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Received for publication May 17, 2005; accepted after revision August 8, 2005.

Published online: September 21, 2005.

doi:10.1016/j.ymthe.2005.03.021

ARTICLE

Specific and Efficient Transduction of *Cochlear* Inner Hair Cells with Recombinant Adeno-associated Virus Type 3 Vector

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Available online 12 May 2005

Recombinant adeno-associated virus (AAV) vectors are of interest for cochlear gene therapy because of their ability to mediate the efficient transfer and long-term stable expression of therapeutic genes in a wide variety of postmitotic tissues with minimal vector-related cytotoxicity. In the present study, seven AAV serotypes (AAV1–5, 7, 8) were used to construct vectors. The expression of EGFP by the chicken β-actin promoter associated with the cytomegalovirus immediate-early enhancer in cochlear cells showed that each of these serotypes successfully targets distinct cochlear cell types. In contrast to the other serotypes, the AAV3 vector specifically transduced cochlear inner hair cells with high efficiency *in vivo*, while the AAV1, 2, 5, 7, and 8 vectors also transduced these and other cell types, including spiral ganglion and spiral ligament cells. There was no loss of cochlear function with respect to evoked auditory brain-stem responses over the range of frequencies tested after the injection of AAV vectors. These findings are of value for further molecular studies of cochlear inner hair cells and for gene replacement strategies to correct recessive genetic hearing loss due to monogenic mutations in these cells.

Key Words: adeno-associated virus, serotype, gene transfer, cochlea, hair cells

INTRODUCTION

The total number of hair cells in the cochlea is finite. They are not renewed and there is very little (if any) redundancy in this population. The irreversible loss of cochlear hair cells is presumed to be a fundamental cause of permanent sensorineural hearing loss. Gene transfer into hair cells presents numerous opportunities for protecting these cells. There is considerable interest in the development of viral vectors to deliver genes to the cochlea to counteract hearing impairment, and recent studies have focused on vectors based on adenovirus [1–3], herpes simplex virus [4–6], lentivirus [7], and adeno-associated virus (AAV) [8,9]. The patterns of vector-encoded transgene expression have been found to differ significantly among vectors. Cochlear hair cells can be efficiently transduced with adenovirus vectors [10–12]. However, these vectors were found to provoke a strong immune response that could damage recipient cells and compromise cochlear function [10,13,14]; they are also incapable of mediating prolonged transgene expression [15,16]. Although AAV vectors might overcome these problems, the transduction of hair cells by AAV2-derived vectors is controversial [8,10,17]. To our knowledge, other AAV serotypes have not yet been tested as cochlear gene transfer vectors *in vitro* or *in vivo*. AAV vectors are of interest in the context of gene therapy because they mediate efficient transfer and long-term stable expression of therapeutic genes in a wide variety of postmitotic tissues with minimal vector-related cytotoxicity.

In this study, we assessed the utility of seven AAV serotypes as vectors with the chicken β-actin promoter associated with a cytomegalovirus immediate-early

1525-0016/$30.00

doi:10.1016/j.ymthe.2005.03.021

enhancer (CAG)-driven enhanced green fluorescent protein (EGFP) gene [18] in the murine cochlea. Vectors were introduced by microinjection through the round window membrane [19]. As a result, we determined that the specific and efficient gene transduction of inner hair cells could be achieved by using AAV type 3 vectors.

RESULTS

Expression Profile of EGFP in the Cochlea

Several cell types line the cochlear duct and support the hair cells (Fig. 1A). We carefully made a small opening in the tympanic bulla and injected vectors derived from the AAV1–4, 7, and 8 pseudotypes into the cochlea of two strains of mice (C57BL/6J and ICR) through the round window membrane (Fig. 1B). The mode of EGFP expression in various murine cochlear hair cells had a close similarity and was essentially equal for both strains. We determined the distribution of AAV vector-mediated EGFP expression throughout the cochlea for all serotypes tested (Table 1). A principal finding is that the inner hair cells in the organ of Corti showed clear evidence of EGFP expression with all of the AAV serotype-derived vectors except for the AAV4 vector (Fig. 2). This result indicates that most of the vectors (AAV1–5, 7, and 8) could efficiently transduce cochlear inner hair cells *in vivo* when slowly infused into the scala tympani. The AAV3-based vector was the most efficient and specific of the serotypes in transducing cochlear inner hair cells (Fig. 3). Transduction with 5×10^{10} genome copies (gc)/cochlea of the AAV3 vector resulted in robust transgene expression in the inner hair cells. The spiral ganglion cells showed significantly higher levels of fluorescence per unit area with the AAV5-based vector (Fig. 2n), and the spiral ligament cells were transduced prominently with the AAV1 and AAV7 vectors (Figs. 2d and 2r). Histological sections of cochleae injected with the AAV4 vector identified EGFP-positive cells predominantly in connective tissue within the mesothelial cells beneath the organ of Corti and in mesenchymal cells lining the perilymphatic fluid spaces (Figs. 2j and 2l). Furthermore, we detected intense expression with the AAV5- and AAV8-based vectors in the inner sulcus cells and in Claudius' cells (Figs. 2p and 2x). We did not detect notable levels of gene expression in the outer hair cells, supporting pillar cells, or stria vascularis cells for any serotype.

Long-term Expression of EGFP

We examined cochlear expression of the EGFP transgene in animals sacrificed at 1–12 weeks. Expression persisted in cochlear tissues for up to 3 months after infusion, while the extent of expression peaked at 2 weeks.

Transgene Activity

We determined the percentage of inner hair cells transduced with the AAV3 vector. The mid- to high-frequency regions of the cochlea were efficiently transduced, as shown in Fig. 3. Almost all of the inner hair cells in the basal and middle cochlear regions were transduced with the AAV3 vector (Fig. 4). Transgene expression was not detected in the hair cells of the apical turn of the cochlea. The predominant expression in the middle and basal cochlear turns is reasonable, as the virus

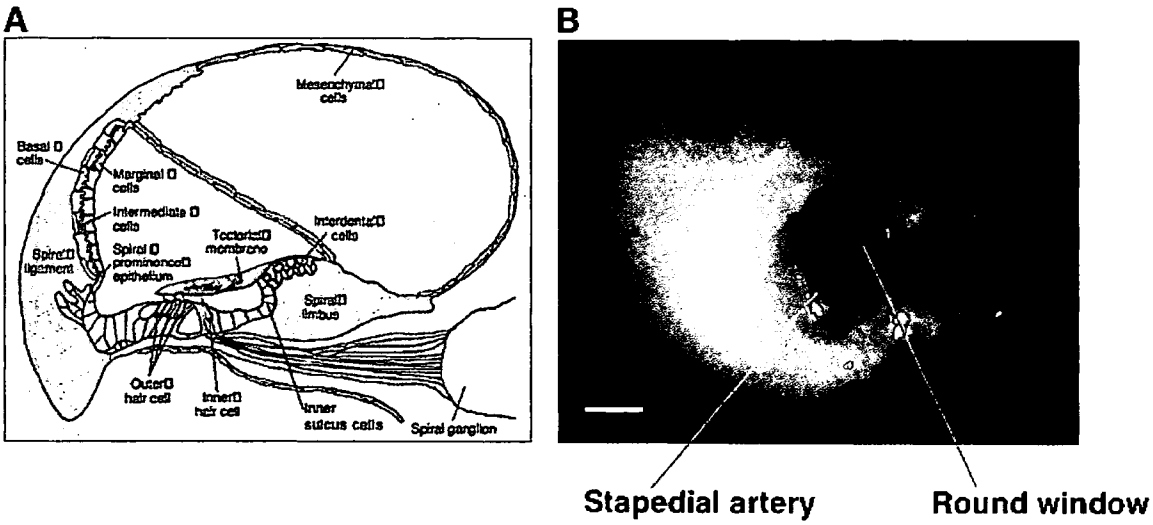


FIG. 1. (A) Schematic diagram of a cross section of the cochlea, demonstrating the scala vestibuli, scala tympani, and scala media or cochlear duct. The organ of Corti rests on the basilar membrane, with the hair cell cilia embedded in the gelatinous tectorial membrane. The outer margin of the cochlear duct contains the stria vascularis. Reproduced, by permission of the publisher, from [44]. (B) Direct visualization of the round window membrane in the right ear. The upper side of the picture is the back of the mouse and the right side is the head of the animal. The stapedial artery, a branch of the internal carotid artery, transverses an open bony semicanal within the round window niche. Bar denotes 500 μm, 15× original magnification.

TABLE 1: Expression of transgene in the mouse cochlea with vectors derived from the AAV1–4, 7, and 8 pseudotypes

Vector	Inner hair cells	Outer hair cells	Spiral ganglion	Stria vascularis	Spiral ligament	Spiral limbus	Reissner's membrane	Inner and outer pillar cells	Inner sulcus cells	Deiter's cells	Claudius' cells	Hensen's cells	Mesenchymal cells
AAV1	+++	–	++	–	++	++	++	–	+	–	–	–	++
AAV2	++	–	+	–	+	+	–	–	–	–	–	–	–
AAV3	++++	–	–	–	–	–	–	–	–	–	–	–	–
AAV4	–	–	–	–	–	–	–	–	–	–	–	–	+
AAV5	+++	–	+++	–	+	++	+	–	++	–	+	–	–
AAV7	+++	–	+	–	+++	++	–	–	+	–	+	–	++
AAV8	++++	–	–	–	+	+	–	–	++	–	+	–	+

The level of expression was graded by fluorescence intensity on a four-level scale (+, ++, +++, ++++) depending on the pixel/unit area count. ++++ means the strongest intensity of EGFP expression, + means the weakest intensity of EGFP expression, while – means no fluorescence.

was slowly infused into the scala tympani adjacent to the most basal turn of the cochlea. The percentage of transduced inner hair cells from the basal (high frequencies) to the apical (low frequencies) cochlear regions is shown in Fig. 4.

