trials as a treatment for stroke, neuroprotective strategies have yet to be proven effective. Reperfusion injury is defined as the enhancement of the damage that occurs in ischemic cells during the reperfusion period. Transient occlusion of the carotid arteries in rodents causes cell death in CA1 pyramidal neurons with a delay of a few days after reperfusion. The mechanisms underlying neuronal death after ischemia involve an excess of the excitatory neurotransmitter glutamate in the synapse. This excess causes a mobilization of free cytosolic calcium in the neuron, leading to calcium-dependent oxygen radical formation, cytoskeletal damage, and protein malfolding [24]. These degenerative events ultimately result in necrotic or apoptotic death in a subset of neurons. Because such neuronal death ensues over days, it is plausible to use protective gene therapy techniques during that interval.

Among the apoptosis repressor genes studied in mammalian cells, the protooncogene *bcl-2* has attracted attention as a potential regulator of neuronal survival. We have previously shown that postischemic injection of an AAV vector is effective for *bcl-2* gene delivery conferring neuroprotection in the gerbil hippocampus [25]. Here we show protocols for adenovirus-free preparation of an AAV vector and methods of gene transfer into gerbil brain for the gene therapy of ischemia-induced neuronal death.

3.2. Brain ischemia procedure

The Mongolian gerbil (*Meriones unguiculatus*) has been used as a model for cerebral ischemia. Since this animal lacks an interconnection between the carotid and the vertebro-basilar circulation, one can easily produce forebrain ischemia by occlusion of the common carotid arteries at the neck [26]. Transient bilateral carotid occlusion between 3 and 5 min can produce an apoptosis-like neuronal cell death in the CA1 area of hippocampus [27].

Three-month-old male Mongolian gerbils (70–90 g) are anesthetized with 3.0% halothane in a mixture of 30% O_2/70% N_2O. The rectal temperature is measured by inserting a thermocouple probe (Unique Medical, Tokyo, Japan) into the anus. To avoid neuronal protection by hypothermia, the rectal temperature is monitored and maintained at 37–38 °C throughout the operation by means of a feedback-controlled infrared heating lamp. Both common carotid arteries are clamped with surgical clips, thereby blocking cerebral blood supply. After a 3-min occlusion, the clips are removed and the skin is sutured.

3.3. Protocol for injection of AAV vector into gerbil brain

Prior to AAV vector injection, gerbils are anesthetized with chloral hydrate and placed in a stereotaxic apparatus. Male Mongolian gerbils are anesthetized with 2.5% chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic frame (Type SR-50; NARISHIGE, Tokyo, Japan). A burr hole is drilled to the pericranium.

Glass micropipettes (30–50 μm in diameter) are fabricated by a CO_2 laser-based micropipette puller (P-2000; Sutter Instrument, Novato, CA) according to the manufacturer's instructions. The glass micropipettes can inject AAV vectors into the hippocampus and provide electrical stimuli and recording. For injection into the CA1/CA2 of hippocampus, a microelectrode is placed 1.5 mm below the pialsurface via a burr hole, 3.0 mm to the right and 2.5 mm posterior to point bregma. Electrical repetitive stimuli (square-wave pulses of 0.3 ms duration, 0.1/s frequency) are delivered to the hippocampal commissure through bipolar tungsten microelectrodes. The electrodes are placed within the hippocampus ipsilateral to the recording site. Extracellular field potentials are recorded by microelectrodes stereotaxically positioned. For the ventricles lateralis, a micropipette is placed 2.2 mm below the pialsurface, 1.2 mm to the right and 0.4 mm posterior to point bregma. The vectors are injected through a glass micropipette at a rate of 1.0 μl/min by using a microinfusion pump. Following injection, the micropipette is left in place for 3 min and withdrawn slowly to prevent reflux. The muscles are sutured together and the scalp incision is closed with sutures.

3.4. Histochemical analysis

Principle. Histochemical studies on brain tissue are often carried out on frozen sections. This is the gentlest method for the preparation of samples and gives good preservation of cell structure and antigens. In this article we describe a free-floating method for use on frozen sections.

Fixation of brain for histochemical analysis. Gerbils are anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg body weight) and perfused with 50 ml of normal saline (0.9% NaCl) followed by 100 ml of fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), containing sucrose at 7.5%). Fixation is performed by transcardiac perfusion. Brains are removed and preserved overnight in fixative at 4 °C. The brains are then transferred to 0.1 M PB containing 15% sucrose and kept in a refrigerator. Coronal sections of the brains are made with a freezing microtome at 50 μm.

Detection of gene expression with X-Gal staining and anti-FLAG antibody. For X-Gal staining, the sections are stained using a Hist Mark X-Gal detection kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as described in the manufacturer's protocol (Fig. 1).

To evaluate the in vivo expression with immunohistochemistry, avidin–biotin complex (ABC) methods are used. Following 1 h of incubation in block solution (3%

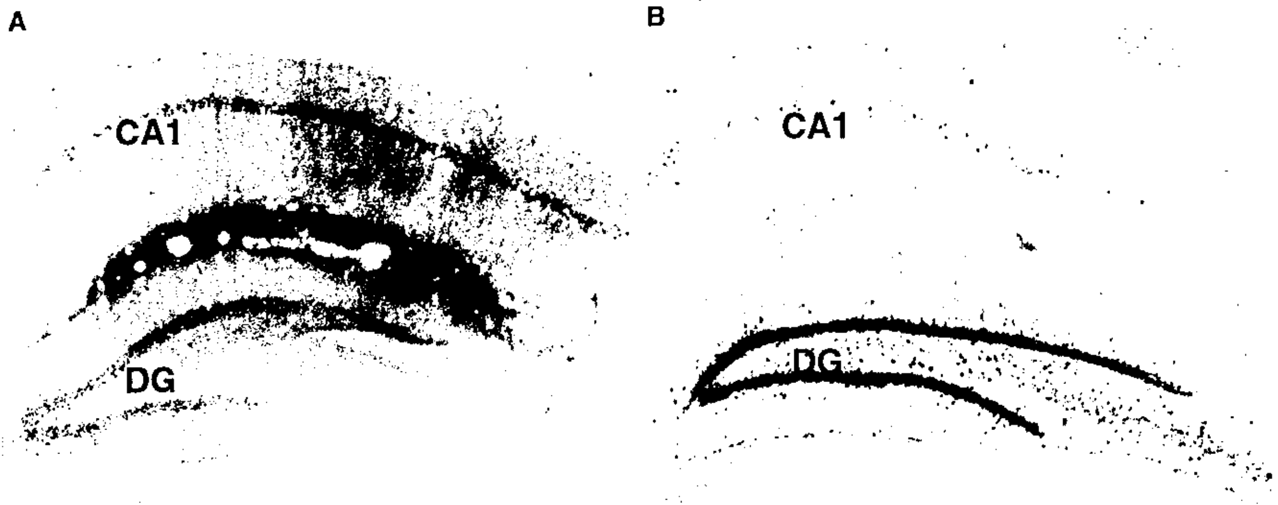

Fig. 1. Transgene expression after injection of rAAV in the gerbil hippocampus. Five days after a 5-μl (4.3×10^9–6.0×10^{10} particles/5 μl) injection of rAAV into the gerbil brain, the transgene product was detected with X-Gal staining assay. Typical staining pattern of the AAV2RSVLZ (A) and AAV5RSVLZ (B) is shown. AAV5RSVLZ revealed a more focused expression pattern of β-galactosidase in the granular cell layer than AAV2RSVLZ. Original magnification, X 80; DG, dentate gyrus; CA1, CA1 pyramidal cell layer.

normal horse serum in PBS), sections are incubated with anti-FLAG M5 antibody (1–10 μg/ml in blocking solution; Kodak, New Haven, CT) overnight at 4 °C. Sections are washed two times for 15 min each in PBS and incubated for 1 h with biotinylated anti-mouse/rabbit IgG. Sections are washed in PBS and further incubated for 1 h with an avidin–biotin complex (ABC Elite Kit; Vector Laboratories, Burlingame, CA) applied according to the instructions of the manufacture. Following a final wash in PBS, the peroxidase reaction is carried out with diaminobenzidine tetrahydrochloride (DAB) (1 mg/ml) in the presence of hydrogen peroxide (H_2O_2) (0.01%). Staining is terminated by washing two times for 5 min each in PB. Sections are then mounted on gelatin-coated glass slides, dehydrated, cleared in xylene, and sealed with cover slides.

Caution: (1) Development times may differ depending on the level of antigen. DAB generally should be developed for 2–10 min; (2) In the presence of nickel ions, the precipitate formed by DAB is gray/black rather than brown. This may enhance the sensitivity of the staining procedure.

Detection of apoptosis. The DNA fragmentation in the hippocampus of the experimental ischemic model is described as a key phenomenon for the apoptotic cell death. DNA nick end labeling of the sections is performed according to the methods of Gavrieli et al. [28] with some modifications. Nuclei of the sections are stripped from proteins by incubation with 20 μg/ml proteinase K in Tris–HCl, pH 6.6, for 15 min at room temperature. Endogenous peroxidase is inactivated by 2% H_2O_2 for 5 min at room temperature. Terminal deoxynucleotidyltransferase (Roche Diagnostics, Indianapolis, IN) and biotinylated dUTP (TACS In Situ Apoptosis Detection Kit; Travigen, Gaithersburg, MD) are diluted in labeling buffer (140 mM sodium cacodylate; 1 mM cobalt chloride in 30 mM Tris–HCl, pH 7.2) at a concentration of 0.15 e.u./ml and 0.8 nmol, respectively. The sections are immersed in the solution and then incubated at 37 °C for 60 min. The reaction is terminated by transferring the sections to the stopping buffer (2× standard saline citrate; 0.3 M NaCl, 30 mM sodium citrate, pH 7.0) for 5 min at room temperature. The sections are rinsed with distilled water and then treated with streptavidin–peroxidase (Travigen) for 10 min at room temperature. Sections are then developed in DAB (1 mg/ml) in the presence of H_2O_2 (0.01%).

Counterstaining. The sections are counterstained to aid in the morphological characterization (Fig. 2). Staining time varies with cell type and must be empirically determined for optical results. Staining times can vary from 5 s to 5 min. Standard steps are as follows: (1) methyl green solution for 5–10 s, (2) two times in deionized water for 10 s each, (3) two times in 95% ethanol for 30 s each, (4) two times in 100% ethanol for 1 min each, and (5) two times in 100% xylene for 5 min each.

4. Genetic therapeutics for Parkinson's disease

Parkinson's disease (PD) is a suitable candidate for gene therapy using rAAV vectors. In rodents and nonhuman primates, a substantial number of striatal neurons can be transduced with high-titer rAAV vectors through simple stereotaxic injection. One potential

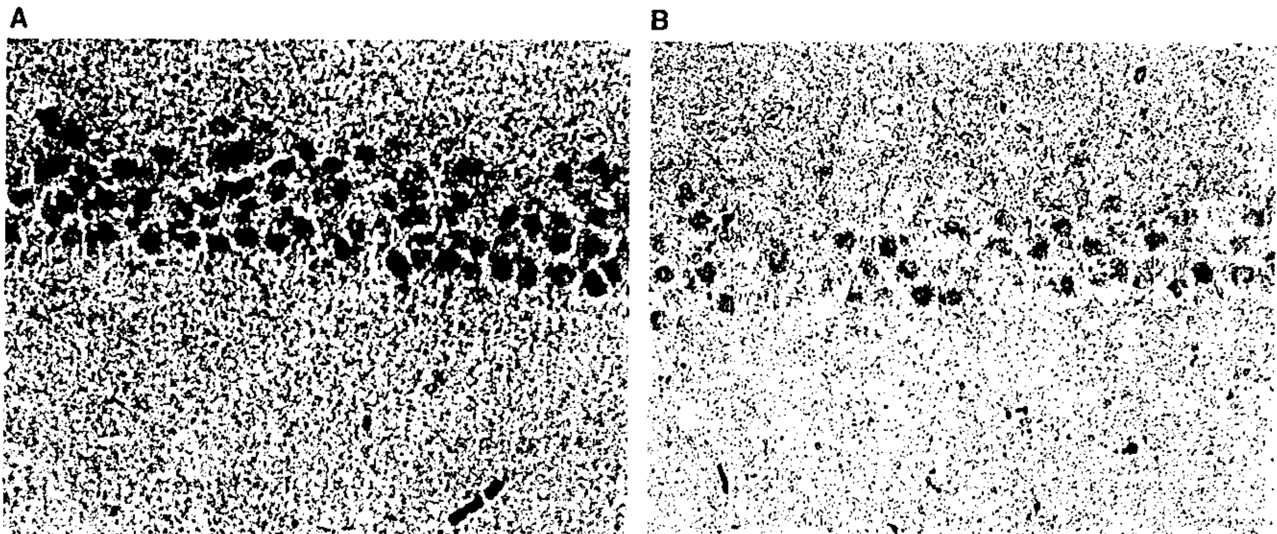

Fig. 2. In situ staining of fragmented DNA 3 days after ischemia in hippocampal CA1 area. DNA fragmentation was detected using the TUNEL technique. (A) The DNA fragmented neurons were densely distributed in the CA1 pyramidal cell layer of control gerbil. (B) With the AAV bcl-2-injected gerbils, far fewer CA1 neurons were positive for DNA fragmentation. The data strongly suggest that AAV bcl-2 transduction was effective in inhibiting DNA fragmentation in CA1 neurons after ischemia.

