enzyme was inactivated with an equal volume of culture medium containing 20% fetal bovine serum. The dissociated cells were added to a modified keratinocyte medium containing 10% fetal bovine serum, tritutated, and seeded with fresh modified keratinocyte medium in a 48-well plate (approximately 10 wells for every 2 cochleas). Cells were cultured at 37°C in a 3% carbon dioxide incubator, and the medium was changed every 3 to 4 days. The cells were subsequently passaged by means of trypsin-EDTA.

**IMMUNOCYTOCHEMICAL ANALYSIS**

Anti-Jagged1 (Santa Cruz Biotechnology, Santa Cruz, California), anti-S100A12 (Laboratory Vision, Fremont, California), and antipancytokeratin14 (Sigma-Aldrich Corp, St Louis, Missouri) primary antibodies were used as cochlear supporting cell markers. Cells were rinsed and fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 30 minutes at 4°C. After washing, the cells were blocked with blocking solution for 30 minutes at room temperature and incubated with secondary antibodies diluted in PBS with 0.1% Triton X-100 overnight at 4°C. After 3 washing cycles, the cells were incubated with the appropriate dilute secondary antibodies (Jackson Immunoresearch, West Grove, Pennsylvania) conjugated to cyanine 3 for 1 hour at room temperature. The cells were then washed and imaged by fluorescence microscopy.

**TRANSFECTION**

The DNA and PEI were diluted in serum-free media with gentle vortexing and incubated at room temperature for 10 minutes before combination. Diluted PEI was added to the DNA solution, vortexed vigorously, and incubated at room temperature for at least 10 minutes before use. Primary cells were plated in 24-well plates the previous day to obtain 70% confluency. On the day of transfection, existing medium was removed and cells were rinsed with serum-free medium. The complex solution containing DNA and PEI was added dropwise into each well (1 μg of DNA per well complexed at various N:P ratios). The cells were incubated at 37°C in a carbon dioxide incubator for 6 hours. After incubation, cells were replaced with fresh complete medium and incubated for 3 days before proceeding to flow cytometry analysis. On the day of harvesting, cells were trypsinized, collected, and fixed in ice-cold 2% paraformaldehyde in PBS. Flow cytometry analysis was carried out and transfection efficiency was assessed on the basis of the percentage of cells expressing the marker gene product of PEI-eGFP. Data were derived from experiments repeated on 3 occasions, with each data point conducted in triplicate. Quantitative data were compared by the t test for unpaired samples.

**SURGICAL PROCEDURES**

Before surgery, all guinea pigs were checked to ensure that they had a normal Preyer reflex and no abnormalities in physical appearance. The guinea pigs were anesthetized with a 40-mg/kg ketamine–3- to 5-mg/kg xylazine mixture given intra-
In addition, 0.05 mg/kg of buprenorphine hydrochloride was injected intramuscularly as an analgesic. Two delivery methods were assessed (n=5 each), using the ventral approach as described. The guinea pig was placed in the supine position. Ventral and paramedian skin incisions were made extending from the jawbone to the collarbone. The jugular vein, submandibular gland, and connective tissue were retracted medially. The tympanic bulla was exposed and opened with forceps, disclosing the cochlea laterally. A small orifice was made in the cochlea bony shell (middle turn), penetrating the scala tympani. In the cochleostomy method, with the use of a microsyringe (Hamilton Company, Reno, Nevada) connected with a 30-gauge needle, 10 µL of the DNA complex solution was injected through the orifice manually over a period of 10 minutes from a microsyringe mounted on a free-standing clamp. The needle was then carefully removed and the orifice was sealed with dental cement on needle retraction to prevent backflow of the vector solution. In the osmotic pump method, a catheter (PUFC-C30-10; Instech Solomon, Plymouth Meeting, Pennsylvania) connected to an osmotic pump (1007D; Alzet, Cupertino, California; flow rate, 0.5 µL/h; reservoir volume, 90 µL; containing 10 µg of DNA complexed with PEI at an N:P ratio of 6) preactivated by overnight incubation at 37°C was inserted into the orifice made in the cochlea bony shell and secured by means of dental cement and a topical tissue adhesive (NEXABAND; Abbott Laboratories, Chicago, Illinois). The osmotic pump reservoir was then inserted subcutaneously under the neck region of the guinea pig. On completion, the cavity in the bulla was sealed with dental cement (Fuji I glass ionomer luting cement; GC America Inc, Alsip, Illinois) and the wound was sutured closed.

