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Adipose Tissue as a Novel Target for *In Vivo* Gene Transfer by Adeno-Associated Viral Vectors

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ABSTRACT

Traditionally, skeletal muscle and liver are the preferred target organs for gene transfer to supply a transgene product into the systemic circulation. In this respect, adipose tissue presents a number of attractive features. However, adipose tissue transduction *in vivo* has not been feasible by conventional methods. To solve this issue, we tested the utility of excipients in adeno-associated virus (AAV) vector-mediated gene transfer and found that Pluronics are suitable for this purpose. In a histological analysis of adipose tissue in *db/db* mice, Pluronic F88 showed the greatest augmentative effect on β-galactosidase expression in combination with the AAV1 vector. When the vector encoding mouse erythropoietin (Epo) was used in the same manner, increased plasma Epo concentrations were observed (230 ± 80 versus 58 ± 14 mU/ml). Moreover, the plasma Epo concentration returned to the normal level after the surgical removal of transduced adipose tissue. No damage was observed in the transduced tissue. Our results indicate that the proposed method is safe and efficient for gene transfer into adipose tissues, thus providing an alternative for supplemental gene therapy.

OVERVIEW SUMMARY

Adipose tissue holds promise as an alternative depot organ in gene transfer approaches. However, no efficient method of gene transfer into adipose tissue *in vivo* has been established. In this study, we explored the utility of excipients to augment gene transfer into the adipose tissue of mice and found that Pluronic F88 was useful for this purpose when combined with AAV serotype 1 vectors. The improvement was also demonstrated with vectors encoding murine erythropoietin, and the mice became polycythemic. Moreover, after removing transduced adipose tissue, plasma erythropoietin levels returned to normal, which suggests the unique advantage of this method.

INTRODUCTION

In supplemental gene therapy, skeletal muscle and the liver have been the preferred targets for gene transfer to supply transgene products into the systemic circulation. However, adipose tissue presents a number of attractive features. Adipose tissue can be found throughout the body and is easily accessible for vector injection. Increasing evidence supports the notion that adipocytes are designed to secrete numerous factors into the systemic circulation (Mohamed-Ali *et al.*, 1998). Further, the majority of adipocytes are considered to be nondividing, which is suitable for achieving long-term expression of transferred genes by the use of nonintegrating vectors such as adeno-associated virus (AAV) vectors (Russell and Kay, 1999). Moreover, the transduced tissue can be safely removed when unexpected events occur, thus adding a unique feature to safety considerations. However, adipose tissue transduction has not been feasible through conventional methods, and few studies have investigated its efficacy *in vivo* (Nagamatsu *et al.*, 2001; Ogata *et al.*, 2004). To overcome these limitations and develop a more practical method, we tested the usefulness of excipients for gene transfer. To achieve efficient and widespread gene transfer, it is essential to assure that the vectors stay within the target tissue for a certain period of time. For this purpose, nonionic surfactants are promising because they have low toxicity and unique features that help stabilize the membrane (American Pharmaceutical Association [AphA] and Royal Pharmaceutical Society of Great Britain [RPSGB], 1986). Moreover,

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improved gene delivery with Pluronic compounds was demonstrated in various applications (Kabanov and Alakhov, 2002; Kabanov *et al.*, 2005). Therefore, we tested the effects of these compounds on *in vivo* gene transfer targeting adipose tissue.

MATERIALS AND METHODS

Cells and plasmids for AAV vector preparation

HEK293 cells, a human embryonic kidney cell line, were maintained as described in a previous report (Fan *et al.*, 1998). Murine erythropoietin (Epo)-encoding plasmids have already been described by Mochizuki *et al.* (2004). AAV vectors of serotype 1 through 5, which encode LacZ or Epo and have a cytomegalovirus (CMV) promoter, were prepared by transient transfection, purified and quantitated as described previously (Matsushita *et al.*, 1998; Mochizuki *et al.*, 2004). Usually, the stocks of AAV vectors contained approximately 1-2 $\times$ 10^{10} genome copies/μl.

In vitro *assessment of potential toxicity of surfactants*

A panel of Pluronics was provided by Asahi Denka (Tokyo, Japan). Tween 80 was obtained from Sigma-Aldrich (St. Louis, MO). The surfactants were dissolved in distilled H_2O to prepare stock solutions at a concentration of 20%. To assess the potential compatibility, we added various concentrations of these surfactants to cultured 293 cells at the time of confluency. After 24 hr, the cells were examined microscopically. The highest concentrations of the surfactants that did not produce any deleterious effect on these cells were tested. The effect of the surfactants on AAV vector capsids was also examined by including a 10% surfactant in the vector stocks (AAV-LacZ with serotype 2 capsid, 2 $\times$ 10^{10} VG/μl) for 24 hr; subsequently, the vector solutions were added to the cultured 293 cells in a 96-well plate at a dose of 2 $\times$ 10^4 VG/cell. The final concentration of the surfactants within the culture medium was 0.05%. Two days later, the infectivity was assessed by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining (Fan *et al.*, 1998).

Analysis of gene expression and of enhancing effect in vivo

For the *in vivo* experiments, the AAV vector solutions containing various concentrations of Pluronic F68, F88, and Tween 80 were injected into the subcutaneous adipose tissues of C57BLKS/J *db/db* mice (Japan SLC, Hamamatsu, Japan) at the age of 10 to 12 weeks. At the time of injection, the average weight of the animals was more than 50 g, and the subcutaneous adipose tissues were well developed. The AAV-CMV-LacZ vectors with various serotypes (serotype 1–5) were tested at a dose of 6 $\times$ 10^{10} VG/body. Two weeks after injection, the mice were killed, and their adipose tissues were enucleated, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), cut into pieces, and then frozen with Tissue-Tek optimal cutting temperature (O.C.T.) compound (Sakura Finetek, Torrance, CA) in dry ice–ethanol, as described (Ogata *et al.*, 2004). When the tissue blocks were sliced for microscopic examination, they were also evaluated macroscopically and photographed. A densitometric analysis of the blocks was performed in order to substantiate the difference in the efficiency of transduction of adipose tissue under various injection conditions. In practice, five areas of the corresponding blocks were chosen and quantified, using Image Gauge software (version 3.0; Fuji Photo Film, Tokyo, Japan). After subtracting the background value, these numbers were analyzed and the statistical significance was evaluated. Experiments were performed with AAV-Epo vectors under conditions optimized with the AAV-LacZ vectors. Later, experiments at a higher vector dose (2 $\times$ 10^{11} VG/body) were included. On the basis of the preliminary experiments, the volume of injection was optimized as 100 μl/body (50 μl per lobe) and kept constant thereafter. Whole blood was collected from the tail vein every 2 weeks. Plasma Epo concentrations were quantified with an enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mannheim, Germany). Complete blood counts were performed with a PC-608 particle counter (Erma, Tokyo, Japan).

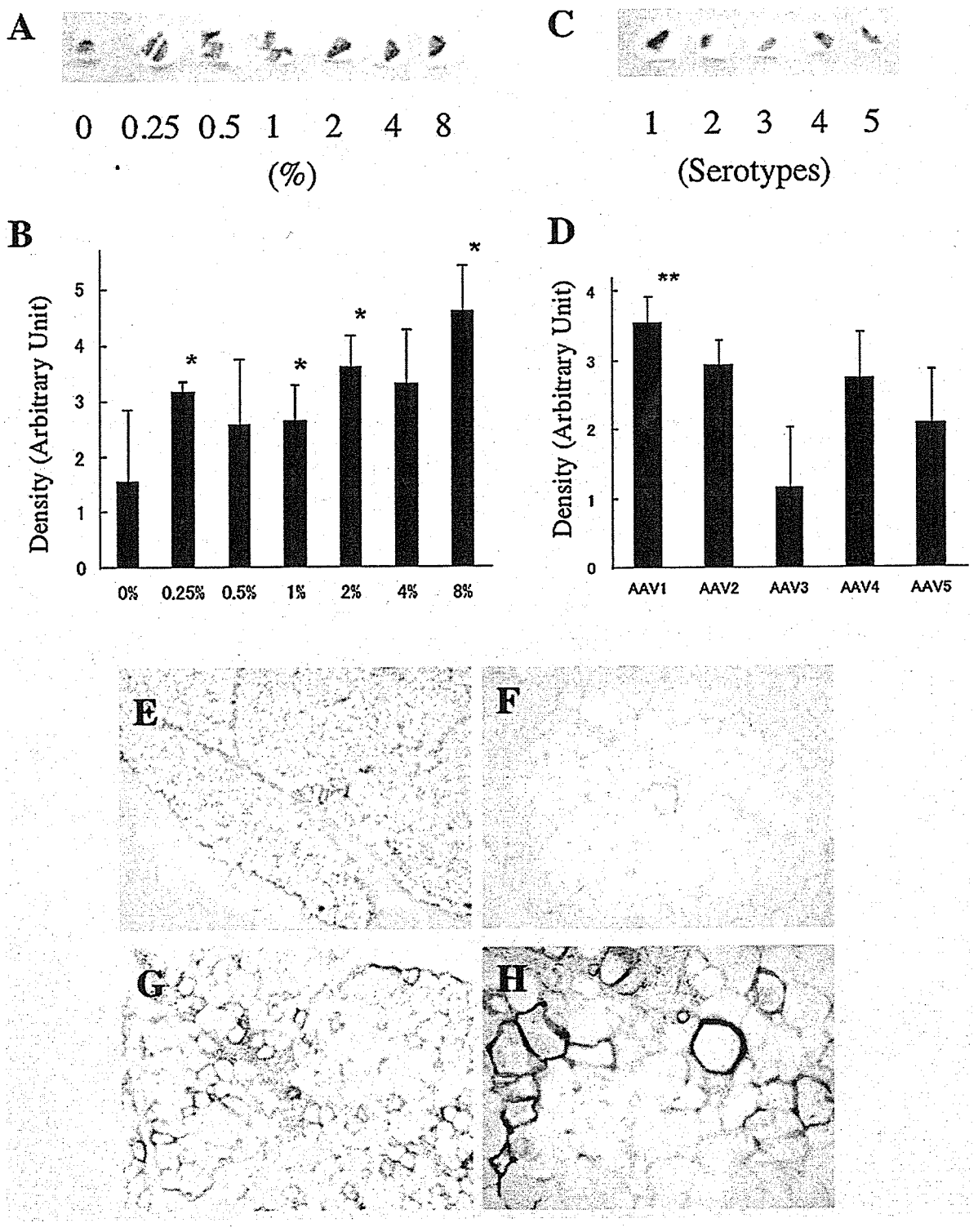
Detection of murine Epo within adipose tissue of db/db *mice*

Adipose tissues were obtained from *db/db* mice at the time of sacrifice or later by operation. For the immunofluorescence study, adipose tissues were fixed and frozen in the presence of the O.C.T. compound in dry ice–ethanol, following which they were reacted with goat anti-human Epo (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat IgG conjugated with Alexa 488 (Invitrogen Molecular Probes, Eugene, OR) at 4°C for 16 hr to visualize murine Epo by fluorescence microscopy, as described in a previous report (Ogata *et al.*, 2004).