strategy is the local production of dopamine (DA) in the striatum induced by restoring DA-synthesizing enzymes. In addition to tyrosine hydroxylase and aromatic l-amino acid decarboxylase (AADC), GTP cyclohydrolase I is necessary for efficient DA production (Fig. 3). Efficient and long-term expression of genes for these three enzymes in the striatum restores local DA production and achieves behavioral recovery in animal

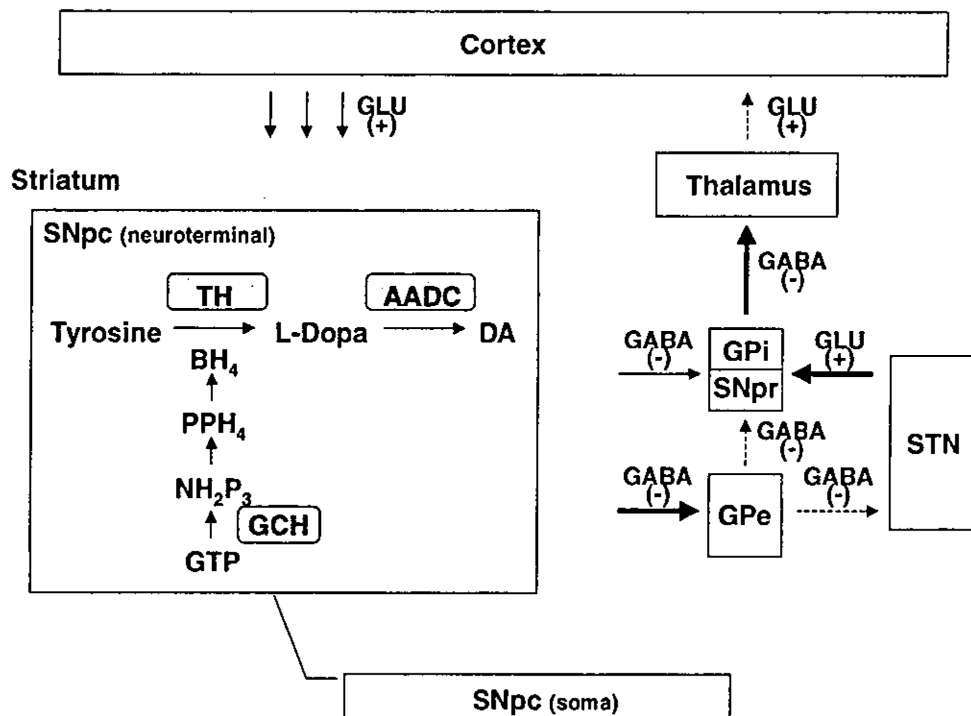

Fig. 3. Functional organization of the basal ganglia in Parkinson's disease. Thick or dashed lines indicate pathways believed to be hyperactive or hypoactive under the pathologic conditions in Parkinson's disease. AADC, aromatic amino acid decarboxylase; DA, dopamine; GABA, γ-aminobutylic acid; BH_4, tetrahydrobiopterin cofactor; GCH, GTP cyclohydrolase I; GLU, glutamate; GPe, external segment of the globus pallidus; GPi, internal segment of the globus pallidus; GTP, guanosine 5′-triphosphate; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; STN, subthalamic nucleus; TH, tyrosine hydroxylase.

models of PD [29]. An alternative approach is to transfer only the gene encoding AADC and administer exogenous L-Dopa [30]. The reduction in AADC might contribute to the loss of L-Dopa therapeutic efficacy so that restoring the decarboxylating capacity might result in a therapeutic gain. Another strategy for gene therapy of PD is to slow or reverse the ongoing degenerative processes by delivering genes of molecules that would block further dopaminergic cell loss. Sustained expression of a glial cell line-derived neurotrophic factor (GDNF) gene in the striatum rescues nigral neurons and leads to functional recovery in a rat model of PD, even when treatment is delayed until after the onset of progressive degeneration [31,32]. We now describe strategies for neuroprotection by GDNF using a rodent model and for restoration of DA synthesis using a primate model.

4.1. Rodent model for neuroprotection by GDNF gene delivery

Principle. Since substantial numbers of DA neurons are already lost before characteristic symptoms appear in PD, a clinically important question is whether GDNF gene delivered in a delayed manner is capable of rescuing DA neurons and improving behavioral performance in animal models with extensive nigrostriatal DA denervation already present. In our study using a rat model, 4 weeks after creation of a unilateral striatal lesion using 6-hydroxydopamine (6-OHDA), animals received injections of rAAV2 vectors expressing GDNF tagged with FLAG peptide (rAAV–GDNF*flag*) or β-galactosidase (rAAV–LacZ) into the lesioned striatum. At that time point, the percentage of tyrosine hydrolase-immunoreative (TH-IR) neurons on the lesioned side had been reduced to 35% of that of the intact side [31]. At 20 weeks after rAAV vector injection, extensive loss of TH-IR cells in the SN (20% of intact side) and TH-IR fibers in the striatum (14% of intact side) was observed in rats injected with rAAV–LacZ. In contrast, injection of rAAV–GDNF*flag* significantly increased numbers of TH-IR neurons in the SN and density of TH-IR fibers in the striatum, reaching nearly 57 and 49% of the contralateral side, respectively. Levels of DA and its metabolites in the striatum were markedly higher in the rAAV–GDNF*flag* group than in the control group. Consistent with anatomical and biochemical changes, significant behavioral recovery was observed from 4 to 20 weeks following rAAV–GDNF*flag* injection. Dual immunostaining for FLAG and TH demonstrated double-positive cells in the SN. In contrast, no β-galactosidase signals could be detected in the SN of rAAV–LacZ-injected rats, although X-Gal positive cells were detected in the striatum. Thus, we suppose that GDNF*flag* protein, rather than rAAV vector, underwent retrograde transport to the SN, although rAAV vector might demonstrate retrograde transport to some extent [33]. Our results indicate that delayed delivery of the GDNF gene using rAAV vectors halts the ongoing degeneration of nigrostriatal pathways, producing functional recovery even after substantial numbers of DA cells have been depleted.

Animals and surgical procedures. Adult male Wistar rats (weight 200–250 g) are maintained in a 12-h light/dark cycle in cages with ad libitum access to food and water. The partial rat model of PD is prepared as described by Sauer and Oertel [34]. Briefly, rats receive stereotaxic injection of 20 μg 6-OHDA calculated as free base (Sigma, St. Louis, MO) dissolved in 4 μl of ascorbate–saline (0.05%) in the left striatum at the following coordinates: anterior–posterior (AP) 1.0 mm, medial–lateral (ML) 3.0 mm, and dorsoventral (DV) −4.5 mm. The stereotaxic coordinates are calculated relative to the bregma and dural surface. After 4 weeks, animals are tested for apomorphine-induced rotation (0.1 mg/kg administered intraperitoneally) and only those animals exhibiting seven or more contralateral rotations/min in a 60-min period are included in further study. To assess the extent of nigrostriatal degeneration, neural tracer cholera toxin subunit B (CTB) (1% in distilled H_2O, 1 μl; List Biological Laboratories, Campbell CA) is injected bilaterally into the striatum (AP = 1 mm, ML = 3.0 mm, DV = −4.5 mm) 3 days before sacrifice. This is performed to retrogradely label the subpopulation of DA neurons in the SN maintaining functional projections to the striatum at 4 weeks after 6-OHDA lesions.

PD animal models are randomly assigned to receive AAV-GDNF*flag*, AAV–LacZ, or vehicle (0.1 M PBS) injection in the left striatum at three different sites (2 μl per site, 10^{13} vector genome copies/ml) using the following coordinates: AP = 1.5, 1.0, and 0.5 mm; ML = 2.6, 3.0, and 3.2 mm; DV = −4.5 mm). The injection rate is set at 1 μl/min and the needle is left in place for an additional 7 min before being slowly retracted. To evaluate retrograde transport of GDNF*flag* protein, some rats are sacrificed for histology at 2, 4, and 8 weeks after AAV injection. Twenty weeks after vector injection, rats are randomly grouped and sacrificed for biochemical or immunohistochemical analysis. In some rats, CTB is injected bilaterally into the striatum 3 days before sacrifice.

Behavioral testing. After AAV vector injection, rats are tested for rotational behavior every 2 weeks by intraperitoneal injection of apomorphine-HCl (0.1 mg/kg). The total number of complete body turns is counted during an observation period of $\geq$60 min, as described previously [29]. Spontaneous limb movement is scored every 4 weeks using the cylinder test method [35]. Rats are placed in a clear glass cylinder large enough for free movement. After they have performed 10 rears during which they place at least one paw on the cylinder wall, the number of both forepaw contacts to the wall of the

cylinder is counted at least 20 times. The data for contralateral forepaw contacts are given as a percentage of total.

Biochemical analysis. The rats are sacrificed by decapitation under sodium pentobarbital anesthesia and brains are immediately dissected and placed on dry ice. The striatum is punched out bilaterally using a sharp-edged, stainless steel tube. Wet tissue samples are weighed and stored at −80 °C until subsequent analysis. Levels of dopamine and its metabolites, HVA and DOPAC, are estimated using HPLC as described previously [29].

Immunohistochemistry. Rats are perfused with PBS followed by ice-cold 4% perfluoroalkoxyl (PFA). Brains are dissected and then postfixed for 4 h in 4% PFA. Coronal sections (30 μm) from the striatum and SN are treated for 30 min in 0.3% H_2O_2 in PBS and blocked with 3% fetal bovine serum in PBS/0.1% Triton X-100 for 1 h. Sections are then incubate with primary antibodies for TH (1:1000, mouse anti-TH monoclonal antibody, Chemicon, Temecula, CA), CTB (1:10,000, goat antiserum to CTB; List Biologic, Campbell, CA), or FLAG (1:1000, anti-FLAG M2 antibody; Sigma) at 4 °C overnight. This is followed by incubation with biotinylated secondary antibodies (to species of primary antibodies) for 1 h at room temperature (1:400; Santa Cruz). Sections are visualized with avidin–biotinylated peroxidase complex procedure (Vectastain ABC kits; Vector Laboratories) using 3,3-diaminobenzidine as a chromogen.

For FLAG/TH double-immunofluorescence staining, sections are incubated with blocking solution containing 5% normal goat serum in PBS for 1 h at room temperature. Sequentially, sections are incubated with monoclonal mouse anti-FLAG antibody (1:250; Sigma) and polyclonal rabbit anti-TH antibody (1:500) overnight at 4 °C. Then, sections are incubated with rhodamine-conjugated goat anti-mouse IgG (1:200; Santa Cruz) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:200; Santa Cruz) for 2 h at room temperature. For CTB/TH double-immunofluorescence staining, anti-CTB (1:5000; goat antiserum to CTB) and mouse anti-TH (1:500) antibodies are used.

For quantitative analyses of TH positive neurons and CTB-labeled neurons, sections at 300-μm intervals throughout the SN bilaterally are counted. Optical density of TH-IR fibers is quantified on sections every 300 μm rostro-caudally throughout the striatum using NIH Image 1.59 software [36].

