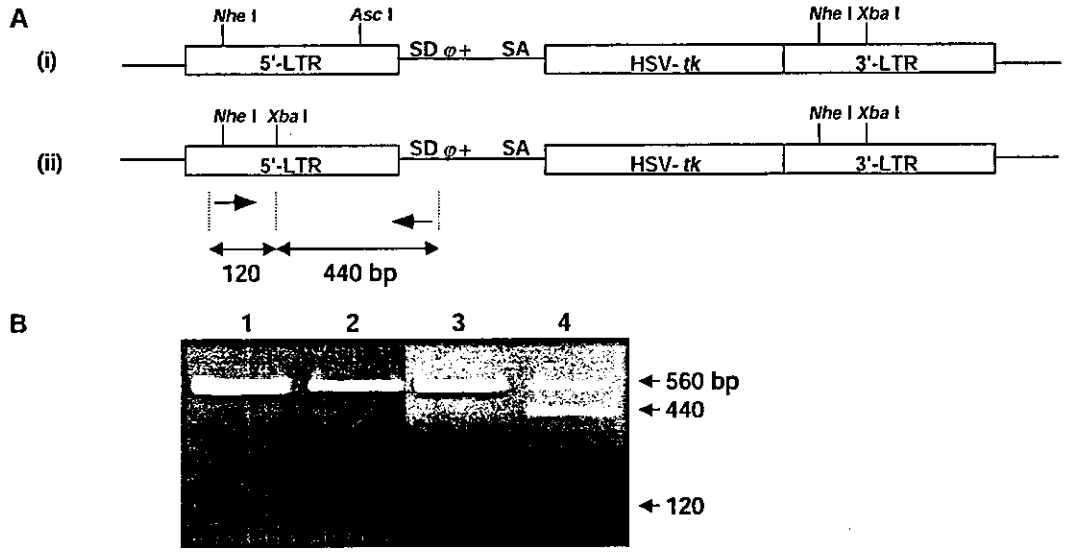

Figure 5. Integration of progeny retrovirus generated from the HSV-*tk* adeno-retroviral vectors. Genomic DNA of subcutaneous 9L tumors transduced with AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA was extracted for PCR analysis to determine if there had been successful integration of the progeny retroviruses. (A) (i) Schematic representation of the retroviral vector incorporated in the adeno-retroviral vector AVC2-GCTK. The 3′ LTR of AVC2.GCTK contains a unique *Xba* I site. (ii) Re-duplication of the 3′ LTR into the 5′ LTR during retroviral replication and proviral integration generates an *Xba* I site within the PCR product. (B) PCR products amplified from AVC2.GCTK DNA (lanes 1 and 2) and high molecular weight DNA purified from 9L cells transduced with the hybrid trans-complementing adeno-retroviruses (lanes 3 and 4). The PCR products in lanes 2 and 4 were digested to completion with *Xba* I

Student's t test). Since groups 3 and 4 contain the VSV-G expression vector, VSV-G-related toxicity might induce different initial growth rates. Intratumoral injection of all the trans-complementing HSV-*tk* adeno-retroviral vectors followed by GCV treatment resulted in complete tumor regression in four of the eight tumors that lasted longer than 4 weeks, while none of the tumors in control animals were eliminated ($p < 0.05$ by the Fisher's exact probability test). Similar results were obtained in a repeat of this experiment.

Enhanced expression of HSV-*tk* in tumors accompanying the generation of retroviral progeny

To determine if the enhanced killing seen following intratumoral injection of the trans-complementing HSV-*tk* adeno-retrovirus was due to the *in situ* generation of HSV-*tk* expressing retrovirus, we estimated the relative copy number of the HSV-*tk* transgene present in the high molecular weight DNA extracted from the tumors of the mice treated as described above (Table 1). The *GAPDH* gene was used as a reference gene to correct for variation in the amounts of DNA. The relative copy number of the HSV-*tk* gene in tumors lacking retroviral production (group 3, AVC2.GCTK, AVC2.null, AxTetVSVG, and AV-rtTA) was similar to that in the control treatment (group 2, AVC2.GCTK), whereas, compared with group 2, it was 89- and 258-fold higher in tumors receiving AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA (group 4).

To determine the expression pattern of the HSV-*tk* transcripts within a tumor, *in situ* hybridization analysis was performed using an RNA probe specific for HSV-*tk*. The HSV-*tk* transcripts in the tumors that did not produce

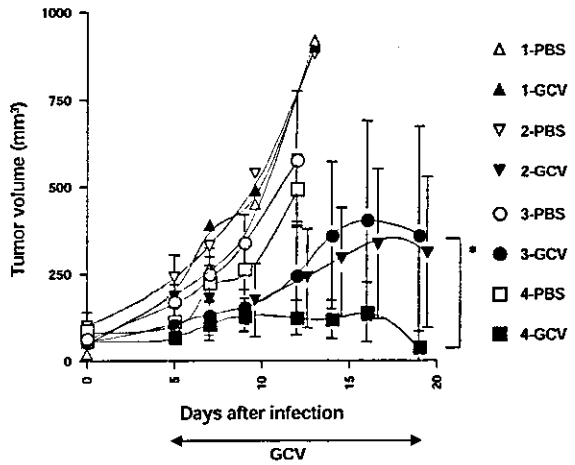

Figure 6. Effects of the hybrid vector system on xenograft growth in athymic mice. To establish subcutaneous tumors, 9L cells were inoculated subcutaneously into BALB/c *nu/nu* mice. Eight days after the inoculation, the established subcutaneous tumors were injected with either the control vector (1, AVC2.null, n = 20), the therapeutic vector (2, AVC2.GCTK, n = 20), a combination of vectors lacking AxTetGP (3, AVC2.GCTK, AxTetVSVG, and AV-rtTA; n = 20), or the complete set of vectors (4, AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA; n = 16). Animals received doxycycline as a 10 mg/ml solution with 5% sucrose via their water bottles for a period of 3 days starting at 24 h after the injection. When the tumor volume reached an average volume of 100 mm^3, half the animals in each group were treated with intraperitoneal injections of GCV at 30 mg/kg or PBS twice a day for 14 consecutive days. Each point represents the average volume of the tumors ± one standard deviation. *$p < 0.05$ by the Student's *t* test. Similar results were obtained in a repeat of this experiment

Table 1. Relative HSV-*tk* transgene copy number in tumors treated with adeno-retroviral trans-complementing viruses as measured by real-time PCR. The relative copy number of the HSV-*tk* gene was determined as the ratio of the copy numbers in tumors treated with AVC2.GCTK alone to the copy numbers in the other treatment group. The copy number of the reference gene *GAPDH* was also determined to correct for variation in the amounts of DNA. The final results are expressed as *N*-fold differences in the HSV-*tk* gene copy number relative to the GAPDH gene

	$2^{\text{corrected}\Delta Ct(GAPDH-TK)}$	
	Expt 1	Expt 2
vector	<0.05	<0.05
AVC2GCTK	1.0	1.0
AVC2GCTK, VSV-G	1.3	3.1
AVC2GCTK, VSV-G, gag-pol	89.3	258

retroviral progeny were restricted to a relatively small area (Figure 7A). In contrast, tumors that had received all of the trans-complementing adeno-retroviruses showed evidence of higher levels and a wider dispersion of the HSV-*tk* RNA signal (Figure 7C). Controls using a sense probe showed no specific reactivity (Figures 7B and 7D).

Discussion

In this study, we sought to engineer adeno-retroviral hybrid vectors that would produce, *in situ* in tumor cells, progeny retrovirus particles that express the therapeutic HSV-*tk* gene. The aim of this system was to improve the distribution of the therapeutic gene, which in turn should reduce the amount of GCV required to kill the transduced cells. We show that a single-step transduction of tumor cells with trans-complementing hybrid adeno-retroviral vectors effectively turns these cells into retrovirus vector-producing cells, which in turn facilitates the transduction of adjacent cells. This significantly increases the expression of the transgene. Moreover, we showed that as a result of the pseudotyped retrovirus production following trans-complementation of the adeno-retroviral hybrid viruses, the transduction efficiency both *in vitro* and *in vivo* was enhanced. In addition, we found that the trans-complementing adeno-retroviral hybrid vector system expressing HSV-*tk* and producing progeny retroviruses reduced by one log the amount of GCV required to induce significant *in vitro* killing of 9L glioma cells. Treatment with this system also completely inhibited the growth of established

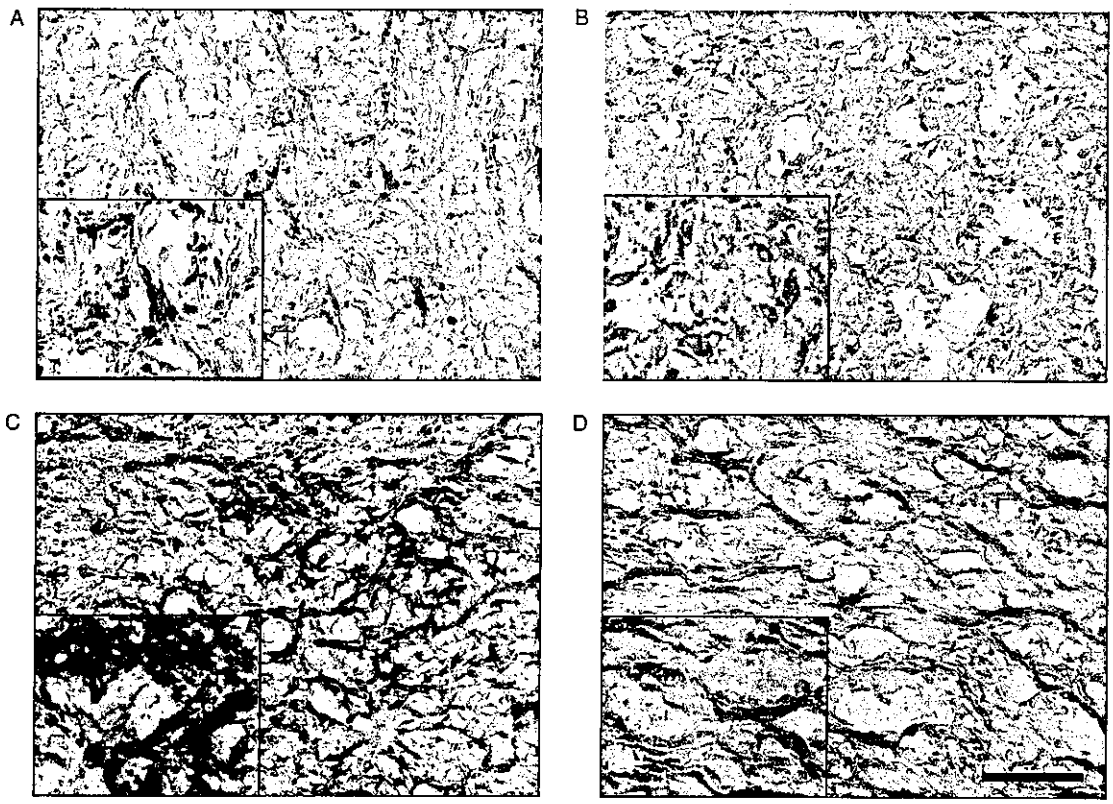

Figure 7. Enhanced expression of HSV-*tk* transcripts in 9L xenografts in athymic mice. Tumors from mice subjected to intratumoral injection with adenovirus along with PBS treatment were removed 14 days after the injection and *in situ* hybridization was performed using an antisense RNA probe specific for the HSV-*tk* transcripts (A and C). In tumor sections from mice treated with AVC2.GCTK, AxTetVSVG, and AV-rtTA (A), HSV-*tk* was rarely detectable. However, in tumor sections from mice treated with the complete set of vectors (AVC2.GCTK, AxTetGP, AxTetVSVG, and AV-rtTA), the HSV-*tk* RNA signal was more intense and shows a wider pattern of dispersion through the tumor section (C). *In situ* hybridization for HSV-*tk* using the anti-sense probe (A and C) or the sense probe (B and D). Results from one of several independent experiments are shown here. Similar results were obtained in the other experiments. Magnification, ×100. Scale bar, 100 μm. Insert, 2× overview

subcutaneous tumors in nude mice, and we showed that this was probably due to the amplification of the HSV-*tk* gene copy number and the consequent enhanced spread of the HSV-*tk* product throughout the tumor.