Detection of murine Epo transcript within adipose tissue by reverse transcription-polymerase chain reaction

Total RNA was isolated from adipose tissue with an RNeasy lipid tissue kit (Qiagen, Hilden, Germany) and was converted to

FIG. 1. Distribution of LacZ expression within adipose tissues. AAV-CMV-LacZ vectors of various serotypes (serotypes 1–5) were tested (n = 3 for each serotype) at a dose of 6 $\times$ 10^{10} VG/body. Two weeks after injection, adipose tissues were removed, stained with X-Gal, and resected in pieces. Tissues were frozen with Tissue-Tek and then cut into 20-μm-thick slices for microscopic analysis. Blocks were also evaluated macroscopically, photographed, and analyzed by densitometry. **(A)** Representative blocks of adipose tissue injected with various concentrations of Pluronic F88. **(B)** Densitometric analysis of the corresponding blocks with various concentrations of Pluronic F88. Asterisks indicate statistical significance ($p < 0.05$) compared with values of tissues without F88 (0% data). **(C)** Blocks of adipose tissue injected with serotypes of AAV-LacZ vectors with 2% F88. **(D)** Densitometric analysis of blocks corresponding to the serotypes of AAV-LacZ vectors. Double asterisks indicate statistical significance ($p < 0.05$) relative to values obtained with the rest of the serotypes. Microscopic analysis of adipose tissue sections transduced with AAV1-LacZ without excipients is shown at low **(E)** and high **(F)** magnifications. Adipose tissues transduced with AAV1-LacZ in the presence of 2% Pluronic F88 are shown at low **(G)** and high **(H)** magnifications.


A
0 0.25 0.5 1 2 4 8
(%)
C
1 2 3 4 5
(Serotypes)
B
Density (Arbitrary Unit)
0% 0.25% 0.5% 1% 2% 4% 8%
D
Density (Arbitrary Unit)
AAV1 AAV2 AAV3 AAV4 AAV5
E
F
G
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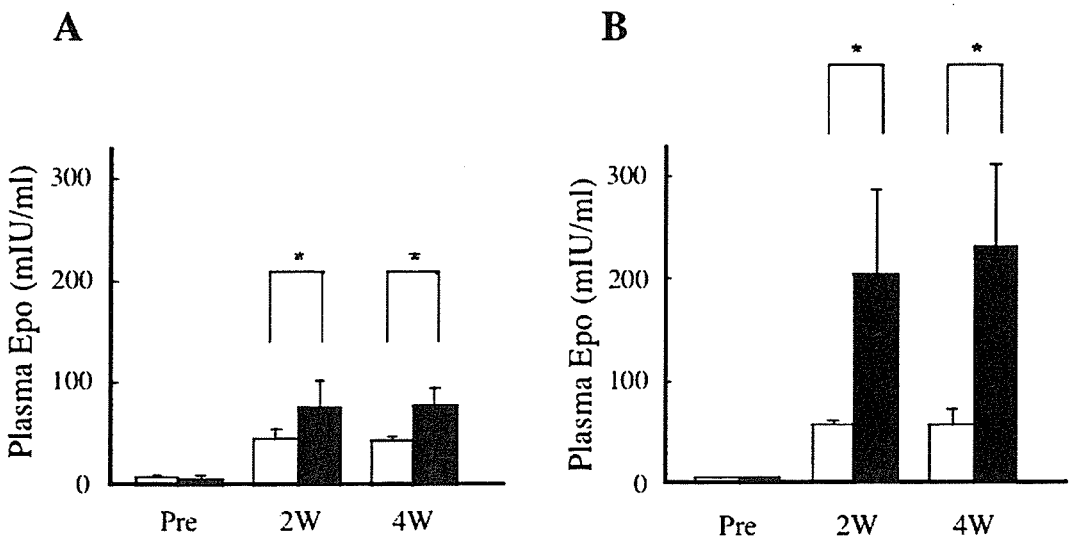

FIG. 2. Plasma Epo concentrations in *db/db* mice after injection of AAV1-Epo vectors into adipose tissue at a dose of (**A**) 6×10^{10} or (**B**) 2×10^{11} VG/body. Open and solid columns represent groups without and with 2% Pluronic F88, respectively, at the time of vector injection into adipose tissue. Each column and error bar indicate, respectively, the mean and SD of the group ($n = 5$). Asterisks indicate significance ($p < 0.05$).

cDNA with reverse transcriptase (SuperScript; Invitrogen, Carlsbad, CA) and oligo(dT) primers in a 20-μl mixture after DNase I (amplification grade; Invitrogen) treatment according to the manufacturer's instructions. Subsequent polymerase chain reaction (PCR) amplification was carried out with 1 μl of cDNA solution in a 50-μl reaction mixture containing 5 units of *Taq* polymerase, 10 m*M* Tris-HCl (pH 8.5), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 100 μM dNTPs in the presence of specific primer pairs (200 n*M*) designed to amplify DNA fragments derived from the transcript of the mouse Epo transgene. Each PCR cycle involved denaturation at 94°C for 15 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. The PCR products were analyzed by agarose gel electrophoresis. The authenticity of the PCR products was confirmed by observing their molecular sizes after agarose gel electrophoresis and by sequencing (ABI PRISM 310 genetic analyzer; Applied Biosystems, Foster City, CA). The primer sequences for mouse Epo were 5′-GTG CAG AAG GTC CCA GAC TGA GTG A-3′ and 5′-TTG GCG TAG ACC CGG AAG AGC TTG-3′. The primers for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Clontech Laboratories (Palo Alto, CA).

Removal of transduced tissue

AAV1-Epo vector solution (2×10^{11} VG/body) including 2% Pluronic F88 was injected into the subcutaneous adipose tissues of *db/db* mice. After 4 weeks, the adipose tissues were removed by standard surgical techniques under anesthesia. Plasma Epo concentrations were followed up 2 weeks thereafter. Four animals were analyzed and monitored.

Data and statistical analysis

Data are presented as means ± SD and were analyzed by Student *t* test after confirming an insignificant difference in variance between groups. $p < 0.05$ was considered statistically significant.

RESULTS

Screening of excipients in vitro

First, *in vitro* screening experiments were performed using nonionic surfactants. We tested Pluronic F68, F88, L72, P85, and Tween 80 for their effects on cell culture. Pluronic F68 and F88 were innocuous to cultured 293 cells at concentrations of up to 10 and 0.3%, respectively. The rest of the surfactants showed deleterious effects on the cultured cells when included at concentrations of 0.1% or higher. Next, we incubated AAV2-LacZ vectors with up to 10% Pluronic F68 and F88 solutions for 1 hr and checked their infectivity in 293 cells at 1×10^5 VG/cell. There were no differences in the infectivity of the vectors treated with these excipients, as assessed by X-Gal staining (data not shown).

Assessment of LacZ expression by use of Pluronics

On the basis of general safety data and *in vitro* experiments, we selected Pluronic F68, Pluronic F88, and Tween 80 as candidates for vector injection into *db/db* mice. Because preliminary experiments indicated the usefulness of Pluronic F88 combined with the AAV1 vector, we tested the usefulness of F88 at various concentrations. Comparison of blocks showed that there appeared to be an augmentation of LacZ expression with increasing concentration (Fig. 1A). The enhanced expression was substantiated by densitometric analysis of the blocks (Fig. 1B). On the basis of the result, we compared the usefulness of serotypes 1 through 5 combined with 2% Pluronic F88. The result showed that AAV1 was the most suitable serotype to trans-

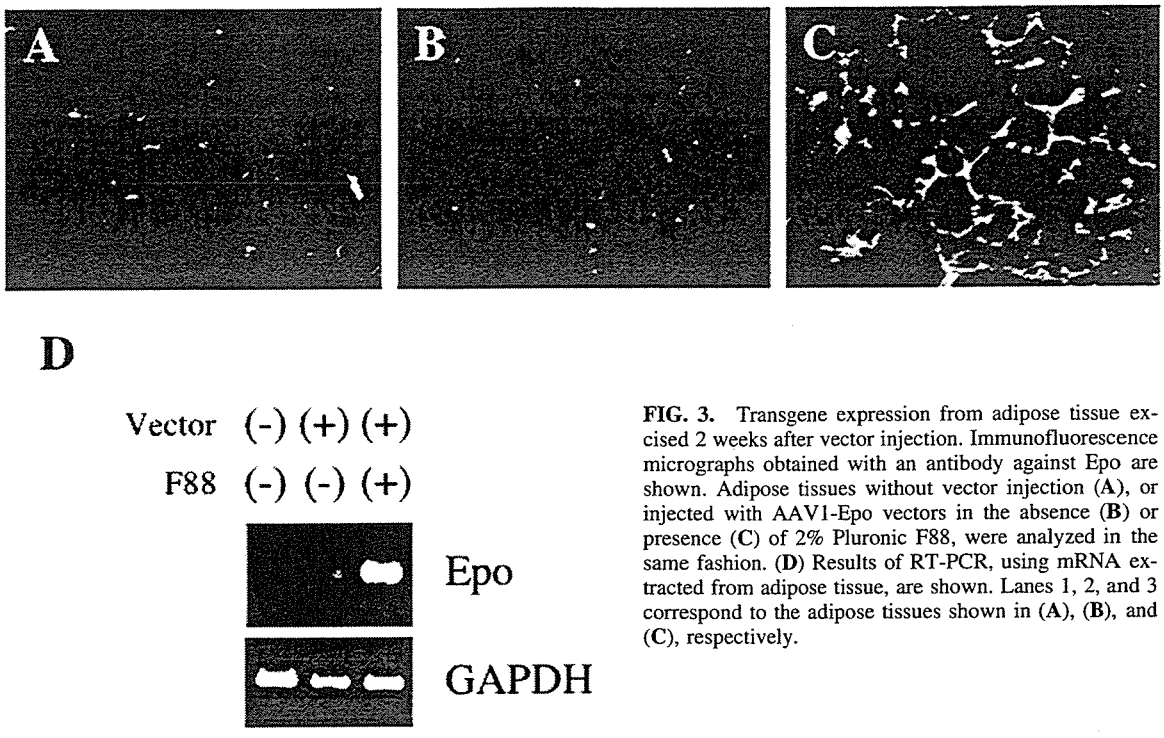

FIG. 3. Transgene expression from adipose tissue excised 2 weeks after vector injection. Immunofluorescence micrographs obtained with an antibody against Epo are shown. Adipose tissues without vector injection (**A**), or injected with AAV1-Epo vectors in the absence (**B**) or presence (**C**) of 2% Pluronic F88, were analyzed in the same fashion. (**D**) Results of RT-PCR, using mRNA extracted from adipose tissue, are shown. Lanes 1, 2, and 3 correspond to the adipose tissues shown in (**A**), (**B**), and (**C**), respectively.

duce adipose tissue *in vivo* (Fig. 1C and D). Enhancement of LacZ expression was also observed microscopically in the presence of Pluronic F88 (Fig. 1G and H) when compared with that of the vector alone (Fig. 1E and F).