4.2. Primate model for restoration of DA synthesis in the striatum

Principle. Encouraging results of rAAV vector-mediated gene transfer of DA-synthesizing enzymes in Parkinsonian rats prompted us to extend preclinical explorations to a primate model of PD. The rAAV vectors efficiently introduced genes for DA-synthesizing enzymes into the striatum of primates, resulting in restoration of motor function with robust transgene expression and elevated DA synthesis in the treated putamen [37]. Compared with rodent models, primate models are more appropriate for evaluation of functions and for examination of transduction efficiency in a larger striatum. Mixtures of three rAAV2 vectors (rAAV–TH/–AADC/–GCH) were stereotaxically injected unilaterally into the putamen of Parkinsonian monkeys. Coexpression of enzymes in the unilateral putamen resulted in marked improvement in manual dexterity on the contralateral side of the rAAV–TH/–AADC/–GCH vector injections. Monkeys picked up raisins better with the contralateral hand, in which tremor had disappeared. The ipsilateral limb remained disabled, suggesting that behavioral recovery of the contralateral limb cannot be explained by spontaneous recovery that may be expected to occur over time. Although only 20% of thalamic projections from basal ganglia decussate in monkeys, unilateral transduction would primarily affect contralateral limb movement. Behavioral recovery persisted throughout the observation period (three monkeys were sacrificed for histological evaluation at 48, 65, and 50 days, respectively, and one monkey was kept alive more than 18 months after rAAV vector injection). After rAAV vector injections, animals demonstrated apomorphine-induced dystonic postures, in which the body and face turned toward the ipsilateral side, and a circling tendency toward the ipsilateral side, indicating reduced DA receptor supersensitivity in the AAV–TH/–AADC/–GCH-injected putamen (Fig. 4). TH-IR, AADC-IR, and GCH-IR cells were present in a large region of the putamen (>90% of the putamen). The optimal TH:AADC:GCH ratio and the minimal volume of transduced region necessary for functional recovery are still to be elucidated for human brains, in which the putamen is approximately 10 times larger than that of the monkeys studied. Except for slight infiltration of mononuclear cells and residual hemosiderin around the needle tract, hematoxylin and eosin staining revealed no signs of cytotoxicity in the rAAV vector-injected putamen. Microdialysis demonstrated that concentrations of DA in the rAAV–TH/–AADC/–GCH vector-injected putamen increased compared with that of the control side. Monkeys did not show any complications related to rAAV vector injection, including dyskinesia.

Neurotoxin treatment. Female cynomolgus macaques (*Macaca fascicularis*), weighing 2–2.5 kg, are chosen as a nonhuman primate model of gene therapy experiments. They are housed in standard conditions of humidity and dark/light cycles with *ad libitum* access to food and water. Some monkeys have been trained to perform a fine motor task consisting of the capture of four raisins

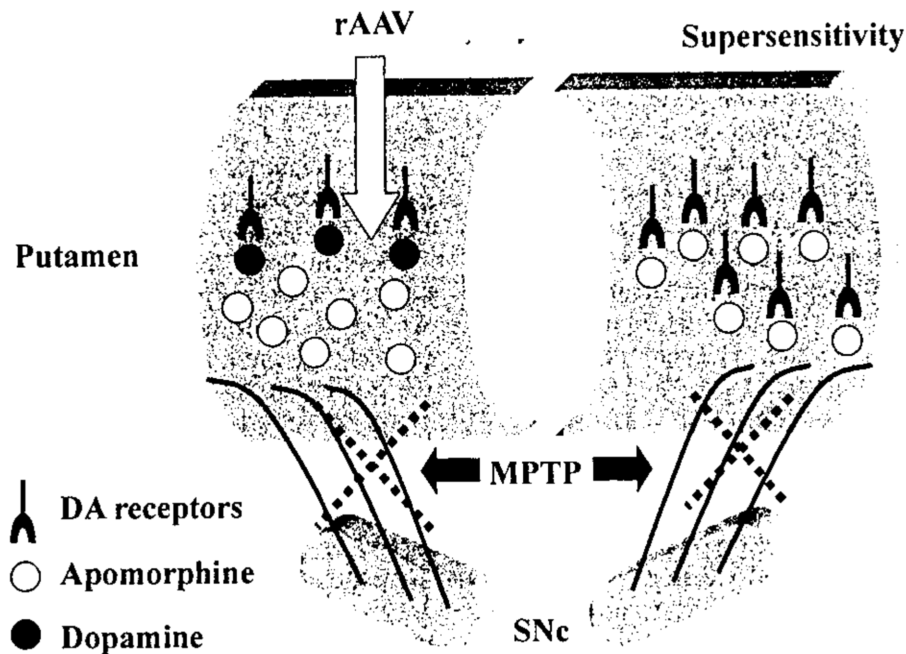

Fig. 4. Schematic representation of mechanisms of turning behavior. MPTP, a selective neurotoxin, induced supersensitivity of DA receptors in the striatum. Upregulation of DA receptors is shown here as simple elevation in receptor numbers, but may involve changes in downstream transduction pathways. This change was ameliorated by production of DA in the rAAV vector-treated side. Systemic administration of apomorphine caused an imbalance between the two striata, resulting in turning of the body axis toward the vector-treated side.

or small pieces of an apple with either of the upper limbs. To make bilateral striatal lesions, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, 0.25 to 0.5 mg/kg of free base; Sigma) in phosphate-buffered saline is injected intravenously once a week until a stable Parkinsonian symptom is achieved. The total dose of MPTP given is 1.0–6.25 mg/kg for 7 consecutive months. To avoid the possibility that spontaneous recovery from acute toxicity of MPTP can mimic the behavioral effect of AAV injection, monkeys are observed for 2 months after the last MPTP treatment.

Surgery and injection of rAAV. All surgical procedures are performed in an aseptic environment with the monkeys under isoflurane (1–2%) anesthesia. The head is placed in a stereotaxic device (Kopf Instruments, Tujunga, CA). Each monkey receives nine injections of AAV vectors in three tracts in the unilateral putamen. The side for injection is randomly assigned. Each injection is 5 μl with a 1:1:1 mixture of AAV–TH, AAV–AADC, and AAV–GCH (1×10^{13} each vectorgenome copies per milliliter). Thus, in total, 1.5×10^{11} genome copies of each vector are injected. Injections are made through a Hamilton microsyringe at a rate of 1 μl/min. The needle is left in the injection site for an additional 5 min to prevent the loss of vectors by back flow. As a control, 15 μl of AAV–LacZ (1×10^{13} vectorgenome copies per milliliter) or PBS is injected into the contralateral putamen. The stereotaxic coordinates of injection sites in the putamen are as follows: Track 1, anterior 18.1 mm, lateral 11 mm, depth 19, 17, and 15 mm from the midpoint of ear bar; Track 2, anterior 16.4 mm, lateral 11.5, depth 20, 18, and 16 mm; and Track 3, anterior 13.4 mm, lateral 12 mm, depth 19, 17, and 15 mm.

Behavioral assessment. Animals are clinically evaluated twice a week using a primate Parkinsonism rating scale (PPRS) [38] and activities are recorded on digital videotapes. PPRS is modeled on the Unified Parkinson's Disease Rating Scale, but developed specifically for nonhuman primates. It scores independently from 0 (normal) to 4 (maximal disability) Parkinsonian features that consist of spatial hypokinesia (moving around cage), bradykinesia, manual dexterity (right arm/left arm), balance, and freezing, thus giving a total maximal score of 24. The disability scores are average values of six evaluations done during the last 3 weeks prior to AAV vector injection and sacrifice.

Hand movements on the fine motor task are analyzed by counting the pixels of digital recordings using an image subtraction method [39]. A video camera is set up in front of a tray on which four raisins are placed in a line from the monkey side to the observer side. While monkeys sequentially pick up the four raisins, the number of pixels representing the hand increase when the hand is coming forward to reach each raisin. Time spent on picking up each raisin is measured before and after the AAV vector injection. To evaluate the functional asymmetry of the striatum, apomorphine (0.2 mg/kg) is administered intramuscularly, and the appearance of circling behavior is analyzed using prolonged video recordings.

5. Concluding remarks

The effective and safe transgene delivery into the brain illustrates its potential for therapeutic application for neurologic disorders. A protocol of clinical trials for PD gene therapy has been submitted to the Recombinant DNA Advisory Committee [40]. They propose to infuse recombinant AAV vectors expressing the two isoforms of the enzyme glutamate decarboxylase (GAD-65 and GAD-67) into the subthalamic nucleus (STN). GAD synthesizes the major inhibitory neurotransmitter in the brain, GABA. The conventional surgical intervention, deep-brain stimulation (DBS) of the STN, has shown remarkable efficacy in even late-stage PD, unlike the early trials associated with recombinant GDNF infusion or cell transplantation approaches in PD. Not only will the gene transfer strategy palliate symptoms by inhibiting STN activity, as with DBS, but also there exists evidence that the vector converts excitatory STN projections to inhibitory projections. However, a long-term effect of STN stimulation has not yet been demonstrated. The mechanism of action of subthalamic stimulation remains to be defined and stimulation of nonmotor territories may be deleterious. The optimal target site in the STN, where GABA synthesis is enhanced, should be identified to ameliorate Parkinsonian symptoms, avoiding dyskinesia and cognitive side effects.

These advancements in rAAV application are also expected to accelerate and facilitate further studies on other neuronal disorders, providing validation for use of rAAVs in human clinical trials. Nerve growth factor gene transfer by intraseptal injections of rAAV to the medial septum increases cholinergic neuron size and protects from age-related, spatial memory deficits in middle-aged rats [41]. This approach may represent a viable therapy for age-related dementia involving dysfunction in cholinergic activity and memory, such as Alzheimer's disease. AAV-based vectors are also being tested in animal models as viable treatments for malignant glioma. Physiological differences between normal tissue and tumors also provide the potential for designing tumor-specific gene therapy. For instance, transgene expression from rAAV vectors with hypoxia-responsive elements can be regulated by hypoxia [42]. The presence of hypoxic cells in human brain tumors is an important factor leading to resistance to radiation therapy. Furthermore, AAV is a safe and effective vector for antiangiogenic gene therapy. Glioblastoma multiforme is one of the most highly vascularized neoplasms in humans with poor prognosis. Intratumoral injection of a high-titer AAV–angiostatin vector renders efficient intracranial tumor suppression and results in long-term survival of treated rats [43]. Single intramuscular injection of an AAV vector expressing angiostatin also effectively suppresses human glioma growth in the brain of nude mice [44]. AAV-mediated antiangiogenesis gene therapy may offer efficient and sustained systemic delivery of the therapeutic product, which in turn effectively suppresses glioma growth in the brain.

The volume of distribution of AAV-2 in the brain can be increased by heparin coinfusion [45,46]. AAV-2 binds to heparan-sulfate proteoglycans on the cell surface. In vivo, attachment of viral particles to cells adjacent to the injection tract limits the distribution of AAV-2 when infused into the central nervous system parenchyma, and heparin coinfusion might decrease the binding of AAV-2 particles to cells in the vicinity of the infusion tract. In addition to improvements in expression cassettes and viral titers and the use of very slow infusion rates, parameters with heparin coinfusion to maximize transduction efficiency and viral spread should be optimized [46]. A nonviral lipid-entrapped, polycation-condensed delivery system for central nervous system gene transfer, in conjunction with AAV-based plasmids has been reported [47]. In vivo gene transfer study using AAV-based plasmid vector in two children with Canavan disease showed no toxicity in human subjects and efficacy of aspartoacylase gene delivery with biochemical and clinical changes. A HSV-1/AAV hybrid system shows high transduction efficiency and stability without immune response [48]. With such recent improvements in the generation of vectors, the regulation of gene expression is now a key issue for successful translation of gene therapy-based treatments into the clinic. The level of the transgene expression may need to be regulated within a narrow therapeutic window for the successful treatment of human disease.