We and others have previously described hybrid vector systems that use adenoviral vectors to deliver retroviral vector and packaging proteins into cells [3–9]. These systems benefit from the efficient gene transfer characteristics of adenoviral vectors along with the stable and long-term gene expression that is typical of retroviral vectors. Initial studies of the co-transduction of adeno-retroviral hybrid vectors showed that transient retrovirus-producing cells can be successfully generated and that these could subsequently cause the transduction of neighboring cells [3,7]. Torrent *et al.* [9] also reported a study with a chimeric vector system that resulted in 10- to 50-fold transgene amplification *in vivo*, although they did not examine the distribution of the transgene or the therapeutic efficacy of their system. In the study reported here, we have altered these hybrid adeno-retroviral vector systems so that the tumor-killing effect of adenovirus-mediated HSV-*tk*/GCV therapy *in situ* is enhanced. Compared with the adenovirus delivery systems that lack retrovirus production, our system provides 89- and 258-fold transgene amplification *in situ* and stronger and wider dispersion of the transgene RNA signal. The enhanced amplification efficiency is most likely due to the *in situ* generated progeny retrovirus particles bearing the VSV-G envelope protein, which has a broader host range and higher transduction efficiency than murine leukemia virus derived retrovirus [7]. Murine leukemia virus derived retroviral vectors have had limited application in gene therapy because of low transduction efficiency of tissues, both *in vitro* and *in vivo*. One study showed that the transduction efficiency of the amphotropic vector into human cancer cells was not dose-dependent and reached a plateau or even decreased, especially at high MOIs [25]. This may be attributed in part to the presence of the envelope protein and non-infectious particles that compete for the receptor of infectious amphotropic viruses. In contrast, the receptor for VSV-G exists in abundance on the cell surface. Thus, our hybrid vector system pseudotyped with the VSV-G envelope glycoprotein is an alternative tool for efficient transduction.

For safety reasons, the retroviral functions in our system were split into different adenoviral backbones. However, there are some reports of studies that use a single helper-dependent adenovirus (HDAd) vector to accommodate large inserts. A vector system that uses an HDAd vector as a carrier to deliver a replication-competent ecotropic retrovirus vector has been developed [26]. An adenovirus/lentivirus hybrid vector on a single HDAd backbone was also developed for stable integration [27]. Although adenovirus-mediated transduction seems to be efficient in most cancers, there are several reports of studies using different viral components that convert tumor cells into retroviral producer cells. One of these employs a single-step system with herpes simplex virus/Epstein-Barr virus hybrid amplicons that converts cells into retrovirus vector producers [28]. Infection of primary gliomas with this system resulted in the production of retrovirus vectors and long-term retention *in vitro*. Poxviral/retroviral chimeric vectors also allow cytoplasmic production of defective retroviral particles [29]. Vaccinia-mediated expression of retroviral vector particles could be observed as early as 3 h post-infection and resulted in the stable transduction of NIH/3T3 cells. An alphavirus/retrovirus hybrid vector has also been used to transduce the retroviral vector packaging cell line [30]. The produced factor IX minigene-containing retroviral vectors were used for stable transduction *in vitro*.

When we used our hybrid vector system to treat a subcutaneous tumor model, we detected enhanced transgene expression together with an increased therapeutic effect *in vivo*. This suggests that the retroviral progeny are efficiently produced *in situ*. Although the efficacy of this system has to be evaluated in orthotopic models, current observations suggest that it is a promising method that has many possible applications in cancer gene therapy.

Acknowledgements

We wish to thank Dr. Tom Shenk for providing dl327 and Drs. Hiroshi Amanuma and Kiyotake Tobita for their support. We also wish to thank Ms. Miyoko Mitsu and Dr. Masahide Koremoto for their technical support and encouragement. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Jichi Medical School young investigator award, by the Uehara Memorial Foundation, by the Osaka Cancer Research Foundation, and by the Nakajima Foundation.

References

1. Ram Z, Culver KW, Oshiro EM, *et al.* Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997; 3: 1354–1361.
2. Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000; **11**: 2389–2401.
3. Feng M, Jackson WH Jr, Goldman CK, *et al.* Stable in vivo gene transduction via a novel adenoviral/retroviral chimeric vector. *Nat Biotechnol* 1997; **15**: 866–870.
4. Bilbao G, Feng M, Rancourt C, Jackson WJ, Curiel DT. Adenoviral/retroviral vector chimeras: a novel strategy to achieve high-efficiency stable transduction in vivo. *FASEB J* 1997; **11**: 624–634.
5. Ramsey WJ, Caplen NJ, Li Q, Higginbotham JN, Shah M, Blaese RM. Adenovirus vectors as transcomplementing templates for the production of replication defective retroviral vectors. *Biochem Biophys Res Commun* 1998; **246**: 912–919.
6. Lin X. Construction of new retroviral producer cells from adenoviral and retroviral vectors. *Gene Ther* 1998; **5**: 1251–1258.
7. Caplen NJ, Higginbotham JN, Scheel JR, *et al.* Adeno-retroviral chimeric viruses as in vivo transducing agents. *Gene Ther* 1999; 6: 454–459.
8. Duisit G, Salvetti A, Moullier P, Cosset FL. Functional characterization of adenoviral/retroviral chimeric vectors and their use for efficient screening of retroviral producer cell lines. *Hum Gene Ther* 1999; **10**: 189–200.

9. Torrent C, Jullien C, Klatzmann D, Perricaudet M, Yeh P. Transgene amplification and persistence after delivery of retroviral vector and packaging functions with E1/E4-deleted adenoviruses. *Cancer Gene Ther* 2000; **7**: 1135–1144.
10. Okada T, Ramsey J, Munir J, Wildner O, Blaese M. Efficient directional cloning of recombinant adenovirus vectors using DNA-protein complex. *Nucleic Acids Res.* 1998; **26**: 1947–1950.
11. Treisman J, Hwu P, Minamoto S, *et al.* Interleukin-2-transduced lymphocytes grow in an autocrine fashion and remain responsive to antigen. *Blood* 1995; **85**: 139–145.
12. Okada T, Shah M, Higginbotham JN, *et al.* AV.TK-mediated killing of subcutaneous tumors in situ results in effective immunization against established secondary intracranial tumor deposits. *Gene Ther* 2001; **8**: 1315–1322.
13. Stein R, Ziff EB. HeLa cell beta-tubulin gene transcription is stimulated by adenovirus 5 in parallel with viral early genes by an E1a-dependent mechanism. *Mol Cell Biol* 1984; **4**: 2792–2801.
14. Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. *Biotechnology* 1992; **20**: 363–390.
15. Yoshida Y, Emi N, Hamada H. VSV-G-pseudotyped retroviral packaging through adenovirus-mediated inducible gene expression. *Biochem Biophys Res Commun* 1997; **232**: 379–382.
16. Cosset FL, Takeuchi Y, Battini JL, Weiss RA, Collins MK. High-titer packaging cells producing recombinant retroviruses resistant to human serum. *J Virol* 1995; **69**: 7430–7436.
17. Benda P, Someda K, Messer J, Sweet WH. Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. *J Neurosurg* 1971; **34**: 310–323.
18. Bethea JR, Gillespie GY, Chung IY, Benveniste EN. Tumor necrosis factor production and receptor expression by a human malignant glioma cell line, D54-MG. *J Neuroimmunol* 1990; **30**: 1–13.
19. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 1977; **36**: 59–74.
20. Wildner O, Candotti F, Krecko EG, Xanthopoulos KG, Ramsey WJ, Blaese RM. Generation of a conditionally neo(r)-containing retroviral producer cell line: effects of neo(r) on retroviral titer and transgene expression. *Gene Ther* 1998; **5**: 684–691.
21. Wigler M, Pellicer A, Silverstein S, Axel R. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* 1978; **14**: 725–731.
22. Onodera M, Yachie A, Nelson DM, Welchlin H, Morgan RA, Blaese RM. A simple and reliable method for screening retroviral producer clones without selectable markers. *Hum Gene Ther* 1997; **8**: 1189–1194.
23. Onodera M, Nelson DM, Yachie A, *et al.* Development of improved adenosine deaminase retroviral vectors. *J Virol* 1998; **72**: 1769–1774.
24. Harstrick A, Casper J, Guba R, Wilke H, Poliwoda H, Schmoll HJ. Comparison of the antitumor activity of cisplatin, carboplatin, and iproplatin against established human testicular cancer cell lines in vivo and in vitro. *Cancer* 1989; **63**: 1079–1083.
25. Arai T, Takada M, Ui M, Iba H. Dose-dependent transduction of vesicular stomatitis virus G protein-pseudotyped retrovirus vector into human solid tumor cell lines and murine fibroblasts. *Virology* 1999; **260**: 109–115.
26. Soifer H, Higo C, Logg CR, *et al.* A novel, helper-dependent, adenovirus-retrovirus hybrid vector: stable transduction by a two-stage mechanism. *Mol Ther* 2002; **5**: 599–608.
27. Kubo S, Mitani K. A new hybrid system capable of efficient lentiviral vector production and stable gene transfer mediated by a single helper-dependent adenoviral vector. *J Virol* 2003; **77**: 2964–2971.
28. Sena-Esteves M, Saeki Y, Camp SM, Chiocca EA, Breakefield XO. Single-step conversion of cells to retrovirus vector producers with herpes simplex virus-Epstein-Barr virus hybrid amplicons. *J Virol* 1999; **73**: 10 426–10 439.
29. Holzer GW, Mayrhofer JA, Gritschenberger W, Dorner F, Falkner FG. Poxviral/retroviral chimeric vectors allow cytoplasmic production of transducing defective retroviral particles. *Virology* 1999; **253**: 107–114.
30. Wahlfors JJ, Morgan RA. Production of minigene-containing retroviral vectors using an alphavirus/retrovirus hybrid vector system. *Hum Gene Ther* 1999; **10**: 1197–1206.

Gene Therapy (2004) 11, 253–259

www.nature.com/gt

RESEARCH ARTICLE

Expression of human coagulation factor VIII in adipocytes transduced with the simian immunodeficiency virus agmTYO1-based vector for hemophilia A gene therapy

K Ogata[1,5], J Mimuro[1,4], J Kikuchi[1], T Tabata[2], Y Ueda[2], M Naito[1], S Madoiwa[1,4], K Takano[1], M Hasegawa[2], K Ozawa[3,4] and Y Sakata[1,4]

[1]*Cell and Molecular Medicine of Center for Molecular Medicine, Jichi Medical School, Tochigi-ken, Japan;* [2]*DNAVEC Research Inc., Ibaraki-ken, Japan;* [3]*Genetic Therapeutics of Center for Molecular Medicine, Jichi Medical School, Tochigi-ken, Japan; and* [4]*Hematology Division of Department of Medicine, Jichi Medical School, Tochigi-ken, Japan*

We demonstrate that transduction of adipocytes with a simian immunodeficiency virus agm TYO1 (SIVagm)-based lentiviral vector carrying the human coagulation factor VIII gene (SIVhFVIII) resulted in expression of the human FVIII transgene in vitro and in db/db mice in vivo. Cultured human adipocytes were transduced with the SIVagm vector carrying the GFP gene in a dose-dependent manner and transduction of adipocytes with SIVhFVIII resulted in efficient expression of human coagulation factor VIII (hFVIII; 320±39.8 ng/10⁶ adipocytes/24 h) in vitro. Based upon successful transduction of adipocytes by SIV vectors carrying the lacZ gene in vivo in mice, the adipose tissue of db/db *mice was transduced with SIVhFVIII. There was a transient appearance of human FVIII in mouse plasma (maximum 1.8 ng/ml) on day 11 after the injection. Transcripts of human FVIII transgene and human FVIII antigen also were detected in the adipose tissue by RT-PCR and immunofluorescence, respectively, on day 14. Emergence of anti-human FVIII antibodies 14 days after the injection of SIVhFVIII may explain the disappearance of human FVIII from the circulation. These results suggest that transduction of the adipocytes with vectors carrying the human FVIII gene may be potentially applicable for gene therapy of hemophilia A.*
Gene Therapy (2004) **11**, 253–259. doi:10.1038/sj.gt.3302174

Keywords: *adipocyte; simian immunodeficiency virus vector; hemophilia*

Introduction

Hemophilia A is an inherited X-linked lifelong bleeding disorder caused by abnormality in the coagulation factor VIII (FVIII) gene.[1,2] The genetic abnormalities result in deficiency of FVIII, which in turn creates a bleeding diathesis, such as life-threatening intracranial bleeding and bleeding in joints and muscles. Hemophilias occur as mild, moderate, or severe, depending on the blood FVIII level of 6% or more, 2–5%, or 1% or less. The current standard therapy is intravenous (i.v.) injection of human plasma-derived FVIII or recombinant FVIII. Aside from certain specific situations, such as preoperative factor coverage, i.v. infusion of FVIII is usually used to treat acute bleeding episodes and prophylactic FVIII i.v. infusion is not recommended. However, maintaining of blood FVIII levels to more than 5% of the normal FVIII concentration may result in significant clinical improvement. Furthermore, if one can increase FVIII levels to more than 1% in severe hemophilia patients, they may have significantly fewer bleeding episodes and improved quality of life. Recombinant FVIII products are now commercially available, but may not be completely free from pathological substances such as prions or as yet unknown viruses. In this regard, gene therapy is being explored as the next generation therapy for hemophilia patients.[1,2]

Adipocytes are terminally differentiated and nondividing cells. They not only store excess energy in the form of fat but also synthesize and secrete a variety of biologically active molecules such as leptin, adiponectin, cytokines, and plasminogen activator inhibitor-1 to the circulation.[3] Subcutaneous adipose tissues are readily accessed for vector administration. In addition, the adipose tissue can be removed surgically if necessary. These characteristics present attractive features of adipocytes for therapeutic gene therapy. In the present study, we use SIVagmTYO1-based vectors to show that SIVagmTYO1vectors can transduce adipocytes *in vitro* and *in vivo*, resulting in therapeutic gene expression, an expression mode that may be applicable to hemophilia gene therapy.