Assessment of murine erythropoietin expression by use of Pluronic F88

On the basis of the findings obtained with LacZ vectors, we used vectors encoding murine erythropoietin (Epo) to demonstrate the enhancement of Epo concentration in a more quantitative manner. Increased plasma Epo concentration was observed 2 and 4 weeks after vector injection, and there was enhancement of Epo in the presence of 2% Pluronic F88 at a dose of 6×10^{10} VG/body (Fig. 2A). At a higher dose of 2×10^{11} VG/body, the enhancement effect was more prominent (230 ± 80 versus 58 ± 14 mU/ml at 4 weeks; Fig. 2B).

Assessment of transgene expression within adipose tissue

In the histological analysis of transduced tissues, a significant enhancement of Epo expression was observed by immunofluorescence when 2% F88 was included in the vector solution (Fig. 3A–C). Results of reverse transcription (RT)-PCR also showed enhanced expression of murine Epo in the presence of 2% F88 (Fig. 3D). Tissue damage or cellular infiltrates were not observed in the transduced adipose tissues throughout the histological evaluation (data not shown).

Effect of removal of transduced tissue

Bilateral lobes of abdominal adipose tissue were selected as a target for transduction. Plasma Epo concentrations were monitored after injection of AAV1-Epo vector (2×10^{11} VG/body) with 2% F88. After 4 weeks of observation, both lobes of the adipose tissue were removed *en bloc*, using standard surgical techniques. A significant decrease in plasma Epo concentration was observed 2 weeks after operation, with a return to the baseline level (Fig. 4). In addition, mice after removal of transduced tissue showed extended survival compared with "non-operated" mice. The survival period of *db/db* mice is summarized in Table 1.

DISCUSSION

In this study, we demonstrated the advantages of using excipients in adipose tissue transduction with AAV vectors. In practice, Pluronics have been widely used as excipients, including for administration to humans (APhA and RPSGB, 1986). Generally, one of the most frequent complications associated with administering a surfactant *in vivo* is hemolysis. With regard to this, it is noteworthy that Pluronics have a membrane-protecting effect on erythrocytes and that Pluronic F68 has long been used as a drug to prevent hemolysis and thrombotic events during extracorporeal circulation (Wright *et al.*, 1963). Further, their efficacy for use in the treatment of vasoocclusive disease in sickle cell anemia is currently being evaluated (Gibbs and Hagemann, 2004). Pluronics are also used in

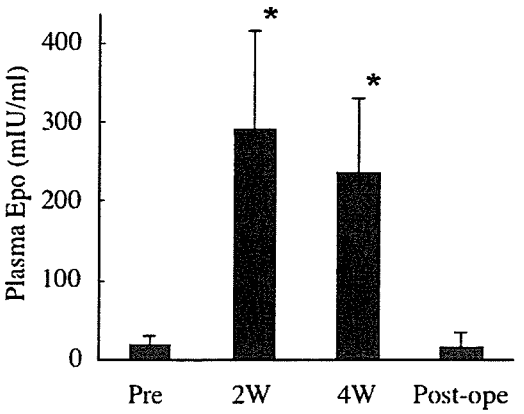

FIG. 4. Plasma Epo concentrations of *db/db* mice after injection and removal of adipose tissue. AAV1-Epo vectors were injected into adipose tissue at 2×10^{11} VG/body with 2% Pluronic F88. Four weeks after injection, transduced adipose tissues were surgically removed and monitored for an additional 2 weeks. Columns and bars indicate, respectively, mean ± SD of the groups ($n = 4$ each). Asterisks indicate significance ($p < 0.05$) relative to concentrations before injection. No significant differences in concentrations were observed 2 weeks after the operation relative to concentrations before injection.

a variety of applications in gene therapy; for example, they are used to augment gene transfer into cultured cells (Gebhart and Kabanov, 2001), to protect skeletal muscle membranes at the time of electroporation *in vivo* (Lee *et al.*, 1992; Hartikka *et al.*, 2001), and to enhance adenovirus-mediated gene transfer into the lungs (Croyle *et al.*, 2001). In addition, it has been postulated that adding a low concentration (0.01%) of Pluronic F68 into the stocks of AAV vectors prevents vector loss by reducing nonspecific attachment (Sommer *et al.*, 2003).

In the present study, the efficiency of gene transfer into adipose tissue was augmented by the use of Pluronic F88. The mechanisms behind this phenomenon are not clear; however, it is known that Pluronics improve the distribution of a solution and its content (APhA and RPSGB, 1986). In a previous study, one member of the Pluronics family (poloxamer 407) was shown to improve the efficiency of adenovirus-mediated gene transfer to arterial smooth muscle cells (Feldman *et al.*, 1997). A reduction in incubation time from 20 to 10 min to attain the same level of gene transfer by including this excipient was also demonstrated. The following two possible mechanisms were postulated for the increase in efficiency of gene transfer: (1) the formation of a transient local reservoir for the sustained release of adenoviral vectors, or (2) acceleration of the uptake of adenoviral vectors produced by the interaction between poloxamer 407 and the cell membrane. On the basis of our observations concerning LacZ expression within adipose tissues (Fig. 1), it can be said that vector distribution was improved and that uptake of the vectors was facilitated. The augmentation was most significant in the case of AAV1 vectors, as assessed on the basis of both LacZ and Epo. There may be a specific advantage in combining the AAV1 capsid with Pluronic F88 when transducing adipose tissue.

A novel action of Pluronics has been reported (Sriadibhatla *et al.*, 2006). In this literature, transcriptional activation of transgenes driven by the CMV promoter or nuclear factor-κB (NF-κB)-responsive elements was demonstrated in the presence of Pluronics. As all the vectors in our current study used the CMV promoter, transcriptional activation through this mechanism might be a concern. Therefore, this issue needs to be taken into consideration. In the literature, all the transcriptional activation was observed in *in vitro* experiments, and Pluronics were continuously present within the culture medium, typically at levels of 0.1% or higher. On the other hand, in our experiments, Pluronics were administered only at the time of vector injection, and the net amount constitutes 0.004% of the total body weight based on the volume and concentration of the vector solution. Moreover, the half-life of Pluronics *in vivo* is estimated as some hours and the majority of the administered material is known to be excreted from the urine within days (APhA and RPSGB, 1986; Gibbs and Hagemann, 2004). Therefore, it is unlikely that transcriptional activation is responsible for gene expression *in vivo* weeks after administration. Nonetheless, this mechanism of action may potentially be useful in order to enhance the outcome of gene therapy approaches *in vivo*. As all the known regulatable gene expression systems share the weakness of toxicity (Goverdhana *et al.*, 2005), safety profiles of Pluronics along with rapid clearance from the body may lead to the development of a novel system for regulatable gene expression *in vivo*. Further studies in this respect may extend the utility of Pluronics in future.

A relatively small number of studies have reported successful gene transfer into adipocytes. There are reports on gene transfer into cultured adipocytes by using viral vectors such as adenovirus (Meunier-Durmort *et al.*, 1996, 1997; Hertzel *et al.*, 2000), lentivirus (Morizono *et al.*, 2003; Carlotti *et al.*, 2004), and retrovirus (Ito *et al.*, 2005). Regarding efficacy *in vivo*, gene transfer into gonadal adipose tissues, using adenoviral vectors, demonstrated clinical efficacy in treating diabetic conditions (Nagamatsu *et al.*, 2001). Successful transduction of adipose tissue by using either simian immunodeficiency viral vector (Ogata *et al.*, 2004) or herpes simplex viral vector (Fradette *et al.*, 2005) was reported. To our knowledge, this is the first report that demonstrates the efficacy of adipocyte-mediated gene transfer by AAV vectors.

In terms of vector dose, adipocyte-mediated gene transfer required a higher vector dose to achieve the same plasma Epo

TABLE 1. LENGTH OF SURVIVAL OF *db/db* MICE

Group	*Survival (weeks after injection)*
AAV1-Epo (no Pluronic F88)[a]	4,[b] 4, 6, >8,[c] >8[c]
AAV1-Epo + 2% Pluronic F88[d]	4, 4, 5, 6, >8[c]
AAV1-Epo + 2% Pluronic F88 + operation[e]	>24, >24, >24, >24

[a]Reflects animals in Fig. 2B (open columns).
[b]This animal became paralyzed at the time of blood collection and subsequently died.
[c]These animals were killed at week 8 for tissue analysis.
[d]Reflects animals in Fig. 2B (solid columns).
[e]Reflects animals in Fig. 4.

concentration as that obtained by muscle- or liver-mediated gene transfer in our previous study (Mochizuki *et al.*, 2004). At a dose of 6×10^{10} VG/body, which was the standard dose for muscle- and liver-mediated gene transfer, the Epo concentration was less prominent; the plasma Epo concentration became comparable at a dose of 2×10^{11} VG/body. Therefore, even after the addition of F88, transduction efficiency was still low in adipose tissue. Whether there are any better methods to augment the efficiency of transduction, including the use of a higher vector dose or other serotype-derived vectors, needs to be investigated further.