Acknowledgments

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References

[1] D.W. Russell, M.A. Kay, Blood 94 (1999) 864–874.
[2] S. Muramatsu, H. Mizukami, N.S. Young, K.E. Brown, Virology 221 (1996) 208–217.
[3] U. Bantel-Schaal, H. Delius, R. Schmidt, H. zur Hausen, J. Virol. 73 (1999) 939–947.
[4] P.E. Monahan, R.J. Samulski, Mol. Med. Today 6 (2000) 433–440.
[5] J.E. Rabinowitz, F. Rolling, C. Li, H. Conrath, W. Xiao, X. Xiao, R.J. Samulski, J. Virol. 76 (2002) 791–801.
[6] B.L. Davidson, C.S. Stein, J.A. Heth, I. Martins, R.M. Kotin, T.A. Derksen, J. Zabner, A. Ghodsi, J.A. Chiorini, Proc. Natl. Acad. Sci. USA 97 (2000) 3428–3432.
[7] J.A. Chiorini, F. Kim, L. Yang, R.M. Kotin, J. Virol. 73 (1999) 1309–1319.
[8] D. Duan, Z. Yan, Y. Yue, W. Ding, J.F. Engelhardt, J. Virol. 75 (2001) 7662–7671.
[9] T. Matsushita, S. Elliger, C. Elliger, G. Podsakoff, L. Villarreal, G.J. Kurtzman, Y. Iwaki, P. Colosi, Gene Ther. 5 (1998) 938–945.
[10] F.L. Graham, J. Smiley, W.C. Russell, R. Nairn, J. Gen. Virol. 36 (1977) 59–74.
[11] X.L. Liu, K.R. Clark, P.R. Johnson, Gene Ther. 6 (1999) 293–299.
[12] K. Tamayose, Y. Hirai, T. Shimada, Hum. Gene Ther. 7 (1996) 507–513.
[13] G.P. Gao, G. Qu, L.Z. Faust, R.K. Engdahl, W. Xiao, J.V. Hughes, P.W. Zoltick, J.M. Wilson, Hum. Gene Ther. 9 (1998) 2353–2362.
[14] G. Chadeuf, D. Favre, J. Tessier, N. Provost, P. Nony, J. Kleinschmidt, P. Moullier, A. Salvetti, J. Gene Med. 2 (2000) 260–268.
[15] T.R. Flotte, X. Barraza-Ortiz, R. Solow, S.A. Afione, B.J. Carter, W.B. Guggino, Gene Ther. 2 (1995) 29–37.
[16] M. Mamounas, M. Leavitt, M. Yu, F. Wong-Staal, Gene Ther. 2 (1995) 429–432.
[17] Y. Ogasawara, H. Mizukami, M. Urabe, A. Kume, Y. Kanegae, I. Saito, J. Monahan, K. Ozawa, J. Gen. Virol. (1999) 2477–2480.
[18] T. Okada, H. Mizukami, M. Urabe, T. Nomoto, T. Matsushita, Y. Hanazono, A. Kume, K. Tobita, K. Ozawa, Biochem. Biophys. Res. Commun. 288 (2001) 62–68.
[19] C. Holscher, M. Horer, J.A. Kleinschmidt, H. Zentgraf, A. Burkle, R. Heilbronn, J. Virol. 68 (1994) 7169–7177.
[20] M. Urabe, K. Shimazaki, Y. Saga, T. Okada, A. Kume, K. Tobita, K. Ozawa, Biochem. Biophys. Res. Commun. 276 (2000) 559–563.
[21] S. Zolotukhin, B.J. Byrne, E. Mason, I. Zolotukhin, M. Potter, K. Chesnut, C. Summerford, R.J. Samulski, N. Muzyczka, Gene Ther. 6 (1999) 973–985.
[22] A. Auricchio, M. Hildinger, E. O'Connor, G.P. Gao, J.M. Wilson, Hum. Gene Ther. 12 (2001) 71–76.
[23] G. Gao, G. Qu, M.S. Burnham, J. Huang, N. Chirmule, B. Joshi, Q.C. Yu, J.A. Marsh, C.M. Conceicao, J.M. Wilson, Hum. Gene Ther. 11 (2000) 2079–2091.
[24] J.M. Lee, G.J. Zipfel, D.W. Choi, Nature 399 (1999) A7–A14.
[25] K. Shimazaki, M. Urabe, J. Monahan, K. Ozawa, N. Kawai, Gene Ther. 7 (2000) 1244–1249.
[26] T. Kirino, Brain Res. 239 (1982) 57–69.
[27] K. Shimazaki, A. Ishida, N. Kawai, Neurosci. Res. 20 (1994) 95–99.
[28] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, J. Cell Biol. 119 (1992) 493–501.
[29] Y. Shen, S.I. Muramatsu, K. Ikeguchi, K.I. Fujimoto, D.S. Fan, M. Ogawa, H. Mizukami, M. Urabe, A. Kume, I. Nagatsu, F. Urano, T. Suzuki, H. Ichinose, T. Nagatsu, J. Monahan, I. Nakano, K. Ozawa, Hum. Gene Ther. 11 (2000) 1509–1519.
[30] R. Sanchez-Pernaute, J. Harvey-White, J. Cunningham, K.S. Bankiewicz, Mol. Ther. 4 (2001) 324–330.
[31] L. Wang, S. Muramatsu, Y. Lu, K. Ikeguchi, K. Fujimoto, T. Okada, H. Mizukami, Y. Hanazono, A. Kume, F. Urano, H. Ichinose, T. Nagatsu, I. Nakano, K. Ozawa, Gene Ther. 9 (2002) 381–389.
[32] J. McGrath, E. Lintz, B.J. Hoffer, G.A. Gerhardt, E.M. Quintero, A.C. Granholm, Cell Transplant. 11 (2002) 215–227.
[33] B.K. Kaspar, B. Vissel, T. Bengoechea, S. Crone, L. Randolph-Moore, R. Muller, E.P. Brandon, D. Schaffer, I.M. Verma, K.F. Lee, S.F. Heinemann, F.H. Gage, Proc. Natl. Acad. Sci. USA 99 (2002) 2320–2325.
[34] H. Sauer, W.H. Oertel, Neuroscience 59 (1994) 401–415.
[35] D. Kirik, C. Rosenblad, A. Bjorklund, R.J. Mandel, J. Neurosci. 20 (2000) 4686–4700.
[36] D.A. Kozlowski, B. Connor, J.L. Tillerson, T. Schallert, M.C. Bohn, Exp. Neurol. 166 (2000) 1–15.
[37] S. Muramatsu, K. Fujimoto, K. Ikeguchi, N. Shizuma, K. Kawasaki, F. Ono, Y. Shen, L. Wang, H. Mizukami, A. Kume, M. Matsumura, I. Nagatsu, F. Urano, H. Ichinose, T. Nagatsu, K. Terao, I. Nakano, K. Ozawa, Hum. Gene Ther. 13 (2002) 345–354.
[38] J.W. Langston, M. Quik, G. Petzinger, M. Jakowec, D.A. Di Monte, Ann. Neurol. 47 (2000) S79–89.
[39] T. Hashimoto, Y. Izawa, H. Yokoyama, T. Kato, T. Moriizumi, J. Neurosci. Methods 91 (1999) 115–122.
[40] M.J. During, M.G. Kaplitt, M.B. Stern, D. Eidelberg, Hum. Gene Ther. 12 (2001) 1589–1591.
[41] R.L. Klein, A.C. Hirko, C.A. Meyers, J.R. Grimes, N. Muzyczka, E.M. Meyer, Brain Res. 875 (2000) 144–151.
[42] H. Ruan, H. Su, L. Hu, K.R. Lamborn, Y.W. Kan, D.F. Deen, Neoplasia 3 (2001) 255–263.
[43] H.I. Ma, S.Z. Lin, Y.H. Chiang, J. Li, S.L. Chen, Y.P. Tsao, X. Xiao, Gene Ther. 9 (2002) 2–11.
[44] H.I. Ma, P. Guo, J. Li, S.Z. Lin, Y.H. Chiang, X. Xiao, S.Y. Cheng, Cancer Res. 62 (2002) 756–763.
[45] J.B. Nguyen, R. Sanchez-Pernaute, J. Cunningham, K.S. Bankiewicz, Neuroreport 12 (2001) 1961–1964.
[46] M.Y. Mastakov, K. Baer, R.M. Kotin, M.J. During, Mol. Ther. 5 (2002) 371–380.
[47] P. Leone, C.G. Janson, L. Bilaniuk, Z. Wang, F. Sorgi, L. Huang, R. Matalon, R. Kaul, Z. Zeng, A. Freese, S.W. McPhee, E. Mee, M.J. During, L. Bilianuk, Ann. Neurol. 48 (2000) 27–38.
[48] L.C. Costantini, D.R. Jacoby, S. Wang, C. Fraefel, X.O. Breakefield, O. Isacson, Hum. Gene Ther. 10 (1999) 2481–2494.

Gene Marking in Adeno-Associated Virus Vector Infected Periosteum Derived Cells for Cartilage Repair

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ABSTRACT. ***Objective.*** To evaluate both the potential for transferring genes to periosteal cells using an adeno-associated virus (AAV) vector and the potential for gene expression after transplantation of those cells to a cartilage defect *in vivo*.

Methods. Periosteum was obtained from the tibia of 6-week-old rabbits and enzymatically digested. The isolated periosteum derived cells were cultured and the subconfluence cells were infected with a recombinant AAV expressing the LacZ gene (AAV-LacZ). Collagen gel containing the LacZ transferred, periosteum derived cells was transplanted into a full thickness articular cartilage defect in 10 rabbits.

Results. Infected cells still growing on the plate continued to express LacZ at least 12 weeks after AAV infection, with the highest percentage of LacZ positive cells reaching 74.4%. The LacZ positive cells were recognized at the transplant sites in 8 out of 10 knees.

Conclusion. Gene expression in periosteum derived cells was sustained *in vitro* for at least 12 weeks using the AAV vector, and for 2 weeks *ex vivo* after transplantation into a cartilage defect. (J Rheumatol 2002;29:2176–80)

Key Indexing Terms:
ADENO-ASSOCIATED VIRUS
PERIOSTEUM DERIVED CELLS
LACZ GENE
CARTILAGE

Mesenchymal stem cells are defined as multipotential cells capable of undergoing repeated mitotic division and differentiating into mesodermal tissue such as bone, cartilage, muscle, adipose tissue, and tendon under appropriate conditions[1]. Periosteal cells are thought to be a type of multipotential cell found in postnatal organs. Their chondrogenic property has made periosteal cells attractive for cartilage repair and periosteal grafts, leading to their use both experimentally and clinically for the treatment of cartilage defects[2].

To promote cartilage repair, implanted cells may need stimulation from various growth factors, especially transforming growth factor-ßI (TGF-ßI)[3]. However, it may be difficult to achieve or maintain a bioactive level of growth factors in a cartilaginous lesion with a single exposure *in vivo*. Sustained gene expression of growth factors *in vivo* is needed to maintain their stimulatory levels and achieve satisfactory cartilage repair.

Recently, there have been several attempts to treat arthritis with gene therapies using vectors such as adenovirus[4], retrovirus[5], herpes simplex virus, or nonviral methods[6] to perform the gene transfer. Among them, adeno-associated virus (AAV) is recognized as having several advantages over the others: an authentic capability of infecting many kinds of cells, no virulency, integration of the transduced gene into the host chromosome, and longevity of gene expression[7]. However, there have been few reports discussing gene delivery to synovial cells or chondrocytes using an AAV vector for the treatment of joint disorders[8-12]. We investigated the ability of the AAV vector to transfer a gene to periosteum derived cells *in vitro* and also the possibility for the gene transferred cells to repair a cartilage defect.

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MATERIALS AND METHODS

Periosteum was obtained from the medial side of the tibia of 6-week-old Japanese white rabbits, and digested with 0.05% trypsin-EDTA and 0.25% collagenase. Subsequently, about 3×10^6 cells were obtained from one piece of the periosteum. The liberated cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) and supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

The AAV vector expressing LacZ gene (AAV-LacZ) was produced as described[13]. Briefly, human fetal kidney cells (293 cells) cultured in DMEM:nutrient mixture F-12 (1:1) (DMEM/F-12; GibcoBRL, New York, NY, USA) that was supplemented with 10% fetal bovine serum (FBS) were used for conjugation of the AAV vector and plasmid LacZ. The 293 subconfluent cells were cotransfected by the calcium phosphate coprecipitation

method with pLacZ, pIM45, and pladeno-1 to produce the AAV inducing LacZ gene (AAV-LacZ). After 48 h, the cells were harvested and lysed in Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0) through 3 cycles of freezing and thawing. One round of sucrose precipitation and 2 rounds of CsCl density gradient ultracentrifugation were performed to isolate the AAV-LacZ from the lysates. The vector titer was determined by quantitative DNA dot-blot hybridization of the Dnase-I resistant fraction.

As a preliminary experiment, the percentage of LacZ positive cells attendant with each virus titer was investigated. The subconfluent cells in a 96 well dish were infected with AAV-LacZ in titers of 10^3, 10^4, 10^5, 10^6 particles/cell, and incubated 1 h. One week after infection, the percentage of LacZ positive cells was calculated and averaged for each virus titer (Figure 1). The mean percentage of LacZ positive cells at each titer was 49.0% ± 9.2% at 10^3, 72.1 ± 4.1% at 10^4, 80.4 ± 8.2% at 10^5, 83.1 ± 5.4% at 10^6 particles/cell, with the optimum titer determined to be 10^5 particles/cell.

After the subconfluent cells in subculture dishes were washed with phosphate buffered saline (PBS), AAV-LacZ (10^5 particles/cell) was added, incubated 1 h, and then cultured for 1 week. One week after infection some of the harvested cells were mixed with 80 µl of 0.25% collagen gel (DME-02; Koken Co. Ltd., Tokyo, Japan) and the cell density was adjusted to 4 × 10^6/ml. The cells embedded in the collagen gel were cultured for 2 days in preparation for transplanting to a cartilage defect.

Intravenous pentobarbital (0.6 ml/kg) was used to anesthetize ten 8-week-old Japanese white rabbits for surgery. One knee of each rabbit had a full thickness defect 5 mm diameter by 3 mm deep drilled into the femoral patellar groove, which was then filled with the collagen gel containing the periosteum derived cells. Periosteum harvested from the contralateral tibia was used as a patch and fixed at the edge of the defect. The other knee received a sham operation as a control, and the defect was filled with either cell-free collagen gel or gel containing non-AAV infected cells. The rabbits were allowed to move freely immediately after surgery.

