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.

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 ($p < 0.01$).

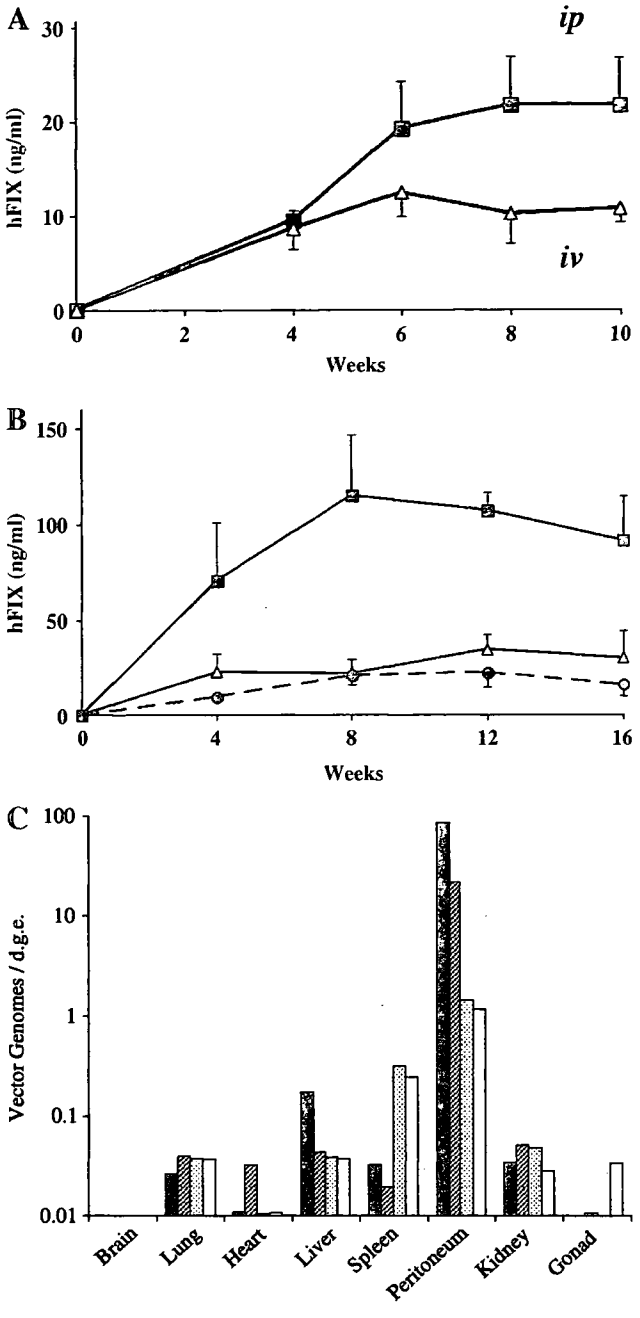

Figure 1. Analysis of C57BL/6 mice after intraperitoneal (*ip*) or intravenous (*iv*) injection of AAV vectors. (A) Plasma hFIX concentration after *ip* (n = 4, closed squares) and *iv* (n = 5, open triangles) administration of the AAV5-CMV-hFIX vector (1×10^{10} genome copies/body weight (g.c./g)) in 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.

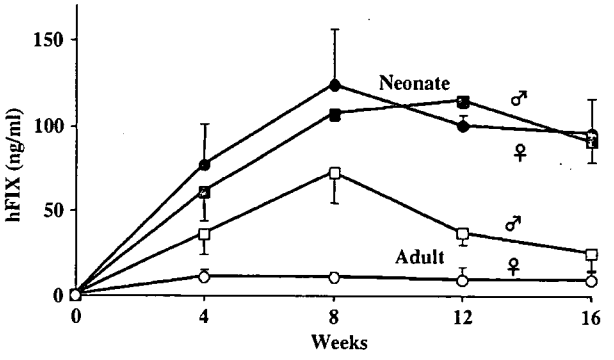

Figure 2. Plasma hFIX concentrations in mice after *ip* injections into different groups. The AAV5-CMV-hFIX vector at a dose of 3×10^{11} g.c./g was injected into C57BL/6 neonatal males (n = 6, closed squares), neonatal females (n = 4, closed circles), adult males (n = 4, open squares), and adult females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

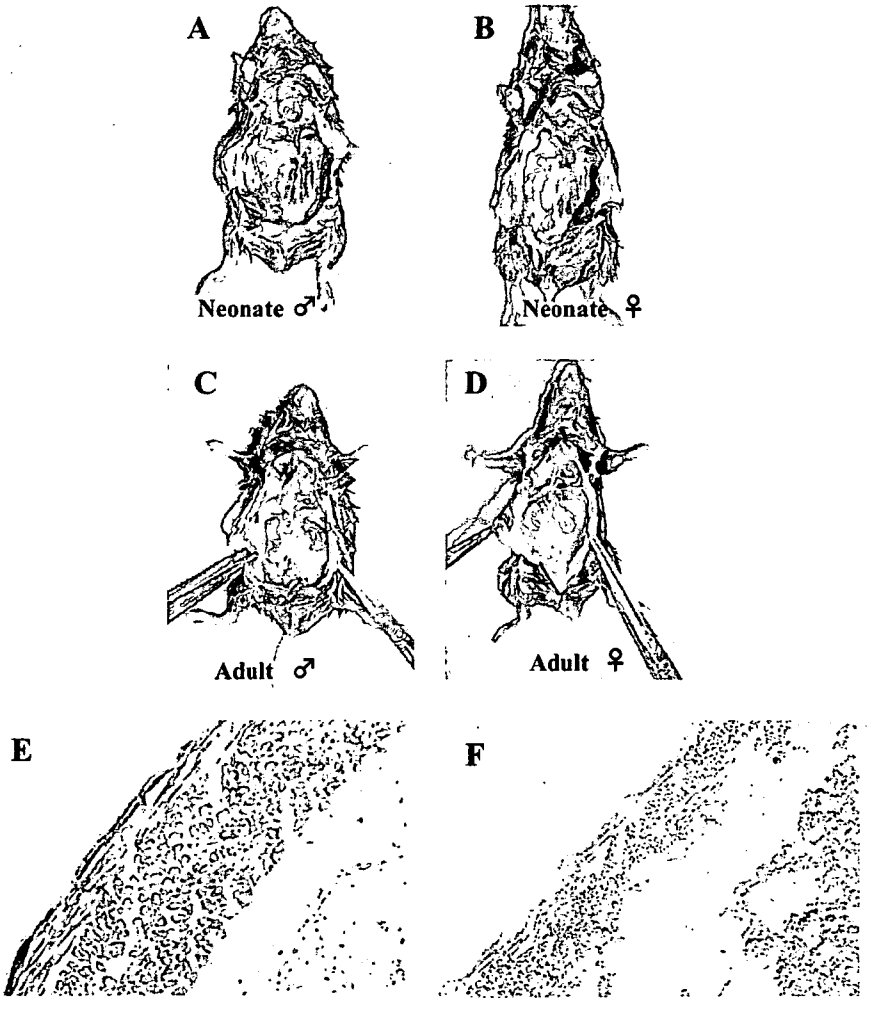

Figure 3. β-Galactosidase expression at 8 weeks after *ip* injection of the AAV5-CMV-LacZ vector at a dose of 1×10^{11} g.c./g in the C57BL/6 mice (A–D). X-gal staining was performed after removal of the intraperitoneal organs. Histochemistry with β-galactosidase performed on tissues from the neonatal male peritoneum after the injection stained the mesothelium (E) and the untransduced control (F) (final magnification x100)

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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 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).