In our series of experiments, all the transduced mice became polycythemic; therefore, transgene-derived Epo was functional (data not shown). Although the Epo concentration was augmented by the addition of Pluronic F88, there was no significant difference in blood hemoglobin levels or red blood cell counts among the groups. This is because the Epo concentrations in the transduced animals were far beyond the physiological dose–response window (Mochizuki *et al.*, 2004), and even modest Epo expression after injecting the vector without Pluronic F88 could result in polycythemic conditions. It is generally difficult to eliminate the possibility that the use of this excipient may alter the tropism of the vector and promote gene transfer to certain remote organ(s). Nonetheless, because removal of the transduced adipose tissue resulted in the elimination of the Epo (Fig. 4), we can exclude this possibility. Whether the tissue specificity of expression is common to all serotypes of AAV is yet to be confirmed. To test the tissue specificity, *db/db* mice are useful because they develop rich adipose tissues and a specific lobe can be completely removed by standard surgical procedures. On the other hand, the limitation of this model lies in the difficulty of long-term transgene expression: these animals were naturally diabetic and susceptible to thromboembolic events when they became polycythemic and eventually lost their lives after 4 weeks (Table 1). In this series of experiments, no clear threshold of Epo level on mortality was recognized, although all the "operated" animals attained long-term survival with normalized values of Epo and blood parameters. Therefore, in order to demonstrate long-term expression, a different transgene needs to be used.

Transducing adipose tissue may have another advantage with respect to immunology. Although the distribution and density of antigen-presenting cells within the adipose tissue remain unknown, it is possible that these cells are relatively scarce in the adipose tissue than in "standard" tissues such as muscle or liver. Therefore, the immune response against transgene product, which is a current hurdle in the field of gene therapy (Zaiss and Muruve, 2005), can partly be overcome by targeting adipose tissue. In our series of experiments, we did not observe any immunological responses to the transgene products or to the transduced adipose tissues. To test this hypothesis, a transgene product that is highly immunogenic to mice should be chosen and the outcome needs to be evaluated.

Adipose tissue is usually abundant in the body, can be easily transduced by simple vector injection, and can be removed safely. For these reasons, it is a potential depot organ for gene transfer. In this sense, there may be a wide range of applications of this method in supplemental gene therapy.

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RESEARCH ARTICLE

Utility of intraperitoneal administration as a route of AAV serotype 5 vector-mediated neonatal gene transfer

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Abstract

Background Gene transfer into a fetus or neonate can be a fundamental approach for treating genetic diseases, particularly disorders that have irreversible manifestations in adulthood. Although the potential utility of this technique has been suggested, the advantages of neonatal gene transfer have not been widely investigated. Here, we tested the usefulness of neonatal gene transfer using adeno-associated virus (AAV) vectors by comparing the administration routes and vector doses.

Methods To determine the optimal administration route, neonates were subjected to intravenous (*iv*) or intraperitoneal (*ip*) injections of AAV5-based vectors encoding the human coagulation factor IX (*hfIX*) gene, and the dose response was examined. To determine the distribution of transgene expression, vectors encoding *lacZ* or luciferase (*luc*) genes were used and assessed by X-gal staining and *in vivo* imaging, respectively. After the observation period, the vector distribution across tissues was quantified.

Results The factor IX concentration was higher in *ip*-injected mice than in *iv*-injected mice. All transgenes administered by *ip* injection were more efficiently expressed in neonates than in adults. The expression was confined to the peritoneal tissue. Interestingly, a sex-related difference was observed in transgene expression in adults, whereas this difference was not apparent in neonates.

Conclusions AAV vector administration to neonates using the *ip* route was clearly advantageous in obtaining robust transgene expression. Vector genomes and transgene expression were observed mainly in the peritoneal tissue. These findings indicate the advantages of neonatal gene therapy and would help in designing strategies for gene therapy using AAV vectors. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords AAV vector; neonatal gene therapy; luciferase; coagulation factor IX

Introduction

Due to its unique properties, the adeno-associated virus (AAV) vector is one of the most promising vehicles for gene therapy. It can efficiently transduce a variety of tissues, and long-term transgene expression can be attained. Therefore, the AAV vector is suitable for supplemental gene therapy, particularly for hemophilia. However, despite the promising results obtained in animals [1–4], insignificant levels of human coagulation factor IX (hFIX)

were observed in humans after intramuscular (*im*) injection of the AAV vector [5,6]. The use of alternative serotypes may possibly improve the therapeutic outcome. To achieve therapeutic levels of hFIX expression, several reports have suggested the necessity of optimizing the serotypes of the AAV vector for each administration route [7–10].

It is also believed that neonatal or fetal gene therapy is potentially useful for improving the therapeutic outcome of genetic diseases. These methods are advantageous for preventing early manifestations of genetic diseases, for transducing organ systems that are not easily accessible in later life [11–13], and for providing robust transgene expression at relatively low vector doses. Moreover, since the neonatal and fetal immune systems are immature, gene transfer during this period may induce tolerance to transgene products [7,14,15].

With regard to the utility of the AAV serotypes for neonatal gene therapy, relatively little information is currently available. Limited utility of the AAV serotype 2 (AAV2) vector for *in utero* gene transfer was previously described [16]. It was reported that an intraperitoneal (*ip*) injection of AAV5-based vectors resulted in transgene expression that is at least 10 times higher than that obtained with an *ip* injection of the AAV2 vector [17]. In this study, based on these reports and our previous observations that demonstrated the advantages of AAV5 in gene transfer experiments [18,19], we compared the efficacy and distribution of transgene expression for evaluating the utility of AAV5-based vectors administered to neonates and adult mice either by an *ip* or intravenous (*iv*) injection.

Materials and methods

Plasmids and AAV vectors

Plasmids for AAV vector production were purchased from Stratagene (La Jolla, CA, USA). pAAV5-CMV-LacZ, a plasmid encoding LacZ, and 5RepCapA, a helper plasmid, were donated by Dr. J. A. Chiorini (National Institutes of Health, Bethesda, MD, USA). pAAV5-CMV-hFIX that contains the hFIX sequence was prepared as previously described [20,21], with the inverted terminal repeat (ITR) sequences changed to those of the AAV5 vector. pAAV5-CMV-Luc, which harbors the firefly luciferase gene, was originally purchased from Promega (Madison, WI, USA), and its ITR sequences were also changed to those of the AAV5 vector. Recombinant AAV vector stocks were prepared in accordance with an adenovirus-free triple-plasmid transfection protocol [22]. After harvest, vector solutions were purified twice on a cesium chloride (CsCl) gradient and quantified by DNA dot blot hybridization. The same vector stock was used in the same series of experiments in order to minimize the variability that could occur due to the potential differences in vector potency.

Animal procedures

All animal experiments were performed in accordance with the standards in the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and the institutional guidelines. Pregnant female C57BL/6 mice were purchased from CLEA Japan, Inc. (Hamamatsu, Japan), and the neonates were subjected to vector injection within 24 h of birth. Isoflurane anesthesia was applied at the time of injection, and the injection volume was kept constant at 20 μl throughout the study. In order to determine a suitable route for administration in neonates, the AAV5-CMV-hFIX vector was injected either intravenously (*iv*, into the jugular vein) or intraperitoneally (*ip*). In order to validate the usefulness, *ip* injections of the AAV5-CMV-hFIX vector at higher doses were tested. In order to assess the tissue distribution of the vector and transgene expression, the AAV5-CMV-LacZ vector ($n = 8$) or the AAV5-CMV-Luc vector ($n = 10$) was injected into the peritoneal cavity. Along with the neonates, an adult group comprising 12-week-old mice were used as adults for *ip* injection, and the AAV5-CMV-hFIX vector ($n = 8$), AAV5-CMV-LacZ vector ($n = 6$), or AAV5-CMV-Luc vector ($n = 10$) was administered. All procedures were performed safely, and animal death was rarely observed following vector injection.

Determination of the plasma concentration of human factor IX

Whole blood was collected from the tail vein by using heparinized capillary tubes. Plasma concentrations of the hFIX protein were determined as described previously [21]. The detection limit of this assay was 1 ng/ml. Normal human plasma stock was used as the standard. This assay system did not react with murine factor IX [21].

Detection and quantitation of vector genomes

Organs were isolated from mice after 16 weeks of vector injection. Tissue samples were frozen in liquid nitrogen and stored at −70 °C. Total DNA was extracted from the tissue samples using the DNeasy tissue kit (Qiagen GmbH, Hilden, Germany). In order to analyze the vector distribution following *ip* administration, total DNA was extracted from various tissues and subjected to quantitative polymerase chain reaction (Q-PCR) using an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA), under conditions that were previously described [23]. The detection limit was 0.01 vector genome copies per diploid genome equivalent (g.c./d.g.e.).

Histochemistry

The mice were sacrificed, and each tissue was obtained at 8 or 10 weeks after the AAV5-CMV-LacZ injection. For microscopic evaluation, the tissues were washed, incubated with phosphate-buffered saline (PBS) containing sucrose (15–30%), frozen in OTC compound (Tissue Tek, Miles Inc., Elkhart, IN, USA) in dry ice/ethanol, attached to polylysine-coated glass slides, and analyzed by standard X-gal staining [24].

Bioluminescence studies

For *in vivo* bioluminescence imaging, the mice were anesthetized with isoflurane, and an aqueous solution of luciferin substrate (150 μg/10 μl/g body weight) was injected into the intraperitoneal cavity 12 min prior to imaging. The mice were placed in a light-tight chamber to maintain complete darkness. Photons transmitted through the tissues were then collected and analyzed using IVIS Imaging Systems and Living Image software (Xenogen Corp., Alameda, CA, USA). Imaging was performed with 5 s of the integration time. The range of the reference pseudocolor scale, representing the light intensity, was kept constant for all mice. For *ex vivo* luciferase analysis, in order to discontinue the follow up of the *in vivo* observation, the representative mice were chosen and sacrificed 10 min after *ip* injection of the luciferin substrate solution (150 μg/10 μl/g body weight), and the internal organs were then separated. Each organ was immediately placed into each well of a 24-well dish containing 1 : 50 dilutions of an aqueous solution of the luciferin substrate (final concentration, 300 μg/ml), and bioluminescence was measured using 60 s of the integration time. The light intensity was calculated based on the weight of the tissue.

Statistical analysis

All data are shown as means ± standard deviation (SD). To compare the means between the two groups, statistical analysis was performed by applying Student's t test after confirming the equality between the variances of the groups. If the variances were unequal, Mann-Whitney U tests were performed. Values of $p < 0.05$ were regarded to be significant.

Results

Comparison of delivery routes for neonatal injection

As shown in Figure 1A, the plasma levels of hFIX were higher in the *ip*-injected group than in the *iv*-injected group. The plasma concentration of hFIX at 8 weeks for the two groups was 21.8 ± 5.0 ng/ml and 10.2 ± 3.1 ng/ml, respectively, and the difference in the hFIX concentration was significant after 6 weeks ($p < 0.01$).