Expression of LacZ in cells remaining in the original culture was evaluated at 3 days and 1, 2, 4, and 12 weeks after AAV-LacZ infection. Cells transplanted into the cartilage defect were evaluated for LacZ expression at 1 and 2 weeks after transplantation. After fixation with PBS containing 2% formaldehyde and 0.2% glutaraldehyde, the specimens were directly stained with X-gal solution. Under a microscope at 100× magnification, 4 visual fields were randomly selected. The total number of cells and the number of LacZ positive cells were counted to determine the percentage of LacZ positive cells.

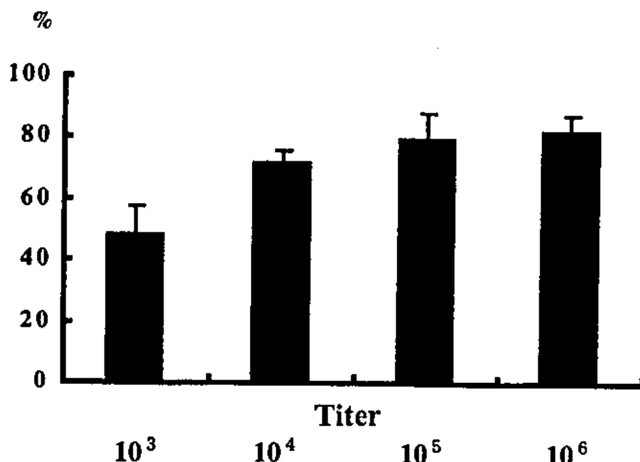


Figure 1. Percentage of LacZ positive cells at different AAV titers: 49.0% ± 9.2% at 10^3, 72.1 ± 4.1% at 10^4, 80.4 ± 8.2% at 10^5, 83.1 ± 5.4% at 10^6 particles/cell.

RESULTS

LacZ expression in vitro. Many blue stained LacZ positive cells were observed in the culture dishes, and this number increased gradually with time (Figure 2). The mean percentage of LacZ positive cells per total cells was 54.2 ± 10.2% at 3 days after infection with AAV-LacZ (Figure 3A), 68.2 ± 3.8% at 1 week (Figure 3B), 73.9 ± 6.0% at 2 weeks, and 74.4 ± 6.9% at 4 weeks (Figure 3C, 3D). By 12 weeks postinfection, the number of LacZ positive cells decreased to 53.2 ± 11.7% (Figure 3E), but gene expression was still strong. There was no cytotoxicity or other side effects of the virus, and there was no difference in cell growth or morphology between AAV infected cells and noninfected cells *in vitro* (data not shown).

LacZ expression after transplant in vivo. The cells implanted in the cartilage defect were enucleated, snap frozen in OCT compound, sectioned thinly with a cryotome, and stained with X-gal. Eight out of 10 rabbits had transplanted cells that strongly expressed LacZ (Table 1). One week after the transplant, LacZ positive cells were observed beneath the periosteum patch (Figure 4A), and the expression of LacZ was sustained for at least 2 weeks after transplant (Figure 4B).

DISCUSSION

One advantage of the AAV vector is its ability for sustained gene expression because of its integration of the target gene

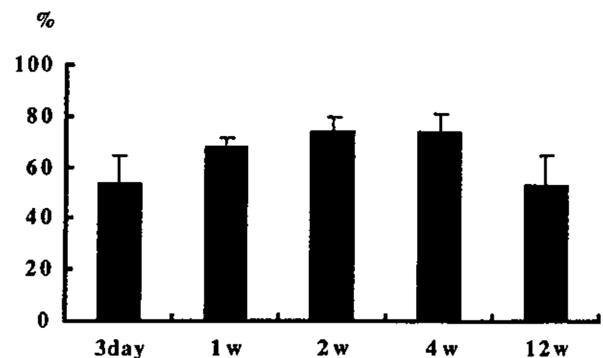
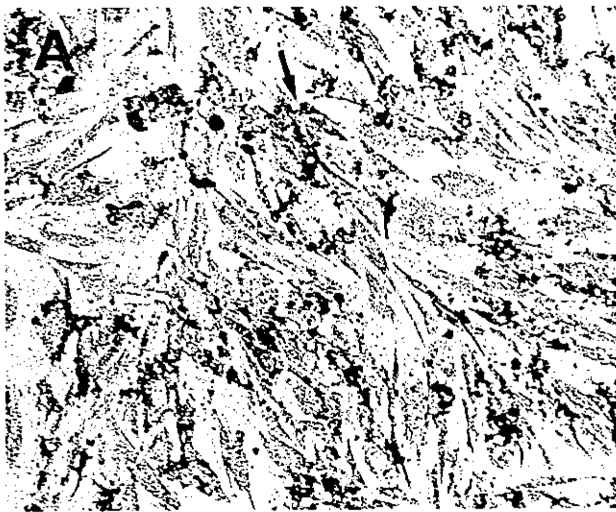
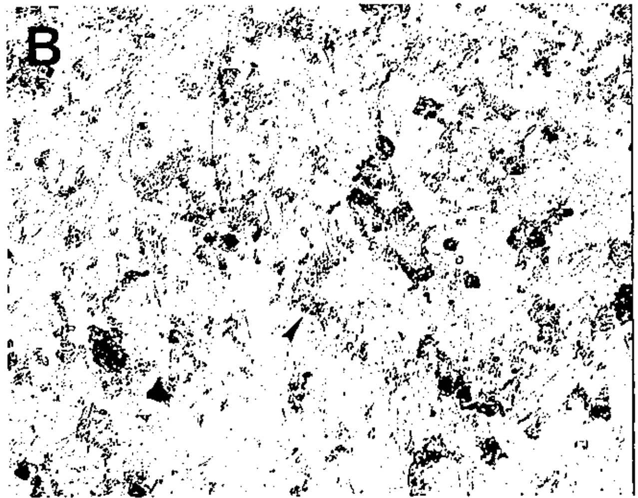


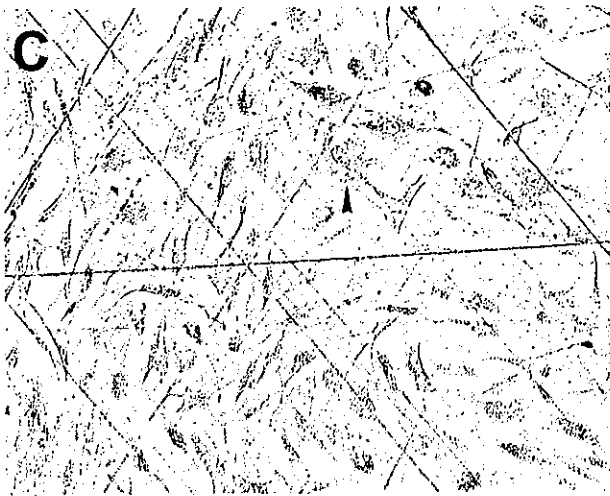
Figure 2. Percentage of LacZ positive cells at different times after infection. The percentage increased gradually with time reaching a maximum at 4 weeks (w: week).

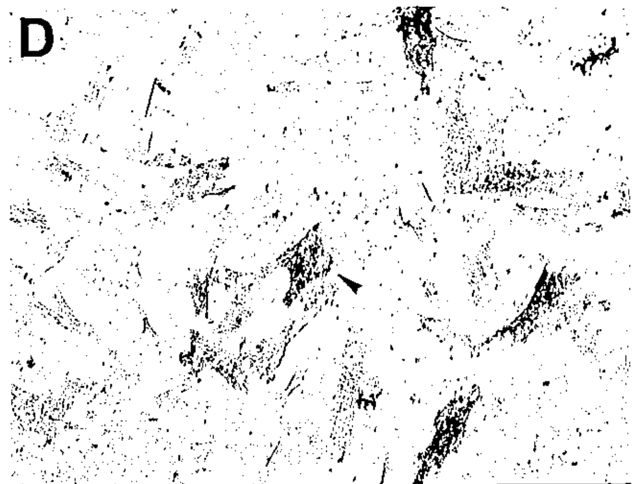
Table 1. LacZ was expressed at 80% of all transplantation sites (n = 10).

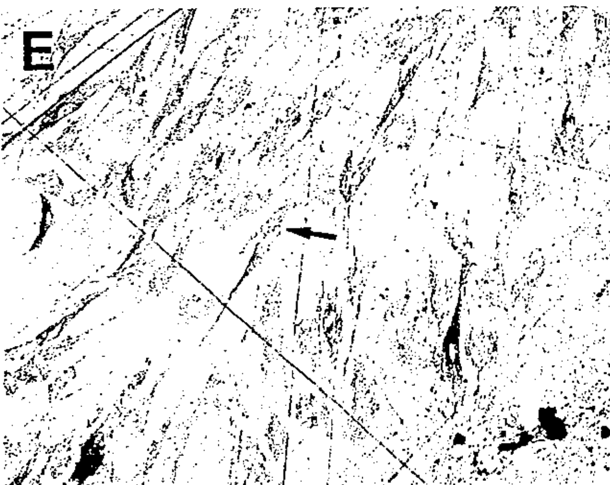
Time After Transplantation	Knees with Transplants	Knees with LacZ + Cells (%)
1 week	5	4 (80)
2 weeks	5	4 (80)
Total	10	8 (80)











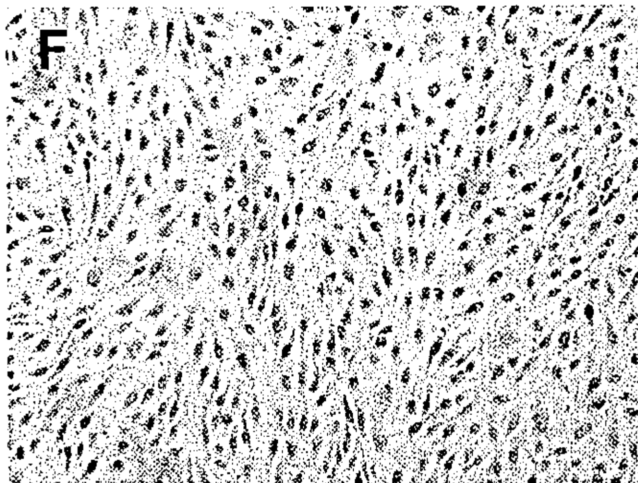


Figure 3. Photomicrographs of periosteum derived cells on culture plate stained with X-gal (100× original magnification). The X-gal staining was performed at 3 days (A) and at 1 (B), 2 (C), 4 (D), and 12 (E) weeks after AAV-LacZ infection. LacZ expression was sustained strongly for at least 12 weeks *in vitro*. Arrows indicate LacZ positive cells. LacZ positive cells were not seen in the control cells (F).