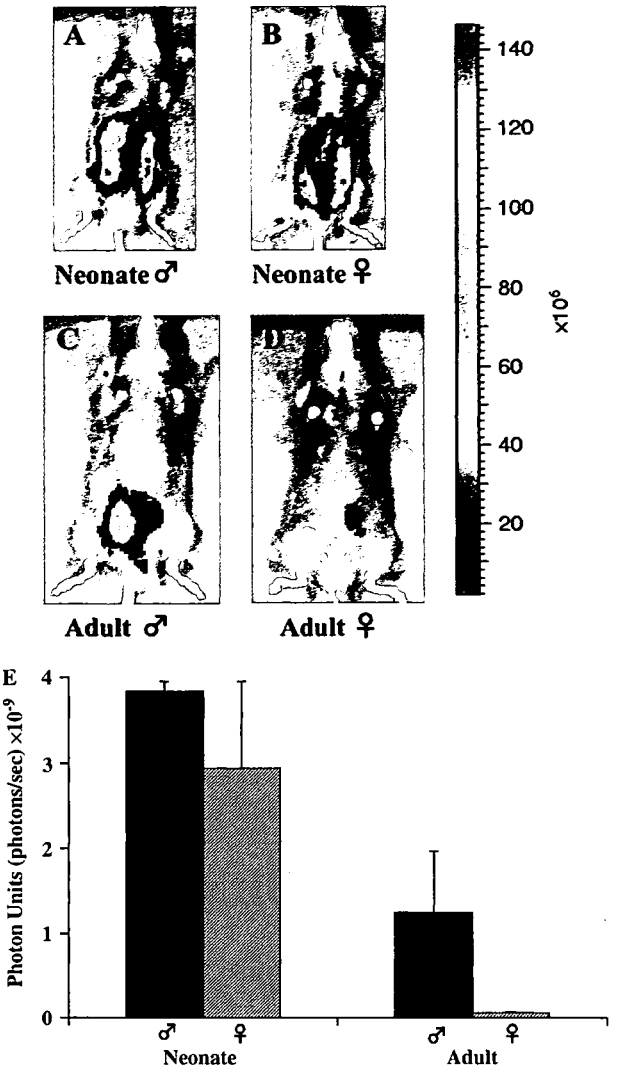

Figure 4. *In vivo* **bioluminescence imaging at 10 weeks after** *ip* **injection of the AAV5-CMV-Luc vector at a dose of 5×10^9 g.c./1.5 g in the C57BL/6 mice (A–D). Images were analyzed under the same condition, and the reference color bar, indicating the photon units (photons/s), is the same for 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)**

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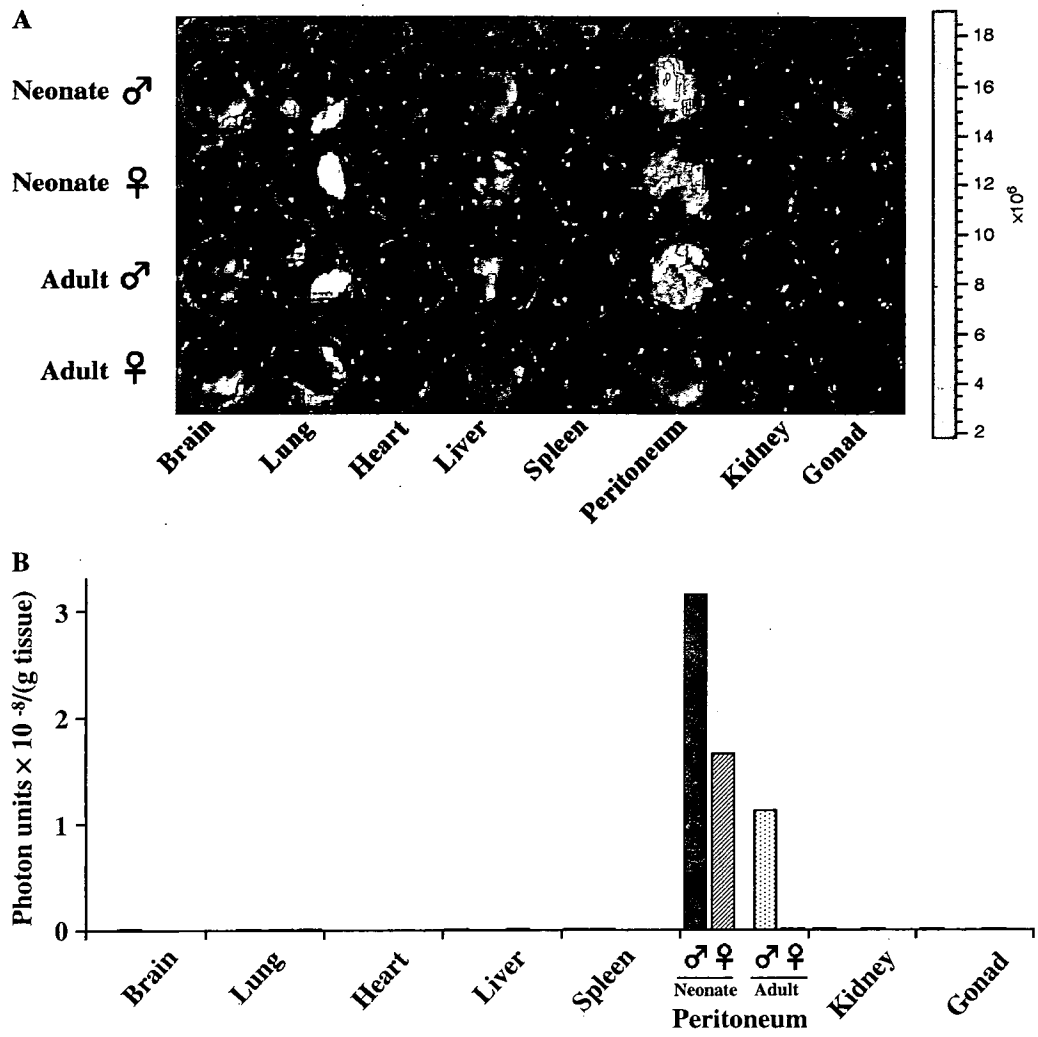

Figure 5. Analysis of tissue-specific expression after *ip* injection of the AAV5-CMV-Luc vector. (A) *Ex vivo* bioluminescence images of injected neonates and adults are shown. Mice were sacrificed at 10 weeks after vector injection and the major organs were extracted and placed into each well of a 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.

Acknowledgements

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SHORT COMMUNICATION

Removal of Empty Capsids from Type 1 Adeno-Associated Virus Vector Stocks by Anion-Exchange Chromatography Potentiates Transgene Expression

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Production of recombinant adeno-associated virus (rAAV) results in substantial quantities of empty capsids or virus-like particles (VLPs), virus protein shells without the vector genome. The contaminating VLPs would interfere with transduction by competing for cell-surface receptors and, when administered *in vivo*, contribute to antigen load, which may elicit a stronger immune response. Density-gradient ultracentrifugation provides a means to separate VLPs from rAAV particles, but is not feasible for large-scale preparations of vectors. Since the compositions of the VLP and vector differ by the single-stranded DNA genome, we hypothesized that the isoelectric point of the vector may differ from that of the VLP. In an attempt to separate type 1 rAAV particles from VLPs by ion-exchange chromatography, we tested a number of buffer systems and found that trimethylammonium sulfate, or $[(CH_3)_4N]_2SO_4$, effectively separated rAAV1 particles from VLPs. The rAAV1-GFP chromatographically separated from VLPs induced stronger GFP expression in HEK293 cells than rAAV1-GFP contaminated with VLPs. The transduction of mouse muscles with rAAV1-SEAP (secreted form of alkaline phosphatase) isolated from VLPs also showed higher serum SEAP levels than rAAV1-SEAP with VLPs. These results suggest that chromatographic separation of rAAV1 from empty capsids increased the efficacy of rAAV1.