Correspondence: Dr J Mimuro, Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical School, Tochigi-ken 329-0498, Japan
[5]*Current address: Department of Laboratory Medicine, Tokyo Medical University, Shinjuku-ku, Tokyo 160-0023, Japan*
Received 17 March 2003; accepted 3 September 2003

Results

Transduction of adipocytes with SIVeGFP and SIVhFVIII in vitro

To assess the *in vitro* transduction efficiency of adipocytes with the SIV vector, human adipocytes were cultured in the presence of increasing concentrations of SIVeGFP for 48 h. After transduction, cells were washed and incubated in medium for 72 h. As shown in Figure 1a (phase-contrast view), cells containing intracellular lipid droplets represent the typical adipocyte morphology. The fluorescent microscopy image showed that eGFP was expressed in lipid droplet-containing adipocytes (Figure 1b). Flow cytometry analysis (Figure 1c) of these cells showed that eGFP expression in human adipocytes increased in a dose-dependent manner. Approximately 62% of adipocytes were efficiently transduced with SIVeGFP at MOI 5×10^3 vg/cell (100 transduction units/cell). We also assessed human FVIII production in the transduced human adipocytes. Cells were incubated in the presence of increasing concentrations of SIVhFVIII. The supernatants were harvested after various incubation times and human FVIII antigen levels were quantified by ELISA. FVIII production from the human adopocytes started on day 5 and increased in a dose- and time-dependent manner (Figure 2). After

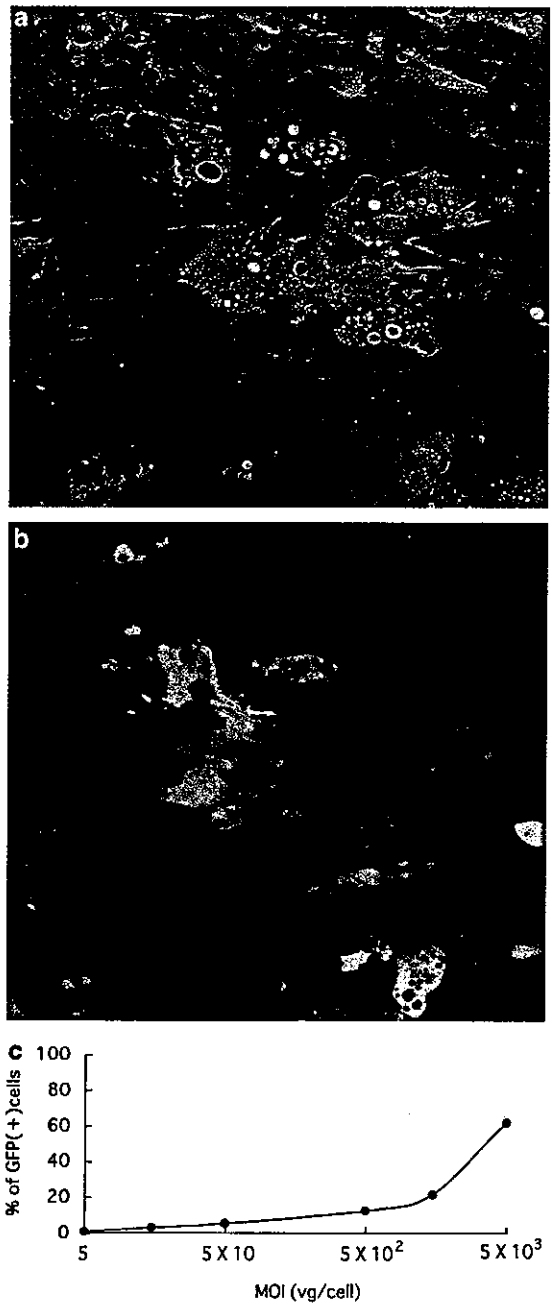

Figure 1 Transduction of human white adipocytes with SIVeGFP. Human white adipocytes (6.7×10^4cells/well) were incubated with increasing concentrations of SIVeGFP for 48 h. After incubation, cells were washed with PBS and incubated in media for 72 h (a, phase contrast). Expression of eGFP was visualized by fluorescence microscopy (b) and flow cytometry (c). The percentages of transduced cells expressing eGFP are shown (c) (mean, n = 2).

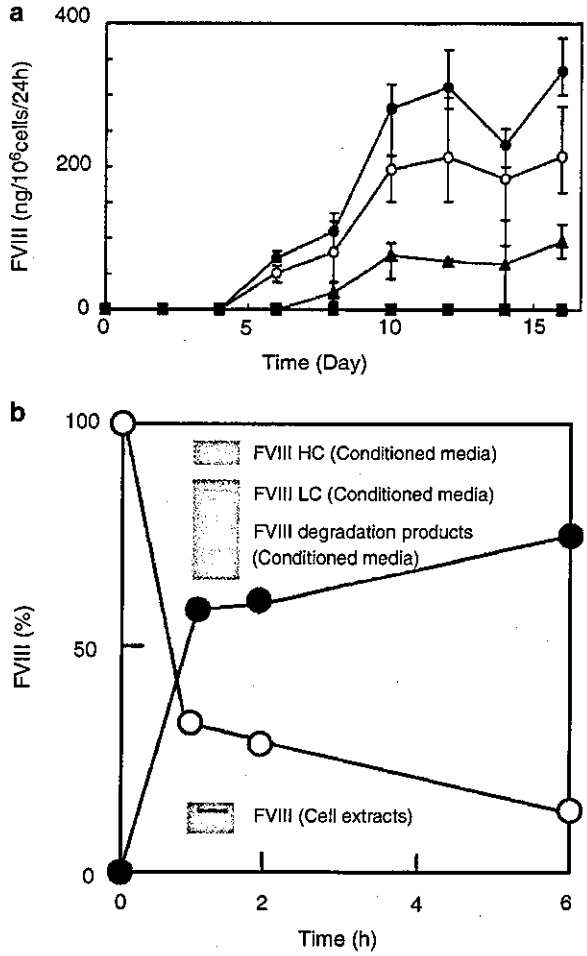

Figure 2 Transduction of human white adipocytes by SIVhFVIII. (a) Human white adipocytes (6.7×10^4 cells/well) were incubated with increasing concentrations of SIVhFVIII (■, 2×10^2 vg/cell; ▲, 6.5×10^2 vg/cell; ^, 2×10^3 vg/cell; ●, 6.5×10^3 vg/cell). After 48 h, cells were washed with PBS and cultured for 14 days. Supernatants were harvested and human FVIII antigen quantified by ELISA, as described in Materials and methods (mean ± s.d., n = 3). (b) Pulse-chase experiments for FVIII production in human adipocytes (5×10^5 cells) were carried out on day 7 after transduction with SIVhFVIII (6.5×10^3 vg/cell). Amounts of [^{35}S]-labeled FVIII in the conditioned media (●) and in the cell extracts () were quantified as described in Materials and methods. Insets show the pertinent portion of the autoradiography of [^{35}S]-labeled FVIII in the cell extracts after pulse labeling and [^{35}S]-labeled FVIII species (heavy chain (HC); light chain (LC); degradation products) in the conditioned media after 1 h chase incubation.

transducing cultured human adipocytes with SIVhFVIII at MOI 6.5×10^3 vg/cell, 320 ± 39.8 ng of human factor VIII was produced from 10^6 adipocytes during a 24 h *in vitro* incubation. To study the secretion of FVIII from transduced adipocytes, pulse-chase experiments were performed. Approximately 53% of human FVIII, expressed in cultured adipocytes, was secreted from adipocytes during 1 h incubation periods, suggesting that adipocytes could secrete expressed FVIII efficiently. FVIII molecules identified in the conditioned media consisted of the heavy chain, the light chain, and degraded FVIII products, and the presence of these FVIII species in the conditioned media were consistent with the previous report.[4]

Transduction of the adipose tissue by SIVlacZ in vivo

To explore the possibility that the SIV vector can transduce adipocytes *in vivo*, SIVlacZ vectors were injected into the subcutaneous adipose tissue of 8-week-old *db/db* mice. Wild-type mice have adipose tissue in the mesenterium and peritesticular regions, but they are generally lean and do not have enough subcutaneous adipose tissues for vector injection. NOD/SCID mice are used frequently for gene therapy research because of their immunodeficiency, but they also have little subcutaneous adipose tissues. Among several types of obese mice used for metabolic disease research, *db/db* mice are well characterized, obese, and diabetic. They become obese by accumulating fat in the subcutaneous and visceral adipose tissues after 4 weeks of age. Thus, *db/db* mice are appropriate for studying *in vivo* transgene expression from subcutaneous adipose tissue. At 2 weeks after the injection, the adipose tissues were excised and processed for detection of β-galactosidase activity. As shown in Figure 3a, the adipose tissue was stained blue homogenously after the X-gal staining in the macroscopic view. In the histology sections of the adipose tissue, β-galactosidase activity was detected in the adipocytes of *db/db* mice (Figure 3b, c). These data suggest that SIV vectors are capable of transducing adipocytes *in vivo* in mice.

Plasma human FVIII levels in SIVhFVIII-injected db/db mice

To evaluate *in vivo* production of human VIII from adipocytes, SIVhFVIII was injected into subcutaneous adipose tissues of *db/db* mice. Mouse plasma was obtained on days 0, 4, 7, 11, 14, 18, 21, and 25 after vector injection and human FVIII levels in mouse plasma were quantified by an ELISA that recognizes only human FVIII. Plasma human FVIII levels (closed circle) increased to 1.8 ng/ml on day 11, but human FVIII antigen was not detectable in the circulation on day 14 after vector injection (Figure 4). Since *db/db* mice are immunocompetent, they may develop antibodies to human FVIII expressed *in vivo*. To explore the possibility that disappearance of human FVIII from the mouse circulation was caused by the presence of antibody against human FVIII in *db/db* mice, a solid-phase EIA for detection of *db/db* mouse antibody to human FVIII was carried out. As shown in Figure 4, anti-human FVIII antibodies (triangle) were detected in *db/db* mouse plasma obtained on day 14 and the levels increased gradually to 6.9 μg/ml by day 25.

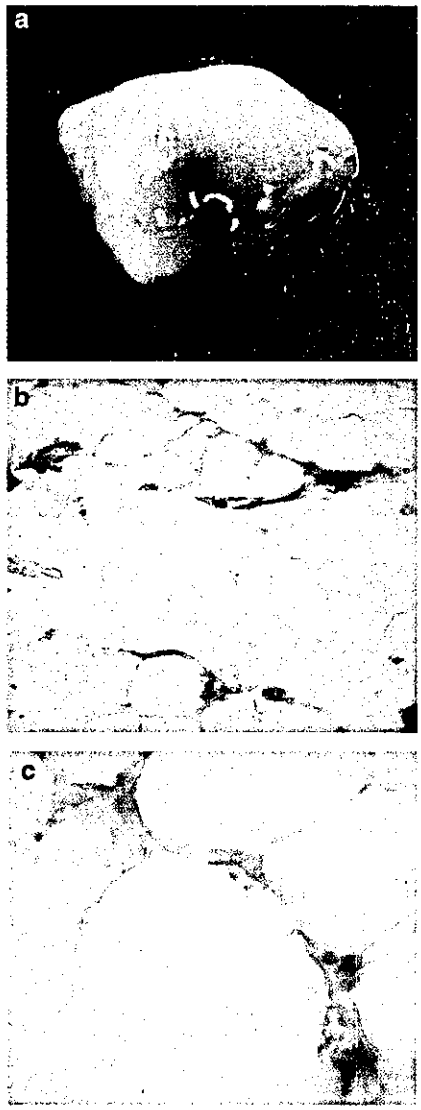

Figure 3 Transduction of the adipose tissues of db/db mouse by SIVlacZ. The SIVlacZ vector (5×10^7) was diluted in PBS and was injected into the subcutaneous adipose tissues of db/db mice. The subcutaneous adipose tissues were excised on day 14 after injection. Tissues were processed for detection of β-galactosidase activity, as described in Materials and methods. The adipose tissue (macroscopic view) was homogenously stained blue (a). β-galactosidase activity was observed in the adipocytes, as reflected by the blue staining in the histology sections (b, c).