Cytotoxicity

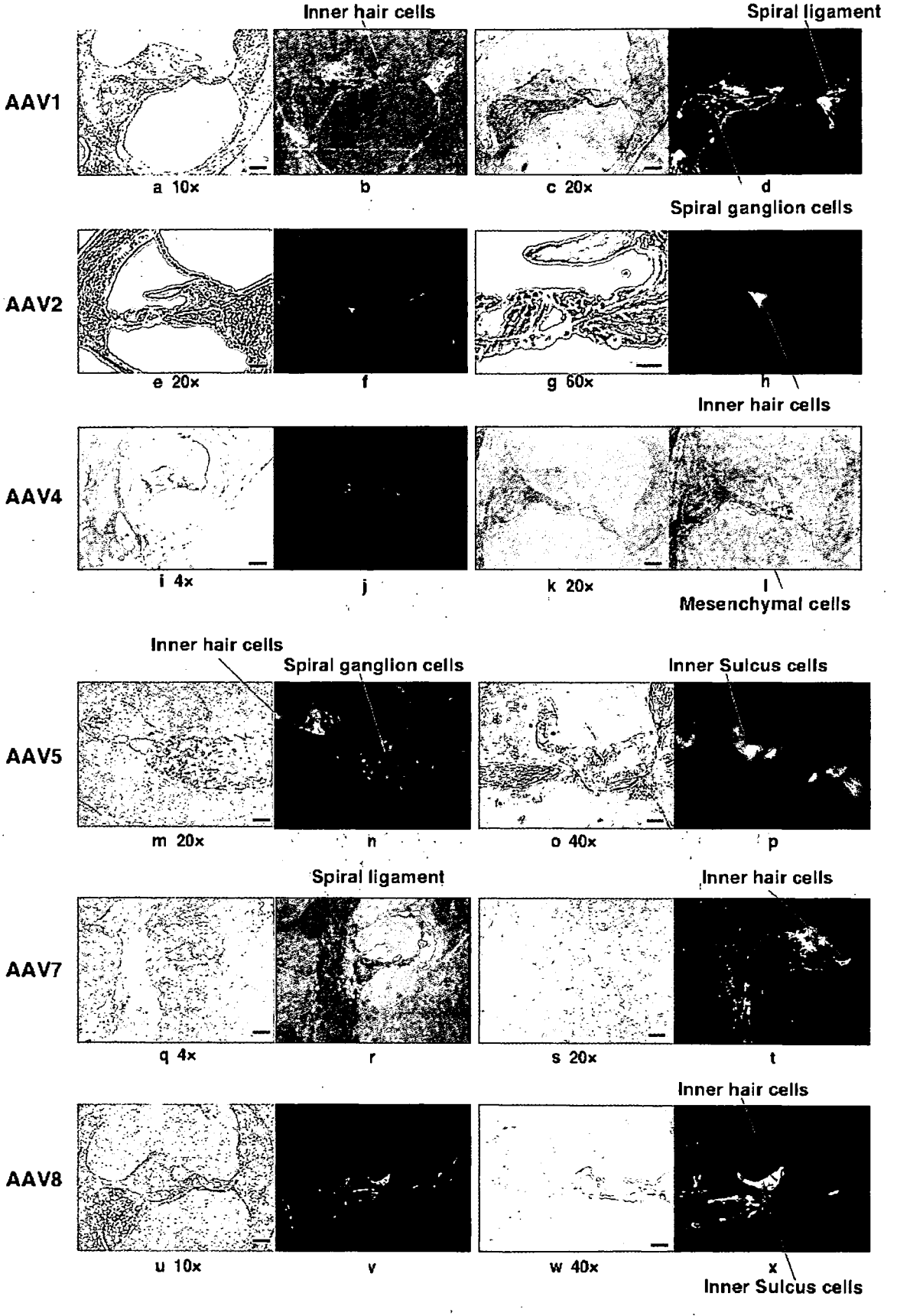
We detected no deleterious effects on the viability of transduced cells. We compared evoked auditory brainstem response (ABR) threshold levels before and after injection, using a two-way repeated measure of the analysis of variance. There was no significant loss in ABR and hence no change in cochlear function for up to 10 days following vector infusion (Figs. 5A and 5B). In addition, the cellular and tissue architecture of experimental cochleae remained intact. There was no evidence of endolymphatic hydrops after AAV vector injection in any of the animals. We observed no significant destruction of the inner or outer hair cells (Fig. 5C).

Discussion

In the present study, we assessed the utility of vectors derived from seven AAV serotypes for gene delivery into the cochlea. Our results showed that the AAV3 vector was the most efficient and specific in transducing cochlear inner hair cells, although these cells could also be transduced with AAV1, 2, 5, 7, and 8 vectors. The transduction efficiency of the spiral ganglion by the AAV5 vector was particularly high, followed by that of the AAV1, AAV2, and AAV7 vectors. The efficient and specific transduction of inner hair cells with the AAV3 vector suggests that it recognizes a unique host range with a distinct cellular receptor. Transduction efficiency is dependent on initial viral binding (a property of the viral capsid), entry, and various postentry processes such as intracellular trafficking and second-strand synthesis [20–22]. The genome size of AAV vectors has also been demonstrated to affect transduction efficiency [23]. Comparisons of the serotypes have indicated that heterogeneity in the capsid-encoding regions and a differential ability to transduce cells may be associated with different receptor and co-receptor requirements for cell entry [24]. However, the receptors and co-receptors of AAV3 have not yet been clearly identified.

In the current study, we found that cochlear inner hair cells could be transduced with six AAV serotypes, although Lalwini *et al.* [8] reported that outer hair cells could be transduced with a low titer (1×10^6 viral particles/ml) of AAV2 *in vivo*. After injecting the AAV2 vector, we found that the spiral ganglion neurons, the inner hair cells, and the cells in the spiral ligament were all transduced. This transduction pattern differs from that reported in previous studies [8,10,17], and this discrepancy might be due to the different delivery methods and dissimilar promoters. Although the CAG promoter directs

doi:10.1016/j.ymthe.2005.03.021



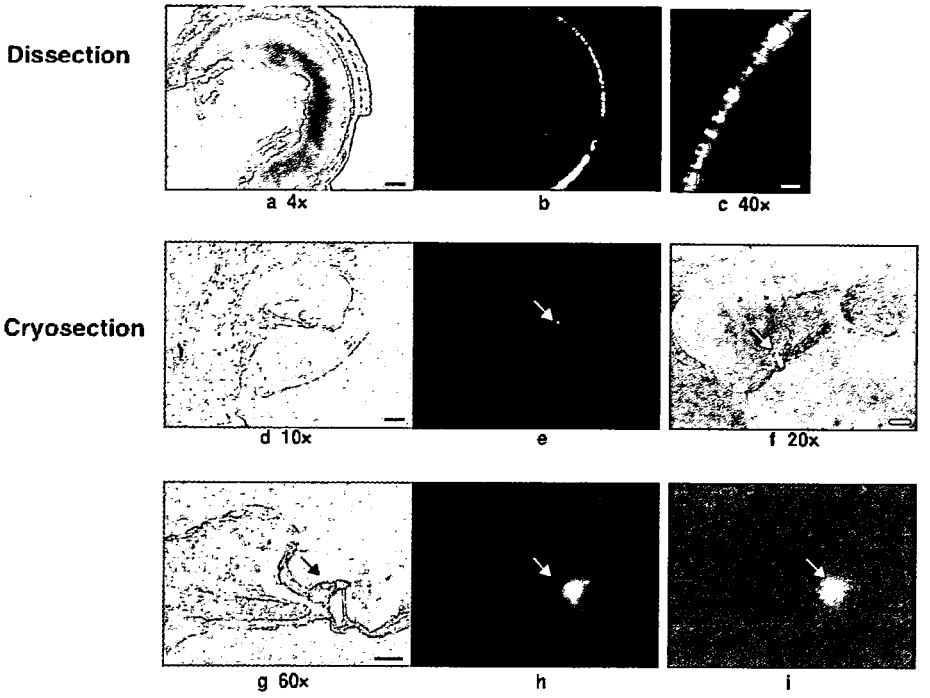


FIG. 3. Cochlear transduction with AAV3-CAG-EGFP. Dissected cochleae and cryosections show transgene expression in inner hair cells. (a) A light photomicrograph of the basal turn of the cochlea is shown, illustrating its laminar structure. (b) A fluorescence photomicrograph of this dissection. (c) A higher magnification view of the dissection shown in (b), illustrating a row of inner hair cells in the organ of Corti expressing EGFP. (d–i) Representative photomicrographs from three magnifications of a radial cochlear cryosection. (d) Light photomicrography of an intact cochlear duct. Fluorescence photomicrography of this duct is shown in (e). (h and i) A higher magnification of (e), illustrating EGFP expression within inner hair cells. Cryosections show transgene expression in the inner hair cells (arrows). Scale bars: 4×, 250 μm; 10×, 100 μm; 20×, 50 μm; 40×, 25 μm; 60×, 25 μm.

higher expression than do the cytomegalovirus (CMV) and EF-1α promoters [25], each promoter drives reporter gene expression in different cell types [26,27].

Cell-specific or -selective infectivity of the viral vectors suggests the presence of various factors to introduce the distinct expression patterns of the transgenes. Spiral ganglion neurons and glial cells can be transduced with a lentivirus–GFP construct *in vitro* but not *in vivo* [7]. The differential transducibility under *in vivo* and *in vitro* conditions reflects a high degree of structural isolation of the spiral ganglion and other cell types—such as the cells on the periphery of the endolymph—from the perilymph into which the viral vector was introduced. The strict separation of the endolymph from the perilymph is maintained by tight junctions that line the boundary between these fluid chambers. The size of the viral particle may contribute to the observed variability in transgene expression promoted by different vectors. The diameters of adenovirus and retrovirus (including lentivirus) particles are approximately 75 nm and greater than 100 nm, respectively, while the diameters of AAV vectors are typically 11–22 nm [28,29]. Thus, the larger size of lentiviruses and adenoviruses may limit their subsequent dissemination from the perilymph into the endolymph. The variable patterns of adenovirus- and lentivirus-mediated gene expression seen with different methods of inoculation may be due to the inoculation route, the volume and number of viral particles, differences in viral preparation, or differences in the method of transgene detection. The introduction of adenovirus vectors by cochleostomy or with an osmotic pump via the round window leads to a more efficient transduction of cochlear hair cells [30–32]. The apical domain (apical membrane and stereocilia) of cells in the sensory epithelium (hair cells and supporting cells) is bathed in endolymph, while the basal–lateral domain is immersed in perilymph. Access of the viral vectors to the endolymphatic space by cochleostomy may facilitate the transduction of hair cells and supporting cells. However, although the cochleostomy procedure has been tested, inoculation into the membranous labyrinth could not be confirmed [32]. In the present study, AAV vectors were found to infect cochlear hair cells easily *in vivo,* via round window injection.