TISSUE PROCESSING AND FLUORESCENCE MICROSCOPY

Cochleas were harvested and fixed in 4% paraformaldehyde in PBS overnight at 4°C. For surface mount preparations, the cochleas were dissected in PBS. The basilar membranes were collected, mounted on glass slides, and then prepared for fluorescence microscopy. For histologic preparations, the cochleas were transferred and stored in 10% EDTA at room temperature for

Figure 1. Schematic drawing of the proton sponge hypothesis postulating the mechanism of polyethylenimine (PEI)–mediated gene transfer. The anionic DNA and the cationic PEI spontaneously collapse into a DNA-vector complex with a net cationic charge. The complex binds to the polyanionic glycosaminoglycans of the cell membrane, leading to endocytosis. Every third atom of PEI is a protonable amino nitrogen atom, making the polymeric network an effective “proton sponge” at virtually any pH. Accumulation of protons is coupled to an influx of Cl− anions within the endosome. This results in a large increase in ionic concentration and substantial water entry, leading to rapid osmotic swelling and membrane rupture. Hence, the DNA is protected against nucleases during trafficking, and translocation into the nucleus is aided.
7 days in the dark with daily solution changes. After decalcification, the cochleas were cryopreserved in 20% sucrose solution at 4°C overnight and infiltrated by a gelatin embedding technique. The gelatin blocks containing the cochlea samples were then trimmed and frozen at −20°C. Cryosections were obtained at 10-µm thickness and mounted on poly-L-lysine-coated slides. Slides were air dried and rinsed in 37°C PBS to remove residual gelatin and tissue mounting medium. Cryosections were incubated at room temperature for 2 hours to detect the presence of T lymphocytes by means of a monoclonal antibody specific for guinea pig T lymphocyte (MCA564, 1:10; AbD Serotec, Oxford, England). After washing, the sections were incubated at room temperature for 1 hour with the appropriate secondary antibody. Negative controls were performed with the use of cochlea sections without primary antibody and sections without secondary antibody. Acoustic deafened cochlea sections were used as positive controls for T-lymphocyte labeling. All surface mounts and cryosections were viewed over a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan) (eGFP, excitation/emission wavelengths, 488/520 nm) or fluorescence microscope (Eclipse E600; Nikon, Tokyo, Japan). Transfection was assessed on the basis of the marker gene product of PEI-eGFP.

PEI-MEDIATED TRANSFECTION IN VITRO

A dissociated cochlear cell culture was derived from the guinea pig sensory epithelium to assess PEI's transfection efficiency in vitro. These cells were immunostained with antibodies specific to cochlea supporting cell markers and shown to express 3 supporting cell markers: Jagged1, cytokeratin, and S100A1 (results not shown). The cells were then transfected by PEI (Figure 2A) to assess the transfection efficiencies at various N:P ratios in order to select the optimum N:P ratio for subsequent in vivo experiments. A steady increase in cells transfected was observed from an N:P ratio of 2 to 6. No significant increase in transfection efficiency was observed between N:P ratios of 6 and 8 (Figure 2B). Hence, in view of the possible cytotoxic effects in vivo with an increasing amount of PEI, an N:P ratio of 6 was used in subsequent in vivo experiments.

PEI-MEDIATED TRANSFECTION IN VIVO

To assess the transfection efficiency of PEI, cochleostomy (single inoculation) was used as the initial method of delivery. Only 3 of 5 injected cochleas showed signs of transfection, and low transfection efficiencies were observed. On inspection of surface mount preparations and cryosections, few transfected cells were observed, and these were mostly mesenchymal cells lining the perilymphatic fluid spaces (Figure 3). To compensate for PEI's low transfection efficiency, sustained delivery was provided with the use of an osmotic pump via cochleostomy. The osmotic pump delivered the vector solution at 0.5 µL/h during a period of 1 week, providing a constant supply of vector particles into the cochlear fluid spaces. At the end of 1 week, all 5 guinea pigs showed improved transfection efficiencies compared with the single inoculation of PEI-eGFP via cochleostomy. Cells that are preferentially targeted include the fibrocytes lining the scala vestibuli and scala tympani, mesenchymal and epithelial cells of Reissner membrane, and fibrocytes in the suprastrial zone of the spiral ligament (Figure 4). No transfection was observed in the organ of Corti and stria vascularis. In addition, absence of T-lymphocyte labeling at various regions within the infused cochleas, including the spiral ganglion cells and the organ of Corti, indicates minimal inflammatory response.