Detection of FVIII transcripts in the adipose tissue of db/db mice

To assess the expression of transgenes in the adipose tissue of *db/db* mice, the adipose tissues were excised on day 14 after injection and subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for detection of human BDD-FVIII transcripts using human FVIII or mouse GAPDH-specific primers. As shown in Figure 5, human FVIII transcripts were detected in the adipose tissue from the SIVhFVIII-injected mice, but not in the adipose tissue from SIVlacZ-injected mice. Mouse GAPDH transcripts were detected in both RNA preparations. These data suggest that the human FVIII transgene was expressed in the mouse adipose tissue.

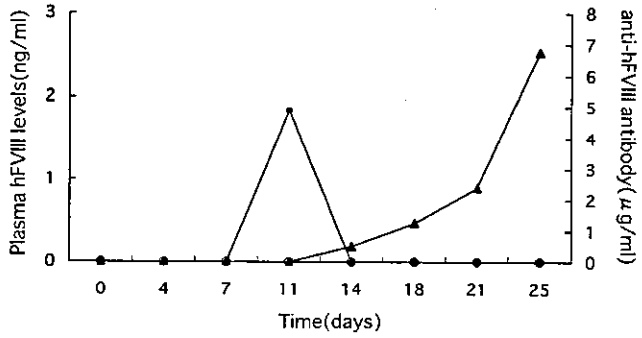

Figure 4 Plasma human FVIII levels in SIVhFVIII-injected db/db mice. Peripheral blood was obtained from SIVhFVIII-injected mice or the SIVlacZ-injected control mice on days 0, 4, 7, 11, 14, 18, 21, and 25 after injection. Human FVIII concentrations in plasma of db/db mice who received SIVhFVIII (closed circle) or SIVlacZ (open circle) injection were determined by ELISA (mean, n = 2). Anti-human FVIII antibodies present in mouse plasma (closed triangle) were quantified by the solid phase EIA as in described in METHODS.

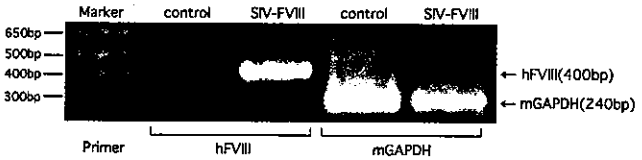

Figure 5 RT-PCR analysis of adipose tissue-derived RNA. RNA was isolated from the murine adipose tissue on day 14 after vector injection. A measure of 100 ng of RNA was subjected to RT-PCR with specific primer pairs for the human BDD-FVIII transcript (human FVIII) or for the mouse GAPDH transcript (mouse GAPDH). Amplified products were analyzed on 2% agarose gels followed by ethidium bromide staining (control, RNA isolated from the SIVlacZ injected adipose tissue; SIVhFVIII, RNA isolated from the SIVhFVIII-injected adipose tissue).

Detection of human FVIII expressed in the adipose tissue of db/db mouse

Mouse adipose tissues were processed for detection of human FVIII antigen, and imaged by immunofluorescence (Figure 6). Human FVIII was observed in adipocytes isolated from SIVhFVIII-injected mice (left), but not in cells from mice that received the SIVlacZ vector (right). These data confirm the notion that the human FVIII was produced from the SIVhFIII-transduced cells *in vivo*.

Discussion

The subcutaneous adipose tissue has attractive features for genetic therapy, such as easy accessibility, active biosynthesis and secretion behavior, and high vascularity.[3] However, adipocytes are terminally differentiated and nondividing cells with a characteristic phenotype expression. A variety of viral and nonviral vectors are used to transduce cells *in vitro* and *in vivo* for gene delivery, but it is still rather difficult to transduce nondividing cells efficiently. Adenoviral vectors are able to transduce nondividing cells including adipocytes,[5,6] but transgene expression by adenoviral vectors is thought to be transient and they are highly immunogenic. In contrast, pseudotyped lentiviral vectors are versatile enough to stably transduce various types of cells including nondividing cells, and were not as immunogenic as the adenoviral vectors.[7–10] But transduction of adipocytes with lentiviral vectors had not been studied yet. There are safety concerns in utilizing HIV-1-based lentivirus vectors for gene therapy clinical trials. In this regard, simian immunodeficiency virus agmTYO1 (SIVagmTYO1)-based vectors are of particular interest. SIVagmTYO1 is an HIV-related lentivirus isolated from the African green monkey and shown to be nonpathogenic to both their natural hosts and to experimentally inoculated Asian macaques.[11] Additionally, due to the use of contaminated blood products, some hemophilia patients are HIV-1 carriers. If an HIV-1-based vector is administered to such patients, the replication-competent lentivirus particles carrying the therapeutic gene may be generated by homologous recombination between the recombinant HIV vector and the wild-type HIV genome. The packaging signal in the HIV vector sequence may be another factor contributing to production of replication-competent lentivirus particles. From this perspective, then, a SIV vector based on the SIVagm TYO1 strain may be a better vehicle for hemophilia gene therapy because SIVagm TYO1 has less than 60% genomic sequence similarity to HIV-1.

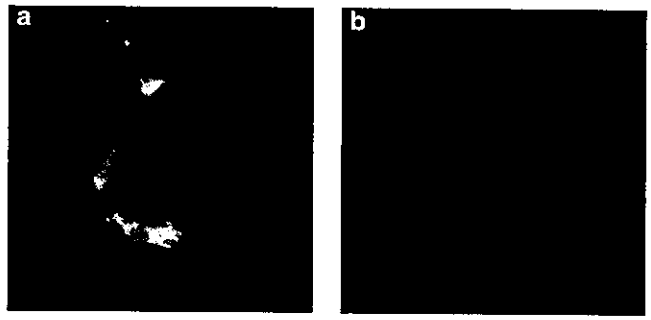

Figure 6 Detection of human FVIII antigen in mouse adipose tissue. The adipose tissue of SIVhFVIII injected db/db mice (a) or SIVlacZ-injected mice (b) were subjected to immunofluorescence staining for human FVIII antigen. Tissue sections were incubated with sheep anti-human FVIII polyclonal antibodies. The bound antibodies were detected by Alexa-Fluor488-conjugated secondary antibody and visualized using a fluorescence microscope (E800, Nikon Co Ltd, Tokyo, Japan).

We have shown that SIV vectors carrying the eGFP, the lac Z, or a therapeutic gene can transduce cultured human adipocytes *in vitro* and mouse adipose tissue *in vivo*. Transduction of the human adipocytes by the SIVeGFP vector *in vitro* was dose-dependent and appeared to be efficient. Production of human FVIII (320 ± 39.8 ng/10^6cells/24 h) from transduced adipocytes at MOI 6.5×10^3 vg/cell *in vitro* was considerable and efficient, raising the possibility of achieving therapeutic levels of plasma FVIII in mice if 10^6 adipocytes were transduced by SIVhFVIII efficiently *in vivo*. Thus, SIVhFVIII 4×10^9 vg was injected to the mouse subcutaneous adipose tissue. Human FVIII was detected in the mouse plasma and this human FVIII level was approximately 1–2% of the normal FVIII level of normal human subjects. The FVIII levels achieved in mice were relatively low, but such increase of the FVIII level would develop clinical effects in hemophilias such as decrease

of bleeding episodes and of the use of FVIII concentrates. Data on clinical trials of hemophilia A gene therapy support this notion.[12] However, this human FVIII level in mouse plasma was lower than that expected from the observed *in vitro* production rate. One contributing factor may be the shorter half-life of human FVIII in mice compared to humans. The half-life of injected human FVIII in mice is approximately 1 h,[13] whereas the half-life in hemophilia patients is closer to 8–12 h. Another possibility may be the inefficient transduction of adipocytes *in vivo* because the vector-containing solution might not diffuse throughout the adipose tissues, resulting in less adipocytes being exposed to the viral vectors. It is also possible that transduction of mouse adipocytes by the SIV vector is less efficient than that of human cells. Production of human FVIII from differentiated 3T3-L1 cells, transduced with the SIVhFVIII vector, was significantly lower than that from human adipocytes (not shown). The observation that anti-human FVIII antibodies were present in the mouse plasma obtained on day 14 and subsequently increased to 6.9 μg/ml by day 25 suggested that human FVIII was rapidly cleared from the mouse circulation by the formation of immune complexes after day 14. Thus, human FVIII was secreted into the circulation and was recognized by the *db/db* mouse immune system. The notion that human FVIII was synthesized in the adipocyts *in vivo* is also supported by detection of human FVIII gene transcripts and products in the adipocytes. In conclusion, adipose tissue is easily accessible and is an appropriate target for gene delivery. Recombinant SIV vectors may be applicable for adipocyte-targeted gene therapy for hemophilia A.

Materials and methods

Cell culture

Human white adipocytes prepared in 24-well culture plates were purchased from Zen-Bio Inc. (Research Triangle Park, NC, USA). These cells were differentiated from preadipocytes isolated from the adipose tissue of 49-year-old healthy subjects undergoing liposuction surgery with informed consent. The cells were shown to express leptin, CCAAT/enhancer-binding protein α, peroxisome proliferator-activated receptor γ, and STATs.[14,15] Human adipocytes were cultured in DMEM/HAM F10 medium supplemented with 3% fetal bovine serum, 15 mM HEPES, biotin (33 μM), pantothenate (17 μM), insulin (100 nM), dexamethazone (1 μM), penicillin 100 U/ml, streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) (Zen-Bio Inc.). 3T3-L1 cells (ATCC) were cultivated in DMEM containing 10% FBS. Differentiation of 3T3-L1 cells to adipocytes was carried out in the medium containing dexamethasone, insulin, and 1-methyl-3 isobutylxantine, as described previously.[16]

Production of SIVagm vectors

Human FVIII cDNA spanning the entire coding region was a generous gift from Dr JA van Mourik (VU University Medical Centre).[17] As the B domain is excised from other FVIII domains upon activation by thrombin and is not essential for coagulation activity of FVIIIa, coding for the B domain was deleted from the human FVIII cDNA by PCR-based mutagenesis (BDD FVIII cDNA), as described previously.[18] The deletion was confirmed by sequencing. The characteristics and production of SIVagm vector used in this study were described previously.[19] Self-inactivating SIVagm vectors are pseudotyped with vesicular stomatitis virus glycoprotein G (VSVG). We constructed a series of gene transfer vectors to express the eGFP gene, the lacZ gene, and the hBDDFVIII gene driven by the cytomegalovirus (CMV) promoter. To produce SIV vectors, 293T cells were transfected with the packaging vector, the gene transfer vector, and pVSVG (Clontech), as described previously.[19] Transduction units of SIVeGFP were determined by infection of SIVeGFP to 293T cells, followed by determination of eGFP expression by FACS analysis. RNA dot blot analysis was performed to quantify the amount of SIVagmTYO1vector genome of vector preparations. When SIVhFVIII was produced in 293T cells, SIVeGFP also was prepared simultaneously and the transduction unit of SIVeGFP and the amount of vector genome were determined. The SIVeGFP preparation was used as the standard to estimate the transduction units of SIVhFVIII.

In vitro culture and transduction of human adipocytes

For transduction, increasing concentrations of the SIV vector were added to human adipocyte cell monolayers in 24-well culture plates, and the cells were incubated at 37°C for 48 h in the presence of 5% CO_2. After incubation, the medium was harvested and changed, according to the manufacturer's instruction. SIVeGFP-transduced adipocytes were analyzed for eGFP expression by fluorescence microscopy and flow cytometry, and the conditioned medium of SIVhFVIII-transduced adipocytes was harvested and subjected to FVIII ELISA. To study the secretion of FVIII from adipocytes transduced with SIVhFVIII, pulse-chase experiments were performed. SIVhFVIII-transduced adipocytes were cultured for 30 min in methionine-deficient DMEM (GIBCO-Invitrogen Japan, Tokyo, Japan) containing 3700 kBq/ml [^{35}S]-methionine (NEN Life Science Products, Inc., Boston, MA, USA), and then cultured in the complete medium (DMEM). After various incubation time periods, conditioned medium and cell extracts were prepared. To isolate [^{35}S]-labeled FVIII molecules, the conditioned media and cell lysates were subjected to immunoprecipitation using sheep polyclonal antibodies against human FVIII and protein A-coupled Sepharose CL-4B (Amersham Pharmacia Biotech, UK). [^{35}S]-labeled FVIII was analyzed by SDS-PAGE followed by autoradiography, and quantified using an image analyzer BAS 2000 (Fujifilm, Tokyo, Japan), as described.[20]

Mice

Experimental *db/db* mice (C57BL/KsJ-db/db) are well characterized obese and diabetic mice caused by the genetic abnormality of the leptin receptor gene,[21] and were purchased from Japan SLC Inc. (Hamamatsu, Japan). The *db/db* mice were kept in a clean P3-level experimental room, and were maintained on a sterile diet and given autoclaved water.

Transduction of mouse adipose tissues by SIV vectors

SIV vectors carrying either the lacZ gene or the hFVIII gene were diluted in PBS and injected into the subcutaneous adipose tissue of the mice. Peripheral

blood (100 μl) was collected from mouse tail veins into tubes containing heparin. Platelet poor plasma was prepared by centrifugation of the peripheral blood at 1000 g for 15 min, and subjected to FVIII ELISA. Some mice were killed on day 14 after the injection for detection of transcripts and products of the transgenes.