Gene transfer into the cochlea through the round window membrane is ideal, because this procedure

FIG. 2. Transduction of the cochleae by AAV1-, AAV2-, AAV4-, AAV5-, AAV7-, and AAV8-based vectors. (a, c, e, g, i, k, m, o, q, s, u, and w) Light photomicrographs of cochlear cryosections. (b, d, f, h, j, l, n, p, r, t, v, and x) Fluorescence photomicrographs (green fluorescence from transgene). The spiral ligament cells were transduced prominently with the AAV1 and AAV7 vectors (d and r). Transgene expression in inner hair cells was detected with AAV1-, AAV2-, AAV5-, AAV7-, and AAV8-based vectors (b, h, n, t, and x). AAV4-based vector faintly transduced mesenchymal cells (j and l). The spiral ganglion cells showed significant levels of fluorescence with the AAV5-based vector (n). Intense fluorescence was detected with the AAV5- and AAV8-based vectors in the inner sulcus cells (p and x). Scale bars: 10×, 100 μm; 20×, 50 μm; 40×, 25 μm; 60×, 25 μm.

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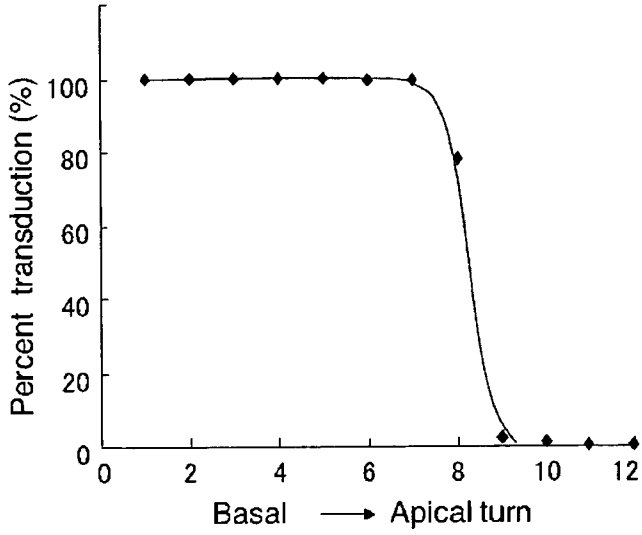

FIG. 4. EGFP expression profile of inner hair cells transduced with AAV3, as shown for a cross section subdivided into 12 segments ranging from the basal (high frequencies) to the apical (low frequencies) cochlear regions.

requires simple surgery without cochlear trauma [19]. Another critical factor in assessing the utility of a gene transfer vector is safety. Factors determining safety include the toxicity of the gene transfer agent itself, the provocation of immune responses, the generation of replication-competent virus, and the risk of creating genetically modified cells by insertional mutagenesis. The cells and tissues within the AAV-EGFP-perfused cochleae were free from inflammation and were generally intact. No pathological changes were observed in the organ of Corti, stria vascularis, or spiral ganglion cells. The long-term expression of EGFP within the cochlear tissues is consistent with data obtained from other animal models and different organ systems [9,33]. Since EGFP is known to introduce cellular toxicity, vectors expressing physiologically therapeutic proteins would achieve longer transduction periods than EGFP. Gene transfer into the inner hair cells presents numerous opportunities for auditory neuroscience. Potential applications include the localization of proteins by expression of tagged constructs, the generation of dominant-negative or antisense knockouts of endogenous proteins, the rescue of mutant phenotypes to identify disease genes, and perhaps even the treatment of auditory disorders. Advances in the molecular basis of auditory diseases have allowed the identification of a number of genetic disorders such as presbycusis, acoustic trauma, and ototoxicity. The development of gene therapy now allows us to evaluate the effects of transferring therapeutic genes into the inner ear by several different strategies. The expression of marker genes in the inner ear tissue has been demonstrated. Further studies will improve our understanding of cochlear function as well as provide for the development of novel therapies for a wide variety of inner ear diseases. Intracochlear gene transfer using AAV vectors has been established as a viable experimental proposition. Future study will include the transfer of functioning genes *in vivo* and the development of alternative vectors. While clinical application may be some way off, it is vital that gene delivery techniques are optimized in anticipation of future need.

In conclusion, the data presented in this paper demonstrate successful gene transfer into several types of cochlear cells *in vivo* with AAV-based vectors. Interestingly, the AAV3 vector promoted inner hair cell-specific transduction. These findings are of value for further molecular studies of the cochlear inner hair cells and for gene replacement strategies to correct hereditary hearing loss due to specific monogenic mutations affecting cochlear inner hair cells.

MATERIALS AND METHODS

Construction and preparation of proviral plasmids. The AAV vector proviral plasmid pAAV2-*LacZ* harbors an *Escherichia coli* β-galactosidase expression cassette with the CMV promoter, the first intron of the human growth hormone gene, and the SV40 early polyadenylation sequence, which are flanked by inverted terminal repeats (ITRs) [34]. The *LacZ* expression cassette of pAAV2-*LacZ* was ligated to *Not*I-excised pAAV5-RNL [35] to form the proviral plasmid pAAV5-*LacZ*. The pAAV2-CAG-EGFP-WPRE construct consists of the EGFP gene under the control of the CAG promoter (the chicken β-actin promoter associated with the cytomegalovirus immediate-early enhancer) and WPRE (woodchuck hepatitis virus posttranscriptional regulatory element) flanked by ITRs. The WPRE cassette augments the stability of transgene mRNA [36] and increases EGFP expression levels, thereby ensuring long-term transgene expression. A *Bam*HI–*Xba*I fragment containing the EGFP cDNA excised from pEGFP-1 and a *Hin*dIII fragment containing the WPRE sequence excised from pBS II SK$^+$WPRE-B11 (a gift from Dr. J. Donello) was ligated to *Xho*I linkers and cloned into an *Xho*I site of pCAGGS (a gift from Dr. J.-I. Miyazaki) to create pCAG-EGFP-WPRE. The EGFP expression cassette from pCAG-EGFP-WPRE was ligated to the *Not*I-excised pAAV2-*LacZ* and pAAV5-RNL [35] to form the proviral plasmids pAAV2-CAG-EGFP-WPRE and pAAV5-CAG-EGFP-WPRE, respectively. The AAV-helper plasmid harbors Rep and Cap. The adenovirus helper plasmid pAdeno5 (identical to pVAE2AE4-5) encodes the entire E2A and E4 regions and the VA RNA I and II genes [37]. Plasmids were purified with the Qiagen plasmid purification kits (Qiagen K.K., Tokyo, Japan).

Recombinant AAV vector production. Vectors derived from the AAV1–4, 7, and 8 pseudotypes were produced with the AAV packaging plasmid pAAV1RepCap (for AAV1) [38], pHLP19 (for AAV2), pAAV3RepCap (for AAV3) [39], pAAV4RepCap (for AAV4) [40], pAAV7RepCap (for AAV7) [41], or pAAV8RepCap (for AAV8) [41] and the AAV proviral plasmid pAAV2-*LacZ* or pAAV2-CAG-EGFP-WPRE. The plasmids pAAV5RepCap [35] and pAAV5-*LacZ*, or pAAV5-CAG-EGFP-WPRE, were used to produce vector with the AAV5 pseudotype [42]. Seven AAV serotype vectors were produced as previously described by the three-plasmid transfection adenovirus-free protocol [37]. Briefly, three days before transfection, 293 cells were plated onto a 10-tray Cell Factory (Nalge Nunc International, Rochester, NY, USA; 6×10^7 cells/10-tray). The cells were cotransfected with 650 μg each of the proviral plasmid, the AAV vector packaging plasmid, and the adenovirus helper plasmid pAdeno5 [34] by the calcium phosphate coprecipitation method. The medium was changed following incubation for 6–8 h at 37°C. Recombinant AAV was harvested 72 h after transfection by three freeze/thaw cycles. The crude viral lysate was purified twice on a cesium chloride two-tier centrifugation