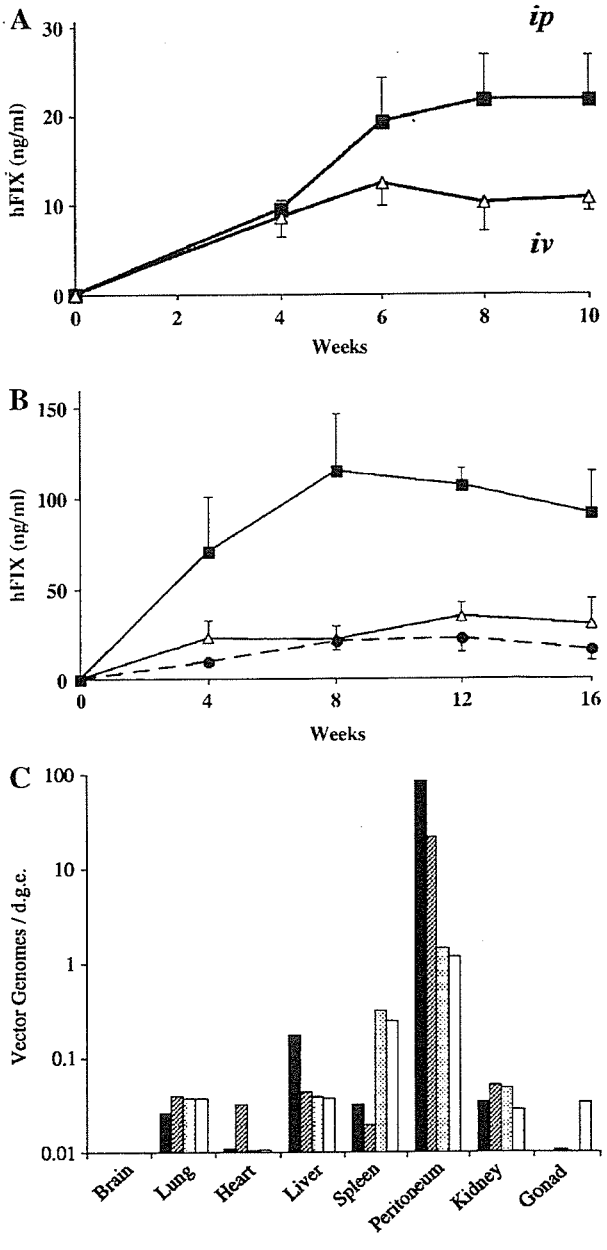

Figure 1. Analysis of C57BL/6 mice after intraperitoneal (*ip*) or intravenous (*iv*) injection of AAV vectors. (A) Plasma hFIX concentration after *ip* (n = 4, closed squares) and *iv* (n = 5, open triangles) administration of the AAV5-CMV-hFIX vector (1×10^{10} genome copies/body weight (g.c./g)) in the C57BL/6 neonatal mice. (B) Plasma hFIX concentration in neonatal mice after *ip* injections at different vector doses. The vector dose was 1×10^{10} g.c./g (closed circles), 3×10^{10} g.c./g (open triangles), or 3×10^{11} g.c./g (closed squares). (C) The number of vector genomes within the tissues at 10 weeks after *ip* injection into neonates. Total DNA (100 ng) was analyzed by Q-PCR, and the results were calculated as vector genomes per diploid genome equivalent (d.g.e.). Closed, hatched, dotted, and open columns indicate the results with neonatal males, neonatal females, adult males, and adult females, respectively

Effect of the vector dose in *ip* administration

As *ip* administration appeared to be more promising than *iv*, we focused on the utility of *ip* in neonates. For this purpose, increasing doses of AAV5-CMV-hFIX vectors were tested. Higher hFIX concentrations were observed in animals with higher vector doses (Figure 1B). In the group with the highest vector dose (3×10^{11} genome copies/body weight (g.c./g)), the plasma hFIX concentrations were approximately 100 ng/ml, which is a therapeutically relevant level for severe hemophilia B, and these concentrations were sustained throughout the observation period.

Tissue distribution of the AAV vector genome

The tissue distribution of the vector genome after the *ip* injection into male mice was analyzed by real-time PCR. Substantial numbers of vector genomes were detected in the peritoneum and to a lesser extent in the liver and other tissues (Figure 1C). Note that the vector genomes are shown on a logarithmic scale.

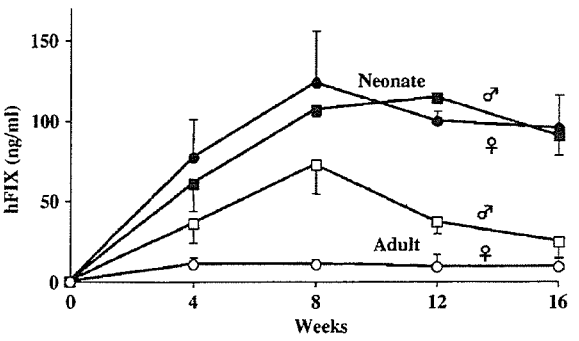

Figure 2. Plasma hFIX concentrations in mice after *ip* injections into different groups. The AAV5-CMV-hFIX vector at a dose of 3×10^{11} g.c./g was injected into C57BL/6 neonatal males (n = 6, closed squares), neonatal females (n = 4, closed circles), adult males (n = 4, open squares), and adult females (n = 4, open circles)

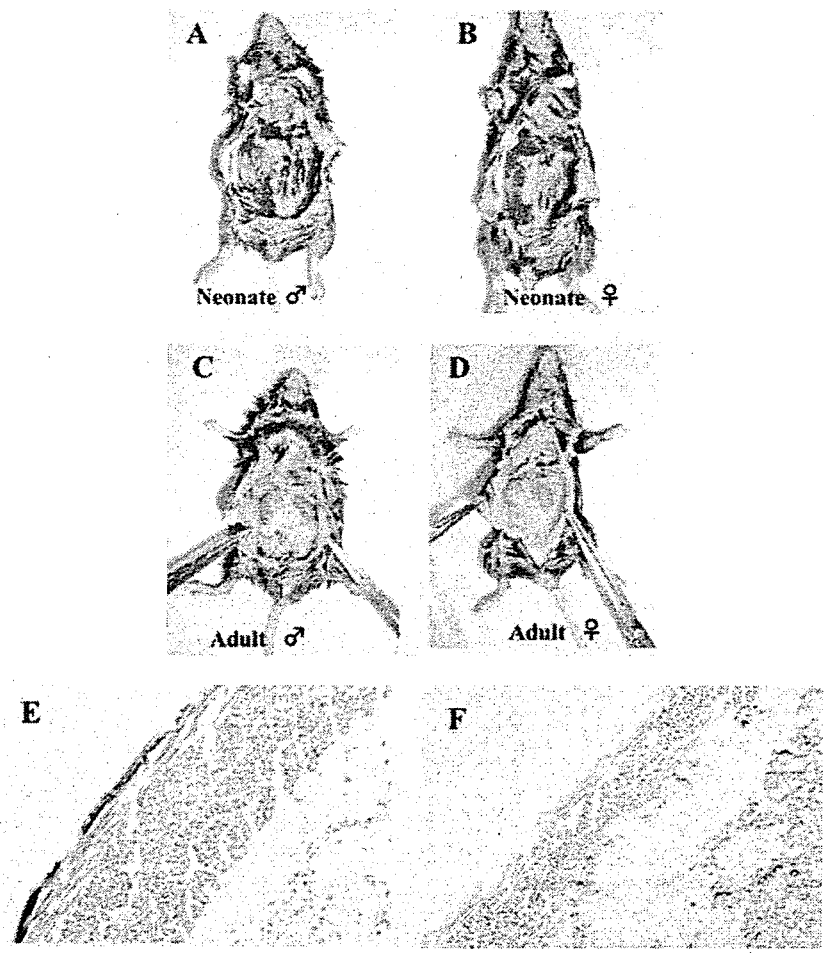

Figure 3. β-Galactosidase expression at 8 weeks after *ip* injection of the AAV5-CMV-LacZ vector at a dose of 1×10^{11} g.c./g in the C57BL/6 mice (A–D). X-gal staining was performed after removal of the intraperitoneal organs. Histochemistry with β-galactosidase performed on tissues from the neonatal male peritoneum after the injection stained the mesothelium (E) and the untransduced control (F) (final magnification ×100)

Influence of sex and age of mice on transgene expression

In order to compare the efficiency with regard to the sex and age of mice during administration, the same dose of the AAV vector based on the body weight (3×10^{11} g.c./g) was administered by *ip* injection to both neonatal and adult mice. As summarized in Figure 2, the plasma levels of hFIX were significantly higher in males than in females when adults were used ($p < 0.05$). On the other hand, there were no sex-related differences in the hFIX concentration in neonates. Moreover, the hFIX levels were much higher in neonates (neonate vs. adult; $p < 0.05$ in males, $p < 0.01$ in females). After 8 weeks, a considerable reduction in the plasma hFIX concentration was observed in adult males.

Tissue distribution of transgene expression following *ip* injection

To evaluate the efficacy and location of transgene expression following *ip* vector administration, 1×10^{11} g.c./g of the AAV5-CMV-LacZ vector was injected into either neonatal or adult mice. After 8 weeks, the mice were sacrificed and their tissues were subjected to X-gal staining. As shown in Figures 3A–3D, β-galactosidase expression was observed in the peritoneum. Robust β-galactosidase expression was observed in both male and female mice in the neonatal group (Figures 3A and 3B). In contrast, in the injected adults, only weak β-galactosidase expression was observed in the male mice, and faint expression was detected in the female mice (Figures 3C and 3D). Other tissues were also analyzed by X-gal staining, and none of these, including liver and kidney, showed positive results (data not shown). Microscopic examination of the peritoneum of neonatally injected male mice revealed β-galactosidase expression in mesothelial cells, while the control mice did not show X-gal positivity (Figures 4E and 4F).