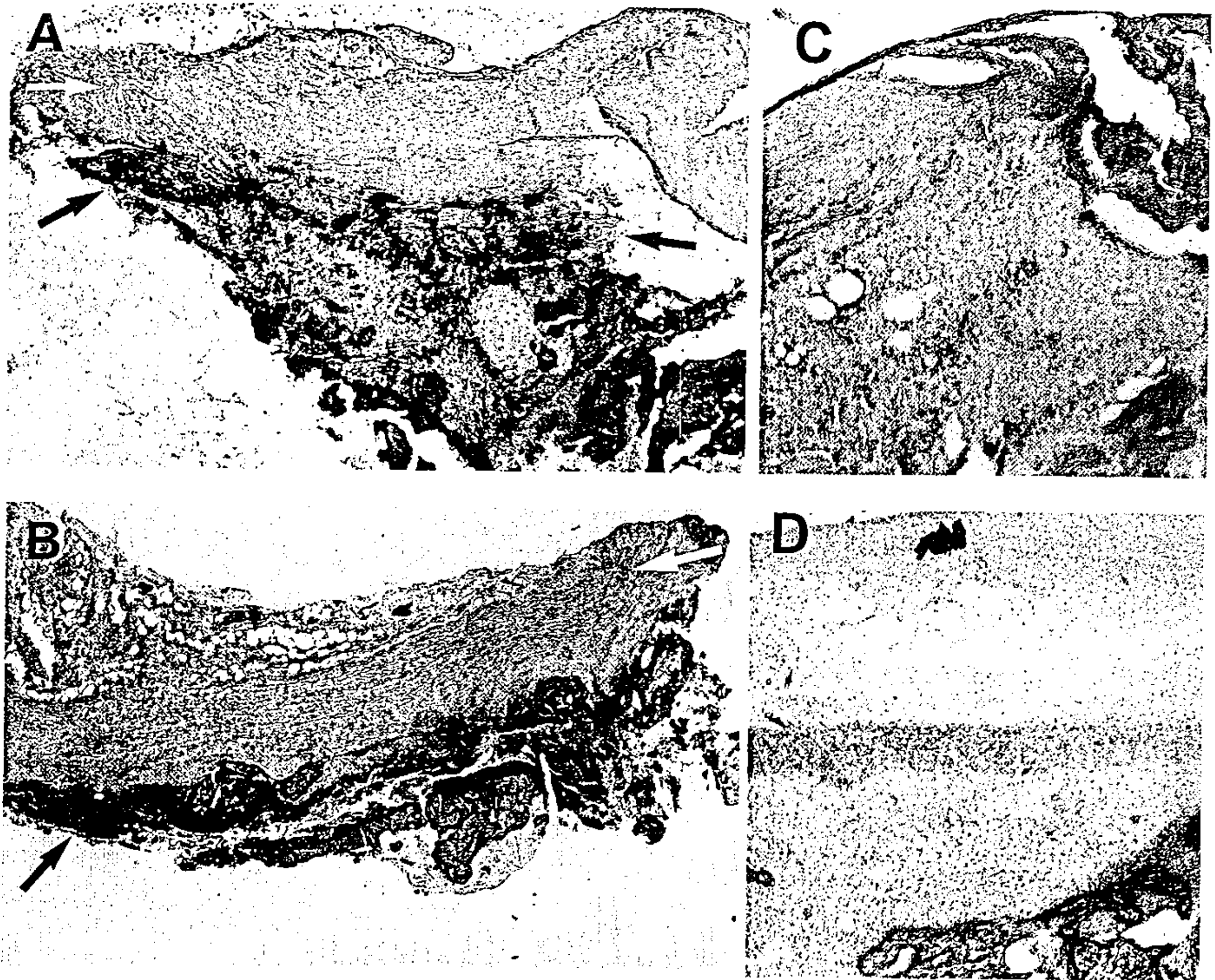


Figure 4. Microphotograph of a specimen obtained from a knee with transplanted periosteum derived cells embedded in collagen gel (20× original magnification). X-gal and H&E stainings were performed on each specimen using frozen sections. The transplanted cells grew at the transplant site and expressed LacZ under the periosteum patch when examined 1 and 2 weeks after transplant (A, B). White arrows indicate the periosteum patch, black arrows indicate LacZ positive periosteum derived cells. In control knees treated with cell-free collagen gel (C) or collagen gel with noninfected cells (D) there were no LacZ positive cells.

into the host chromosome[14]. We confirmed continued gene expression for 12 weeks *in vitro*, whereas in a previous study using another vector, the longest term of transferred gene expression was about 8 weeks[4,5], which indicates the superior ability of the AAV vector for sustained gene expression.

The advantage of *ex vivo* over *in vivo* gene delivery is the ability to limit the area of gene expression to the cartilage defect alone. Because of the strong infection potential of AAV, it is not certain where the AAV will infect using *in vivo* gene transfer. For example, synovium is easier to infect than cartilage since AAV has difficulty passing through the extracellular matrix[8-10]. Kang, *et al* performed *ex vivo* gene transfer to chondrocytes using a retroviral vector and confirmed LacZ expression for 4 weeks *in vivo*, but the percentage of LacZ positive cells was less than 10%[15], whereas this study had considerably more LacZ positive cells confirmed at the transplant site.

In a previous study using an AAV vector, the synovium[8,9,11,12] and the cartilage[9,10] were chosen as the target tissue for gene transfer. However, there have been no studies on gene transfer to periosteum derived cells using an AAV vector both *in vitro* and *in vivo*. We used periosteum derived cells, which can be obtained easily and maintain their chondrogenic potential throughout expansion regardless of donor age[16]. Periosteum derived cells were stained strongly at immunohistochemistry using anti-fibroblast antibody (Biomeda Corp., Hayward, CA, USA), so immunohis-

tochemical type of these cells was defined to be fibroblasts (data not shown).

We confirmed sustained gene expression in periosteum derived cells *in vitro* using an AAV vector, and also confirmed the usefulness of AAV as an *ex vivo* gene transfer agent, making this the first study demonstrating gene transfer to periosteum derived cells using an AAV vector. In the future, this method may be used for repairing cartilage defects or for treating osteoarthritis.

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REFERENCES

1. Caplan AI. The mesengenic process. Clin Plast Surg 1994; 21:429-35.
2. O'Driscoll SW, Keeley FW, Salter RB. The chondrogenic potential of free autogenous periosteal grafts for biological resurfacing of major full-thickness defects in joint surfaces under influence of continuous passive motion. An experimental investigation in the rabbit. J Bone Joint Surg Am 1986;68:1017-35.
3. O'Driscoll SW, Recklies AD, Poole AR. Chondrogenesis in periosteal explants. J Bone Joint Surg Am 1994;76:1042-51.
4. Roessler BJ, Allen ED, Wilson JM, Hartman JW, Davidson BL. Adenoviral-mediated gene transfer to rabbit synovium in vivo. J Clin Invest 1993;92:1085-92.
5. Ladner UM, Roberts CR, Franklin BN, et al. Human IL-1Ra gene transfer into human synovial fibroblasts is chondroprotective. J Immunol 1997;158:3492-8.
6. Oligino TJ, Yao Q, Ghivizzani SC, Robbins P. Vector systems for gene tranfer to joints. Clin Orthop 2000;379S:S17-30.
7. Flotte TR, Carter BJ. Adeno-associated virus vectors for gene therapy. Gene Ther 1995;2:357-62.
8. Pan RY, Xiao X, Chen SL, et al. Disease-inducible transgene expression from a recombinant adeno-associated virus vector in a rat arthritis model. J Virol 1999;73:3410-7.
9. Goater J, Müller R, Kollias G, et al. Empirical advantages of adeno associated viral vectors for in vivo gene therapy for arthritis. J Rheumatol 2000;27:983-9.
10. Arai Y, Kubo T, Fushiki S, et al. Gene delivery to human chondrocytes by an adeno associated virus vector. J Rheumatol 2000;27:979-82.
11. Watanabe S, Imagawa T, Boivin GP, Gao G, Wilson JM, Hirsch R. Adeno-associated virus mediates long-term gene transfer and delivery of chondroprotective IL-4 to murine synovium. Mol Ther 2000;2:147-52.
12. Zhang HG, Xie J, Yang P, et al. Adeno-associated virus production of soluble tumor necrosis factor receptor neutralizes necrosis factor α and reduces arthritis. Hum Gene Ther 2000;11:2431-42.
13. Xin KQ, Urabe M, Yang J, et al. A novel recombinant adeno-associated virus vaccine induces a long-term humoral immune response to HIV. Hum Gene Ther 2001;12:1047-61.
14. Kearns WG, Afione SA, Fulmer SB, et al. Recombinant adeno-associated virus (AAV-CFTR) vectors do not integrate in a site-specific fashion in an immortalized epithelial cell line. Gene Ther 1996;3:748-55.
15. Kang R, Marui T, Ghivizzani SC, et al. Ex vivo gene transfer to chondrocytes in full-thickness articular cartilage defects: a feasibility study. Osteoarthritis Cartilage 1997;5:139-43.
16. Bari CD, Dell'Accio F, Luyten FP. Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. Arthritis Rheum 2001;44:85-95.

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Recombinant adeno-associated viral vectors bring gene therapy for Parkinson's disease closer to reality

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Abstract The recombinant adeno-associated viral (rAAV) vector is a powerful tool for delivering therapeutic genes into mammalian brains. In rodents and non-human primates, a substantial number of striatal neurons can be transduced with high titer rAAV vectors by simple stereotaxic injection. Efficient and long-term expression of genes for dopamine (DA)-synthesizing enzymes in the striatum restored local DA production and achieved behavioral recovery in animal models of Parkinson's disease (PD). Moreover, sustained expression of a glial cell line-derived neurotrophic factor gene in the striatum rescued nigral neurons and led to functional recovery in a rat model of PD, even when treatment was delayed until after the onset of progressive degeneration. These results suggest that gene therapy using rAAV vectors may become a novel and feasible treatment for PD.

Key words Parkinson's disease · adeno-associated virus · gene therapy

Introduction

Recent advances in gene transfer methods, especially the development of better viral vectors, have expanded the potential of gene therapy for the treatment of a wide range of genetic and acquired diseases. Among the various viral vectors used in gene therapy, the recombinant adeno-associated viral (rAAV) vector is one of the most attractive candidates for applying this method to neurodegenerative disorders, such as Parkinson's disease (PD) [27], because efficient transduction of neurons can be achieved without significant side effects using rAAV vectors [1, 16, 18, 23, 24, 28, 33, 34].

Apart from the genetic mutations described in some familial cases, the cause of PD largely remains unknown, and it seems that multiple factors including environmental factors may contribute to the development of sporadic PD. Currently, two strategies are being pursued for gene therapy of PD, although neither involves the correction of specific genetic abnormalities [7, 31]. One possible approach is to restore dopamine (DA) production by delivering the genes of DA-synthesizing enzymes to the striatum in order to relieve the motor symptoms of PD. Cardinal motor symptoms, including resting tremor, muscular rigidity, and bradykinesia, are caused by a marked decrease in the striatal DA content secondary to progressive loss of nigrostriatal dopaminergic neurons, and DA replacement therapy is important for functional recovery. The second strategy is to block or slow the ongoing degenerative process by transduction of the genes for growth factors, antioxidant molecules, or antiapoptotic molecules [26]. Glial cell line-derived neurotrophic factor (GDNF), the most potent survival factor for dopaminergic neurons, is a strong candidate for this neuroprotective strategy [5, 7].

rAAV vectors

AAVs are small, non-enveloped, single-stranded DNA viruses of the *Parvoviridae* family, which have been as-

signed to the genus *Dependovirus* because productive infection requires coinfection with a helper virus such as adenovirus (Ad) or herpes virus [3]. AAVs have been isolated from a variety of different species, including primates, dogs, cows, horses, sheep, and chickens, in which these viruses appear to be non-pathogenic. So far, six primate AAVs (AAV-1 to 6) have been cloned and sequenced [2, 9, 10, 29, 32, 35, 37]. Among them, AAV-2 has been the most extensively studied and is a major focus of gene therapy vector development. The genome of AAV-2 consists of two large open reading frames (ORFs) that are flanked by two inverted terminal repeats (ITR). These ITRs are 145-nt sequences that function in *cis* as the origin of replication and are required for the packaging of progeny AAV DNA into virus particles. The left ORF encodes four nonstructural Rep proteins, which are essential for replication of the AAV genome and for its targeted integration into the host chromosome during latent infection. The right ORF encodes three viral capsid proteins, which are known as VP1, VP2, and VP3. Conventional methods of rAAV vector production involve double-plasmid transfection followed by infection with helper Ad. The vector plasmid contains a transgene expression cassette flanked by the AAV ITRs, while the packaging plasmid expresses the Rep and VP proteins in *trans* to supply the required AAV helper functions. More recently, a helper-free system has been developed to avoid undesirable contamination with wild-type Ad (Fig. 1) [25]. This system uses a helper plasmid that contains the minimum Ad genomic sequences necessary to perform helper functions (E1A, E1B, E2, E4ORF6, VA RNA), and has the ability to produce pure, high-titer rAAV vectors that offer safe, efficient, and long-term transgene expression in non-dividing cells without eliciting a substantial immune response.

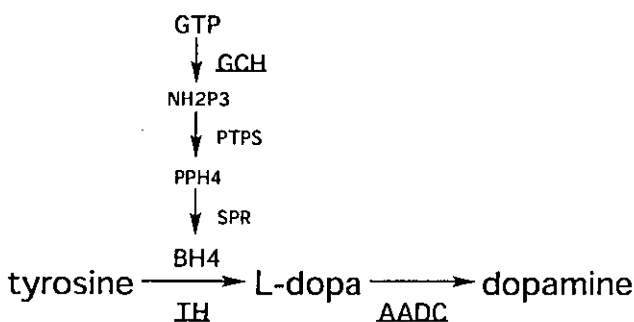

Fig. 1 Biosynthetic pathway of dopamine. Three enzymes (TH, AADC and GCH) are necessary for efficient production of dopamine. GCH is a rate-limiting enzyme for the biosynthesis of BH4, the essential cofactor of TH (*TH* tyrosine hydroxylase, *AADC* aromatic-L-amino-acid decarboxylase, *BH4* tetrahydrobiopterin, *GTP* guanosine triphosphate, *GCH* GTP cyclohydrolase I, *NH2P3* d-erythro-7,8-dihydroneopterin triphosphate, *PPH4* 6-pyruvoyl tetrahydropterin, *PTPS* 6-pyruvoyl tetrahydropterin synthase, *SPR* sepiapterin reductase).