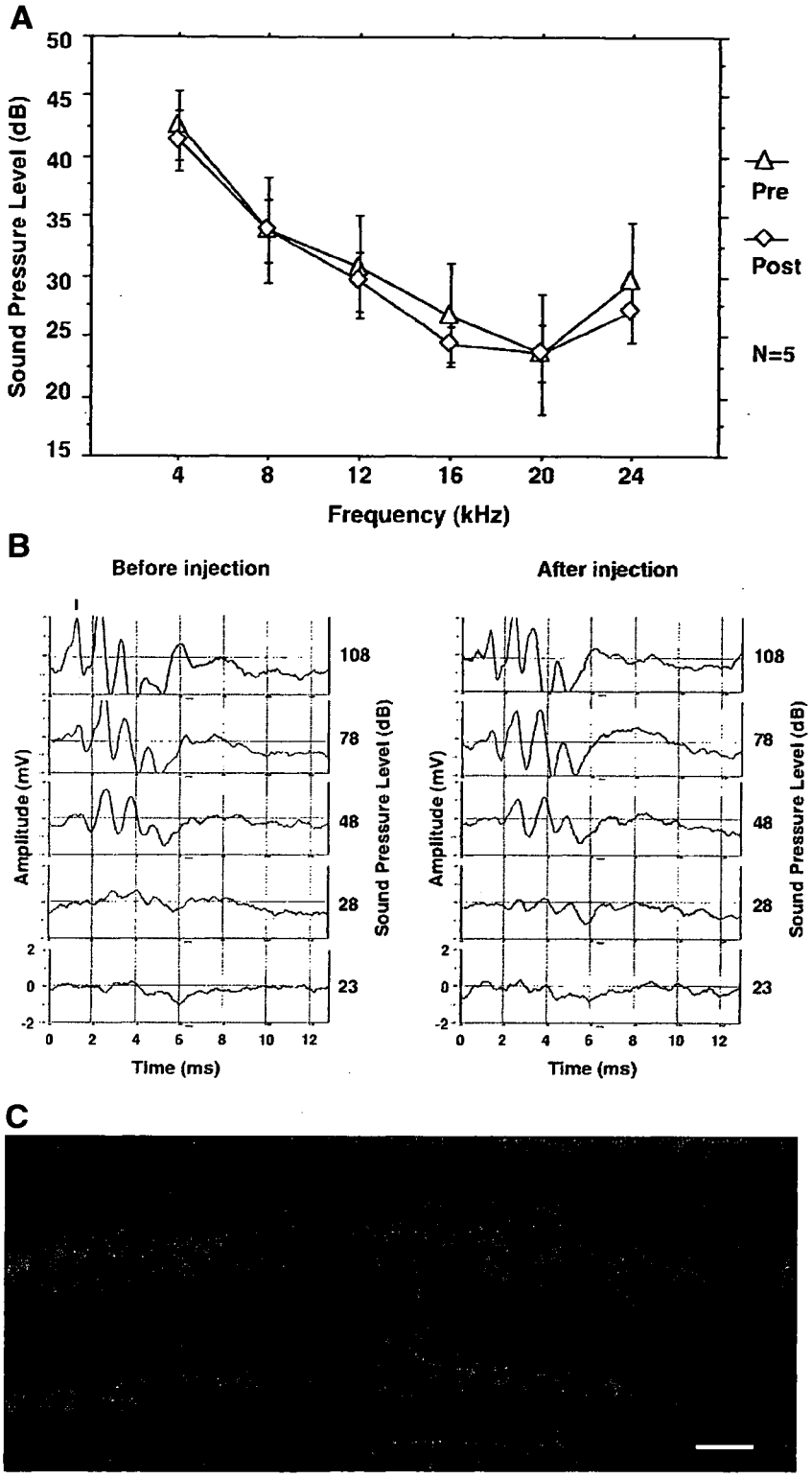

FIG. 5. (A) ABR threshold (mean ± SD) at each frequency tested preoperatively (pre) versus postoperatively (post). (B) Example of ABR waveforms in C57BL/6J at various stimuli (16 kHz; 108 dB, 78 dB, 48 dB, 28 dB, and 23 dB). ABR were tested in the transduced ear prior to viral injection and 10 days after injection. Wave I was measured to analyze the activity of the cochlea. (C) F-actin staining showing that no outer hair cells were lost from inoculated cochleae. Original magnification 40×; scale bar, 25 μm.

gradient as described previously [24]. The viral stock was treated with DNase and titrated by quantitative real-time PCR with plasmid standards [43].

Surgical procedures and cochlear perfusions. All animal studies were performed in accordance with the guidelines issued by the committee on animal research of Jichi Medical School and approved by its ethics

doi:10.1016/j.ymthe.2005.03.021

committee. Sixty female C57BL/6J mice (4 weeks of age; CLEA Japan, Tokyo, Japan) and 40 male ICR mice (2 months of age; Japan SLC, Shizuoka, Japan) were utilized. The mice were initially anesthetized with ketamine (50 mg/kg) and the analgesic xylazine (5 mg/kg). A postauricular approach was used to expose the tympanic bony bulla. A small opening (2 mm) in the tympanic bulla was carefully made to allow access to the round window membrane. In the tested groups, 5 μl AAV vector solution (5×10^{10} gc) was microinjected into the cochlea through the round window over 10 min with a glass micropipette (40 μm in diameter) fitted on a Univentor 801 syringe pump (Serial No. 170182, High Precision Instruments, Univentor Ltd., Malta) [19]. A small plug of muscle was used to seal the cochlea and the surgical wound was closed in layers and dressed with antibiotic ointment. Five mice of each strain received control cochlear perfusions with artificial perilymph (145 mM NaCl, 2.7 mM KCl, 2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 5 mM Hepes) alone. Each AAV-EGFP serotype was injected into five mice of each strain. Another 20 C57BL/6J mice were injected with the AAV3 vector to study long-term expression.

Cochlear function assessment using ABR. To assess the physiological status of experimental ears, auditory thresholds were determined with multiple frequency and intensity tone bursts by performing ABR audiometry with Tucker–Davis Technologies and Scope v3.6.9 software (Power Lab/200; ADInstruments, Castle Hill, Australia). Tone pipes were introduced into the operated ears of the anesthetized mice, and evoked potentials were recorded using needle electrodes inserted through the skin. ABR were elicited and measured 256 times at 4, 8, 12, 16, 20, and 24 kHz frequencies with tone bursts in systematic 5-dB steps. The rise/fall times for the tone bursts were 0.1 ms rise/ms flat (cosine gate). Free-field system was used as a calibration procedure. Wave I was measured to analyze the activity from the cochlea. The lowest stimulus level that yielded a detectable ABR waveform was defined as the threshold. ABR were tested in the infused ear prior to surgery and 10 days postsurgery. Data were statistically analyzed using repeated-measures analysis of variance followed by paired Student's t test performed with StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Values of $P < 0.05$ were considered significant.

Histology. Cochlear transgene expression patterns were determined for all AAV serotypes by visualizing EGFP expression. The animals were sacrificed 10 days after injection, and intracardiac perfusion was performed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The cochleae were harvested and the stapes footplates were removed. For AAV3-mediated transduction, the animals (five mice for each time point) were sacrificed 1, 2, 4, 8, or 12 weeks after inoculation. Postfixation was carried out in 4% PFA for 4 h at 4°C, and decalcification was performed in 10% EDTA for 12 days at room temperature. The cochlear half-turns were microdissected and processed and the other half-turns were prepared by cryosection (10 μm) to detect EGFP expression by using an Olympus IX70 (Olympus Corp., Tokyo, Japan) fluorescence microscope with a standard fluorescein isothiocyanate filter set and Studio Lite software (Olympus Corp.). Cells that exhibited fluorescence were considered positive for transgene expression. The level of expression was graded by fluorescence intensity on a four-point scale (+, ++, +++, ++++) depending on the pixel/unit area count. Hair cell counts were carried out with dissected cochlea.

ACKNOWLEDGMENTS

The authors thank Avigen, Inc. (Alameda, CA, USA) for providing pAAV-LacZ, pHLP19, and pAdeno; Dr. John A. Chiorini for pAAV4RepCap (identical to pSV40oriAAV4-2), pAAV5-RNL, and pAAV5RepCap (identical to 5RepCapB); and Dr. James M. Wilson for pAAV7RepCap and pAAV8RepCap. We also thank Dr. John E. Donello (Infectious Disease Laboratory, The Salk Institute for Biological Studies) for providing pBS II SK+WPRE-B11 and Dr. Jun-Ichi Miyazaki (Osaka University Graduate School of Medicine) for pCAGGS. The authors also thank Mr. Takeshi Hayakawa (Bio Research Center Co., Ltd.), Ms. Miyoko Mitsu, and Ms. Kiyomi Aoki for their encouragement and technical support. This study was supported in part by (1) grants from the Ministry of Health, Labor, and Welfare of Japan; (2) Grants-in-Aid for Scientific Research; (3) a grant from the 21 Century COE Program; and (4) the High-Tech Research Center Project for Private Universities matching fund subsidy from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

RECEIVED FOR PUBLICATION NOVEMBER 1, 2004; ACCEPTED MARCH 24, 2005.

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HUMAN GENE THERAPY 16:1413–1421 (December 2005)

Repair of Articular Cartilage Defect by Intraarticular Administration of Basic Fibroblast Growth Factor Gene, Using Adeno-Associated Virus Vector

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ABSTRACT

The objective of this study was to establish the potency of adeno-associated virus (AAV) as a viral vector to transport the basic fibroblast growth factor (bFGF) gene into synovial tissue, and to evaluate the consequent repair of articular cartilage defects. In the *in vitro* study, LacZ- and bFGF-encoding genes were transduced into rabbit synoviocytes by recombinant adeno-associated virus (AAV) vector, and the cells were cultured for 2 weeks. The percentage of successfully transduced LacZ-positive cells was assessed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining, and the concentration of bFGF in the culture supernatant was confirmed by bFGF-specific enzyme-linked immunosorbent assay. In the *in vivo* study, 12- to 14-week-old Japanese white rabbits (all female) were used. AAV-bFGF was administered into an artificially created full-thickness defect (5 mm in diameter and 3 mm deep) in the patellar groove of the distal femur. Cartilage repair was subsequently monitored at 4, 8, and 12 weeks, by macroscopic and histological examination, and results were graded on the basis of semiquantitative scores. *lacZ* gene expression in synoviocytes reached more than 93% within the first 2 weeks, and the mean bFGF concentration in the culture supernatant of the bFGF gene-transduced group was significantly increased ($p < 0.01$). Semiquantitative macroscopic and histological assessment indicated that the average score was significantly better in the bFGF-transduced group throughout the observation period, suggesting better cartilage repair. These results demonstrate that gene transfer into synoviocytes, using the AAV vector, was a potent method of gene transduction. Moreover, after intraarticular administration of AAV-bFGF, constant expression of bFGF in the knee joints resulted in substantial cartilage regeneration that, with further long-term study, could possibly merit consideration for clinical application.