COMMENT

Since inner-ear gene delivery was reported in 1996, much work and great progress have been achieved in the field of inner-ear gene therapy. Numerous studies have been published investigating the use of various vectors, genes, and routes of delivery for the treatment of hearing-related disorders or protection of hearing function. Much progress has been reported in hair cell protection, regeneration, and functional restoration with the use of viral vectors in gene therapy protocols. The most commonly used viral vectors are the adenovirus and adeno-associated virus. Compared with viral vec-
tors, there have been far fewer reports on the use of nonviral vectors in inner-ear gene delivery studies. These studies were carried out in the late 1990s and early 2000s4,6 with cationic liposome as the delivery vector. Since then, limited work involving the use of nonviral vectors in inner-ear gene delivery studies has been reported. However, with safety issues being the top priority in clinical trials, and rising concerns with the use of viral vectors, nonviral vectors remain a relevant approach to be further explored for inner-ear gene therapy.

Clearly, the lower transfection efficiency of nonviral vectors limits their potential in competing with viral counterparts. Besides the selection of vector used for inner-ear gene delivery, the vectors' route of delivery into the cochlea also plays an important role in determining gene transfer efficiency. On the basis of our results comparing the transfection efficiencies of the various routes of delivery (B.T.G.T. and R.R., unpublished data, 2007) and the results reported by others,26,27 the delivery methods could be ranked according to transfection efficiency, with the osmotic pump method being at the top of the list, followed by cochleostomy, round window membrane inoculation, and finally the absorbable gelatin sponge method (delivery through intact round window membrane), the least invasive and the least effective. In nonviral vector delivery studies, the route of delivery is especially important owing to its generally lower transfection efficiency, and selection of an ideal delivery method could compensate for such a disadvantage. In our experiments, prolonged and sustained delivery using an osmotic pump improved the transfection efficiency of PEI and presents a possible way to augment the transfection efficiency of nonviral cochlear gene transfer.

Our results displayed eGFP expression restricted to the perilymphatic fluid spaces in both sets of experiments and are consistent with previous gene delivery models reported.28,29 This is likely due to vector inoculation via the scala tympani, which exposes only the walls of the perilymphatic fluid spaces and the basolateral domain of the organ of Corti to the vector particles. This excludes the spiral ligament and the apical surface of the organ of Corti cells. Therefore, contact between the vector particles inoculated and regions of transfection is particularly important to obtain successful transfection within the scala media because there is no dissemination of vector particles from the perilymphatic into the endolymphatic fluid space after inoculation. This is especially true for nonviral PEI or cationic liposomes–DNA complexes (approximately 300-600 nm), which tend to be larger than viral vector particles such as adenovirus and adeno-associated virus (<100 nm). To reach the cells in the organ of Corti within the endolymphatic fluid space, inoculation of the vector solution into the scala media is critical. However, locating the scala media from the exterior of the cochlea bony shell or the round window membrane is challenging because of the relatively small size of the scala media even in the guinea pig model. Two possible techniques reported are vector inoculation into the endolymphatic sac30 and cochleostomy through the fenestra via a microcannula reaching the scala media.31 Targeting the scala media for vector inoculation would aid the transfection of cells in the organ of Corti and would also reduce the amount of vector solution required, thus improving efficiency and reducing possible cytotoxicity. However, limited transfection in the cochlea apical turns and hair cell loss associated with scala media inoculation are 2 major drawbacks that may limit its future clinical application.

Because of PEI's potential cytotoxicity,32,33 particular attention was paid to the cellular structure of the inoculated cochlea. Regions in the vicinity of the inoculation site underwent trauma, and cytostructural damage was observed near the site of catheter implantation and cochleostomy. As distance increased from the inocula-

Figure 3. Cochlea transfected with polyethyleneimine–enhanced green fluorescent protein reporter gene DNA complex 3 days after administration of complex solution by cochleostomy, showing representative fluorescence confocal images of cryosections (A) and surface mounts of basilar membrane (B). Sparse and weak transfection is observed in the various cochlear turns, and transfection is restricted to perilymphatic fluid spaces. Arrowheads indicate transfected cells on the lining of perilymphatic fluid spaces.
tion site toward the extreme apical and basal turns, the cochlea displayed intact cellular and tissue cytoarchitecture with no pathologic changes. The cytostructural damage observed near the implantation site was likely due to catheter implantation rather than the vector particles. Previous in vivo studies involving viral vectors showed apparent lymphocytic infiltration in the cochlea.15,34 In our case, no cellular infiltration was observed and there was absence of positive T-lymphocyte staining at various regions including the organ of Corti and spiral ganglion cells. Hence, with the current experimental dosage administered, there was minimal cytotoxic effect and inflammatory response in the infused cochlea.