Key Words: AAV vector, empty capsid, antichaotropic ion, chromatography

Recombinant adeno-associated virus (rAAV) is one of the promising gene transfer vectors and efficiently transduces neurons, hepatocytes, and skeletal muscle in rodent, dog, and nonhuman primate models [1]. AAV vectors produced with serotype 1 capsid protein transduce skeletal muscles particularly well compared to serotypes 2, 4, 5, and 6 [2]. Obtaining clinically meaningful levels of a therapeutic protein depends on several factors, including the amount of particles administered. A human clinical trial using rAAV2 expressing coagulation factor IX (up to 10^{13} particles/kg) in hemophilia B patients has been conducted, resulting in a partial, but transient, amelioration of symptoms. Thus, extrapolating from these earlier studies, more than 10^{15} particles of rAAV2 would be required for the complete correction of hemorrhagic tendency [3]. Using serotypes with higher biological activities may reduce the dose; even so, large animal studies comparing the efficacy of rAAV1 and other serotypes indicated that large particle numbers of rAAV1 would still be required for human application [2,4].

rAAV is usually produced by plasmid transfection of HEK293 cells with two or three plasmids: AAV helper plasmid encoding *rep* and *cap* genes devoid of inverted terminal repeat (ITR) sequences, AAV vector plasmid harboring the therapeutic gene between the ITRs, and a plasmid containing a minimal set of adenovirus helper genes, E2A, VARNA, and E4orf6. Since the structural and nonstructural genes, as well as the *cis*-acting elements of AAV type 1 and AAV type 2, are highly conserved, it is possible to package the gene of interest between the type 2 ITRs into the coexpressed type 1 capsid [5], which is composed of VP1, VP2, and VP3 polypeptides with a stoichiometry of 1:1:10. In transfected HEK293 cells, the expression of the three structural proteins forms virus-like particles (VLPs) or empty capsids independent of vector DNA replication and packaging. The maturation of particles occurs during vector DNA replication and

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particles with vector genomes appear [6]. However, the fraction of VLPs that acquire vector DNA remains a minor component of the total particles in the cell. The ratio of empty to filled particles can range from 10:1 to 4:1 [7,8]. Without a DNA payload, the presence of VLPs in the rAAV stocks would diminish the effect by competing for cell-mediated processes, such as receptor binding and uptake, as well as providing a source of antigen that may elicit a stronger immune response *in vivo* against AAV vectors [9]. It is therefore desirable to eliminate empty capsids from rAAV vector stocks. The only established method to isolate rAAV from empty capsids is density gradient ultracentrifugation using CsCl or other density materials, which relies on the difference in the buoyant density between DNA-filled and empty particles. However, ultracentrifugation is not readily adaptable to the large-scale preparation of rAAV, especially for clinical grade material. In addition to density gradient centrifugation, other physicochemical differences may lead to exploitable processes for separating VLPs from vector particles. Although affinity column chromatography does not distinguish between vector and VLP, the process is scalable and also provides a higher level of purification than CsCl ultracentrifugation [7,10,11]. In addition, chromatography can preserve more infectious rAAV particles [10].

All members of the *Parvoviridae* are structurally similar and have linear, single-stranded DNA genomes. It is possible that the presence of encapsidated DNA alters the isoelectric point (p*I*) of the AAV particles. We postulated that if the p*I* of rAAV differs from that of the empty capsid, then rAAV separation from empty capsid is possible using high-resolution chromatography.

For starting materials, we used empty and filled rAAV1 particles independently obtained by CsCl density ultracentrifugation and subsequent chromatography, as described in the Fig. 1 legend. We characterized each type of particle by density, DNA content, protein composition, and biological activity (data not shown). We confirmed the purity of rAAV or empty particles by silver staining of the samples resolved on an SDS–PAGE gel (Fig. 1A). We examined the elution profile of rAAV1 containing the green fluorescent protein (GFP) gene and type 1 empty capsid on a high-resolution anion-exchange column, Mini Q 4.6/50 PE (Amersham Biosciences, Piscataway, NJ, USA). Full rAAV particles, equivalent to 5×10^{10} vector genomes (vg), or an equivalent quantity of empty particles, were bound to the column in a low-salt buffer of 20 mM Tris–HCl (pH 8.4), 20 mM NaCl, and 4% glycerol; they were eluted with a linear 20–300 mM NaCl gradient at pH 8.4. Although there is overlap at the base of the peaks, Fig. 1A shows that the empty particles (broken line) eluted at a lower salt concentration than rAAV (solid line). While the resolution of the empty and filled particle fractions was not optimal, the ability to elute the two types of particles selectively was a very encouraging result. To increase the resolution of the eluted particle peaks, we surveyed an extensive range of elution buffers and found that the use of so-called antichaotropic ions, such as NH_4^+, $(CH_3)_4N^+$, PO_4^{3-}, and SO_4^{2-}, was capable of resolving rAAV from empty capsids better than using NaCl gradients. Fig. 1B shows a representative chromatogram of the mixture of rAAV1-GFP particles and VLPs eluted with a linear 20–300 mM Na^+ or $(CH_3)_4N^+$ gradient. Among the buffers we tested, $[(CH_3)_4N]_2SO_4$ or trimethylammonium sulfate most effectively separated the rAAV particles (F) from empty capsids (E). We also examined a weaker anion, $(C_2H_5)_4N^+$, for the separation of rAAV, which more efficiently separated the rAAV particles from empty capsids. However, the solution containing $(C_2H_5)_4N^+$ was viscous and disrupted the rAAV particles. NH_4^+ also isolated rAAV1 from empty particles. The ammonium ion, however, is volatile at high pH and the ammonium solution is not stable over time.

The pH of the buffers is also important for chromatography. The elution of rAAV and empty particles at different pH is shown in Fig. 1C. We loaded the mixture of purified rAAV and empty particles onto the column and eluted them with a linear 20–300 mM $(CH_3)_4N^+$ gradient at pH 7.5, 8.0, 8.5, or 9.0. The separation of the two peaks was better at pH 8.5 or 9.0 than at lower pH. Since the empty and filled AAV particles are unstable at a higher pH [12], we used buffers at pH 8.5 in the subsequent experiments.