OVERVIEW SUMMARY

Adeno-associated virus (AAV) is well known as a dynamic gene transporter with a number of biological advantages, such as a lack of virulence in the wild type and the ability to maintain continuous local expression of the therapeutic gene. It is also known that integration of the basic fibroblast growth factor (bFGF) gene into tissues, such as synoviocytes, by intraarticular administration has the potential to produce lasting expression and sustained secretion of the growth factor, leading to regeneration of cartilage. In this study, using AAV as a viral vector, we have demonstrated that the bFGF cache was notably enlarged in bFGF-transduced synoviocytes, using rabbits as laboratory animals, *in vitro* and *in vivo*. Our study clearly demonstrates that, with intraarticular administration of AAV-bFGF, a high efficiency of transduction can be obtained with a complementary elevated level of cartilage repair that has definite clinical potential in the treatment of cartilaginous diseases.

INTRODUCTION

ARTICULAR CARTILAGE is a highly differentiated tissue with limited capacity for self-repair. Thus, it is extremely vulnerable to traumatic erosion or defect, and osteoarthritic degeneration, which often lead to joint dysfunction associated with pain and/or limitation in range of motion. Current treat-

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ment for osteoarthritis consists of (1) conservative therapy such as use of short-acting nonsteroidal antiinflammatory drugs, intraarticular injection of steroids, and/or other agents such as hyaluronic acid, and (2) surgical intervention such as high-tibial osteotomy and surface replacement. However, these treatments do not always relieve joint pain completely and, moreover, do not aid regeneration of cartilage. Thus, cartilage repair at an early stage, if possible, would appear to be a more fundamental method of preventing the irreversible detrimental outcome of total joint failure. In the past, some efforts have been made to repair osteochondral defects by methods such as treatment by transplantation of cultured chondrocytes (Brittberg *et al.*, 1994) or, more recently, *ex vivo* transplantation of cultured chondrocytes (Yokoo *et al.*, 2005); however, both methods had their disadvantages. In the former, when a large quantity of chondrocytes from normal articular cartilage is required, donor sites have limited capacity to provide them. The latter study overcomes the donor problem, but presents other disadvantages such as infection during culture, and complexity of the method itself.

Other studies have reported that basic fibroblast growth factor (bFGF) is one of the most potent substances for proliferation and differentiation of chondrocytes, triggering a cascade of events in the cartilage repair process (Cuevas *et al.*, 1988; Hunziker and Rosenberg, 1996; Shida *et al.*, 1996; Weisser *et al.*, 2001). With this knowledge, it can be hypothesized that to maintain a certain level of bFGF for a specific time period at a chondral defect site could prove to be advantageous. Application of gene therapy could provide the answer, and cases of cartilage repair using naked DNA or viral vectors as gene transporters have been reported (Arai *et al.*, 1997; Baragi *et al.*, 1997; Kang *et al.*, 1997; Doherty *et al.*, 1998).

However, such gene transduction has also proved problematic, with a low level of transduction efficiency, lack of capacity to maintain long-term expression of the therapeutic gene, and difficulty in maintaining adequate safety levels. In other studies, the adeno-associated virus (AAV) has been recognized as a powerful tool with which to transduce genes into target cells and tissues (Kaplitt *et al.*, 1994; Berns and Giraud, 1995; Xiao *et al.*, 1997; Schwarz, 2000), with several advantages over other virus vectors. The benefits include a lack of virulence in the wild type, an inherent inability to replicate itself, an ability to transduce nondividing cells and to integrate into a host genome, and long-term expression of the transduced gene. Furthermore, Goater *et al.* (2000) have shown there is a substantial integration rate of AAV into synovial tissue, but there has not been any quantitative analysis so far. Synovial tissue is the most abundant tissue in an articular joint, which makes it a formidable host for viral transfection.

From the advantages demonstrated by AAV, there would appear to be a distinct possibility that cartilage repair could be accomplished by transduction of the bFGF gene using AAV as a viral vector, and we hypothesizd that if the high level of integration into chondrocytes, as shown by Yokoo *et al.* (2005), could also be achieved in synoviocytes via intraarticular administration of AAV-bGFG, cartilage regeneration may result.

Therefore, in this study, we set out to evaluate the potency of the AAV vector, and to investigate whether articular cartilage repair is possible. Two methods were proposed: first, *in vitro*, to observe the efficiency of AAV as a gene transporter when targeted at synoviocytes, and second, *in vivo*, to determine whether cartilage regeneration is possible by intraarticular administration of AAV-bFGF into rabbit knee joints with chondral defects.

PILOT STUDIES

In vivo studies by Goater *et al.* (2000) confirmed high induction rates of AAV in synovial tissues. Our previous *ex vivo* studies (Kobayashi *et al.*, 2002; Yokoo *et al.*, 2005) also demonstrated that gene transduction was effective in both synoviocytes and chondrocytes. To reconfirm and establish our method protocol for the main experiments, a series of preliminary studies, in which AAV-LacZ was administered into synovial tissues of both knee joints of two rabbits, was performed. Subsequent 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining confirmed both macroscopically and histologically, that gene tranduction was indeed effective and that a substantial transduction rate could be expected when synoviocytes were targeted by AAV. In another series, the optimal viral concentration was also determined by administering AAV-LacZ at various concentrations. The minimal dose was 1×10^5 particles, and the transfection rate was optimized at 1×10^9 particles (100 μl of solution containing 1×10^{10} particles/ml), after which the visible percentage of LacZ-positive cells reached a plateau. However, because of the inherent nature of a single synovium, we were unable to perform a precise quantitative analysis of LacZ-positive synoviocytes (details of the data obtained are not shown).

MATERIALS AND METHODS

AAV vector production

Two different adeno-associated viral constructs were prepared for the study: AAV-LacZ, carrying the bacterial β-galactosidase gene, a marker gene that can be detected by X-Gal staining; and AAV-bFGF, carrying the bFGF gene, which harbors a nuclear localization signal, under the regulation of the cytomegalovirus (CMV) immediate-early promoter. The AAV subtype 2 vector plasmid used was derived from the vector plasmid pW1 (hereafter referred to as pLacZ), which carries the *lacZ* gene (Price *et al.*, 1987). Recombinant bFGF gene was obtained from Takeda Pharmaceutical (Osaka, Japan; GenBank accession number X07285). A fragment containing bFGF cDNA was amplified by polymerase chain reaction (PCR) using the following primer pairs (*Eco*RI and *Xho*I sites are underlined): 5′-ATGAATTCATGGCTGCCGGCAGCATCACTTCGCTT-3′ and 5′-ATCTCGAGAGAGTCAGCTCTTAGCAGAC-3′. The fragment was subcloned between the *Eco*RI and *Xho*I sites of the pLacZ AAV vector plasmid to replace the *lacZ* gene (pbFGF). pIM45 is an AAV helper plasmid carrying subtype 2 AAV *rep* and *cap* genes, which are required for replication and caspid formation of AAV vectors. pladeno-1, a plasmid containing the E2A, E4, and VA genes of the adenovirus genome, was used in place of helper adenovirus for AAV production.

Subconfluent human fetal kidney cells (293 cells) were cotransfected by the calcium phosphate coprecipitation method

with pbFGF, pIM45, and pladeno-1 to produce the AAV inducing bFGF gene (AAV-bFGF). After 48 hr, cells were harvested and lysed in Tris-HCl buffer (10 m*M* Tris-HCl, 150 m*M* NaCl, pH 8.0) through three cycles of freezing and thawing. One round of sucrose precipitation and two rounds of CsCl density gradient ultracentrifugation were performed to isolate AAV-bFGF from the lysates. The vector titer was determined by quantitative DNA dot-blot hybridization of the DNase I-resistant fraction.

In vitro *study: examination of the potency of AAV as a viral vector when targeted at synoviocytes*

Isolation of synoviocytes. Five Japanese white rabbits (Oriental Yeast, Tokyo, Japan), 12 weeks old, weighing an average of 2.1 kg, were used for the study. Under intravenous anesthesia with pentobarbital sodium (Somnopentyl; Schering-Plough Animal Health, Union, NJ), synovial tissues were harvested from the knee joint, washed three times in phosphate-buffered saline (PBS), and cut into small pieces. These pieces were then treated with 0.25% collagenase (type II collagenase; Worthington Biochemical, Lakewood, NJ) for 2 hr at 37°C. The treated synoviocytes were washed three times with PBS and centrifuged for 5 min at 1500 rpm, and then equally divided into four separate flat-bottomed plates (Falcon [diameter, 30 mm]; BD Biosciences Discovery Labware, Bedford, MA) at 1×10^5 cells per plate in 1.0 ml of Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) and antibiotics (penicillin G [100 U/ml] and streptomycin [0.1 mg/ml]; GIBCO-BRL Invitrogen, Carlsbad, CA) (DMEM–FCS), at 37°C in a 5% CO_2–air atmosphere for 48 hr.

Gene transduction into synoviocytes. After 48 hr in growth medium, the synoviocyte cultures were removed and washed once with serum-free DMEM. A 500-μl volume of serum-free DMEM containing AAV-LacZ was added to the control group culture plate, and 500 μl of serum-free DMEM containing AAV-bFGF was added to the gene-transduced group culture plate. This was to enable quantification of transgene expression at the optimal number of viral particles (10^7 particles per cell) determined from pilot studies (data not shown).

Five samples from the bFGF-transduced group and five from the control group (*lacZ*-transduced group) were used for the experiment to determine the efficiency of gene transduction *in vitro*. Culture medium was not exchanged during the examination period of 2 weeks. 3, 7, and 14 days after transduction, supernatant was removed, and LacZ expression in the LacZ group was assessed by the X-Gal staining technique (Yokoo *et al.*, 2005). The efficiency of gene transduction was calculated as the average percentage of X-Gal-positive cells per total living cells, determined by viewing three randomly selected fields (magnification, ×100) with an optical microscope.