In vivo and *ex vivo* analysis using bioluminescence

To quantify the distribution of transgene expression, the AAV5-CMV-Luc vectors were administered *ip* to neonatal and adult mice at an equivalent vector dose based on the body weight (3×10^9 g.c./g). Luciferase expression was observed by *in vivo* bioluminescence imaging 10 weeks after the vector injection (Figures 4A–4D). Quantitative results of *in vivo* bioluminescence are shown in Figure 4E. In neonates, no sex-related difference was found in luciferase expression ($3.8 \times 10^9 \pm 1.2 \times 10^8$ photons/s and $2.9 \times 10^9 \pm 1.0 \times 10^9$ photons/s for the males and females, respectively, $p = 0.13$). In contrast, a significant difference in distribution and quantitation was observed in adults ($1.3 \times 10^9 \pm 7.2 \times 10^8$ photons/s and $5.3 \times 10^7 \pm 1.6 \times 10^7$ photons/s for males and

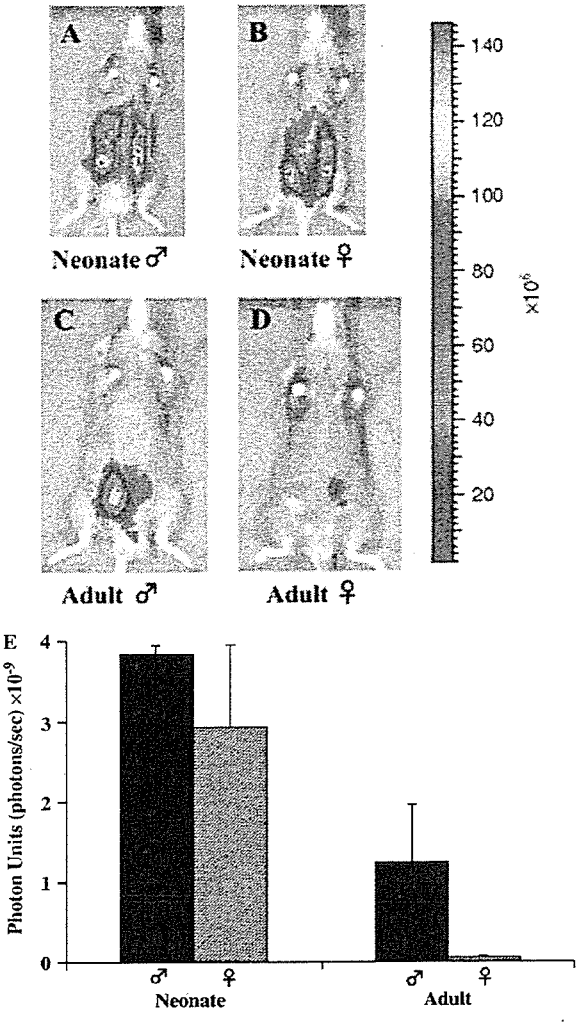

Figure 4. ***In vivo*** **bioluminescence imaging at 10 weeks after** ***ip*** **injection of the AAV5-CMV-Luc vector at a dose of 5×10^9 g.c./1.5 g in the C57BL/6 mice (A–D). Images were analyzed under the same condition, and the reference color bar, indicating the photon units (photons/s), is the same for all mice. (E) Quantitative results of** ***in vivo*** **bioluminescence imaging in neonatal males (n = 6, closed columns) and females (n = 4, hatched column), and adult males (n = 5, dotted column) and females (n = 5, open column), are shown. Mice were transduced with 5×10^9 g.c./1.5 g of the AAV5-CMV-Luc vector (2.5×10^8 g.c./μl). The ordinate indicates the photon units (photon/s)**

females, respectively, $p < 0.05$). In order to identify the tissues responsible for luciferase expression, an *ex vivo* bioluminescence analysis was performed at 10 weeks after the vector injection; this demonstrated that the luciferase expression was localized in the peritoneum (Figure 5A). As shown on the pseudocolor scale, the white color showed background of the assay and did not reflect luciferase expression. A luminometric analysis of individual tissues from representative animals revealed a difference in the expression in the peritoneum among the injected neonates and adults (3.1×10^8 and 1.6×10^8 photons/s/g for male and female neonates, respectively; 1.1×10^8 and 7.9×10^4 photons/s/g for male and female adults, respectively) (Figure 5B).

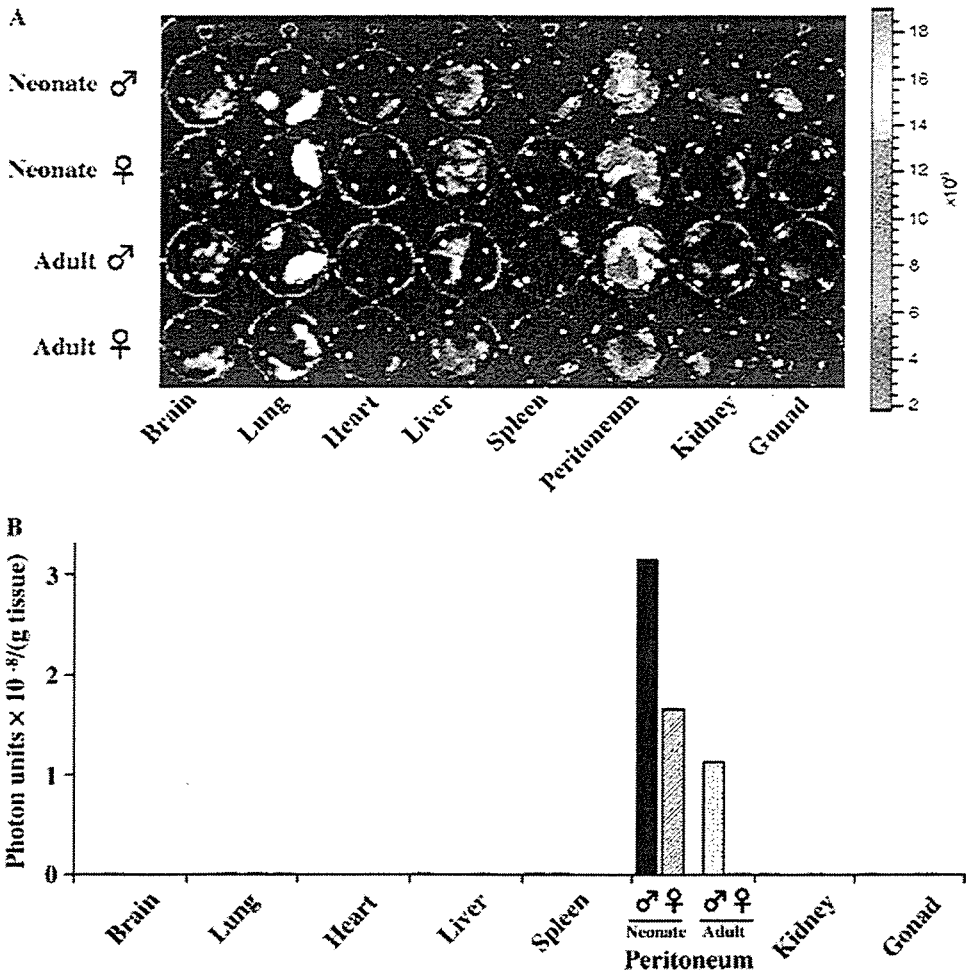

Figure 5. Analysis of tissue-specific expression after *ip* injection of the AAV5-CMV-Luc vector. (A) *Ex vivo* bioluminescence images of injected neonates and adults are shown. Mice were sacrificed at 10 weeks after vector injection and the major organs were extracted and placed into each well of a 24-well dish containing luciferin substrate solution in order to measure the individual bioluminescence. (B) Quantitative results of transgene expression are as indicated in (A). The ordinate shows the photon units (photons/s)

Discussion

In this study, we tested the utility of neonatal gene transfer by using AAV5-based vectors. All genes tested – *lacZ*, *hfIX*, and *luc* – demonstrated robust transgene expression after *ip* injection. The advantage of neonatal gene transfer was clearly demonstrated by the plasma hFIX levels after injecting both adult and neonatal mice with equivalent doses of the AAV-CMV-hFIX vector (3×10^{11} g.c./g). Throughout the observation period, a higher hFIX concentration was detected in neonates than in adults; therapeutic levels of hFIX were maintained even after maturation (Figure 2). Another comparison using vectors encoding luciferase at an equivalent vector dose also resulted in a higher transgene expression in neonates (Figure 4). These data support the advantages of neonatal gene transfer.

Neonatal gene delivery in mice is technically difficult due to their size. In this study, we demonstrated the usefulness of *ip* injections as a route of vector delivery. On the other hand, we did not include the *im* route in this series of experiments because the injection volume was strictly limited in neonates. However, this latter method is apparently an attractive route of administration in clinical applications. Therefore, the efficacy of *im* administration requires further analysis in larger animal models.

In this study, transgene expression was mostly confined to the peritoneum after *ip* injection into neonates. This was confirmed by different modes of detection. In addition, the vector genome distribution was mostly comparable to the level of transgene expression. However, in a previous report, transgene expression was also observed in tissues other than the peritoneum when fetuses were injected [17]. Since the vector system and the promoter were the same, the difference in tissue distribution may be related to the age at the time of injection, vector dose, technical details, or other unrecognized factors. At present, the mechanism responsible for tissue specificity is not clear. The abundance of receptor molecules, such as platelet-derived

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growth factor (PDGF) receptors [27], may contribute to this phenomenon. Using other vector systems may result in different tissue specificity. Recently, transgene expression in the whole peritoneal cavity was observed by *ip* administration of polyethylenimine (PEI)/DNA complexes [28]. Further, in neonates, a long-term expression was observed in factor IX concentration, whereas in adult males a sharp decrease was observed at 12 weeks and later (Figure 3). When the peritoneum was analyzed, only the surface epithelium of the peritoneal tissue was transduced (Figure 4E), and it appeared to be responsible for continuously supplying the transgene product at a therapeutic level. These cells contain an extremely high copy number of transgenes even after a prolonged period of time (Figure 2C). The copy number of the vector genome within the peritoneum appears to be underestimated thus far because the whole peritoneal tissue was used for DNA extraction prior to Q-PCR. The presence of an extremely high copy number of vector genomes within the peritoneum is possibly related to the robust and persistent transgene expression in neonatal gene transfer. The mechanism for the persistence of high copy number and transgene expression is interesting and may offer important insights into the biology of the AAV vector.

Interestingly, a sex-related difference in transgene expression within the peritoneal tissues was observed after *ip* injection into adult mice regardless of the transgene. In a previous study, a sex-related difference in transgene expression was demonstrated in the liver, and an androgen-dependent pathway appeared to be involved [25,26]. We have also demonstrated an overwhelming sex-related difference in liver transduction efficiency in a mouse model [19]. Based on our knowledge, this is the first report that demonstrates a sex-related difference in transgene expression in tissues other than the liver. At present, it is not clear whether the same mechanism is involved in the peritoneal tissue. The difference may be a drawback when an attempt is made to transfer genes into females. However, our results indicate that this problem can be circumvented if neonates are targeted for gene therapy.

Neonatal gene transfer is also advantageous from an immunological point of view. Due to the immaturity of the neonatal immune system, tolerance to an 'immunogenic' transgene product can be induced. Recently, neonatal and fetal gene transfer experiments using adenoviral and retroviral vectors demonstrated the induction of tolerance to transgene products [14,15]. In our series of experiments, it is difficult to prove this point because all transgenes were expressed for a long period even in adults. Nonetheless, divergent levels of transgene expression between adults and neonates may reflect a difference in immunology, and needs to be analyzed in the future.