DA synthesis in the striatum

Current therapy for PD aims to increase the striatal DA level by oral administration of L-DOPA. However, this pharmacotherapy becomes less effective with progression of the disease because of reduced conversion of L-DOPA to DA. Frequent administration of high doses of L-DOPA leads to oscillations in motor performance and causes some adverse effects such as hallucinations due to dopaminergic stimulation of the mesolimbic system. For efficient synthesis of DA, three enzymes are necessary (Fig. 2). Tyrosine hydroxylase (TH) is the rate-limiting enzyme that converts tyrosine to L-DOPA, after which aromatic L-amino acid decarboxylase (AADC) converts L-DOPA to DA. In addition, guanosine triphosphate cyclohydrolase I (GCH) is the rate-limiting enzyme for the synthesis of an essential co-factor of TH, which is known as tetrahydrobiopterin (BH_4) [30]. These enzymes undergo anterograde transport from the substantia nigra to the striatum and severe loss of dopaminergic nerve terminals in advanced PD is associated with a profound decrease of their activities.

We investigated whether rAAV vector-mediated gene transfer of DA-synthesizing enzymes could restore local DA production in the striatum and achieve behavioral recovery in animal models of PD [28, 34]. Mixtures of three separate rAAV vectors that expressed TH, AADC, and GCH, respectively (rAAV-TH, rAAV-AADC, and rAAV-GCH), were injected into the striatum of hemiparkinsonian rats that were created by injection of a selective neurotoxin, 6-hydroxydopamine (6-OHDA), into the medial forebrain bundle. The rAAV vectors efficiently transduced striatal cells, most of which were neu-

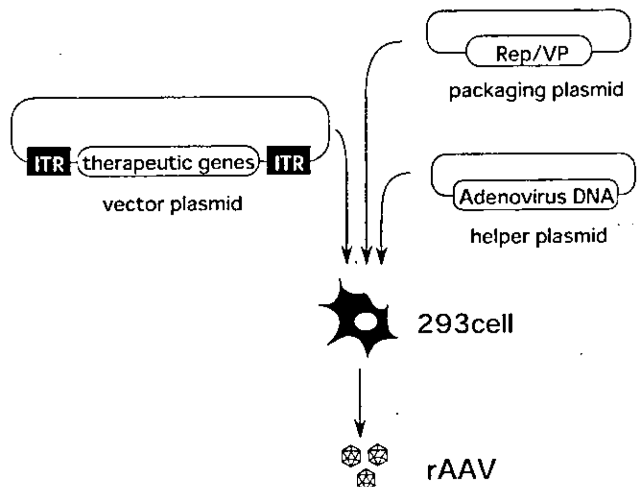

Fig. 2 Helper-free rAAV vector production system. Cells (293, a human embryonic kidney cell line) are transfected with three kinds of plasmid. The vector plasmid contains an expression cassette between the ITRs, the packaging plasmid provides the *rep* and *vp* regions of AAV, and the helper plasmid contains adenovirus sequences necessary for helper function.

rons. Cotransduction with the three rAAV vectors resulted in more DA production and better behavioral recovery when compared with rats receiving the rAAV-TH vector alone or the rAAV-TH and rAAV-AADC vectors. These findings in rats prompted us to examine a primate model of PD.

A selective toxin for dopaminergic neurons, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), was injected intravenously once a week until a stable parkinsonian syndrome was achieved in cynomolgus monkeys (*Macaca fascicularis*). Then a mixture of the three rAAV vectors expressing TH, AADC, and GCH was stereotaxically injected into the unilateral putamen. Coexpression of these three enzymes in the putamen led to a marked improvement of manual dexterity on the contralateral side to that injected with the vectors, and this improvement persisted throughout the observation period (four monkeys: 48 days, 65 days, 50 days, and > 17 months, each). TH-immunoreactive (TH-IR), AADC-IR, and GCH-IR cells were present in > 90 % of the putamen. Except for slight infiltration of mononuclear cells and residual hemosiderin around the needle track, examination of hematoxylin & eosin stained sections showed no signs of cytotoxicity in the rAAV vector-injected putamen. Microdialysis demonstrated that the DA concentration in the vectors-injected putamen was increased compared with that on the control side. The monkeys did not show any complications related to rAAV vector injection, including dyskinesia.

Neuroprotective effect of GDNF

GDNF is a small glycoprotein that provides strong trophic support for dopaminergic neurons [15, 22]. However, application of GDNF protein has been limited because of its short half-life and poor ability to cross the blood-brain barrier. An attempt to treat PD patients by direct injection of GDNF protein into the ventricles was unsuccessful [20]. Moreover, because PD is a slowly progressive disorder, GDNF would need to be delivered continuously. Recombinant viral vectors are powerful tools for achieving sustained GDNF production in the brain. Previous studies have demonstrated that delivery of the GDNF gene via rAAV [24, 23, 18], adenoviral [4, 11–14, 21], or lentiviral [19] vectors can protect nigral DA neurons in rodent and primate models of PD when administration is done before or shortly after injection of neurotoxin. However, a substantial number of dopaminergic neurons have already been lost before characteristic symptoms appear in clinical PD.

We used a rat PD model to investigate whether chronic delivery of the GDNF gene using the rAAV vector was effective even from a later phase of the degenerative process [36]. Four weeks after striatal lesioning with 6-OHDA, an rAAV vector expressing GDNF tagged with the FLAG peptide (rAAV-GDNF*flag*) was injected into the lesioned striatum. We found that GDNF*flag* protein was expressed in the striatum, and showed retrograde transport to DA neurons in the SN. In addition, ongoing degeneration of the nigrostriatal pathways was halted and functional recovery occurred even after a substantial number of dopamergic neurons had been lost.

Discussion

PD is regarded as a disease suitable for gene therapy or cell transplantation. In contrast to other neurological disorders, such as Alzheimer's disease and lipid storage diseases that broadly affect the brain, PD is essentially confined to the well-defined nigrostriatal dopaminergic system. In addition, stereotaxic surgery techniques have been established in clinical practice to approach the basal ganglia, and well-characterized rodent and primate models are available for testing this therapeutic approach, although whether neurotoxin-induced PD models faithfully reflect the human disease has not been sufficiently verified.

Developments in the field of neural or embryonic stem cell research have raised hopes of creating novel cell replacement therapies for PD, but are also associated with both scientific and ethical concerns [6]. In most cases, dopaminergic cells are transplanted ectopically into the striatum instead of into the substantia nigra. If the main mechanism underlying any functional recovery achieved by these cell replacement therapies is restoration of dopaminergic neurotransmission, then rAAV vector-mediated gene delivery of DA synthesizing enzymes is a much simpler and more straightforward approach. The significant functional recovery achieved in our primate model provides some optimism for the development of gene therapy for PD.

Neuroprotective gene therapy with neurotrophic factors has also shown promise as a novel treatment of PD. Neuroimaging and genetic analysis of some familial PD cases has recently provided an opportunity to detect at-risk individuals before the onset of characteristic symptoms [8, 17]. Early gene therapy with GDNF may be applicable to such at-risk individuals as well as to PD patients.

Although no adverse effects were observed in our experiments, the development of vector constructs that allow regulation of gene expression seems to be necessary to avoid over production of DA or GDNF. In facts, studies on rAAV vectors with a regulatable promoter are underway in several laboratories. For DA replacement, gene transfer of AADC alone in combination with oral administration of L-DOPA could be a shortcut to starting clinical trials [1, 33]. With this method, patients would still need to take L-DOPA for the control of PD

symptoms, but DA production could be regulated by the dose of L-DOPA.

In conclusion, gene therapy using rAAV vectors seems to be feasible for PD and may almost be ready to bear fruit clinically.

References

1. Bankiewicz KS, Eberling JL, Kohutnicka M, Jagust W, Pivirotto P, Bringas J, Cunningham J, Budinger TF, Harvey-White J (2000) Convection-enhanced delivery of AAV vector in parkinsonian monkeys, in vivo detection of gene expression and restoration of dopaminergic function using pro-drug approach. Exp Neurol 164:2–14
2. Bantel-Schaal U, Delius H, Schmidt R, zur Hausen H (1999) Human adeno-associated virus type 5 is only distantly related to other known primate helper-dependent parvoviruses. J Virol 73:939–947
3. Berns KI, Bergoin M, Bloom M, Lederman M, Muzyczka N, Siegl G, Tal J, Tattersall P (1995) Family Parvoviridae. In: Murphy FA, Fauquet CM, Bishop DHL, Ghabrial SA, Jarvis AW, Martelli GP, Mayo MA, Summers MD (eds) Virus Taxonomy. Classification and Nomenclature of Viruses, Springer-Verlag, New York, pp 169–178
4. Bilang-Bleuel A, Revah F, Colin P, Locquet I, Robert JJ, Mallet J, Horellou P (1997) Intrastriatal injection of an adenoviral vector expressing glial-cell-line-derived neurotrophic factor prevents dopaminergic neuron degeneration and behavioral impairment in a rat model of Parkinson disease. Proc Natl Acad Sci USA 94:8818–8823
5. Bjorklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ (2000) Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. Brain Res 886:82–98
6. Bjorklund A, Lindvall O (2000) Cell replacement therapies for central nervous system disorders. Nat Neurosci 3:537–544
7. Bohn MC (2000) Parkinson's disease: a neurodegenerative disease particularly amenable to gene therapy. Mol Ther 1:494–496
8. Brooks DJ (2000) Morphological and functional imaging studies on the diagnosis and progression of Parkinson's disease. J Neurol 247 (Suppl 2):II11–18
9. Chiorini JA, Kim F, Yang L, Kotin RM (1999) Cloning and characterization of adeno-associated virus type 5. J Virol 73:1309–1319
10. Chiorini JA, Yang L, Liu Y, Safer B, Kotin RM (1997) Cloning of adeno-associated virus type 4 (AAV4) and generation of recombinant AAV4 particles. J Virol 71:6823–6833
11. Choi-Lundberg DL, Lin Q, Chang YN, Chiang YL, Hay CM, Mohajeri H, Davidson BL, Bohn MC (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. Science 275:838–841
12. Choi-Lundberg DL, Lin Q, Schallert T, Crippens D, Davidson BL, Chang YN, Chiang YL, Qian J, Bardwaj L, Bohn MC (1998) Behavioral and cellular protection of rat dopaminergic neurons by an adenoviral vector encoding glial cell line-derived neurotrophic factor. Exp Neurol 154:261–275
13. Connor B, Kozlowski DA, Schallert T, Tillerson JL, Davidson BL, Bohn MC (1999) Differential effects of glial cell line-derived neurotrophic factor (GDNF) in the striatum and substantia nigra of the aged parkinsonian rat. Gene Ther 6:1936–1951
14. Connor B, Kozlowski DA, Unnerstall JR, Elsworth JD, Tillerson JL, Schallert T, Bohn MC (2001) Glial cell line-derived neurotrophic factor (GDNF) gene delivery protects dopaminergic terminals from degeneration. Exp Neurol 169:83–95
15. Fan D, Ogawa M, Ikeguchi K, Fujimoto K, Urabe M, Kume A, Nishizawa M, Matsushita N, Kiuchi K, Ichinose H, Nagatsu T, Kurtzman GJ, Nakano I, Ozawa K (1998) Prevention of dopaminergic neuron death by adeno-associated virus vector-mediated GDNF gene transfer in rat mesencephalic cells in vitro. Neurosci Lett 248:61–64
16. Fan D, Ogawa M, Fujimoto K, Ikeguchi K, Ogasawara Y, Urabe M, Nishizawa M, Nakano I, Yoshida M, Nagatsu I, Ichinose H, Nagatsu T, Kurtzman GJ, Ozawa K (1998) Behavioral recovery in 6-hydroxydopamine-lesioned rats by cotransduction of striatum with tyrosine hydroxylase and aromatic L-amino acid decarboxylase genes using two separate adeno-associated virus vectors. Hum Gene Ther 9:2527–2535
17. Gasser T (2001) Genetics of Parkinson's disease. J Neurol 248:833–840
18. Kirik D, Rosenblad C, Bjorklund A, Mandel RJ (2000) Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J Neurosci 20:4686–4700
19. Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen EY, Palfi S, Roitberg BZ, Brown WD, Holden JE, Pyzalski R, Taylor MD, Carvey P, Ling Z, Trono D, Hantraye P, Deglon N, Aebischer P (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. Science 290:767–773
20. Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, Cochran EJ, Mufson EJ, Penn R, Goetz CG, Comella CD (1999) Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. Ann Neurol 46:419–424
21. Kozlowski DA, Connor B, Tillerson JL, Schallert T, Bohn MC (2000) Delivery of a GDNF gene into the substantia nigra after a progressive 6-OHDA lesion maintains functional nigrostriatal connections. Exp Neurol 166:1–15
22. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130–1132
23. Mandel RJ, Snyder RO, Leff SE (1999) Recombinant adeno-associated viral vector-mediated glial cell line-derived neurotrophic factor gene transfer protects nigral dopamine neurons after onset of progressive degeneration in a rat model of Parkinson's disease. Exp Neurol 160:205–214
24. Mandel RJ, Spratt SK, Snyder RO, Leff SE (1997) Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. Proc Natl Acad Sci USA 94:14083–14088