Measurement of bFGF concentration in culture medium. Five samples from the bFGF-transduced group and five from the control group (*lacZ*-transduced group) were used to determine the accumulation of bFGF in culture supernatant. The culture medium of bFGF-transduced or control chondrocytes was not exchanged at each sampling. 3, 7, and 14 days after transduction, culture supernatants were collected from all four bFGF-transduced or control group culture wells and, after centrifugation, were stored at −80°C until analysis. The bFGF concentration in culture supernatants of both groups were measured by enzyme-linked immunosorbent assay (ELISA), using a bFGF-specific ELISA kit (Quantikine; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

In vivo *study: cartilage regeneration by intraarticular administration of AAV-bFGF*

Intraarticular administration of AAV-bFGF into articular cartilage defect. Surgery and postoperative management were carried out under certification of the Yokohama City University Animal Center (Yokohama City, Japan) and with the approval of the Animal Care Committee.

Twenty-four Japanese white rabbits were used for the *in vivo* study. They were anesthetized as required by intravenous injection of pentobarbital sodium, and both knees were sterilized for surgery. A 2.5-cm medial parapatellar incision was made and the patella was dislocated laterally. A full-thickness defect (diameter, 5 mm; depth, 3 mm) was artificially created in the patellar groove of the anterior surface of the distal femur, using a hand drill bit; 1×10^9 particles (100 μl of solution containing 1×10^{10} particles/ml) of AAV-bFGF, which had been shown to be the optimal amount in our pilot study, was administered with a microsyringe, and the dislocated patella was reduced to its original position immediately. The same procedure was repeated for the contralateral knee. In the control group, the same procedure was followed, but 100 μl of phosphate-buffered saline (PBS) solution was administered instead of AAV-bFGF. The rabbits were allowed freedom of movement immediately after surgery. Four rabbits from each group were subsequently killed at 4, 8, and 12 weeks for macroscopic and histological examination of the articular surface of both knees.

Macroscopic and histological evaluation of repaired cartilage. To enable comparative examination of the articular components, four animals (both knees) from the AAV-bFGF group, and an equivalent number from the PBS (control) group, were sacrificed at 4, 8, and 12 weeks. Macroscopic assessment and photography were followed by *en bloc* resection of the distal end of the femur. The specimens were fixed with 10% buffered formaldehyde for 1 week, followed by decalcification with 0.5 *M* EDTA solution for 2 to 3 weeks. Sagittal sections through cartilaginous tissue and bone were prepared and stained with hematoxylin and eosin (H&E) and safranin O. Macroscopic findings for each specimen were evaluated semiquantitively according to the scale described by Kumagai *et al.* (2003). The Kumagai Scale for Macroscopic Assessment of Cartilage Surface is an established system of parameters for grading specimens in color (0–3), connection of new tissue to adjacent cartilage (0–2), size of remaining defect (0–5), depression of the defect (0–4), and depth of the defect (0–2). The overall score scales from zero, suggesting full repair, to 16, indicating no repair.

Likewise, histological findings for each specimen were evaluated semiquantitatively according to the scale described by Wakitani *et al.* (1994). The Wakitani Scale for Histological As-

sessment of Cartilage Defects is an established system of parameters for grading specimens in cell morphology (0–4), matrix staining (0–3), surface regularity (0–3), thickness of cartilage (0–2), and integration of donor with adjacent host cartilage (0–2). The overall score scales from zero, suggesting full repair, to 14, indicating no repair.

Evaluation of supernatant bFGF concentration after in vivo *study.* At the time of sacrifice, synovial tissue was harvested for examination of bFGF concentration after *in vivo* administration of AAV-bFGF. Harvested tissues were treated with 0.25% type II collagenase as previously described, and cultured for 1 week at 37°C without exchange of medium. Supernatant of the synoviocyte cultures from the bFGF group and from the control group were analyzed at 4, 8, and 12 weeks and evaluated by the ELISA method.

Immunohistological assessment for type II collagen in cell matrix. Immunohistological assessment targeted the type II collagen seen in the extracellular matrix of the regenerated cartilage, using rabbit anti-type II collagen antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical analysis. Data were expressed as means ± standard deviation (SD). The statistical significance of differences was calculated with StatView version J-5.0 (SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) and the Mann–Whitney *U* test were used for analyzing statistical significance. p Values less than 0.05 were considered significant.

RESULTS

In vitro *study: examination of the potency of AAV as a viral vector when targeted at synoviocytes*

Efficiency of gene transduction into synoviocytes: LacZ expression. Values for synoviocytes transfected with AAV-LacZ were determined at intervals of 3, 7, and 14 days after transduction. The percentage of LacZ-positive cells among total living cells was determined to be 55.8 ± 6.0 (mean ± SD), 83.4 ± 3.8, and 92.0 ± 1.6%, respectively, at an optimal dose of 10^7 particles per cell (Table 1). The percentage of successfully transduced synoviocytes increased in a vector dose-dependent manner up to an optimal figure of 10^7 particles per cell, when approximately 100% efficiency was achieved. Doses of viral particles above that level failed to improve the transduction rate. Microscopic examination did not reveal any evidence of cell death or cytopathic change in the transduced cells.

bFGF gene expression in transduced synoviocytes. bFGF production was detected in both bFGF-transduced and control cells. bFGF concentration in the culture supernatant was 31.2 ± 7.8, 80.5 ± 16.4, and 120.8 ± 22.5 ng/ml at 3, 7, and 14 days after transduction, respectively, in bFGF-transduced cells (Table 1). In control cells, the bFGF concentration was 22.6 ± 9.2, 23.1 ± 6.4, and 28.4 ± 7.8 ng/ml at 3, 7, and 14 days after transduction, respectively. The bFGF concentration was significantly greater in bFGF-transduced cells than in control cells at each sampling time point ($p < 0.01$).

In vivo *study: cartilage regeneration by intraarticular administration of AAV-bFGF*

Macroscopic findings on cartilage regeneration. Observation of the articular cartilage defect site showed regeneration in both the bFGF-transduced and control groups. At 4 weeks, cartilage regeneration was not obvious in the control group (Fig. 1A), whereas partial coverage by regenerated cartilage was seen in the AAV-bFGF group (Fig. 1B). At 8 weeks, the difference in cartilage regeneration became more apparent (Fig. 1C and D) and at 12 weeks, the margin between the regenerated tissue and the original cartilage was not distinguishable in the AAV-bFGF group (Fig. 1F), whereas the margin was clearly visible in the control group (Fig. 1E).

Histological findings on cartilage regeneration. At 4 weeks after administration of AAV-bFGF, tissues obtained from the bFGF-transduced group showed slight formation of a chondral layer in the deep part of the cartilage defect. Tissue was composed of round chondrocytes with weakly safranin O-stained extracellular matrix (Fig. 2B). There was no integration of the edges of regenerated tissue with normal adjacent cartilage or reconstitution of the osteochondral junction in any specimen. In the control group, the extracellular matrix was hardly stained with safranin O (Fig. 2A), and no cartilage regeneration was

TABLE 1. TIME-DEPENDENT LacZ EXPRESSION AND SUPERNATANT bFGF CONCENTRATION IN CULTURED SYNOVIOCYTES[a]

Days after transfection	*Percentage of LacZ-positive cells*	*bFGF concentration (ng/ml)*	
		AAV-bFGF group[b]	*Control group*[b]
3	55.8 ± 6.0	31.2 ± 7.8	22.6 ± 9.2
7	83.4 ± 3.8	80.5 ± 16.4	23.1 ± 6.4
14	92.0 ± 1.6	120.8 ± 22.5	28.4 ± 7.8

[a]LacZ expression was assessed by X-Gal staining 3, 7, and 14 days after AAV-LacZ transduction of chondrocytes. Values represent mean percentages ± SD. bFGF concentration of the supernatant was analyzed by ELISA 3, 7, and 14 days after AAV-bFGF transduction of synoviocytes. Values represent mean concentrations (ng/ml) ± SD. $n = 5$.

[b]$p < 0.01$.

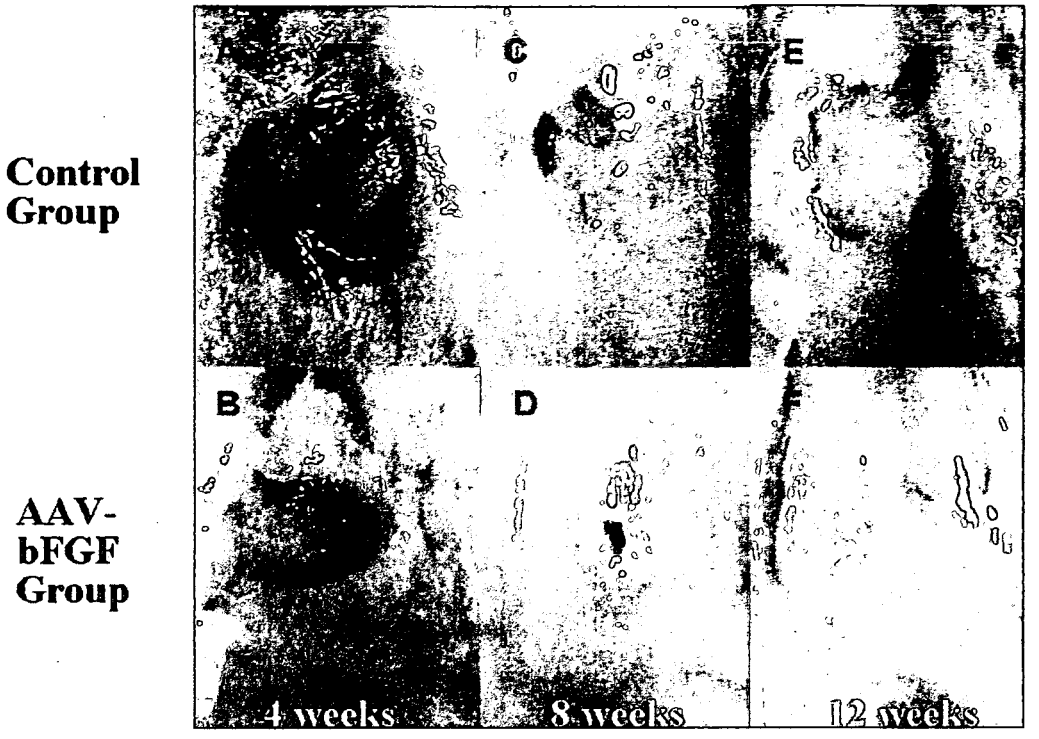

FIG. 1. Macroscopic photographs showing cartilage repair after interarticular administration of AAV-bFGF and control at 4, 8, and 12 weeks after operation. At 12 weeks in the AAV-bFGF group, almost complete repair is seen, whereas in the control group, the margin between the newly formed cartilage and the surrounding normal cartilage is obvious.

seen. The tissues showed inflammatory granulation of the defect area, and any bone may have been partly obscured because of apoptosis and replacement of fibrous tissues.