Enzyme immunosorbent assay (ELISA) for human FVIII antigen

Since human FVIII clotting activity could not be quantified directly in the *db/db* mice because of the presence of endogenous murine FVIII, human FVIII expressed in *db/db* mice was quantified by a human FVIII-specific ELISA, as described previously.[22] Briefly, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 1 μg/ml mouse monoclonal antibody to human FVIII (Chemo-Sero Institute, Kumamoto, Japan). After blocking with 5% casein in PBS, mouse plasma samples or pooled normal human plasma in Tris-buffered saline (TBS) containing 0.1% Tween 20, 1% casein were added. After 16 h incubation at 4°C, human FVIII bound to the plates was detected with sheep anti-human FVIII polyclonal antibodies (Cedarlane Laboratories Ltd, Homby, Ontario, Canada) and horseradish peroxidase-conjugated rabbit anti-sheep IgG.

Detection of anti-human FVIII mouse antibody

Microtiter plates were coated with purified human FVIII in PBS for 16 h. After blocking with 5% casein, FVIII-coated microtiter plates were incubated with mouse plasma at 10–1000-fold dilutions or monoclonal antibodies raised against human FVIII as the standard. Mouse IgG bound to human FVIII was detected by HRP-conjugated anti-mouse IgG, followed by incubation with HRP substrate ABTS (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA).

Detection of β-galactosidase and human FVIII in the mouse adipose tissue

Mouse adipose tissues were obtained from *db/db* mice. The adipose tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, and then incubated in PBS containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 0.01% Na deoxycholate, 0.1% Triton X-100 for 1 h. The tissues were again washed, incubated with PBS containing sucrose (10–30%), and frozen with OTC compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA) in dry ice/ethanol. Tissue sections were made at −35°C and attached to polylysine-coated glass slides. For the immunofluorescence study, the adipose tissues were fixed with 4% paraformaldehyde in PBS for 2 h at 4°C, incubated with PBS containing sucrose (10–30%), and then frozen in the presence of OCT compound in dry ice/ethanol. Sections were prepared from frozen tissues at −35°C and attached to polylysine-coated glass slides. For the detection of human FVIII, tissue sections were blocked with 1% bovine serum albumin in PBS. Samples were incubated with polyclonal anti-human FVIII antibody at 4°C for 16 h. After washing in PBS, cells were incubated with donkey anti-sheep IgG antibody conjugated with Alexa488 (Molecular Probes, Eugene, OR, USA) at 4°C for 16 h for visualization of human FVIII by fluorescence microscopy.

Detection of the BDD-FVIII transcript by RT-PCR

RNA was isolated from the adipose tissue by the acid-guanidine method and was reverse transcribed to cDNA using reverse transcriptase (Superscript, Invitrogen Japan, Tokyo, Japan) and oligo-(dT) primers in a 20 μl mixture (QIAGEN Japan, Tokyo, Japan) after DNase I (Amplification grade, Invitrogen) treatment. Subsequent PCR amplification was carried out with 1 μl of cDNA solution in a 50 μl reaction mixture containing 5 U of Taq polymerase, 10 mmol/l Tris-HCl (pH 8.5), 50 mM KCl, 1.5 mM $MgCl_2$, and 100 μM dNTPs in the presence of specific primer pairs (200 nM) designed to amplify the DNA fragments derived from the transcript of the BDD-FVIII transgene. Each PCR cycle consisted of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The PCR products were analyzed by agarose gel electrophoresis. Authenticity of PCR products was confirmed by their molecular sizes after agarose gel electrophopresis, and by sequencing. The primer sequences for human FVIII are ATTGGAG-CACAGACTGACTT and ATATGGTATCATCATAGTCA (400 bp). Primers for mouse GAPDH were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

Acknowledgements

We thank Dr JA van Mourik for the full-length FVIII cDNA and Dr Nakagaki (Chemo Sero Institute) for monoclonal antibodies to human FVIII. We also thank Dr DJ Stearns-Kurosawa (IdEst, Inc) for critical reading and editing of this manuscript, and Ms Fumino Muroi for technical assistance. This work is supported by Grants-in-aid for Scientific Research #12670687 to JM and #13671078 to SM from the Ministry of Education and Science, and by Health and Labour Sciences Research Grants for Research on HIV/AIDS to KO and to YS from the Ministry of Health, Labour and Welfare.

References

1 Hoyer LW. Hemophilia A. *N Engl J Med* 1994; **330**: 38–47.
2 Kay MA, High K. Gene therapy for the hemophilias. *Proc Natl Acad Sci USA* 1999; **96**: 9973–9975.
3 Mohamed-Ali V, Pinkney JH, Coppack SW. Adipose tissue as an endocrine and paracrine organ. *Int J Obes Relat Metab Disord* 1998; **22**: 1145–1158.
4 Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem* 1988: 6352–6362.
5 Levine JA, Eberhardt NL, Jensen MD, O'Brien T. Adenoviral-mediated gene transfer to human adipocytes *in vitro*, and human adipose tissue *ex vivo* and rabbit femoral adipose tissue *in vivo*. *J Nutr Sci Vitaminol* 1998; **44**: 569–572.
6 Nagamatsu S *et al*. Adnovirus-medicated proproinsulin gene transfer into adipose tissues ameliorates hyperglycemia in obese diabetic KKA^y mice. *FEBS Lett* 2001; **509**: 106–110.
7 Miyoshi H *et al*. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science* 1999; **283**: 682–686.
8 Chen W *et al*. Lentiviral vector transduction of hematopoietic stem cells that mediate long-term reconstitution of lethally irradiated mice. *Stem Cells* 2000; **18**: 352–359.
9 Woods NB *et al*. Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood* 2000; **96**: 3725–3733.

10 Naldini L *et al*. *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; **272**: 263–267.
11 Honjo S *et al*. Experimental infection of African green monkeys and cynomolgus monkeys with a SIVAGM strain isolated from a healthy African green monkey. *J Med Primatol* 1990; **19**: 9–20.
12 Roth DA, The factor FIII transkaryotic therapy study group *et al*. Implantation of non-viral *ex vivo* genetically modified autologous dermal fibroblaststhat express B-domain deleted human factor VIII in 12 severe hemophilia A study subjects. *Blood* 2002; **100** (Suppl): 116a (abstract #430).
13 Dwarki VJ *et al*. Gene therapy for hemophilia A: production of therapeutic levels of human factor VIII *in vivo* in mice. *Proc Natl Acad Sci USA* 1995; **14**: 1023–1027.
14 Halvorsen YC *et al*. Thiazolinediones and glucocortidoids synergistically induce differentiation of human adipose tissue stromal cells: biochemical, cellular, and molecular analysis. *Metabolism* 2001; **50**: 407–413.
15 Harp JB, Franklin D, Vanderpuije AA, Gimble JM. Differential expression of signal transducers and activators of transcription during human adipogenesis. *Biochem Biophys Res Commun* 2001; **281**: 907–912.
16 Weiss GH, Rosen OM, Rubin CS. Regulation of fatty acid synthetase concentration and activity during adipocyte differentiation. *J Biol Chem* 1980; **255**: 4751–4757.
17 Mertens K *et al*. Biological activity of recombinant factor VIII variants lacking the central B-domain and the heavy-chain sequence Lys713-Arg740: discordant *in vitro* and *in vivo* activity. *Br J Haematol* 1993; **85**: 133–142.
18 Lind P *et al*. Novel forms of B-domain-deleted recombinant factor VIII molecules. Construction and biochemical characterization. *Eur J Biochem* 1995; **232**: 19–27.
19 Nakajima T *et al*. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum Gene Ther* 2000; **11**: 1863–1874.
20 Naito M *et al*. Defective sorting to secretory vesicles in the trans Golgi network is partly responsible for protein C deficiency: molecular mechanisms of impaired secretion of abnormal protein C R169W, R352W, and G376D. *Circ Res* 2003; **92**: 865–872.
21 Chua Jr S-C *et al*. Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. *Science* 1996; **271**: 994–996.
22 Yonemura H *et al*. Efficient production of recombinant human factor VIII by co-expression of the heavy and light chains. *Protein Eng* 1993; **6**: 669–674.

Gene Therapy (2004) 11, 1081–1086

www.nature.com/gt

RESEARCH ARTICLE

Long-term correction of hyperphenylalaninemia by AAV-mediated gene transfer leads to behavioral recovery in phenylketonuria mice

S Mochizuki[1,2], H Mizukami[1], T Ogura[1], S Kure[3], A Ichinohe[3], K Kojima[3], Y Matsubara[3], E Kobayahi[4], T Okada[1], A Hoshika[2], K Ozawa[1,5] and A Kume[1]

[1]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan; [2]Department of Pediatrics, Tokyo Medical University, Tokyo, Japan; [3]Department of Medical Genetics, Tohoku University Graduate School of Medicine, Sendai, Japan; [4]Division of Organ Replacement, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan; and [5]Division of Hematology, Department of Medicine, Jichi Medical School, Tochigi, Japan

Classical phenylketonuria (PKU) is a metabolic disorder caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). If untreated, accumulation of phenylalanine will damage the developing brain of affected individuals, leading to severe mental retardation. Here, we show that a liver-directed PAH gene transfer brought about long-term correction of hyperphenylalaninemia and behavioral improvement in a mouse model of PKU. A recombinant adeno-associated virus (AAV) vector carrying the murine PAH cDNA was constructed and administered to PAH-deficient mice (strain PAH^{enu2}) via the portal vein. Within 2 weeks of treatment, the hyperphenylalaninemic phenotype improved and completely normalized in the animals treated with higher vector doses. The therapeutic effect persisted for 40 weeks in male mice, while serum phenylalanine concentrations in female animals gradually returned to pretreatment levels. Notably, this long-term correction of hyperphenylalaninemia was associated with a reversal of hypoactivity observed in PAH^{enu2} mice. While locomotory activity over 24 h and exploratory behavior were significantly decreased in untreated PAH^{enu2} mice compared with the age-matched controls, these indices were completely normalized in 12-month-old male PKU mice with lowered serum phenylalanine. These results demonstrate that AAV-mediated liver transduction ameliorated the PKU phenotype, including central nervous system dysfunctions.

Gene Therapy (2004) **11**, 1081–1086. doi:10.1038/sj.gt.3302262; Published online 1 April 2004

Keywords: *phenylketonuria; adeno-associated virus vector; hyperphenylalaninemia; behavioral recovery*

Introduction

Classical phenylketonuria (PKU; McKusick OMIM 261600) is an autosomal recessive disorder resulting from a deficiency of the liver enzyme phenylalanine hydroxylase (PAH; EC 1. 14.16.1).[1] PAH converts phenylalanine (Phe) to tyrosine with the aid of tetrahydrobiopterin (BH_4), and a deficiency of this enzyme causes accumulation of Phe and abnormal metabolites in the body fluids. If untreated, this condition irreversibly damages the central nervous system (CNS) of the patient, resulting in severe mental retardation. Conventional therapy for PKU consists of dietary restriction of Phe, which can prevent neuronal damage if initiated very early in life. However, the strict and complicated diet is often associated with poor compliance, particularly in adolescents and young adults. Premature termination of the diet leads to declined neuropsychological function, and noncompliance in pregnant women with PKU can produce devastating defects in the offspring referred to as 'maternal PKU syndrome'. A permanent cure is therefore awaited to liberate patients from dietary restrictions, and gene therapy is an attractive novel approach to this goal.

However, previous preclinical studies of PKU gene therapy have revealed that a long-term cure of PKU is a formidable task. Generally, recombinant retroviral vectors cannot deliver the normal PAH gene to the liver at sufficient levels to overcome hyperphenylalaninemia.[2,3] Adenoviral-mediated PAH gene transfer achieved a complete reduction of serum Phe in PKU animals, but the therapeutic effects did not persist and the vector was not effectively readministered due to immune responses against the virus.[4,5] On the other hand, adeno-associated virus (AAV) vectors comprise another class of gene delivery vehicles, which have been shown to stably transduce nondividing cells such as hepatocytes, muscle fibers and neurons.[6–8]

In this study, we evaluated a recombinant AAV vector carrying the PAH gene in a mouse model of PKU (PAH^{enu2} strain).[9–11] A missense mutation (F263S) in the PAH gene was introduced into BTBR mouse strain by chemical mutagenesis, resulting in a loss of enzyme activity. Consequently, the homozygous PAH^{enu2} mice

Correspondence: Dr A Kume, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1 Yakushiji, Minamikawachi, Tochigi 329-0498, Japan
Received 24 December 2003; accepted 11 February 2004; published online 1 April 2004

share many phenotypic characteristics with human PKU patients, such as profound hyperphenylalaninemia (>20 mg/dl; normal 1–2 mg/dl), behavioral disturbances and hypopigmentation. Previous work suggested that at least 10% of normal PAH activity would be required to prevent hyperphenylalaninemia in PKU mice.[4,5]

Results

Construction of the recombinant AAV vector

We first evaluated vectors derived from AAV serotypes 1 through 5. Recombinant AAV vectors containing the mouse erythropoietin (Epo) gene were infused into the mouse portal vein, and the serum Epo levels were determined. Among them, the AAV5-derived virion yielded the highest Epo concentration (unpublished results).[12,13] Next, we tested several promoters to drive the Epo gene in the context of AAV5. We found that the CAG promoter was the strongest in transgene expression in the liver (unpublished results).[14] This promoter consists of the human cytomegalovirus (CMV) immediate-early enhancer, the chicken β-actin promoter, and a chicken β-actin/rabbit β-globin composite intron.