25. Matsushita T, Elliger S, Elliger C, Podsakoff G, Villarreal L, Kurtzman GJ, Iwaki Y, Colosi P (1998) Adeno-associated virus vectors can be efficiently produced without helper virus. Gene Ther 5:938–945
26. Mochizuki H, Hayakawa H, Migita M, Shibata M, Tanaka R, Suzuki A, Shimo-Nakanishi Y, Urabe T, Yamada M, Tamayose K, Shimada T, Miura M, Mizuno Y (2001) An AAV-derived Apaf-1 dominant negative inhibitor prevents MPTP toxicity as antiapoptotic gene therapy for Parkinson's disease. Proc Natl Acad Sci USA 98:10918–10923
27. Monahan PE, Samulski RJ (2000) AAV vectors: is clinical success on the horizon? Gene Ther 7:24–30
28. Muramatsu S, Fujimoto K, Ikeguchi K, Shizuma N, Kawasaki K, Ono F, Shen Y, Wang L, Mizukami H, Kume A, Matsumura M, Nagatsu I, Urano F, Ichinose H, Nagatsu T, Terao K, Nakano I, Ozawa K (2002) Behavioral recovery in a primate model of Parkinson's disease by triple transduction of striatal cells with adeno-associated viral vectors expressing dopamine-synthesizing enzymes. Hum Gene Ther 13:345–354
29. Muramatsu S, Mizukami H, Young NS, Brown KE (1996) Nucleotide sequencing and generation of an infectious clone of adeno-associated virus 3. Virology 221:208–217
30. Nagatsu T, Ichinose H (1999) Molecular biology of catecholamine-related enzymes in relation to Parkinson's disease. Cell Mol Neurobiol 19:57–66
31. Ozawa K, Fan D, Shen Y, Muramatsu S, Fujimoto K, Ikeguchi K, Ogawa M, Urabe M, Kume A, Nakano I (2000) Gene therapy of Parkinson's disease using adeno-associated virus (AAV) vectors. J Neural Transm Suppl 58:181–191
32. Rutledge EA, Halbert CL, Russell DW (1998) Infectious clones and vectors derived from adeno-associated virus (AAV) serotypes other than AAV type 2. J Virol 72:309–319
33. Sanchez-Pernaute R, Harvey-White J, Cunningham J, Bankiewicz KS (2001) Functional effect of adeno-associated virus mediated gene transfer of aromatic L-amino acid decarboxylase into the striatum of 6-OHDA-lesioned rats. Mol Ther 4:324–330
34. Shen Y, Muramatsu S, Ikeguchi K, Fujimoto K, Fan D, Ogawa M, Mizukami H, Urabe M, Kume A, Nagatsu I, Urano F, Suzuki T, Ichinose H, Nagatsu T, Monahan J, Nakano I, Ozawa K (2000) Triple transduction with adeno-associated virus vectors expressing tyrosine hydroxylase, aromatic-L-amino-acid decarboxylase, and GTP cyclohydrolase I for gene therapy of Parkinson's disease. Hum Gene Ther 11:1509–1519
35. Srivastava A, Lusby EW, Berns KI (1983) Nucleotide sequence and organization of the adeno-associated virus 2 genome. J Virol 45:555–564
36. Wang L, Muramatsu S, Lu Y, Ikeguchi K, Fujimoto K, Okada T, Mizukami H, Hanazono Y, Kume A, Urano F, Ichinose H, Nagatsu T, Nakano I, Ozawa K (2002) Delayed delivery of AAV-GDNF prevents nigral neurodegeneration and promotes functional recovery in a rat model of Parkinson's disease. Gene Ther 9:381–389
37. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM (1999) Gene therapy vectors based on adeno-associated virus type 1. J Virol 73:3994–4003

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Oral Administration of Recombinant Adeno-Associated Virus Elicits Human Immunodeficiency Virus-Specific Immune Responses

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ABSTRACT

Oral vaccines can induce both systemic and mucosal immunity. Mucosal immunity, especially regional cell-mediated immunity, plays an important role in protecting individuals from infectious diseases such as acquired immunodeficiency syndrome. In this study, a recombinant adeno-associated virus vector expressing human immunodeficiency virus type 1 *env* gene (AAV-HIV) was orally administered to BALB/c mice. Systemic and regional immunity was induced in the mice. Furthermore, the immunization significantly reduced viral load after an intrarectal challenge with a recombinant vaccinia virus expressing HIV *env* gene. Moreover, we also show that dendritic cells might contribute to the AAV-HIV vector-induced immune responses.

OVERVIEW SUMMARY

The ability of an adeno-associated virus vector expressing the *env* gene from human immunodeficiency virus type 1 (AAV-HIV) to induce a mucosal immune response when administered orally was studied. A single oral administration of AAV-HIV stimulated strong HIV-specific humoral immunity and generated MHC class I-restricted cytotoxic T lymphocytes in BALB/c mice. This response significantly reduced viral load after intrarectal challenge with a recombinant vaccinia virus expressing the HIV *env* gene. These AAV-HIV-infected bone marrow-derived dendritic cells (DCs) induced antigen-specific interferon γ secretion of $CD8^+$ T cells when reinfused *in vivo*. These results demonstrate that oral administration of an AAV-based HIV vaccine elicits mucosal, humoral, and cell-mediated immune responses, and suggest that antigen-presenting cells, such as DCs, contribute to this AAV-HIV vaccine-induced immunity.

INTRODUCTION

Adeno-associated virus (AAV) vectors have been widely used for gene therapy in animal models (During *et al.*, 1998; Fan *et al.*, 1998) and have entered human clinical trials (Kay *et al.*, 2000). AAVs have generally been used to transfect host cells with therapeutically useful syngeneic genes. The transfected cells then transcribe and translate the gene of interest, and produce the encoded protein. Because the self-protein does not elicit a strong immune response, its production can be maintained for a prolonged period. We (Xin *et al.*, 2001) and others (Manning *et al.*, 1997; Clark and Johnson, 1998; During *et al.*, 1998; Brockstedt *et al.*, 1999; Sarukhan *et al.*, 2001) have explored, using AAV as vectors, the introduction of foreign genes encoding immunogenic proteins for the purpose of vaccination.

The use of AAV to stimulate an anti-human immunodeficiency virus (HIV) response is one of many immunization strategies being evaluated for the induction of immunity against

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HIV. A live attenuated *nef*-deleted HIV vaccine was shown to induce a strong protective immune response against HIV infection (Daniel *et al.*, 1992). However, these attenuated mutants appear to retain residual virulence and thus have not been used in healthy individuals (Murphey-Corb, 1997). Modified vaccinia virus vector vaccines (Robinson *et al.*, 1999) administered alone or in combination with protein- and DNA-based vaccines can generate protective immunity in nonhuman primates, and are immunogenic in humans (Belshe *et al.*, 1998). Other viral vectors, including adenovirus (Yoshida *et al.*, 2001), rabies virus (Schnell *et al.*, 2000), flavivirus (Mandl *et al.*, 1998), Friend murine leukemia virus (Matano *et al.*, 2000), Venezuelan equine encephalitis virus (Caley *et al.*, 1999), and bacillus Calmette-Guérin (Aldovini and Young, 1991), have induced satisfactory levels of immunity against HIV. In preclinical studies, some of these viral vectors have induced protective immunity in nonhuman primates (Robinson *et al.*, 1999; Matano *et al.*, 2000). However, *in vivo* pathogenicity of these vectors has limited their use (Hollon, 2000). AAV is the only nonpathogenic, currently available viral vector that has been shown to mediate long-term gene expression without toxicity *in vivo*. Using this vector system, exogenous genes have been efficiently transfected into a number of tissues, including brain (Fan *et al.*, 1998), muscle (Herzog *et al.*, 1997), lung (Flotte *et al.*, 1993), gut (During *et al.*, 1998), liver (Snyder *et al.*, 1997), and eye (Lewin *et al.*, 1998). Human clinical trials of AAV for the treatment of hemophiliacs have been conducted (Kay *et al.*, 2000).

AAV is a small, single-stranded DNA virus lacking an envelope. The virus requires a helper virus to facilitate efficient replication. The AAV genome is known to integrate into the human genome at specific sites, most frequently in chromosome 19q (Kotin *et al.*, 1990). With recombinant AAV vectors, however, maintenance of gene expression occurs as a result of both episomal persistence and integration into nonchromosome 19q locations (Kearns *et al.*, 1996).

Most currently available HIV vaccines have been designed to elicit systemic immune responses. Indeed, we previously reported that systemic immunization with AAV-HIV triggered an HIV-specific antibody and cell-mediated immunity (CMI) response (Xin *et al.*, 2001). However, mucosal immunization can play an important role in protecting individuals from infectious diseases, such as AIDS. Because AAV remains stable and infectious at a range of pH and temperatures, it is a suitable vector for oral administration. In this study, AAV-HIV was administered orally to BALB/c mice. These animals were then challenged with a recombinant vaccinia virus expressing HIV-1 *env* gene. The mechanisms involved in the observed responses were evaluated.

MATERIALS AND METHODS

Plasmid DNA

The AAV subtype 2 vector plasmid used in this study was derived from the vector plasmid pW1 (hereafter referred to as pLacZ), which contains the *lacZ* gene, as previously described (Xin *et al.*, 2001). A fragment containing HIV *env* and *rev* genes derived from the HIV_{IIIB} strain was subcloned between the *Eco*RI and *Xho*I sites of the pLacZ AAV vector plasmid to replace the *lacZ* gene (pHIV). pIM45 is an AAV helper plasmid containing subtype 2 AAV *rep* and *cap* genes, which are required for replication and capsid 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 vector production. *Escherichia coli* DH5α was used for plasmid DNA amplification. Plasmid DNA was extracted with an EndoFree plasmid kit (Qiagen, Valencia, CA). Protein expression of pHIV was confirmed by Western blot, using a monoclonal antibody against the HIV_{IIIB} envelope (No. 810; NIH AIDS Research and Reference Reagent Program, Rockville, MD) as a first antibody and horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody as a second antibody.

Cell culture and AAV vector production

HEK 293 cells were maintained in of Dulbecco's modified Eagle's medium–nutrient mixture F-12 (DMEM/F-12 [1:1, v/v]; GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a 5% CO_2–air incubator. Subconfluent HEK 293 cells were cotransfected by the calcium phosphate coprecipitation method with the vector plasmid (pLacZ or pHIV), pIM45, and pladeno-1. After 48 hr, the cells were harvested and lysed in Tris buffer (10 m*M* Tris-HCl, 150 m*M* NaCl, pH 8.0) by three cycles of freezing and thawing. One round of sucrose precipitation and two rounds of CsCl density gradient ultracentrifugation were sufficient to isolate the AAV vectors (AAV-HIV and AAV-LacZ) from the lysates. CsCl was removed by several washes with 50 m*M* HEPES–0.15 *M* NaCl, using an Ultrafree-4 column (with Biomax-50 membrane; Millipore, Bedford, MA) (Sferra *et al.*, 2000). The vector titer was determined by quantitative DNA dot-blot hybridization of the DNase I-resistant fraction.

Animals and immunization

Eight-week-old BALB/c female mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in the animal center of Yokohama City University (Yokohama, Japan), where they were kept on a 12-hr day–night cycle. Each group consisted of 10 mice. The AAV vector was diluted with phosphate-buffered saline (PBS) to give 10^9–10^{12} particles/ml in a final volume of 0.1 ml. Mice were fasted overnight before immunization. Mice were anesthetized with diethyl ether and, about 20 sec later, 100 μl of AAV vector was administered via oral gavage, using a 21-gauge feeding needle (Popper & Sons, New Hyde Park, NY).

Detection of HIV mRNA in immunized mouse tissues

On days 1, 3, 7, and 14 after oral administration of 10^{10} particles of AAV-HIV vector or AAV-LacZ vector, total RNA was extracted from mouse small intestine, intestinal lymph nodes, and spleen, using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer instructions. Briefly, 50 mg of tissue of 1 ml of TRIzol reagent was homogenized, and total RNA was treated with RNase-free DNase I after phenol–chloroform extraction. cDNA was then synthesized, using random hexamers. The HIV *env* gene region was amplified with primers 5'-GGAGCAGCAGGAAGCACTA-3' and 5'-TCCCTGCCTAACTCTA-3') to produce a 575-bp fragment of the HIV-1 *env* gene.