Our final goal was to develop a chromatographic method for the purification of a large quantity of type 1 rAAV particles free of empty particles. We next tested the separation of approximately 10^{13} vg of rAAV1-GFP from empty particles. We produced rAAV particles and released them from HEK293 cells, as described in the legend to Fig. 1. After low-speed centrifugation, we again centrifuged the cleared cellular lysate for 10 min at 30,000*g* at 4°C and filtered the supernatant through 0.45- and 0.2-μm membrane filters. We diluted the lysate four times with a dilution buffer of 20 mM Tris–HCl (pH 8.4), 2 mM $MgCl_2$, and 4% glycerol and loaded it onto a 10-mm × 60-cm Tricorn column (Amersham Biosciences) packed with POROS HQ 50-μm medium (Applied Biosystems, Foster City, CA, USA). rAAV1 was eluted with a linear 50–400 mM NaCl gradient (250 ml). We collected the fractions containing rAAV1 and diluted them threefold with the dilution buffer and loaded them onto the second anion-exchange column (5 mm × 10 cm) packed with POROS HQ 10-μm matrix (Applied Biosystems). The rAAV1 was again eluted with a linear 50–400 mM NaCl gradient (25 ml). We further purified the rAAV1 by gel filtration column chromatography, as described in the Fig. 1 legend. We mixed the fractions containing rAAV together and diluted them with 4 volumes of the dilution buffer and loaded them onto a high-resolution column (5 mm × 20 cm) filled with POROS HQ 10-μm equilibrated with 25 mM *N*-methyldiethanolamine (pH 8.5) and 10 mM $[(CH_3)_4N]_2SO_4$. Bound viral particles were eluted

with a 10–125 mM $[(CH_3)_4N]_2SO_4$ gradient over 38 ml at a flow rate of 0.5 ml/min. A representative chromatogram (Fig. 2A) shows that the two peaks were observed as expected. The peak that appeared earlier or later corresponded to empty capsids or rAAV particles. The analysis of each fraction by Western blotting with an anti-type 5 VP antibody, which was cross-reactive with type 1 VP protein (middle), revealed that the first, larger peak contained much more AAV VP protein (fractions 19 through 22). The second, smaller peak also contained VP protein, although the amount was smaller (fractions 23 through 26). Quantification using real-time PCR indicated that the majority of rAAV vector genome was in fractions 23 through 26 (bottom), corresponding to the second peak fractions. Electron microscopy of a sample from the pooled peak fractions confirmed that the earlier peak corresponded to empty capsids and the later one corresponded to rAAV particles (insets in Fig. 2A). Since a single run was not sufficient to separate completely the empty from the full capsids, we repeated the high-resolution chromatography step. After the first separation, more than 90% of contaminating empty capsids was removed. The second run was able to eliminate empty particles further and we obtained a rAAV stock with less than 5% empty particles. Table 1 summarizes the recovery of rAAV1 particles after high-resolution column chromatography for the removal of empty particles. After two rounds of chromatography, we were able to recover approximately 50% of rAAV1-GFP.

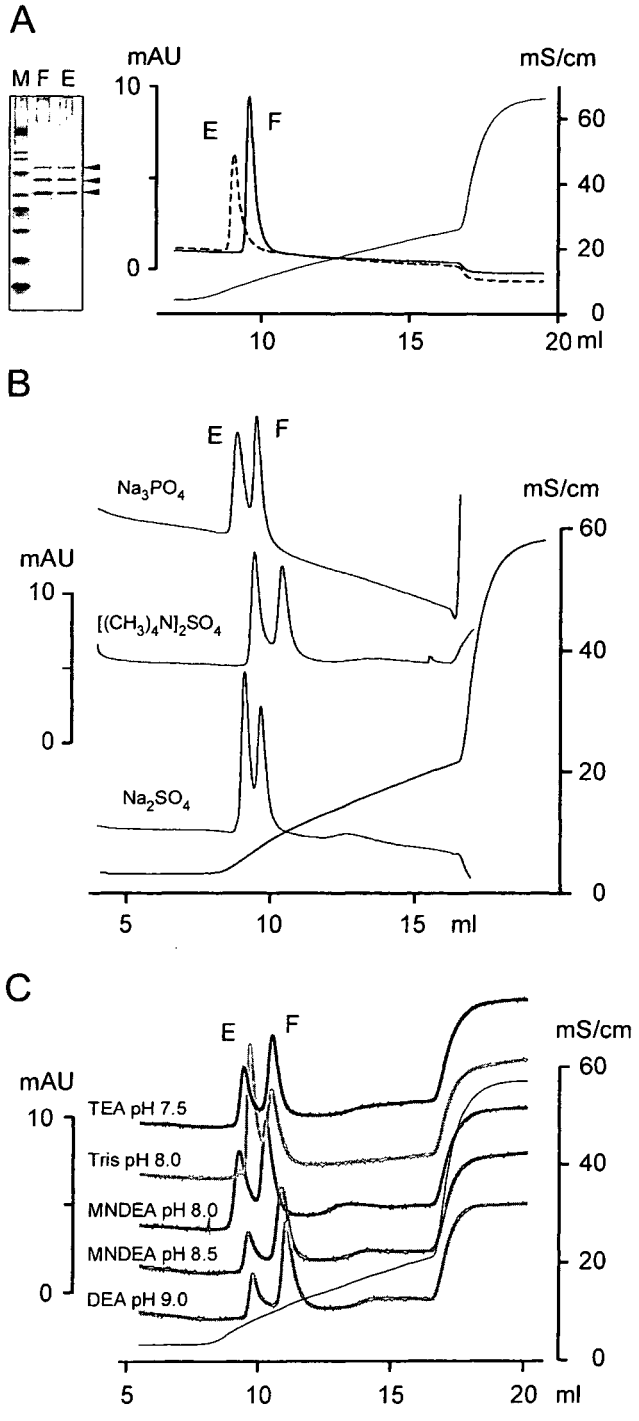

FIG. 1. (A) Elution profile of DNA-filled or rAAV1 and empty particles on a high-resolution anion-exchange column. For the production of rAAV1-GFP, HEK293 cells at 80% confluency (approximately 10^5 cells/cm^2) in a 225-cm^2 flask were cotransfected with 26.7 μg of an AAV vector plasmid harboring a humanized GFP gene (Stratagene, Palo Alto, CA, USA) under the control of the cytomegalovirus immediate early gene promoter (CMV) between the type 2 ITRs, 26.7 μg of an AAV1 helper plasmid carrying type 2 *rep* and type 1 *cap* genes [5], and 26.7 μg of an adenovirus helper plasmid using the calcium precipitation method. Two days after transfection, the cells were pelleted by centrifugation and lysed in 2 ml (per 225-cm^2 flask) of lysis buffer (20 mM Tris–HCl (pH 8.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (Merck, Darmstadt, Germany), 60 U/ml benzonase (Merck)) and incubated at 37°C for 30 min. After low-speed centrifugation, solid CsCl was added to the lysate to produce a buoyant density of 1.36 g/cm^2 and the samples were centrifuged for 24 h at 36,000 rpm at 21°C in an SW40Ti rotor (Beckman Coulter, Fullerton, CA, USA). rAAV1-containing fractions were collected and spun once again. rAAV1-GFP was then loaded on a gel-filtration column (HiPrep 16/60 Sephacryl S-300 HR; Amersham Biosciences) preequilibrated with 50 mM Hepes (pH 7.4), 0.3 M NaCl, 2 mM $MgCl_2$ to eliminate further cellular contaminants. Type 1 empty capsids were also generated in 293 cells transfected with a type 1 AAV helper plasmid alone and purified as for rAAV particles except for the CsCl density of 1.30 g/cm^2. Their purity was confirmed by silver staining of the SDS–PAGE gel using the SilverQuest silver staining kit (Invitrogen, Carlsbad, CA, USA). Arrows indicate VP1, VP2, and VP3 polypeptides. Approximately 5×10^{10} vg of rAAV1-GFP (F) or an equivalent quantity of type 1 empty particles (E) was loaded onto a Mini Q 4.6/50 PE column (Amersham Biosciences) controlled by an ÄKTA FPLC system (Amersham Biosciences). The bound particles were eluted over 10 ml with a linear 20 to 300 mM NaCl gradient at pH 8.4. The profile is represented as the absorbance at 280 nm (mAU). Buffer conductance (mS/cm) is indicated by the thin line. M, molecular weight standards. (B) Chromatogram of the mixture of AAV1 and empty particles in antichaotropic buffers with a 20 to 300 mM Na^+ or $[(CH_3)_4N]^+_2$ gradient. The earlier elution from the column represents empty particles (E) and DNA-filled or rAAV1 (F) eluted at a higher salt concentration. (C) The effect of pH on the elution of rAAV1 and empty particles. A buffer of 25 mM triethanolamine (TEA) at pH 7.5, Trizma (Tris) at pH 8.0, *N*-methyldiethanolamine (NMDEA) at pH 8.0 or 8.5, or diethanolamine (DEA) at pH 9.0 with a 10 to 150 mM $[(CH_3)_4N]_2SO_4$ gradient was used for elution.