At 8 weeks, in the bFGF-transduced group, the extracellular matrix was more distinctly stained with safranin O, and cartilage regeneration was seen not only in the deep part, but also in the superficial layer (Fig. 2D). Both edges of the regenerated cartilage showed almost complete integration with normal adjacent cartilage, and reconstitution of the osteochondral junction was seen in five specimens. In Fig. 2C, tissues from the control group were essentially the same as those seen at 4 weeks. Although safranin O staining appears stronger, there is no apparent formation of hyaline cartilage and no construct of mature cartilage tissue is visible.

In the bFGF-transduced group at 12 weeks, the intensity and thickness of the extracellular matrix were increased when compared with the findings at 4 and 8 weeks, and the microstructure of the regenerated tissue resembled the surrounding normal cartilage (Fig. 2F). There was reconstitution of the osteochondral junction in most specimens, and formation of the surface layer of articular cartilage was distinctly more pronounced in contrast to that shown in the control (Fig. 2E). In the control group, formation of hyaline cartilage-like tissue was seen in three specimens, but others presented formation of fibrous cartilage. Extracellular matrix stained well with safranin O in three specimens, but was less compared with the bFGF group. There was no reconstitution of the osteochondral junction in any specimen.

Semiquantitive macroscopic and histological analyses. Semiquantitive analysis using the Kumagai Scale showed a significant difference in the two groups ($p < 0.01$) as detailed in Table 2. At 4, 8, and 12 weeks, average macroscopic scores in the AAV-bFGF group were 9.25 ± 2.21, 2.25 ± 0.70, and 0.25 ± 0.12 points, and the surface of the regenerated cartilage closely resembled normal cartilage in the bFGF-transduced group. On the other hand, macroscopic scores in the control group showed an average of 12.0 ± 2.34, 8.50 ± 1.72, and 5.00 ± 1.28 points at 4, 8, and 12 weeks, respectively, and regenerated cartilage could still be distinguished from surrounding normal cartilage even at 12 weeks. No sign of osteoarthrosis, such as erosion of cartilage or formation of osteophytes, was recognized in any of the joint surfaces during the observation period. Tumor formation was also not recognized, but there was a slight hyperplastic change in the lateral condyle in one knee joint of the bFGF group. No hyperplasia of synovium was seen in any of the joints of the bFGF-transduced group.

Histological analysis using the Wakitani score showed 6.75 ± 1.15, 2.25 ± 0.67, and 0.75 ± 0.33 points at 4, 8, and 12 weeks after AAV-bFGF administration, respectively (Table 2). In the control group, the score was 13.5 ± 1.0, 11.0 ± 1.5, and 5.00 ± 2.00 points at 4, 8, and 12 weeks respectively. The scores in both groups decreased throughout the experimental period. However, the score in the bFGF-transduced group became significantly lower than that in the control group over time ($p < 0.01$).

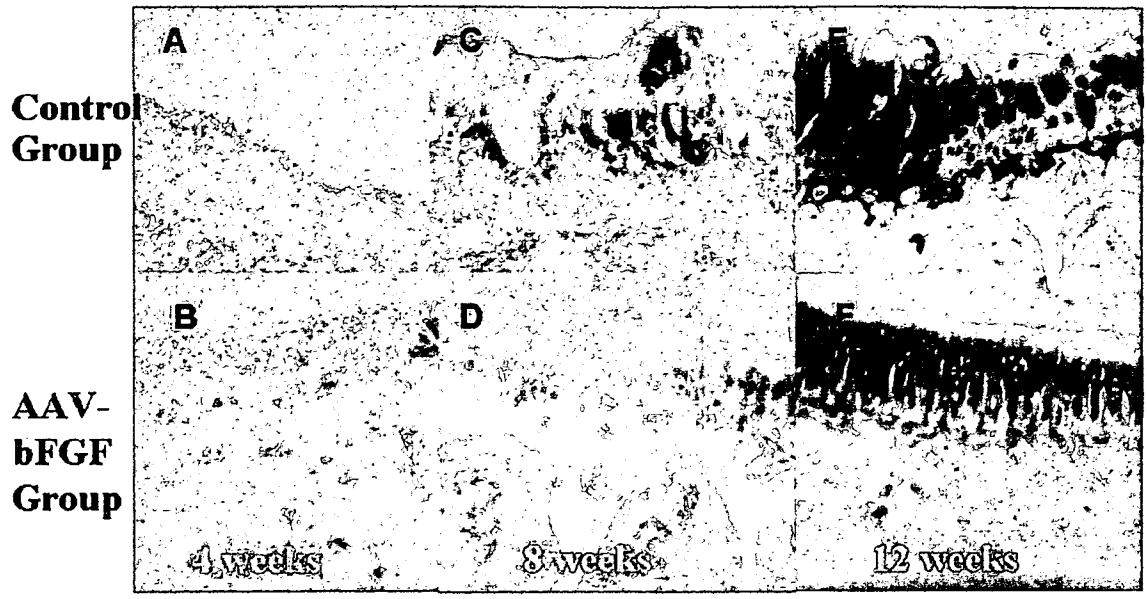

FIG. 2. Histological assessment of prepared sagittal sections using safranin O and hematoxylin staining. At 4 weeks (**A**) control group extracellular matrix is hardly stained, no cartilage regeneration is seen, but the tissues show inflammatory granulation of the defect area, which may obscure any bone because of apoptosis and replacement of fibrous tissue. (**B**) bFGF-transduced group matrix is lightly stained and the deep part is composed of rounded chondrocytes. At 8 weeks (**C**) partial chondrocyte repair is seen, with limited metachromasy. (**D**) The matrix is more distinctly stained and cartilage regeneration has advanced to the superficial layer. At 12 weeks (**E**) generation of chondrocyte-like cells with irregular matrix formation is seen, but no reconstitution of the bone–cartilage junction is apparent. (**F**) Note the intensely stained matrix, reconstitution of the osteochondral junction, and formation of the surface layer of the articular cartilage.

bFGF concentration in synovial cell culture after in vivo *administration of AAV-bFGF.* bFGF production was detected in both bFGF-transduced and control cells. bFGF concentration in the culture supernatant was 133.8 ± 15.6, 122.5 ± 19.3, and 94.0 ± 8.4 ng/ml at 4, 8, and 12 weeks after transduction, respectively, in bFGF-transduced cells (Fig. 3). In control cells, bFGF concentration was 34.0 ± 7.8, 19.5 ± 6.3, and 21.0 ± 2.7 ng/ml at 4, 8, and 12 weeks after transduction, respectively. bFGF concentration was significantly greater in bFGF -transduced cells than in control cells on all days of sampling ($p < 0.01$).

Immunohistological assessment of the cell matrix. Immunohistological assessment of the extracellular matrix was made in the AAV-bFGF group at 8 and 12 weeks. The antibody was targeted at extracellular matrix, and clear staining was seen at both 8 and 12 weeks, when compared with the control group (Fig. 4).

DISCUSSION

Because of its limited self-repairing nature, regeneration of cartilage is a difficult task, and clinical attempts have been made

TABLE 2. SEMIQUANTITIVE MACROSCOPIC AND HISTOLOGICAL ANALYSIS OF bFGF-TRANSDUCED CARTILAGE[a]

Weeks after transduction	*Macroscopic score*[b]		*Histological score*[b]	
	AAV-bFGF-F	*Control*	*AAV-bFGF*	*Control*
4	9.25 ± 2.21	12.0 ± 2.34	6.75 ± 1.15	13.5 ± 1.00
8	2.25 ± 0.70	8.50 ± 1.72	2.25 ± 0.67	11.0 ± 1.50
12	0.25 ± 0.12	5.00 ± 1.28	0.75 ± 0.33	5.00 ± 2.00

[a]Semiquantitive macroscopic and histological scores comparing the AAV-bFGF group and control group at 4, 8, and 12 weeks after administration. Macroscopic scoring system: 0 points, maximum with complete repair; 16 points, minimum with no repair. Histological scoring system: maximum and minimum scores are 0 and 14 points, respectively. $n = 8$.

[b]$p < 0.01$.

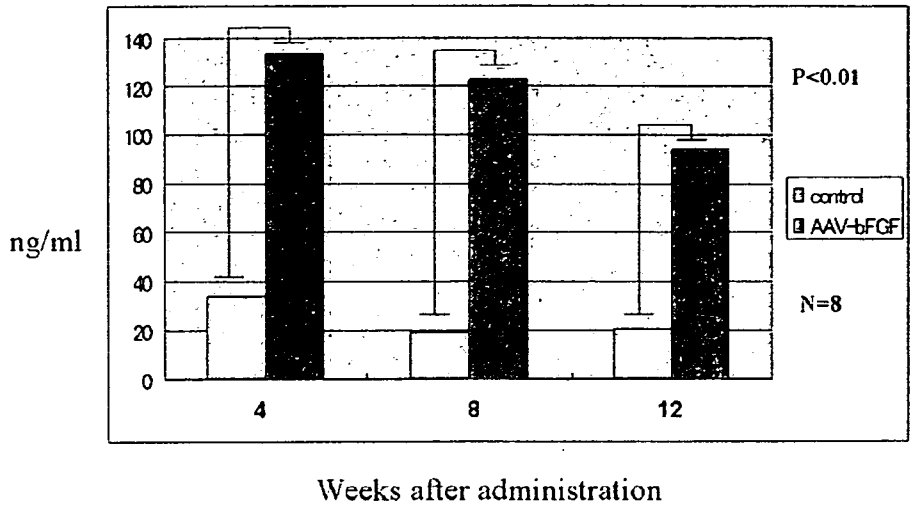

FIG. 3. bFGF concentration in culture supernatant of bFGF-transduced synoviocytes 4, 8, and 12 weeks after AAV-bFGF administration. Culture supernatants of control and bFGF-transduced cells were collected 7 days after harvesting and culture, and bFGF concentration was determined by ELISA. Transduction of the bFGF gene significantly elevates the secretion of bFGF ($*p < 0.01$).

to repair human cartilage, such as implantation of autologous chondrocytes (Richardson *et al.*, 1999), which have proven successful. In these cases, autologous chondrocyte grafts, harvested from non-weight-bearing areas, were cultured *in vitro* and then reinserted into the cartilage defect with coverage by a periosteal flap. However, because of the limited amount of graft tissue that can be obtained from the donor site, the method is limited to small local cartilage defects. Another successful method used clinically is mosaicplasty, used in diseases such as osteochondritis dissecans, but again, patients are limited to those who suffer only from small local cartilage defects. Given these limitations, a more fundamental method for cartilage repair would seem to be needed.