In this study, the effects of PEI-mediated gene transfer on auditory brainstem response (ABR) were not evaluated; however, the effects of sustained delivery with an osmotic pump on hearing have been investigated extensively by several groups. Carvalho and Lalwani35 conducted ABR studies on the osmotic pump infusion method and demonstrated ABR threshold preservation at low frequencies. Although elevated ABR thresholds in the middle and high frequency due to surgical trauma were observed, 1 animal did show significant recovery of thresholds after a month. Luebke and coworkers36,37 monitored the distortion product otoacoustic emissions measurements throughout the infusion period of an adenoviral vector and found no loss in cochlear function at all frequencies assessed. Hence, on the basis of these previous findings, combined with the minimal cytotoxic effects and inflammatory response found in our study, we believe the infusion period of the PEI vector solution via the osmotic pump will be well tolerated with minimal functional compromise on hearing.

Polyethylenimine is less efficient than viral vectors in delivering DNA and initiating gene expression, particularly in cochlear gene delivery studies. However, strategies to improve transgene expression levels that match

Figure 4. Cochlea transfected with polyethylenimine–enhanced green fluorescent protein reporter gene DNA complex (PEI-eGFP) 1 week after sustained release of complex solution by an osmotic pump, showing representative fluorescence confocal images of cryosections (A, C, and D) and surface mounts of basilar membranes (B). A and B, Improvement in transfection efficiency compared with a single inoculation of PEI-eGFP vector solution. Transfection is observed on the lining of the perilymphatic fluid spaces. C and D, Transfection is restricted to regions outside the scala media (SM). Double-headed arrow indicates suprastrial zone of the spiral ligament; arrowheads, scala vestibuli (SV) lining; arrow, Reissner membrane.
or come close to levels observed with viral vectors could be used. One such strategy, as shown in this study, is the choice of delivery method. Because PEIs are polymers, they provide great flexibility and ease in altering their structure to improve transfection efficiency. Some strategies previously reported include conjugating groups to improve stability, or incorporating receptor-specific ligands to target receptors on the cell surface. In addition, PEI can be produced in almost any quantity in large-scale polymerization reactors, and purification is easy and inexpensive compared with that of viruses. This is important because large quantities, high reproducibility, and acceptable cost are required for direct clinical applications. Polyethyleneimine and DNA may form complexes that include more than 1 plasmid or DNA of virtually any size; however, this is limited by aggregation as concentration increases. Nonviral gene delivery is important because large quantities, high reproducibility, and acceptable cost are required for direct clinical applications. Polyethylenimine and DNA may form complexes that include more than 1 plasmid or DNA of virtually any size; however, this is limited by aggregation as concentration increases. Nonviral gene delivery systems at therapeutic doses require high concentrations of polymer-DNA complexes; hence, preparation protocol and conditions such as pH, ionic strength, and temperature should be empirically controlled and stabilization measures implemented for extended circulation times.

Because of compact complex formation, PEI works well for cell uptake and nucleic protection; however, dissociation is difficult and poses difficulties in releasing the gene material within the cytosol. These 2 opposing phenomena could be resolved with the introduction of pH- or temperature-sensitive groups onto polymer chains to produce environment-sensitive polymers capable of targeted delivery and intracellular trafficking of DNA at the releasing stage.

In conclusion, we demonstrated successful PEI-mediated cochlear gene transfer, displayed improved transfection efficiency with sustained release of the vector solution, and identified the osmotic pump infusion method as a way of augmenting the nonviral vector’s transfection efficiency. Currently, no ideal vector exists in cochlear gene therapy, and different systems need to be integrated and optimized to overcome many obstacles that limit the success of nonviral gene delivery. Hence, this study presents a novel and safe approach for nonviral gene transfer in the cochlea using linear PEI as an alternative to viral vectors.

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