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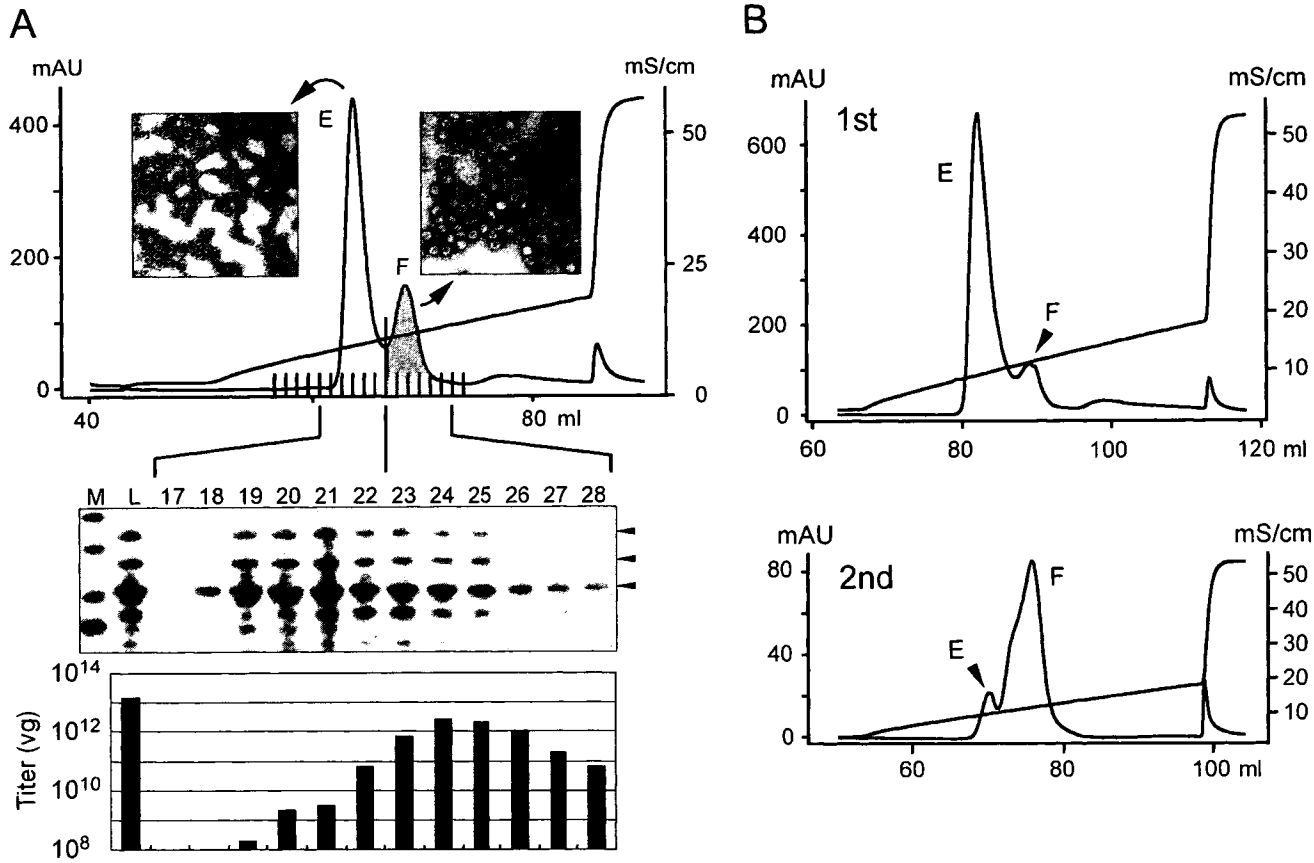

FIG. 2. (A) A representative chromatogram of a rAAV1-GFP preparation. Approximately 10^{13} vg of vector particles were generated and purified as described in the legend to Fig. 1 and finally loaded onto a 5-mm × 20-cm Tricorn column (Amersham Biosciences) packed with POROS HQ 10-μm matrix (Applied Biosystems) equilibrated with 25 mM *N*-methyldiethanolamine (pH 8.5) and 10 mM $[(CH_3)_4N]_2SO_4$. Bound viral particles were eluted with a 10–125 mM $[(CH_3)_4N]_2SO_4$ gradient over 38 ml at a flow rate of 0.5 ml/min. F and E indicate filled and empty particles, respectively. Electron microscopy of negatively stained samples from each peak is shown as an inset. After 1-ml fractionation samples were analyzed on a 4–12% NuPAGE gel (Invitrogen), the separated proteins were transferred to a Durapore membrane (Millipore, Bedford, MA, USA) and incubated with a rabbit polyclonal anti-type 5 VP antibody. After incubation with a secondary anti-rabbit immunoglobulin G labeled with horseradish peroxidase (Pierce, Milwaukee, WI, USA), chemiluminescent signals were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce) (middle). The fraction number is indicated above each lane. VP1, VP2, and VP3 capsid proteins are indicated by arrows. A sample from each fraction was also analyzed by real-time PCR to quantify the GFP vector DNA using a primer set specific to the CMV promoter, as previously described [15]. M, molecular weight standard; L, loaded sample. (B) An example of separation of rAAV1-GFP from empty particles by two runs of the high-resolution column chromatography. The first run was able to eliminate more than 90% of the contaminating empty capsids (E) from rAAV1-GFP (F). Reloading of the eluate from the first run further removed the contaminating empty particles.

We assessed the biological activity of the rAAV1-GFP isolated by column chromatography. We infected HEK293 cells with rAAV1-GFP samples, before or after chromatographic removal of empty particles, at the particle per cell numbers indicated (Fig. 3A). Seven days after transduction, we examined the cells under a fluorescence microscope. To quantify the GFP fluorescence, we also analyzed the cells by flow cytometry as described [13]. The analysis gave the percentage positive cells and the average GFP fluorescence, which refers to the average fluorescence intensity in the subpopulation of GFP-positive cells. The fluorescence volume represents a summation of GFP fluorescence within the subpopulation of GFP-positive cells, which was calculated to be equal to the fraction of GFP-positive cells in the sample population times the mean fluorescence intensity. When HEK293 cells were infected with either rAAV1-GFP at more than 10^4 vg per cell, both vectors transduced almost all the infected cells. However, the volume of GFP

TABLE 1: Recovery of rAAV1-GFP after removal of empty capsids

Preparations	Load	After 1st run (%)	After 2nd run (%)
#1	1.2×10^{13}	7.6×10^{12} (63.3)	5.0×10^{12} (41.7)
#2	3.3×10^{13}	2.4×10^{13} (72.7)	1.7×10^{13} (51.5)
#3	1.3×10^{13}	8.6×10^{12} (66.2)	6.8×10^{12} (52.3)

Number of rAAV1 particles was determined by real-time PCR. The percent recovery was calculated by dividing the number of rAAV1 particles loaded onto the first high resolution column by the number of rAAV particles recovered after chromatography.