With numerous studies concerning growth factors, and discovery of potent gene transporters, methods to transport these genes into target organs have come to light (Kaplitt *et al.*, 1994; Berns and Giraud, 1995; Xiao *et al.*, 1997; Schwarz, 2000). Thus, gene therapy has become one of the most powerful tools for treatment of degenerative disorders, but still has several problems: the first, and perhaps most important, is that of safety—that is, in terms of virus virulence and their ability to self-reproduce; the second concerns their capacity to integrate into the host genome; and the third is the issue of long-term gene expression.

Adeno-associated virus (AAV), which was first discovered in research of its hosting virus, the adenovirus, has several bi-

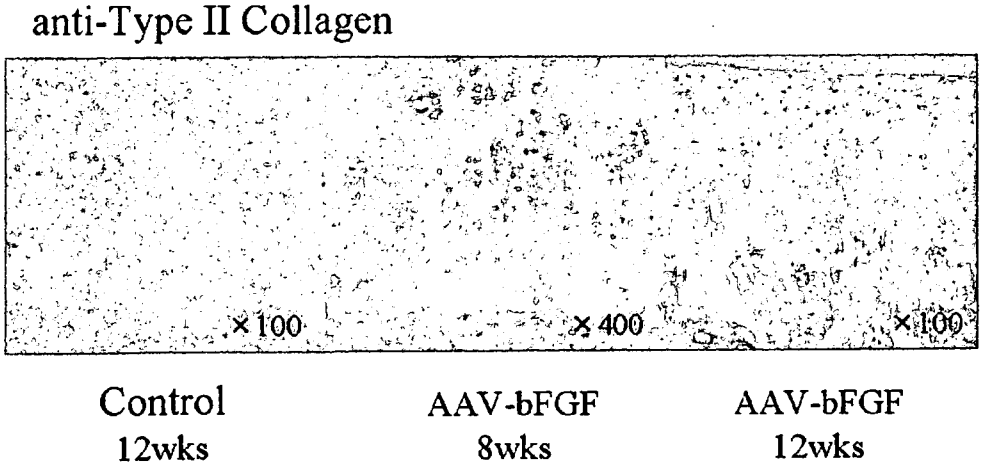

FIG. 4. Immunohistological staining for type II collagen. Staining is not seen in the negative control at 12 weeks. Staining of the extracellular matrix is seen at 8 weeks, and is most strong 12 weeks after AAV-bFGF administration.

ological advantages over other viral gene transporters, and the only apparent disadvantage is its small size, which may limit the size of the gene it can carry. It is most important to obtain high-efficiency transduction and continuous local expression of the therapeutic gene for repair of cartilage defects by gene therapy. Several studies have demonstrated the high ability of the AAV as a gene transporter, and its ability to express the transduced gene for a certain length of time (Kessler *et al.*, 1996; Fisher *et al.*, 1997; Herzog *et al.*, 1997). There are also reports of the utility of the AAV vector in the treatment of joint diseases, in which it demonstrated high-efficiency gene delivery into the synovium *in vivo* (Goater *et al.*, 2000), and gene transduction into cultured chondrocytes *in vitro* (Arai *et al.*, 2000). In our previous study, *ex vivo* gene transfer to periosteum-derived cells, using an AAV vector, induced *lacZ* gene expression for 4 weeks *in vivo* (Kobayashi *et al.*, 2002). However, the *ex vivo* method is complex, and may not be practical in terms of clinical therapy. On the other hand, delivering genes directly to the surface of abnormal articular cartilage could result in long-term treatment to accelerate cartilage repair.

Augmentation of cartilage repair by application of basic fibroblast growth factor (bFGF) has been reported to be efficient (Shida *et al.*, 1996; Cuevas *et al.*, 1988). Weisser *et al.* (2001) documented that by transplanting chondrocytes treated with several growth factors, positive effects on cartilage repair were observed only in bFGF-treated chondrocyte implants, and Fujimoto *et al.* (1999) showed that *in situ* delivery of bFGF also enhanced the ability to repair cartilage. Other studies have also shown that exogenous bFGF induced proliferation of chondrocytes, maturation of cartilage, and differentiation of mesenchymal cells, and stimulated synthesis of cartilaginous matrix (Cuevas *et al.*, 1988; Shida *et al.*, 1996). Otsuka *et al.* (1997) described how continuous administration of bFGF, using an osmotic pump, had a clear beneficial effect on repair of cartilage defects.

After considering the above-described findings, we were led to believe that achieving integration of the bFGF gene into intraarticular tissues, such as synoviocytes, by intraarticular administration could result in long-term expression and prolonged secretion of the growth factor, and culminate in regeneration of cartilage.

In this study, high-efficiency *lacZ* gene transduction to synoviocytes was obtained *in vitro*, and bFGF gene expression was detected in both bFGF-transduced and control cells. Subsequently, gene expression *in vivo* was sustained for at least 12 weeks without any adverse effects, and the findings suggest that *in vivo* gene transfer to articular cartilage defects, using the AAV vector, was successful.

We have shown that bFGF secretion was significantly increased in bFGF-transduced synoviocytes compared with the control groups, both *in vitro* and *in vivo*. Furthermore, repair at a relatively early stage was noticeably different between the bFGF-transduced and control cartilage tissue, and the eventual histological appearance of the transfected site in the bFGF-transduced group showed full repair compared with the control group, where the difference was still visible.

The results demonstrate that repair of full-thickness defects in rabbit articular cartilage can be enhanced by intraarticular administration of AAV-bFGF, by a procedure that is much less complex compared with the orthodox *ex vivo* method, and that is equally, if not more, effective.

Hence, it can be concluded that continuous bFGF secretion by gene transfer was extremely effective in promoting cartilage repair, demonstrating that this approach could in future be used clinically as a potent method for repair of cartilaginous diseases. However, further long-term studies will be necessary to assess the final outcome of regenerated articular cartilage.

ACKNOWLEDGMENTS

Supported in part by grants from the Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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Received for publication August 3, 2005; accepted after revision October 12, 2005.

Published online: November 4, 2005.

ARTHRITIS & RHEUMATISM
Vol. 52, No. 1, January 2005, pp 164–170
DOI 10.1002/art.20739

Repair of Articular Cartilage Defect by Autologous Transplantation of Basic Fibroblast Growth Factor Gene–Transduced Chondrocytes With Adeno-Associated Virus Vector

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Objective. **To examine the effects of basic fibroblast growth factor (bFGF) gene–transduced chondrocytes on the repair of articular cartilage defects.**

Methods. **LacZ gene or bFGF gene was transduced into primary isolated rabbit chondrocytes with the use of a recombinant adeno-associated virus (AAV) vector. These gene-transduced chondrocytes were embedded in collagen gel and transplanted into a full-thickness defect in the articular cartilage of the patellar groove of a rabbit. The efficiency of gene transduction was assessed according to the percentage of LacZ-positive cells among the total number of living cells. The concentration of bFGF in the culture supernatant was measured by enzyme-linked immunosorbent assay to confirm the production by bFGF gene–transduced chondrocytes. At 4, 8, and 12 weeks after transplantation, cartilage repair was evaluated histologically and graded semiquantitatively using a histologic scoring system ranging from 0 (complete regeneration) to 14 (no regeneration) points.**

Results. **LacZ gene expression by chondrocytes was maintained until 8 weeks in >85% of the in vitro population. LacZ-positive cells were found at the transplant sites for at least 4 weeks after surgery. The mean concentration of bFGF was significantly increased in bFGF gene–transduced cells compared with control cells ($P < 0.01$). Semiquantitative histologic scoring indicated that the total score was significantly lower in the bFGF-transduced group than in the control group throughout the observation period.**

Conclusion. **These results demonstrated that gene transfer to chondrocytes by an ex vivo method was established with the AAV vector, and transplantation of bFGF gene–transduced chondrocytes had a clear beneficial effect on the repair of rabbit articular cartilage defects.**

Damage of articular cartilage leads to joint dysfunction associated with pain or limited range of motion and usually progresses to degeneration of the articular surface, resulting in osteoarthritis. It is well recognized that articular cartilage is a highly differentiated tissue with a limited capacity for self-repair. Current therapy for osteoarthritis consists of short-acting antiinflammatory drugs, intraarticular injection of steroids or other agents, such as hyaluronic acid, and surgical intervention. However, these treatments may not relieve joint pain completely. Therefore, cartilage repair seems to be essential for the prevention of a catastrophic outcome in a joint. Several studies describing the successful repair of osteochondral defects by the transplantation of cultured chondrocytes have been reported (1). However, a major problem with cartilage repair by autologous chondrocyte transplantation is that a large quantity of chondrocytes from normal articular cartilage is required, whereas donor sites have a limited capacity to provide chondrocytes.

Supported by a grant-in-aid for Scientific Research and by the Yokohama Foundation for the Advancement of Medical Science.

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Submitted for publication February 4, 2004; accepted in revised form September 27, 2004.