Based on these results, we constructed an AAV vector as shown in Figure 1 (AAV5/CAG-mPAH). A recombinant AAV plasmid pAAV5/CAG-mPAH was comprised of the CAG promoter, the murine PAH cDNA and the SV40 late polyadenylation signal flanked by the AAV5 inverted terminal repeats (ITRs shown as hairpin loops in Figure 1). The vector DNA was then packaged into the AAV5 capsid through an adenovirus-free, transient transfection protocol.[15]

Correction of hyperphenylalaninemia

For liver-targeted gene transfer, the vector was injected into 5–7-week-old PAH^{enu2} mice via the portal vein. We injected male PKU mice with 3×10^{12} vector genomes (vg) ($n=3$), 1×10^{13} vg ($n=4$), 3×10^{13} vg ($n=3$) or 1×10^{14} vg ($n=3$) of AAV5/CAG-mPAH per animal. Female PKU mice were infused with 1×10^{13} vg ($n=4$), 3×10^{13} vg ($n=4$) or 1×10^{14} vg ($n=5$) per animal.

Serum Phe levels were determined prior to the infusion, biweekly until 12 weeks postinfusion, and every 4 weeks thereafter (Figure 2). Before gene transfer (week 0), all PAH-deficient mice showed profound hyperphenylalaninemia (33.7±3.4 mg/dl; range 29.3–43.5 mg/dl; $n=27$). The degree of hyperphenylalaninemia was not significantly different between males (33.2±2.6 mg/dl; $n=14$) and females (34.3±4.1 mg/dl; $n=13$). Figure 2a shows the kinetics of blood Phe in male PKU mice receiving different doses of AAV5/CAG-mPAH. A striking decrease in serum Phe was observed 2-4 weeks after gene transfer. With the lowest vector dose (3×10^{12} vg), serum Phe was only slightly lowered after 2 weeks (from 35.0±1.6 to 28.1±7.0 mg/dl; $P=0.18$ by paired t-test), but was significantly lowered after 4 weeks (15.6±6.9 mg/dl; $P=0.027$ by paired t-test). With higher vector doses (1×10^{13}, 3×10^{13} and 1×10^{14} vg), the serum Phe level was clearly lowered ($P=0.001$, 0.006 and 0.002 by paired t-test, respectively) to a therapeutic range (<10 mg/dl) in 2 weeks. At 4 weeks postinfusion, each cohort of male mice recorded the lowest serum Phe. In particular, it was completely normalized in the mice treated with 3×10^{13} vg (1.4±0.5 mg/dl) and 1×10^{14} vg (1.2±0.5 mg/dl) of AAV5/CAG-mPAH.

The reduced serum Phe levels were stably maintained for 40 weeks. Complete correction of hyperphenylalaninemia (<2 mg/dl) persisted in the mice treated with the highest vector dose (1×10^{14} vg), and the mice receiving

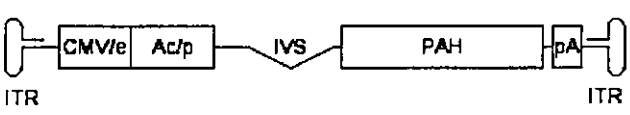

Figure 1 Structure of the AAV5/CAG-mPAH vector. The vector consisted of a CMV immediate-early enhancer (CMV/e), the chicken β-actin promoter (Ac/p), a chicken β-actin/rabbit β-globin composite intron (IVS), the 1.4 kb murine PAH cDNA (PAH) and the SV40 late polyadenylation signal (pA) flanked by the AAV5 inverted terminal repeats (ITRs shown as hairpin loops).

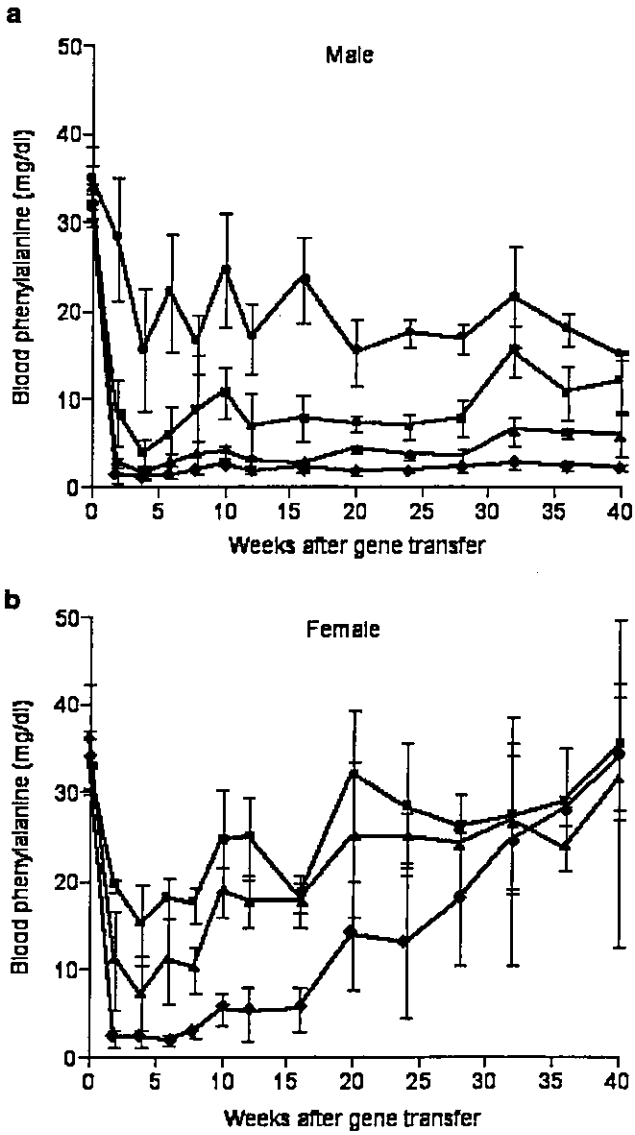

Figure 2 Persistence of the recombinant AAV-mediated correction of hyperphenylalaninemia in male (a) and female (b) PKU mice. Serum Phe concentration was determined prior to vector infusion (week 0) and periodically after gene transfer, and represented as the mean±s.d. for each treatment group. The applied vector dose was 3×10^{12} vg (circles), 1×10^{13} vg (squares), 3×10^{13} vg (triangles) or 1×10^{14} vg (diamonds) per animal.

the second highest dose (3×10^{13} vg) stayed in a well-controlled therapeutic range (<6 mg/dl). Mice receiving lower vector doses (3×10^{12} and 1×10^{13} vg) showed moderate correction of hyperphenylalaninemia, with significant long-term efficacy of the single AAV infusion.

Figure 2b shows the kinetics of serum Phe in female PKU mice after receiving 1×10^{13}, 3×10^{13} or 1×10^{14} vg of AAV5/CAG-mPAH. The vector administration was effective in the female PKU mice, too, but the dose–response and duration were different from the male mice; that is, about three times more vector was required for the female mice to exhibit an equivalent reduction in serum Phe (Figure 3). At 4 weeks postinfusion when the reduction was at its maximum, 1×10^{13} vg of AAV5/CAG-mPAH lowered serum Phe by 50% in the female mice, while the same level of reduction was achieved by 3×10^{12} vg in the males. Similarly, an 80% reduction was achieved by 3×10^{13} vg in the females, whereas only 1×10^{13} vg were required in the males. Complete correction of hyperphenylalaninemia was achieved by 1×10^{14} vg in the females, while it was achieved by 3×10^{13} vg as well as 1×10^{14} vg in the males. As for duration, the therapeutic effect did not persist in the female PKU mice as seen in the males. Serum Phe levels in each female cohort remained low until 8 weeks post-gene transfer, but gradually rose thereafter. With vector doses of 1×10^{13} and 3×10^{13} vg, serum Phe was greater than 20 mg/dl at 20 weeks, and returned to the pretreatment level at 40 weeks. With the highest dose (1×10^{14} vg), serum Phe was kept below 10 mg/dl until 16 weeks, then gradually increased and returned to the pretreatment level at 40 weeks.

Although we did not kill the animals for enzyme assay, previous studies on adenoviral-mediated gene transfer to PAH^{enu2} mice allowed us to estimate the PAH activity accomplished by our vector. These studies showed, in good agreement, that the threshold PAH activity to correct hyperphenylalaninemia was about 10% of normal mice.[4,5] As shown in Figure 3, male PKU mice given 3×10^{12} vg and females given 1×10^{13} vg of the vector showed 50–60% reduction in serum Phe; we speculate that these mice would express about 5% of normal PAH activity. On the other hand, male PKU mice given 3×10^{13} or 1×10^{14} vg and females given 1×10^{14} vg completely recovered from hyperphenylalaninemia, hence their liver PAH activities would be 10% of normal or greater. Male PKU mice given 1×10^{13} vg (ca. 90% reduction in serum Phe) and females given 3×10^{13} vg of AAV (ca. 80% reduction) would have 5–10% of normal PAH activity.

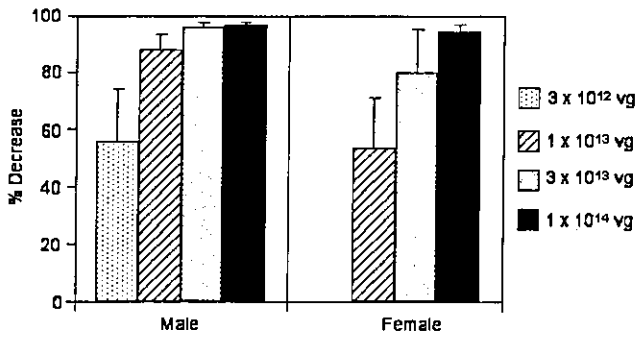

Figure 3 Vector dose-dependent reduction of serum Phe in PKU mice. Percent reduction of serum Phe was calculated by the following formula: {(serum Phe at week 0)−(serum Phe at 4 weeks)} × 100/(serum Phe at week 0). Bars represent the mean ± s.d. of % reduction of serum Phe in PKU mice treated with 1×10^{12} vg (dotted bar), 1×10^{13} vg (hatched bar), 3×10^{13} vg (gray bar), or 1×10^{14} vg of AAV5/CAG-mPAH (black bar).

Correction of hypopigmentation

Associated with extended reductions in serum Phe, hypopigmentation in the AAV-treated PKU mice was ameliorated. While the coat color of untreated mice remained grayish brown, hair darkening in the mice receiving higher vector doses was observed 2 weeks post-transduction, and the mice grew black hair in 4 weeks which was indistinguishable from that of wild-type (WT) BTBR mice (Figure 4). Male PKU mice with reduced serum Phe retained black hair throughout the observation period, while female PKU mice lost pigmentation as the therapeutic effect diminished.