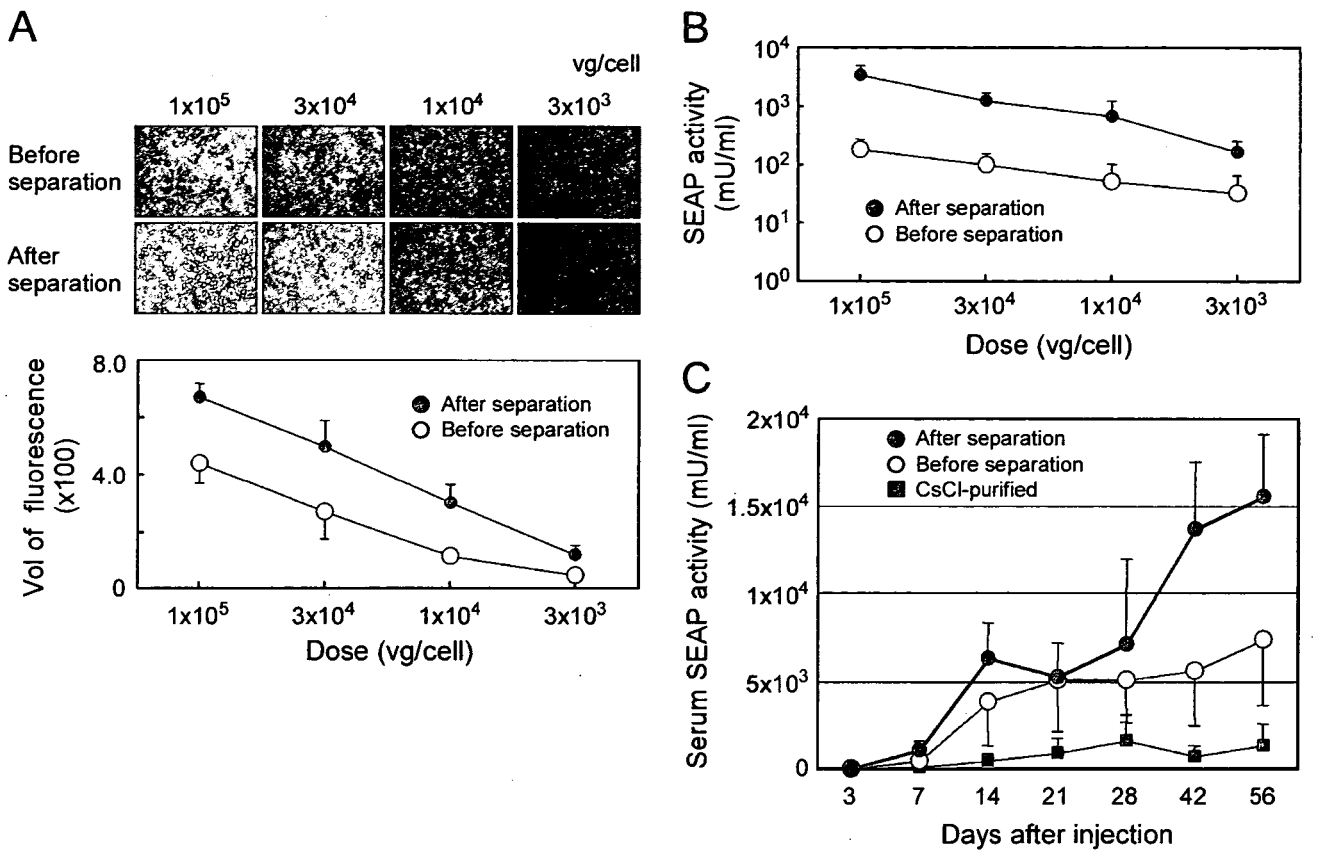

FIG. 3. (A) Transduction of HEK293 cells with rAAV1-GFP chromatographically separated from empty particles. 293 cells were infected with rAAV1-GFP before or after column chromatography intended to separate empty particles at the doses indicated. The GFP-expressing cells were analyzed by flow cytometry. The volume of GFP fluorescence was obtained by calculating (the fraction of GFP-positive cells) × (the average GFP fluorescence). Data represent means and standard deviation of experiments performed in triplicate. (B) The SEAP activity of the culture supernatant after infection of HEK293 cells with rAAV1-SEAP contaminated with or without empty particles. HEK293 cells were infected with type 1 SEAP vector before or after chromatographic removal of empty capsids at doses ranging from 1×10^5 through 3×10^3 vg per cell in triplicate. Results are expressed as means ± SD. (C) The serum SEAP levels after injection of rAAV1-SEAP into mouse muscles. A total of 10^{10} vg of rAAV1-SEAP particles before or after high-resolution chromatography or rAAV1-SEAP purified by CsCl ultracentrifugation was injected into mouse tibialis anterior muscles in triplicate and blood was taken from 3 through 56 days after injection.

fluorescence obtained by rAAV1-GFP separated from empty capsids was larger than that by rAAV contaminated with empty particles. We also infected HEK293 cells with rAAV1 expressing the human secreted alkaline phosphatase (SEAP). We excised the SEAP gene from pSEAP2-Basic (Clontech, Mountain View, CA, USA) with *Nru*I and *Sal*I and blunt-ended the resulting 1.8-kb fragment and inserted it between the type 2 ITRs. We used the resulting plasmid for transfection of HEK293 cells and purified rAAV1-SEAP as described above. We measured the SEAP activity in the culture supernatants 1 week after infection by using the SEAP Report Gene Assay (Roche Diagnostics, GmbH, Penzberh, Germany) according to the manufacturer's instructions. The rAAV1-SEAP separated from empty particles induced higher SEAP levels than rAAV1-SEAP contaminated with empty capsids at the doses tested (Fig. 3B). These results suggested that contaminating empty capsids interfered with the transduction of HEK293 cells by rAAV1.

To investigate the efficacy of rAAV1 *in vivo*, we injected rAAV1-SEAP (10^{10} vg) into mouse tibialis anterior muscles in triplicate. We used rAAV1-SEAP before chromatographic separation of empty capsids and CsCl-banded rAAV1-SEAP as controls. Fig. 3C shows the time course of the serum SEAP levels after the injection of SEAP vectors. rAAV1-SEAP purified by anion-exchange chromatography induced the highest levels of serum SEAP activity. The rAAV1-SEAP purified by column chromatography, but contaminated with empty particles, expressed lower levels of SEAP. CsCl-banded SEAP vector showed the lowest level, although the difference in the SEAP activity among the three groups was not statistically significant due to the small number of animals employed. The serum SEAP level at 56 days postinjection with the rAAV1 vector from which empty capsids were removed by chromatography was 10 times higher than that with the rAAV-SEAP from which empty capsids were excluded by CsCl ultracentrifugation, which may be due

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to the impurity and/or the damage of CsCl-purified rAAV1 [14]. These results again indicated that the removal of empty particles from rAAV1 stocks by chromatography potentiated the SEAP expression in the muscles.

In summary, we report here a method for the selective removal of empty capsids from type 1 AAV vector. The chromatographic separation obtained pure rAAV1 stocks contaminated with less than 5% empty capsids. This method can remove empty capsids without the loss of the efficacy of rAAV1 and is easily scalable to a large volume. It will be useful for the purification of large quantities of rAAV1 for large-animal or human applications.