In conclusion, our findings support the efficacy of neonatal gene therapy and would help to design strategies for neonatal gene therapy using AAV vectors.

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Viral-Mediated Temporally Controlled Dopamine Production in a Rat Model of Parkinson Disease

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Regulation of gene expression is necessary to avoid possible adverse effects of gene therapy due to excess synthesis of transgene products. To reduce transgene expression, we developed a viral vector-mediated somatic regulation system using inducible Cre recombinase. A recombinant adeno-associated virus (AAV) vector expressing Cre recombinase fused to a mutated ligand-binding domain of the estrogen receptor α (CreER^{T2}) was delivered along with AAV vectors expressing dopamine-synthesizing enzymes to rats of a Parkinson disease model. Treatment with 4-hydroxytamoxifen, a synthetic estrogen receptor modulator, activated Cre recombinase within the transduced neurons and induced selective excision of the tyrosine hydroxylase (TH) coding sequence flanked by loxP sites, leading to a reduction in transgene-mediated dopamine synthesis. Using this strategy, aromatic L-amino acid decarboxylase (AADC) activity was retained so that L-3,4-dihydroxyphenylalanine (L-dopa), a substrate for AADC, could be converted to dopamine in the striatum and the therapeutic effects of L-dopa preserved, even after reduction of TH expression in the case of dopamine overproduction. Our data demonstrate that viral vector-mediated inducible Cre recombinase can serve as an *in vivo* molecular switch, allowing spatial and temporal control of transgene expression, thereby potentially increasing the safety of gene therapy.

Key Words: adeno-associated virus, gene therapy, gene regulation, tamoxifen, Cre recombinase, Parkinson disease, dopamine

INTRODUCTION

Advances in gene transfer methods, in particular the development of improved viral vectors, have expanded the potential of gene therapy to treat a wide range of genetic and acquired diseases. Efficient and long-term expression of therapeutic genes within the central nervous system has been demonstrated in preclinical studies aimed at treating neurodegenerative disorders, including Parkinson disease (PD) [1,2]. PD is a progressive movement disorder characterized by selective degeneration of dopaminergic neurons within the substantia nigra, which project to the striatum. As the dopamine content of the striatum decreases severely, its replacement becomes an important strategy to alleviate motor impairment of the disease. One such strategy is gene therapy to restore the local production of dopamine. Recombinant adeno-associated virus (AAV) vector-mediated gene transfer of dopamine-synthesizing enzymes, such as tyrosine hydroxylase (TH) and guanosine triphosphate cyclohydrolase I (GCH), with or without aromatic L-amino acid decarboxylase (AADC), has induced behavioral recovery in animal models of PD [3–5]. Before clinical trials examining this therapy can commence, however, it is desirable to have a mechanism by which

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dopamine synthesis can be controlled by regulation of gene expression.

Methods utilizing the properties of bacteriophage P1 site-specific Cre recombinase have been developed in recent years as a means of generating somatic mutations [6]. Regulation of Cre recombinase activity, achieved by fusing Cre with mutated hormone-binding domains of various steroid receptors, has been used in various transgenic applications [7,8]. A chimeric protein known as CreER^{T2}, obtained by fusing Cre to a mutated ligand binding domain of the human estrogen receptor α, is particularly useful. Cell-specific expression of CreER^{T2} in transgenic mice allows efficient tamoxifen-dependent Cre-mediated recombination at loci flanked by loxP sites, without background activity [9]. In the present study, we demonstrate that stereotaxic injection of recombinant AAV vectors expressing dopamine-synthesizing enzymes and CreER^{T2} enables spatiotemporal control of dopamine levels within the brains of rats of a PD model. Our results indicate that these vectors may have a number of applications in gene therapy.

RESULTS

Viral-Mediated Temporally Controlled Cre-Mediated Recombination

We generated AAV vectors expressing either Cre recombinase containing a nuclear localization signal (AAV-Cre) or tamoxifen-dependent Cre recombinase (AAV-CreER^{T2}). To engineer a reporter system, we designed an AAV-EGFP/Red vector to express a destabilized variant of red fluorescent protein (DsRed-Express DR) only after Cre-mediated recombination of a loxP-flanked DNA segment encoding a destabilized, red-shifted variant of green fluorescent protein (d2EGFP) (Fig. 1A). To determine the efficacy of viral-mediated recombination, we infected HEK293 cells with AAV-EGFP/Red and either AAV-Cre or AAV-CreER^{T2} (Fig. 1B). Co-infection of AAV-Cre and AAV-EGFP/Red resulted in expression of DsRed-Express-DR, while only d2EGFP was expressed in control cells infected with AAV-EGFP/Red alone. Co-infection with the reporter vector and AAV-CreER^{T2} induced DsRed-Express-DR expression in almost all 4-hydroxytamoxifen (4-OHT)-treated cells. Although we detected slight background expression of DsRed-Express-DR in the absence of 4-OHT, we observed only a limited number of these cells (<1%), indicating that CreER^{T2} activity is tightly regulated in these virally transduced cells. To test the potential use of AAV-CreER^{T2} *in vivo*, we used stereotaxic injections to deliver AAV-CreER^{T2} into the brains of reporter mice [10]. These mice were engineered to express a red-shifted variant of the wild-type green fluorescent protein (EGFP) only after Cre-mediated excision of a loxP DNA fragment. After 5 consecutive days of 4-OHT treatment (1 mg by intraperitoneal injection), we observed numerous EGFP-expressing cells in AAV-injected brains, all of which coexpressed Cre recombinase (Fig. 1C). In the absence of treatment with 4-OHT, only a few cells (<0.1% of Cre-positive cells) expressed EGFP in the vicinity of AAV-CreER^{T2}-injected sites (data not shown). These data indicate that floxed DNA segments are efficiently excised *in vivo* by combining AAV-CreER^{T2} injection with 4-OHT treatment.

Temporally Controlled Reduction of Dopamine Synthesis

We generated AAV vectors expressing each of the three dopamine-synthesizing enzymes (TH, AADC, and GCH). In the TH-expressing vector, two loxP sites flanked the TH coding sequence (AAV-floxed TH). We infected HEK293 cells with these dopamine-synthesizing vectors (AAV-floxed TH, AAV-AADC, and AAV-GCH) in combination

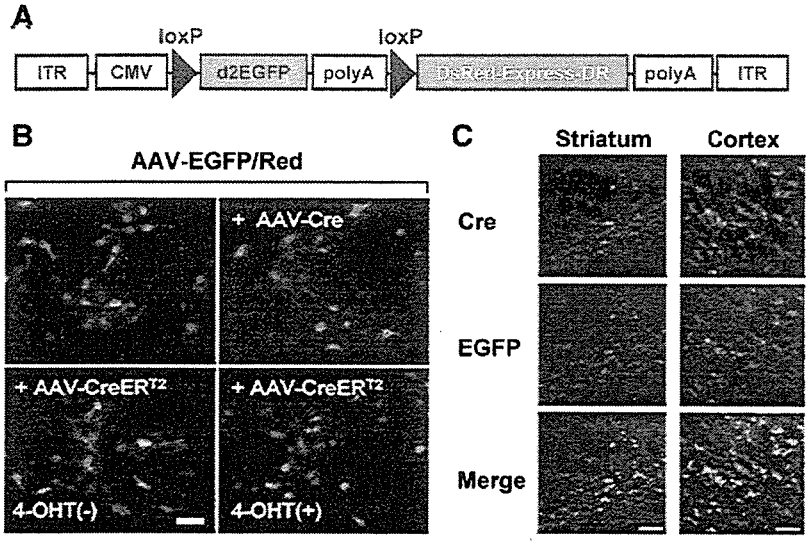

FIG. 1. Viral vector-mediated Cre-dependent floxed DNA excision. (A) Illustration of the AAV-EGFP/Red vector construct. A DsRed-Express-DR marker was placed downstream of the d2EGFP marker with a SV40 poly(A) sequence flanked by loxP sites. ITR, inverted terminal repeat; CMV, human cytomegalovirus immediate-early promoter followed by the first intron of human growth hormone. (B) 4-OHT-induced Cre-dependent recombination. HEK293 cells were infected with AAV-EGFP/Red and either AAV-Cre or AAV-CreER^{T2}. 4-OHT was added to the medium 5 h after infection. Fluorescence was observed 48 h after infection. Bar, 40 μm. (C) EGFP expression in the AAV-CreER^{T2}-injected striatum and cortex of transgenic mice. 4-OHT (1 mg) was administered intraperitoneally 1 week after vector injection every day for 5 days until the mice were killed. In these mice, the stop fragment was flanked by loxP sites and placed between the EGFP sequence and the *Gt(ROSA)26Sor* promoter. Bar, 40 μm.

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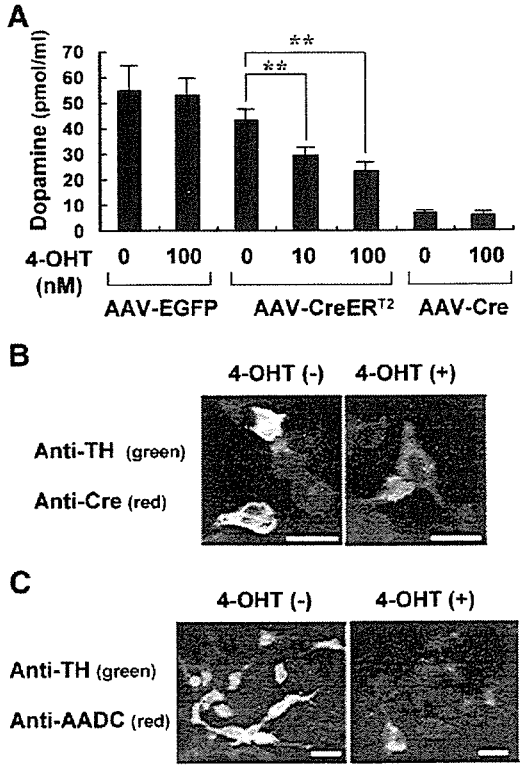

FIG. 2. Reduced dopamine synthesis after 4-OHT-induced ablation of a floxed TH transgene. HEK293 cells were infected with dopamine-synthesizing vectors (AAV-floxed TH, AAV-AADC, and AAV-GCH) in combination with AAV-CreER^{T2} or control vectors. (A) Dopamine content in the culture medium was significantly reduced in the presence of 4-OHT. $^{**}P < 0.01$, $n = 4$. (B) TH (green) and CreER^{T2} (red) immunocytochemistry was performed 48 h after vector infection. Yellow fluorescence in the merged image indicates colocalization. In the presence of 4-OHT, CreER^{T2} translocated to the nucleus. TH was not expressed in cells positive for nuclear CreER^{T2}. Bar, 20 μm. (C) TH (green) and AADC (red) immunocytochemistry. Note the reduced number of TH-positive cells in the presence of 4-OHT. Bar, 20 μm.

with AAV-CreER^{T2} or control vectors. We found that treatment with 4-OHT significantly reduced dopamine synthesis (Fig. 2A). Immunocytochemistry demonstrated coexpression of TH and CreER^{T2} in the cytoplasm in the absence of 4-OHT and an absence of TH expression when CreER^{T2} was translocated into the nucleus in the presence of 4-OHT (Fig. 2B). The expression of AADC was not reduced by the presence of 4-OHT (Fig. 2C). Dual labeling showed that more than 80% of the TH-immunoreactive (TH-IR) cells were also positive for AADC (251 of 300) and Cre (242 of 300) in the absence of 4-OHT-treatment.