Recovery from hypoactivity following PAH gene transfer

Along with persistent correction of hyperphenylalaninemia and hypopigmentation, we observed behavioral

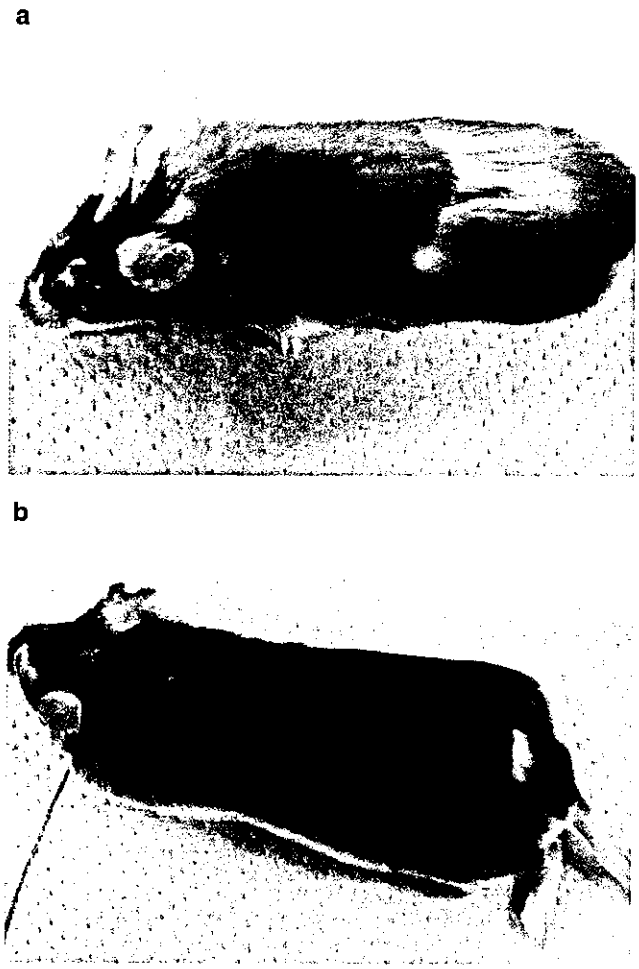

Figure 4 Correction of hypopigmentation in PKU mouse following PAH gene transfer. (a) Untreated PKU mouse showing grayish brown hair, easily distinguished from wild-type and PAH$^{+/-}$ heterozygous BTBR mice. (b) By 8 weeks after PAH gene transfer, the PKU mouse with complete correction of hyperphenylalaninemia recovered black coat color and was indistinguishable from normal BTBR mice.

recovery in AAV-treated PKU mice. Consistent with previous studies showing abnormal behavior and cognitive deficits in PAH^{enu2} mice,[9,16,17] we found that untreated PKU (PAH$^{-/-}$) mice were relatively hypoactive compared with WT (PAH$^{+/+}$) and heterozygous carrier (PAH$^{+/-}$) animals. The hypoactivity became apparent with aging, and the difference was significant among animals aged 10 months or older. Figure 5 shows the results of behavior tests on the 12-month-old animals. As for total locomotion over 24 h, the untreated PKU mice displayed about 70% of normal activity (Figure 5a, $P<0.01$ by Student's *t*-test). On the other hand, the AAV-administered male mice without hyperphenylalaninemia exhibited significantly higher 24-h locomotion than the untreated mice (Figure 5a, $P=0.001$ by Student's *t*-test). Indeed, the AAV-treated animals showed a normal activity level in this test.

Similarly, PAH gene transfer improved the PKU animals' exploratory activity in a novel environment.

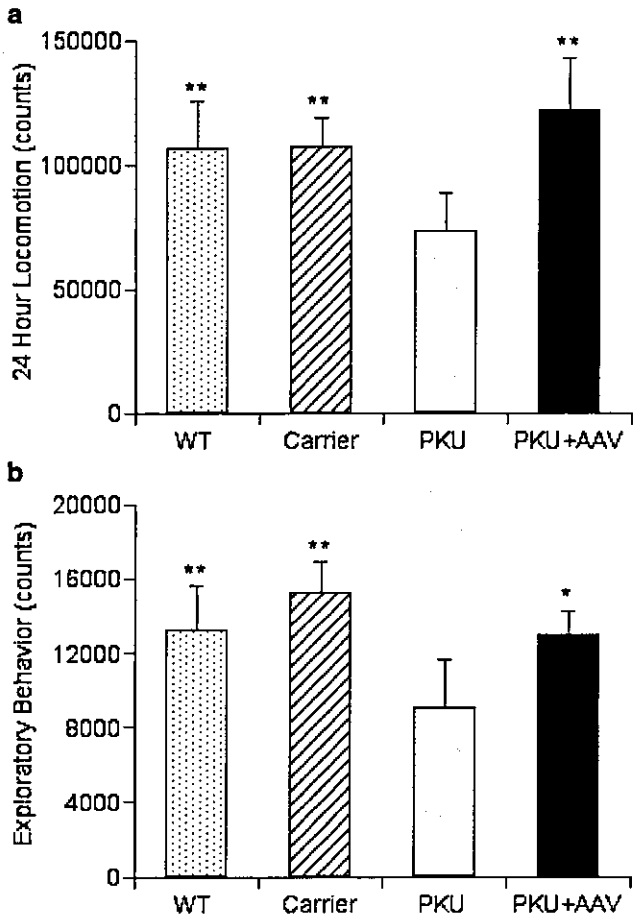

*Figure 5 Recovery from hypoactivity following PAH gene transfer. (a) Total locomotion over 24 h. Mice were placed under an infrared sensor and ambulatory activity was recorded consecutively for 24 h. Wild-type (WT), heterozygous and AAV-treated PKU mice exhibited significantly higher locomotory activity than untreated PKU mice (**$P<0.01$ by Student's t-test). (b) Exploratory behavior. Mice were placed in a novel cage under a sensor and ambulatory activity was quantified during the first 2 h in the chamber. This test showed significantly higher performance by WT, heterozygous and AAV-treated PKU mice than untreated PKU animals (**$P<0.01$ and *$P<0.05$ by Student's t-test). Bars represent the mean ± s.d. of WT mice (WT; dotted bar), heterozygous mice (Carrier; hatched bar), untreated PKU mice (PKU; gray bar), and AAV-transduced PKU mice (PKU + AAV; black bar).*

When settled in a novel cage, the untreated PKU males showed 60–70% of normal exploratory activity (Figure 5b, $P<0.01$). On the other hand, PKU mice that had recovered from hyperphenylalaninemia explored as vigorously as WT animals, and their activity level was significantly greater than that of untreated PKU mice ($P=0.015$). These results clearly indicate that the PAH gene transfer improved the CNS function of PKU mice in addition to correction of hyperphenylalaninemia.

Discussion

In this study, we demonstrated that AAV-mediated transduction of the PKU mouse liver brought about a long-term cure of the disease. A single infusion of AAV5/CAG-mPAH completely normalized the hyperphenylalaninemic phenotype in male PKU mice, and the longevity of the therapeutic effect was superior to any other gene delivery vehicle thus far. Although not thoroughly investigated, the result suggests that the transgene was transcriptionally active during observation, and that no significant immune response was elicited against the transduced hepatocytes in the animals. In addition, infusion of very large amounts of AAV did not show any toxicity in the treated mice. The vector safety and viability may be further improved by adopting recently developed purification methods, such as iodixanol gradient, affinity or ion-exchange chromatography.[18–21]

A major problem we encountered in this study was that the same AAV vector was less effective in female PKU mice. About three times more vector was required to achieve an equivalent reduction of serum Phe seen in males, and the therapeutic effect was shorter in duration. The underlying mechanisms for these female-specific phenomena are currently unknown. Davidoff *et al*[22] recently reported similar observations that AAV2- and AAV5-derived vectors less efficiently transduced livers of female mice than males. They suggested that the difference was due to an androgen-dependent pathway for augmenting hepatocyte transduction, but its mode of action is undetermined. Since precise molecular events involved in recombinant AAV-mediated transduction remain obscure, the critical step accounting for the observed sex difference is also a mystery. Androgen may augment the uptake of AAV particles into the cell or traffic them to the nucleus; alternatively, it may stabilize the AAV genome in an episomal state, or enhance vector integration into the host chromosome. Of these possibilities, the last one is less likely, because only a small fraction (<10%) of recombinant AAV genome was reportedly integrated into the mouse hepatocytes.[23] If there is an androgen-dependent mechanism to retain the AAV genome in an episomal state in the liver, lack of such machinery would allow gradual loss of the vector DNA in females, thereby transgene-derived PAH activity would descend over time as we observed. Other possibilities accounting for the lower therapeutic efficacy include transcriptional silencing and an immune response against AAV-transduced hepatocytes, although the latter is unlikely to occur only in female mice.

In genetic treatment of autosomal and acquired disorders, sex-dependent transduction raises a novel issue. Development of more efficient vectors may over-

come this problem, or other approaches can be considered. In terms of PKU, the disease-associated pathology is caused by accumulated Phe in the body fluids. Thus, it can be prevented by 'heterologous gene therapy', ie targeting tissues other than hepatocytes. Several investigators have exploited this strategy because of difficulties with liver transduction and safety concerns. Christensen *et al*[24] transduced primary keratinocytes with genes for PAH and GTP cyclohydrolase I, which is the rate-limiting enzyme in BH_4 biosynthesis. They showed that the cells cleared excess Phe in the culture medium, and suggested that engraftment of enough of these cells may function as a metabolic sink for detoxification. Harding *et al*[25] investigated the potential of skeletal muscle as a PAH-expressing organ. Using a transgenic technique, they created mice expressing PAH in the skeletal muscle but not in the liver. These mice showed hyperphenylalaninemia at baseline, but serum Phe significantly decreased when the animals were supplemented with BH_4. A similar approach to bone marrow cells was unsuccessful,[26] and careful consideration is required in translating these transgenic studies into human applications.

A novel finding in this study was that AAV infusion lead to behavioral improvement in addition to correction of hyperphenylalaninemia and hypopigmentation. To our knowledge, this is the first demonstration that a gene-based approach to PKU actually benefited CNS function. It has been reported that free amino acid and amine contents are dramatically reduced in the PAH^{enu2} mouse brain, as in untreated human PKU patients.[27,28] Presumably, the observed hypoactivity in older PKU mice was associated with the abnormal synthesis of biogenic amines, whereas the abnormality was reversed in AAV-treated PKU animals with normal serum Phe. We speculate that the behavioral recovery in these mice represents an analogous situation in which dietary restriction of Phe can improve some neuropsychiatric symptoms in untreated PKU patients. It is of particular interest whether an earlier genetic intervention can prevent irreversible neuronal defects in PKU and preserve more sophisticated CNS function such as memory. The AAV vectors and PAH^{enu2} mice will provide an attractive system to address such prompting questions.

Materials and methods

AAV vector construction

To isolate murine PAH cDNA (GenBank Accession # NM008777), liver mRNA was prepared from a C57BL/6J mouse (from Clea Japan, Tokyo, Japan) with Isogen reagent (Nippon Gene, Toyama, Japan) and an mRNA Purification kit (Amersham Pharmacia Biotech, Little Chalfont, UK). The PAH cDNA was cloned by reverse transcriptase-directed polymerase chain reaction using a Superscript II cDNA synthesis kit (Invitrogen, Grand Island, NY, USA). The CAG promoter was derived from pCAGGS (a gift from Dr J Miyazaki, Osaka University, Osaka, Japan).[14] The AAV5 vector plasmid pAAV5LacZ and a helper plasmid 5RepCapA were generous gifts from Dr JA Chiorini (National Institutes of Health, Bethesda, MD, USA).[12] To construct a recombinant AAV5 vector plasmid for PAH expression, the expression cassette of pAAV5LacZ was replaced with the CAG promoter, the murine PAH cDNA and the SV40 late polyadenylation signal, and the plasmid was referred to as pAAV5/CAG-mPAH (Figure 1).

Recombinant AAV stocks were propagated according to an adenovirus-free, three-plasmid transfection protocol described previously.[15] Briefly, subconfluent 293 cells (4×10^8 cells per 10 trays) in Cell Factories 10 (Nunc, Roskilde, Denmark) were cotransfected with 650 μg of the vector plasmid pAAV5/CAG-mPAH, 650 μg of the AAV helper plasmid 5RepCapA and 650 μg of the adenoviral helper plasmid pLadeno1 (identical to pVAE2AE4-2 in Matsushita *et al*;[15] kindly provided by Avigen, Alameda, CA, USA) by using the calcium phosphate precipitation method for a period of 6 h. Cells were harvested 72 h after transfection and lysed by three freeze–thaw cycles. The crude viral lysate was incubated with Benzonase (Merck KGaA, Darmstadt, Germany) and centrifuged. Finally, the clear supernatant was subjected to two rounds of CsCl density-gradient ultracentrifugation for purification. The physical titer of the viral stock was determined by DNA dot blot and hybridization with the murine PAH cDNA probe, along with plasmid standards. Typically, we obtained 5×10^{13} vg of AAV5/CAG-mPAH from a culture container (10 trays).

Transduction of mouse liver

All animal experiments were carried out in accordance with our institutional guidelines. PAH^{enu2} mice were generous gifts from Dr T Shiga (University of Tsukuba, Tsukuba, Japan), and a colony was established at Jichi Medical School (Tochigi, Japan). PKU mice used for *in vivo* gene transfer were 5–7 weeks of age. Mice were anesthetized with isoflurane inhalation followed by laparotomy. A 300 μl of saline suspension containing 3×10^{12}–1×10^{14} vg of AAV5/CAG-mPAH was slowly injected into the portal vein using an insulin syringe with a 29-gauge needle (Terumo, Tokyo, Japan).

Serum Phe assay

Serum Phe was measured by an enzymatic microfluorometric assay using an Enzaplate PKU-R kit (Bayer Medical, Tokyo, Japan). Mice were tail phlebotomized and the blood was spotted onto a mass-screening grade paper filter (#545, provided by Advantec Toyo, Tokyo, Japan). A 3 mm diameter disc was punched out from the dried blood spot and placed in a 96-well plate. Phe was eluted from the disc and incubated with Phe dehydrogenase, an NAD-dependent enzyme, and resazurin. The enzyme reaction produces NADH, which in turn converts resazurin to resorufin with the aid of diaphorase. The resultant resorufin was measured on a Fluoroskan Ascent plate reader (Labsystems, Helsinki, Finland) with a 544/590 nm filter set.