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REGULAR ARTICLE

Phenotype correction of hemophilia A mice with adeno-associated virus vectors carrying the B domain-deleted canine factor VIII gene☆

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Abstract Adeno-associated virus (AAV) vectors carrying the B domain-deleted canine FVIII (BDD cFVIII) gene utilizing the β-actin minimum promoter (167b) pseudotyped with serotype 1 (AAV1-β-actin-cFVIII) and serotype 8 (AAV8-β-actin-cFVIII) were developed to express cFVIII in hemophilia A mice. FVIII clotting activities measured by the APTT method increased in hemophilia A mice with intramuscular injection of AAV1-β-actin-cFVIII in a dose-dependent manner. Therapeutic FVIII levels ($2.9 \pm 1.0\%$) in hemophilia A mice with the AAV1-β-actin-cFVIII dose of 1×10^{12} gc/body were achieved, suggesting partial correction of the phenotype with AAV1-β-actin-cFVIII vectors. FVIII clotting activity levels in hemophilia A mice with intravenous injection of AAV8-β-actin-cFVIII also were increased dose-dependently, achieving therapeutic FVIII levels (5–90%) in hemophilia A mice with the AAV8-β-actin-cFVIII doses of $1–3 \times 10^{11}$ gc/body and supernormal FVIII levels (180–670%) in hemophilia A mice with the AAV8-β-actin-cFVIII dose of 1×10^{12} gc/body. Transduction of the liver with AAV8-β-actin-cFVIII is superior to transduction of skeletal

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muscles with AAV1cFVIII regarding the FVIII production and antibody formation. These data suggested that both AAV1 and AAV8 vectors carrying the FVIII gene utilizing a minimum promoter have a potential for hemophilia A gene therapy.

Introduction

Hemophilia A is an inherited X-linked life-threatening bleeding disorder caused by abnormalities in the factor VIII (FVIII) gene that lead to deficiency of FVIII and bleeding diathesis. Hemophilia is considered suitable for gene therapy because it is caused by a single gene abnormality and therapeutic coagulation factor levels may well be in a wide range (5–100%) without strict gene regulation [1–3]. Gene therapy is expected to provide an alternative to current FVIII supplemental therapy because it may be able to prevent lethal intracranial bleeding episodes and provide a good quality of life without bleeding. Among a variety of vectors, Adeno-associated virus (AAV) vectors are thought to be ideal for transfer of therapeutic genes since they are derived from non-pathogenic viruses and have been demonstrated to provide sustained transgene expression in non-dividing cells with little toxicity, although delivery of the FVIII gene using AAV vectors is limited by their small packaging capacity [4].

The dual AAV vector system that utilized two AAV2 vectors separately carrying the FVIII heavy chain gene and the FVIII light chain gene was successful for expressing functional FVIII molecules and correction of phenotypes of hemophilia A mice [5]. A recent report has shown that construction of single AAV vectors carrying the 4.5-kb B domain-deleted (BDD) canine FVIII (cFVIII) gene can be packaged in AAV vectors using the 543-base (b) DNA fragments composed of the insulin-like growth factor binding protein (IGBP) promoter, an enhancer element, and an intron, although the packaging efficiency for incorporation of the cFVIII gene into the AAV vectors was low [6].

The liver would appear to be the appropriate target organ for transduction because FVIII is physiologically synthesized in this organ. However, if any adverse reaction to the therapy occurs, removal of the liver would be an unacceptable solution. In fact, minor liver dysfunction upon AAV2 vector injection into the hepatic artery was reported in clinical trials for hemophilia B gene therapy, whereas no liver dysfunction was observed upon injection of the same vector into the skeletal muscles in the same series of clinical trials [7,8]. Although the precise mechanisms of these phenomena have not yet been elucidated, the T cell response to viral capsid was thought to be one of the causes of the liver injuries [8]. In this respect, surgically removable organs such as skeletal muscles may well be the alternative target organs.

To explore the possibility that skeletal muscles transduced with AAV vectors could produce FVIII resulting in increase of FVIII levels in the circulation, we packaged the BDD FVIII gene in AAV vectors using the same promoter and compare production of FVIII in the skeletal muscles and in the liver that were transduced with AAV vectors. We developed AAV vectors carrying the BDD cFVIII gene utilizing the β-actin minimum promoter (167b) and tried to express canine FVIII in hemophilia A mice. Recent studies on recombinant AAV vectors have shown that AAV serotypes have tropism, suggesting that a specific AAV serotype vector can be used for gene delivery to certain organs [3,6,9]. AAV serotype 1 (AAV1) may be the best AAV serotype for transduction of skeletal muscles and the AAV serotype 8 (AAV8) is superior to other AAV serotypes for transduction of the liver [6,9], thus in this study, we constructed AAV1 and AAV8 vectors carrying the BDD cFVIII gene and studied efficacies of these vectors for FVIII transgene expression, long term transgene expression, and neutralizing antibody formation to the transgene products in the hemophilia A mice.

Materials and methods

Vector construction

The full-length human FVIII (hFVIII) cDNA was a generous gift from Dr. J.A. van Mourik (Blood Coagulation, Sanquin, Amsterdam, Netherlands) and the human B domain deleted (BDD) FVIII (hFVIII) cDNA was generated by PCR-based mutagenesis as described [10,11]. The canine FVIII (cFVIII) cDNA was a generous gift from Dr. Brownlee (Chemical Pathology Unit, University of Oxford, UK) and the BDD canFVIII cDNA also was generated by PCR-based mutagenesis. The intervening amino acid sequence of the heavy chain and the light chain of BDD cFVIII was $RSFS^{743}$-Q^{1630}NPPVSK. The CAG promoter is a chimeric promoter, composed of the CMV enhancer, the chicken β-actin promoter,

and an intron, was derived from pCAGGS [12]. The chicken β-actin minimum promoter (−155~+12, 167 b) was generated by PCR, cloned in pCR2.1 TOPO (Invitrogen), and sequenced. Plasmid vector p1.1c, composed of the CMV promoter, human growth hormone gene intron 1, and the SV40 polyadenylation signal sequences, was kindly supplied by Avigen Inc. The DNA fragments spanning the CMV promoter and the human growth hormone intron of p1.1c were replaced with the CAG promoter, the phosphoglycerokinase 1 (PGK1) promoter, or the β-actin minimum promoter DNA fragments to make plasmid p1.1CAG, p1.1PGK1, or p1.1β-actin, respectively. The DNA fragments encoding the BDD hFVIII cDNA or the BDD cFVIII cDNA were cloned in the downstream of the respective promoter sequences of these plasmids to make p1.1CMV-hFVIII, p1.1CAG-hFVIII, p1.1PGK1-hFVIII, p1.1β-actin-hFVIII, and p1.1β-actin-cFVIII, respectively. The Lac Z gene was cloned in the downstream of the β-actin promoter to make plasmid p1.1β-actin Lac Z. DNA fragments spanning the promoter, the LacZ gene, and the poly adenylation signal sequence of pAAV2 Lac Z (Stratagene) were replaced with DNA fragments spanning the β-actin promoter, the BDD cFVIII cDNA, and the SV40 poly adenylation signal sequences of p1.1β-actin-cFVIII to make the gene transfer vector pAAV2-β-actin-cFVIII in which these DNA fragments were flanked by ITR sequences of AAV serotype 2 (AAV2) as described previously [9,13]. The gene transfer vector pAAV2-β-actin-Lac Z equipped with AAV2 ITRs was also constructed. The chimeric packaging plasmid for AAV8 capsid pseudotyping was a generous gift from Dr. James M. Wilson (Division of Medical Genetics, Department of Medicine, University of Pennsylvania, Philadelphia, PA) [6]. The packaging plasmid composed of the AAV2 rep gene and the cap gene derived from AAV1 for AAV1 capsid pseudotyping was described previously [9].