Reduction of Dopamine Production in a Rat Model

We next tested whether the vector-mediated Cre-dependent regulation of transgene expression observed in culture could be extended to animal models. We obtained hemiparkinsonian rats by injecting a selective neurotoxin, 6-hydroxydopamine (6-OHDA), into the left medial forebrain bundle. The animals then received a mixture of AAV-CreER^{T2}, AAV-floxed TH, AAV-AADC, and AAV-GCH into their lesioned striatum, after which two-thirds were further treated with 4-OHT (4 mg/kg by intraperitoneal injection for 5 days) during the course of experimentation. Control rats were injected with AAV-LacZ alone or with AAV-Cre plus AAV-floxed TH, AAV-AADC, and AAV-GCH. To evaluate abnormal motor functions associated with depletion of dopamine in the striatum, we repeated quantification of apomorphine-induced rotation, as well

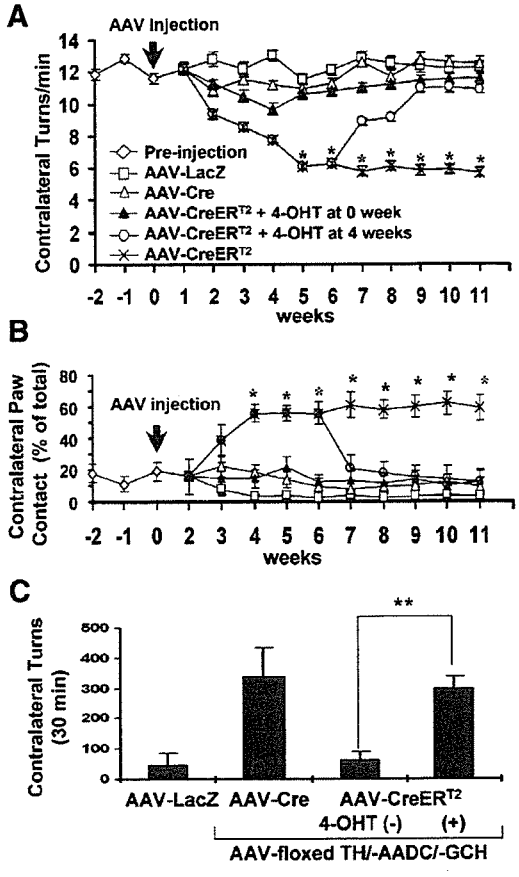

FIG. 3. Temporal control of dopamine synthesis in a rat model of PD transduced with AAV vectors. Sixty hemiparkinsonian rats were generated by 6-OHDA injection. Thirty-six received a mixture of AAV-CreER^{T2}, AAV-floxed TH, AAV-AADC, and AAV-GCH, after which they were divided into three groups of 12. Two of the groups were treated with 4-OHT (4 mg/kg by intraperitoneal injection for 5 days), at the same time or 4 weeks after vector injection. Control PD rats were injected with AAV-LacZ alone ($n = 12$) or AAV-Cre ($n = 12$), instead of AAV-CreER^{T2} with AAV-floxed TH, AAV-AADC, and AAV-GCH. (A) The total number of complete body turns induced by apomorphine was counted for each rat, and (B) spontaneous limb use was scored using the cylinder test. $^{*}P < 0.05$. (C) Efficient conversion of L-dopa to dopamine by AADC. L-Dopa (5 mg/kg) was administered to 4-OHT-treated rats and AAV-Cre-injected rats. Contralateral turning in response to L-dopa was counted for 30 min. $^{**}P < 0.01$. Legend symbols are as shown in A and B.

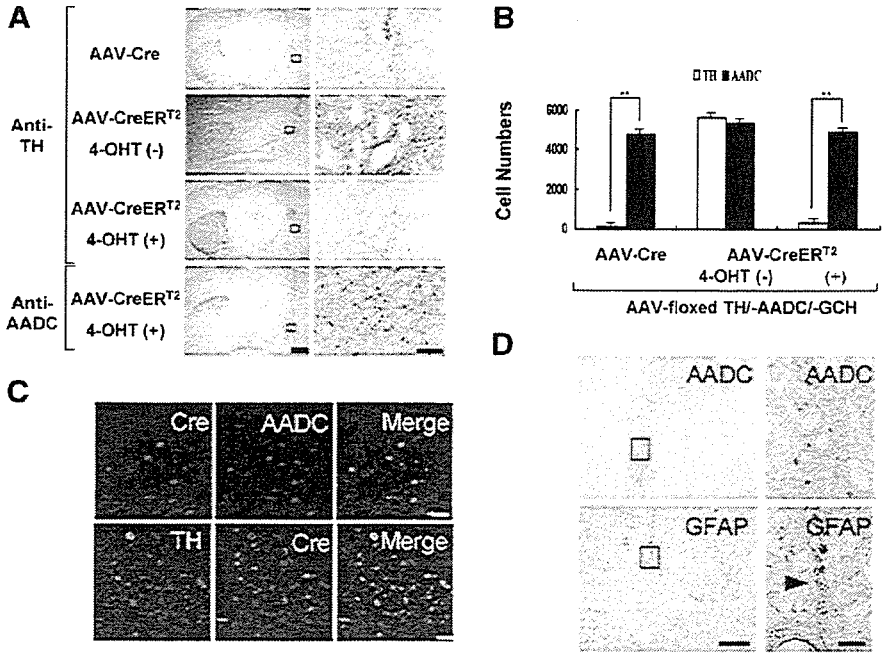

FIG. 4. Selective ablation of the TH transgene induced by treatment with 4-OHT. (A) Immunohistochemical staining for TH or AADC in the brains of 6-OHDA-lesioned rats 12 weeks after stereotaxic injection of AAV-Cre or AAV-CreER^{T2}, with or without 4-OHT treatment. AAV vectors were injected into the lesioned side of the striatum (right side of the photos). High-power-magnified images of the vector injection sites (squares in the left column) are shown in the right column. Representative photographs are also shown. Bar, 1.5 mm (left column), 100 μm (right column). (B) Number of immunoreactive (IR) cells against TH or AADC in the multiple AAV vector-injected striatum. The number of cells in 11 sections per rat (n = 3 for each group) was counted. The numbers of TH-IR cells and AADC-IR cells in AAV-CreER^{T2}-injected rats given 4-OHT 0 or 4 weeks after vector injection were indistinguishable and the results pooled for comparison with other groups. $^{**}P < 0.01$. (C) Efficient cotransduction of AAV vectors, as determined by dual immunofluorescence staining of the 6-OHDA-lesioned striatum. The majority of Cre-IR cells were also positive for TH and AADC. Bar, 20 μm. (D) Parallel striatal sections immunostained for glial fibrillary acidic protein (GFAP) or AADC. Striatal cells were transduced without obvious reactive astrocytosis. Residual hemosiderin was observed along the needle tract. On the right are magnified views of the boxes on the left. Bars: 0.5 mm, left; 50 μm, right.

as the cylinder test, weekly until the rats were killed. In the absence of 4-OHT, we observed behavioral recovery in rats that received both AAV-CreER^{T2} and AAV vectors expressing dopamine-synthesizing enzymes. Following 4-OHT treatment, these rats regressed, demonstrating impaired behavior (Figs. 3A and 3B). No recovery occurred in AAV-CreER^{T2}-injected rats treated with 4-OHT at the same time as vector injection or in AAV-Cre- or AAV-LacZ-injected rats. Contralateral turning in response to L-3,4-dihydroxyphenylalanine (L-dopa, 5 mg/kg) was not significantly reduced in 4-OHT-treated rats or AAV-Cre injected rats, indicating efficient conversion of L-dopa to dopamine in the striatum due to preservation of AADC activity (Fig. 3C).

Immunohistochemistry showed fewer TH-IR cells in rats that received AAV-Cre or AAV-CreER^{T2} plus 4-OHT, compared to injected rats not treated with 4-OHT (Figs. 4A and 4B). The numbers of AADC-immunoreactive cells,

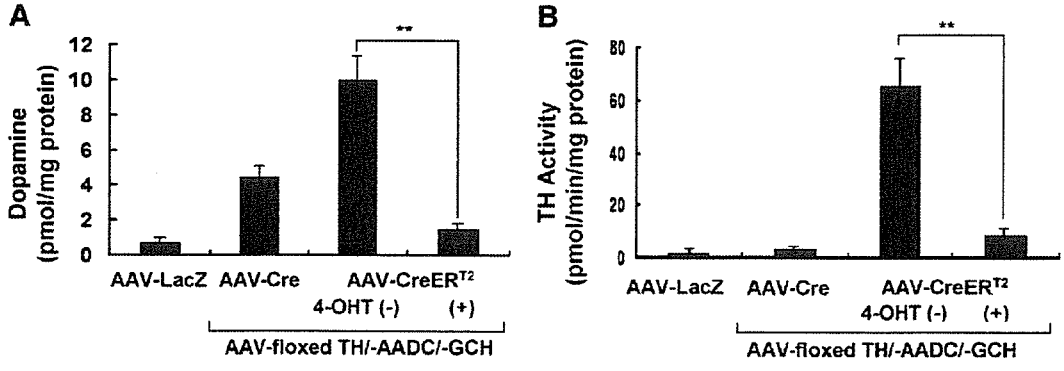

FIG. 5. Reduction of dopamine synthesis in 4-OHT-treated rats. Significantly less (A) dopamine content and (B) TH activity were observed in the lesioned striatum of 4-OHT-treated rats 12 weeks after vector injection, compared to 4-OHT-untreated rats. $^{**}P < 0.01$, n = 4.