Mouse behavior tests

Mice were tested at 12 months of age. To measure locomotory activity over 24 h, the home cage of the mouse was placed under an infrared sensor that detects thermal radiation from animals (Supermex; Muromachi Kikai, Tokyo, Japan).[29] Ambulation was scored by a personal computer interfaced to the sensor. Alternatively, exploratory behavior was tested by placing the mouse in a novel cage under the infrared sensor.

Ambulatory activity was quantified during the first 2 h in the chamber.

Acknowledgements

We are grateful to Dr JA Chiorini for pAAV5LacZ and 5RepCapA, Dr J Miyazaki for pCAGGS, Dr T Shiga for PAH^{enu2} mice, Avigen for pLadeno1, and Advantec Toyo for #545 filter paper. We also thank Dr Y Hakamata for technical assistance in the animal experiments. This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare, Japan.

References

1 Scriver CR, Kaufman S. Hyperphenylalaninemia: phenylalanine hydroxylase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The Metabolic and Molecular Basis of Inherited Diseases*. McGraw-Hill: New York, 2001, pp 1667–1724.
2 Liu T-J, Kay MA, Darlington GJ, Woo SLC. Reconstitution of enzymatic activity in hepatocytes of phenylalanine hydroxylase-deficient mice. *Somat Cell Mol Genet* 1992; **18**: 89–96.
3 Eisensmith RC, Woo SLC. Gene therapy for phenylketonuria. *Eur J Pediatr* 1996; **155** (Suppl 1): S16–S19.
4 Fang B *et al*. Gene therapy for phenylketonuria: phenotypic correction in a genetically deficient mouse model by adenovirus-mediated hepatic gene transfer. *Gene Therapy* 1994; **1**: 247–254.
5 Nagasaki Y *et al*. Reversal of hypopigmentation in phenylketonuria mice by adenovirus-mediated gene transfer. *Pediatr Res* 1999; **45**: 465–473.
6 Nathwani AC *et al*. Sustained high-level expression of human factor IX (hFIX) after liver-targeted delivery of recombinant adeno-associated virus encoding the hFIX gene in rhesus macaques. *Blood* 2002; **100**: 1662–1669.
7 Kay MA *et al*. Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; **24**: 257–261.
8 Muramatsu S *et al*. 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* 2002; **13**: 345–354.
9 Shedlovsky A, McDonald JD, Symula D, Dove WF. Mouse models of human phenylketonuria. *Genetics* 1993; **134**: 1205–1210.
10 McDonald JD, Charlton CK. Characterization of mutations at the mouse phenylalanine hydroxylase locus. *Genomics* 1997; **39**: 402–405.
11 McDonald JD *et al*. The phenylketonuria mouse model: a meeting review. *Mol Genet Metab* 2002; **76**: 256–261.
12 Chiorini JA, Kim F, Yang L, Kotin RM. Cloning and characterization of adeno-associated virus type 5. *J Virol* 1999; **73**: 1309–1319.
13 Mingozzi F *et al*. Improved hepatic gene transfer by using an adeno-associated virus serotype 5 vector. *J Virol* 2002; **76**: 10497–10502.
14 Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; **108**: 193–200.
15 Matsushita T *et al*. Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Therapy* 1998; **5**: 938–945.
16 Zagreda L *et al*. Cognitive deficits in a genetic mouse model of the most common biochemical cause of human mental retardation. *J Neurosci* 1999; **19**: 6175–6182.
17 Cabib S *et al*. The behavioral profile of severe mental retardation in a genetic mouse model of phenylketonuria. *Behav Genet* 2003; **33**: 301–310.
18 Hermens WT *et al*. Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum Gene Ther* 1999; **10**: 1885–1891.
19 Zolotukhin S *et al*. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Therapy* 1999; **6**: 973–985.
20 Auricchio A, O'Connor E, Hildinger M, Wilson JM. A single-step affinity column for purification of serotype-5 based adeno-associated viral vectors. *Mol Ther* 2001; **4**: 372–374.
21 Kaludov N, Handelman B, Chiorini JA. Scalable purification of adeno-associated virus type 2, 4, or 5 using ion-exchange chromatography. *Hum Gene Ther* 2002; **13**: 1235–1243.
22 Davidoff AM *et al*. Sex significantly influences transduction of murine liver by recombinant adeno-associated viral vectors through an androgen-dependent pathway. *Blood* 2003; **102**: 480–488.
23 Nakai H *et al*. Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction *in vivo*. *J Virol* 2001; **75**: 6969–6976.
24 Christensen R, Kolvraa S, Blaese RM, Jensen TG. Development of a skin-based metabolic sink for phenylalanine by overexpression of phenylalanine hydroxylase and GTP cyclohydrolase in primary human keratinocytes. *Gene Therapy* 2000; **7**: 1971–1978.
25 Harding CO *et al*. Metabolic engineering as therapy for inborn errors of metabolism – development of mice with phenylalanine hydroxylase in muscle. *Gene Therapy* 1998; **5**: 677–683.
26 Harding CO *et al*. Expression of phenylalanine hydroxylase (PAH) in erythrogenic bone marrow does not correct hyperphenylalaninemia in *Pah^{enu2}* mice. *J Gene Med* 2003; **5**: 984–993.
27 Puglisi-Allegra S *et al*. Dramatic brain aminergic deficit in a genetic mouse model of phenylketonuria. *NeuroReport* 2000; **11**: 1361–1364.
28 Pascucci T, Ventura R, Puglisi-Allegra S, Cabib S. Deficits in brain serotonin synthesis in a genetic mouse model of phenylketonuria. *NeuroReport* 2002; **13**: 2561–2564.
29 Masuo Y, Matsumoto Y, Morita S, Noguchi J. A novel method for counting spontaneous motor activity in the rat. *Brain Res Protoc* 1997; **1**: 321–326.

The Journal of Molecular Biology Research, Protocols, Reviews, and Applications

Molecular Biotechnology

Editor-in-Chief: John M. Walker

Volume 27, Number 1, May 2004

Featured in this issue:

HUMANA PRESS

RESEARCH

Separate Control of Rep and Cap Expression Using Mutant and Wild-Type LoxP Sequences and Improved Packaging System for Adeno-Associated Virus Vector Production

*Hiroaki Mizukami, Takashi Okada, Yoji Ogasawara, Takashi Matsushita, Masashi Urabe, Akihiro Kume, and Keiya Ozawa**

Abstract

Adeno-associated virus (AAV) vectors are a practical choice for gene transfer, and demand for them is increasing. To cope with the necessity in the near future, we have developed a number of approaches to establish packaging cell lines for the production of AAV vectors. In our previous study, a highly regulated expression of large Rep proteins was obtained by using the Cre–loxP switching system. Therefore, in the present study, to regulate Cap expression as well, we developed an inducible expression system for both Rep and Cap proteins by using an additional set of mutant loxP sequences. The mutants possess two base alterations in the spacer region of loxP and recombine specifically with the same counterpart in the presence of Cre. By using two separate plasmids, one with mutant and the other with wild-type loxP sequences, the expression of two different proteins can be induced simultaneously by Cre recombinase. When the LacZ-encoding plasmid vector was used as a packaging model, a significant packaging titer of 2.1×10^{10} genome copies per 10-cm dish was obtained. These results indicate the importance of controlling Cap expression, in addition to Rep, to achieve an optimum production rate for AAV vectors.

Index Entries: Cre–loxP; mutant loxP; dependovirus; AAV vector; packaging cell line; 293 cells.

1. Introduction

Adeno-associated viruses (AAVs) are currently being investigated as a gene transfer vector for a variety of applications. Several diseases are thought to be prime candidates for AAV vector-medicated therapeutic intervention; clinical trials are already set out for the correction of hemophilia B *(1)*, and for Parkinson's disease in the near future *(2,3)*. However, one drawback to the use of AAV is difficulty in making large-scale preparations. To improve the process of preparation, we have developed packaging cell lines for AAV *(4,5)*. Early studies indicate that in addition to Rep, relatively large amounts of Cap proteins should be expressed to achieve a high titer of virus production, despite the fact that constitutive expression of these proteins has cytotoxic consequences *(6,7)*. Therefore, controlling the expression profiles for these proteins has vital significance. For this purpose, the Cre–loxP system is one of the best-known approaches as an induction system, and in our previous study we used loxP sequences to regulate Rep expression *(4)*. However, there was a limitation to this approach in that Cap expression could not be regulated efficiently; only Rep expression could be regulated. To control Cap expression in addition to Rep expression, we used mutant loxP sequences along with the wild type. These mutant loxP sequences are shown to recombine specifically with each

*Author to whom all correspondence and reprint requests should be addressed: Dr. Keiya Ozawa, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, 3311-1, Yakushiji, Minamikawachi-machi, Kawachi-gun, Tochigi, 329-0498, Japan.E-mail: kozawa@ms2.jichi.ac.jp

other, but less efficiently with wild-type sequences on treatment with Cre *(8)*. In the present study, we compared the efficiency of recombination and designed plasmids to express optimal amounts of Rep and Cap proteins on Cre treatment. By optimizing these parameters, we developed a packaging cell line with improved production rate compared with our prototype cell line.

2. Materials and Methods

2.1. Cells and Plasmids

A human embryonic kidney cell line, known as 293 cells *(9)*, was maintained as described previously *(4)*. Plasmid ploxlox (a gift from Dr. Jamey D. Marth), which contains two adjacent loxP sequences in the same direction, was used as a backbone for the wild-type loxP *(10)*. To make plas-mids with mutant loxP, the sequences corresponding to the spacer region of loxP were mutated by synthesizing oligonucleotides based on published sequence information (**Fig. 1A**) *(8)*. Briefly, the spacer region of wild-type loxP constitutes ATGTATGC; for the loxP (V) and loxP(S), the sequences correspond to 5'-ATGT GTAC-3' and 5'-AAGTATCC-3', respectively. The CAG promoter (a gift from Dr. J Miyazaki, Osaka University, Japan) *(11)*, neomycin resistance gene, blasticidin S resistance gene (Invitrogen Corp., Carlsbad, CA), bacterial LacZ sequence, and AAV sequences corresponding to *p5*, *rep*, and *cap* genes were excised and ligated to complete plasmids named CAPBPL, CAVBVL, CASBSL, CAPBPC, and p5SNSR, respectively (*see* **Fig. 1B**), using standard techniques as reported previously *(4)*.

2.2. Induction of Recombination and Demonstration of Gene Expression

Plasmids encoding *LacZ* gene with the "stuffer" sequences between the two loxP sequences (CAPBPL, CAVBVL, CASBSL) were introduced into 293 cells using a standard calcium phosphate transfection technique *(12)*. Briefly, 1 μg of plasmid was mixed with 150 μL of 0.3*M* $CaCl_2$ and 2X HBS buffer and added to a single 6-well chamber. Six hours later, the medium was replenished. To assess the efficiency of recombination, a Cre-expressing adenovirus vector (AxCANCre, a gift from Dr. I. Saito) was applied to the culture thereafter at an MOI of 1 *(13)*. At various time-points, cells were dislodged, lysed, and β-galactosidase activity was measured by orthonitrophenyl-β-galactosidase assay (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. Lysates were then subjected to Western analyses, either with anti-Rep (clone 303.9, Progen, Heidelberg, Germany) or anti-Cap (clone B1, Progen, Heidelberg, Germany), as reported previously *(4)*.

2.3. Development of Clones

Seven micrograms of each plasmid were used to transfect one 10-cm dish of 293 cells using a standard calcium-phosphate method at 70% of confluence. Forty-eight hours later, the cells were replated to several dishes and exposed to the selection medium containing both 800 μg/mL of G418 and 10 μg/mL of blasticidin S. The selection medium was replenished every 3 d. After 2 to 3 wk of selection, individual clones were recovered and amplified in 12-well plates in the presence of a half concentration of the selection medium of G418 and Blasticidin S. When a clone grew to semiconfluence in a 12-well plate, it was assumed to be established and was subjected to the analysis for packaging titer. The clones were numbered according to the order of establishment.

2.4. Titration of Vector Production

Established clones were further expanded, replated in new 12-well plates, then transfected with 0.5μg of the vector plasmid containing the *LacZ* gene cassette (driven by cytomegalovirus [CMV] promoter) flanked by two ITR sequences. Six hours after transfection, the medium was replenished, and Cre-expressing adenoviruses (AxCANCre) were added at an MOI of 1. Forty-eight hours later, cells were collected, subjected to three cycles of freeze–thawing, and treated with deoxyribonuclease I (Takara Bio, Inc., Ohtsu, Japan) for 30 min at 370°C as indicated by the manufacturer. The samples were quantified using dot-blot analysis. Known copy numbers of LacZ-expressing plasmid was used as controls.