AAV vector production

Viral vectors were packaged with AAV1 or AAV8 capsid by pseudotyping. The FVIII gene or the Lac Z gene located in the downstream of the β-actin minimum promoter and flanked by AAV2 ITRs was packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid, the adenovirus helper plasmid pHelper (Strategene, La Jolla, CA), and gene transfer plasmid vectors (pAAV2-β-actin-cFVIII or pAAV2-β-actin-Lac Z) as described previously [9,13]. For virus vector purification, the DNAse (Benzonase, Merck Japan, Tokyo, Japan)-treated virus particle containing samples were subjected to two rounds of iodixyanol-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM EDTA at 21 °C as described [9]. Titration of recombinant AAV vectors was carried out by quantitative dot-blot hybridization using the ^{32}P-labeled probes [9,13].

Analysis of the ß-actin minimum promoter activity

Expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII by the calcium phosphate coprecipitation method was studied to show that the β-actin minimum promoter had an enough FVIII expression activity. After incubation with the DNA containing media for 6 h, HEK 293 cells were incubated further in DMEM/HAM F-12 media supplemented with 10% fetal bovine serum for 48 h at 37 °C in the presence of 5% CO_2. FVIII clotting activities in the conditioned media of HEK 293 cells harvested after 48 h incubation were quantified by the activated partial thromboplastin time (APTT) method using FVIII deficient plasma. FVIII activities were expressed as the percentages of normal control plasma.

Animal experiments

FVIII-deficient mice (Hemophilia A mice) with targeted destruction of exon 16 of the FVIIII gene were previously reported by Bi et al. [14] and generously given to us by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA). J1 ES cells were used for targeted destruction of the FVIII gene and blastcysts derived from C57BL/6 mice were used to generate chimaeras [14]. C57BL/6 wild-type mice were purchased from SLC Inc. Mice were maintained in a standard lighting condition in a clean room. All surgical procedures were carried out in accordance with guidelines approved by the institutional Animal Care and Concern Committee at Jichi Medical School [15]. Male hemophilia A mice and male wild-type C57BL/6 mice were used in the experiments. Blood was drawn from the cervical vein plexus of mice and mixed with 1 / 10 volume of 3.8% sodium citrate, and then platelet-poor plasma was prepared by centrifugation. AAV8 vectors were injected intravenously into the cervical vein plexus while AAV1 vectors were injected directly to the skeletal muscles of lower extremities of mice under anesthesia with isoflurene [15]. Cyclophosphamide (100 μg/body/day, SIGMA-ALDRICH Japan, Tokyo, Japan) and tachrolimus (12.5 μg/body/day, Fujisawa Pharmaceuticals Co., Tokyo, Japan) were

given (s.c.) to mice daily after vector injection as the immunosuppressant.

Analysis of cFVIII expression in mice

FVIII activities were measured by the activated partial thromboplastin time (APTT) method used for determination of plasma FVIII activities of hemophilia patients utilizing human FVIII-deficient plasma as described [15]. Antigen levels of cFVIII in mouse plasma were determined by ELISA (Asserachrom FVIIIC: Ag, Diagnostica Stago, Parsipanny, NJ) as described [6]. Analyses of neutralizing antibodies against cFVIII developed in mice were performed by the Bethesda method as described using FVIII deficient plasma and normal canine plasma. Detection of the transcripts of cFVIII transgene was performed by RT-PCR [10,11]. RNA was isolated from the mouse organs using an RNA isolation kit (RNeasy Protect kit; Qiagen Inc., Valencia, CA). DNAse I (Amplification grade, Invitrogen, Carlsbad, CA)-treated and heat-treated RNA samples were subjected to RT-PCR using a pair of primers (sense: 5′-GTTGGAGCACAAACTGACTTCC-3′, antisense: 5′-CAATTGAGAAGGTGTCATCATACTC-3′) for cFVIII and an RT-PCR kit (SuperScript One-Step RT-PCR System, Invitrogen). PCR amplification (25–30 cycles) for cFVIII was performed as described [10,11]. A primer pair for mouse GAPDH mRNA (R&D Systems, Inc., Minneapolis, MN) was used instead of cFVIII primers in the control RT-PCR experiments. For detection of cFVIII molecules in mouse tissues by immunohistochemistry, the skeletal muscles and the liver were fixed with 4% paraformaldehyde in phosphate-buffered saline (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 −25 °C and attached to poly-lysine coated glass slides. For detection of FVIII, tissue sections were blocked with 1% rabbit serum in PBS containing Triton-X 100 (0.1%) and incubated with sheep polyclonal anti-human FVIII antibodies (Cedarlane Laboratories Ltd, Homby, Ontario, Canada) at 4 °C for 16 h [10,11]. After washing in PBS, sections were incubated with biotin-conjugated rabbit anti-sheep IgG antibody followed by the ABC reagents (Vectastain ABC Elite kit; Vector, Burlingame, CA) and a DAB kit (Vector).

Detection of ß-galactosidase in mouse tissues

To analyze Lac Z gene expression in mice injected with AAV-β-actin-Lac Z, mouse tissues were fixed with 2% paraformaldehyde in PBS for 5 min, washed with PBS, incubated with PBS containing sucrose (10–30%), and frozen with OTC compound (Tissue-Tek; Miles, Inc., Elkhart, IN) in dry ice/ethanol. Sections, prepared from frozen tissues at −25 °C and attached to poly-lysine coated glass slides, were 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 at 25 °C for 1 h [10]. Some sections further were processed for the Feulgen reaction (red purple) to visualize nuclei.

Results

Expression of FVIII by the ß-actin minimum promoter

We studied expression of hFVIII in HEK293 cells transfected with plasmid vectors p1.1β-actin-hFVIII, p1.1CMV-hFVIII, p1.1CAG-hFVIII, and p1.1PGK1-hFVIII. FVIII clotting activities detected in the conditioned medium of HEK293 cells are shown in Fig. 1. Expression of FVIII driven by the β-actin minimum promoter (167 b) in the 293 cells was approximately 1/3–1/2 of that by the CMV promoter (1 kb) or the CAG promoter (1.7 kb). Although the β-actin minimum promoter is weaker than the CMV promoter and the CAG promoter, it

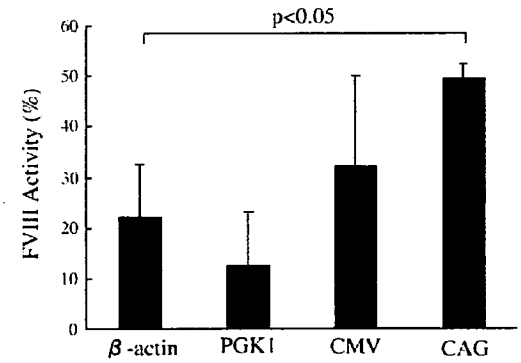

Figure 1 FVIII expression by the β-actin minimum promoter in vitro. FVIII clotting activities expressed in the conditioned media of HEK 293 cells transfected with p1.1CMV-FVIII, p1.1 CAG-FVIII, p1.1 PGK1-FVIII, or p1.1 β-actin-FVIII after 48 h incubation are shown. FVIII clotting activities in the conditioned media were quantified by the APTT method using FVIII deficient plasma and FVIII clotting activities were expressed as the percentages of normal control plasma. There were no FVIII activities in the conditioned media of HEK 293 cells with mock transfection. There was a significant difference of FVIII activity levels in the conditioned media of HEK 293 cells transfected with p1.1 CAG-FVIII and those in the conditioned media of HEK 293 cells transfected with p1.1 β-actin-FVIII ($n=4$, Student's t-test, $p<0.05$).