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G. 知的財産の出願・登録状況（予定を含む）

研究は現在、モデル動物を用いて、治療実験にむけた条件検討中にある。動物実験の結果が出た段階で、自治医科大学病態治療研究センター分子病態研究部（大森司講師）とDNAVEC株式会社（井上誠様）と、知的財産権の出願についての随時協議を行う予定である。

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

THE JOURNAL OF GENE MEDICINE
J Gene Med 2009; **11**: 1020–1029.
Published online 15 September 2009 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/jgm.1391

RESEARCH ARTICLE

Liver-restricted expression of the canine factor VIII gene facilitates prevention of inhibitor formation in factor VIII-deficient mice

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Received: 18 December 2008
Revised: 15 July 2009
Accepted: 20 July 2009

Abstract

Background Gene therapy for hemophilia A with adeno-associated virus (AAV) vectors involves difficulties in the efficient expression of factor VIII (FVIII) and in antibody formation against transgene-derived FVIII.

Methods AAV8 vectors carrying the canine B domain deleted FVIII (cFVIII) gene under the control of the ubiquitous β-actin promoter, the liver-specific human α1 anti-trypsin promoter (HAAT) and the liver-specific hepatic control region (HCR) enhancer/human α1 anti-trypsin promoter complex (HCRHAAT) were used for the expression of cFVIII in FVIII deficient (*fviii*$^{-/-}$) mice.

Results Addition of the hepatic control region enhancer element to the HAAT promoter successfully augmented HAAT promoter activity without loss of liver-specificity *in vivo*. Using this enhancer/promoter complex, a high cFVIII transgene expression was achieved, resulting in increased blood cFVIII activities to more than 100% of the normal canine FVIII levels in *fviii*$^{-/-}$ mice at a 1 : 10 lower dose of the AAV8 vector carrying the cFVIII gene driven by the HAAT promoter. Under short-term immunosuppression, neutralizing antibodies against cFVIII developed in only one out of six mice when the HAAT promoter was used for cFVIII expression, whereas all the mice developed neutralizing antibodies against cFVIII when the β-actin promoter was used for cFVIII expression. No neutralizing antibodies against cFVIII developed in *fviii*$^{-/-}$ mice that received the AAV8 vector carrying the cFVIII gene driven by the HCRHAAT enhancer/promoter complex without immunosuppression.

Conclusions These data suggest that AAV8 vector-mediated liver-restricted cFVIII gene expression is sufficient for immune hypo-responsiveness to transgene-derived cFVIII in *fviii*$^{-/-}$ mice.

Keywords adeno-associated virus vector; factor VIII; gene therapy; hemophilia A

Introduction

Hemophilia A is an inherited X-linked bleeding disorder caused by abnormalities in the coagulation factor VIII (FVIII) gene. The genetic

abnormalities result in FVIII deficiency, which in turn creates bleeding diathesis, such as life-threatening bleeding in the brain or harmful bleeding in joints and muscles. The current standard therapy involves the intravenous injection of monoclonal antibody-purified coagulation factor concentrates from plasma or recombinant coagulation factors. Hemophiliacs are not free from the risks of life-threatening intracranial bleeding and harmful bleeding. Therefore, gene therapy enabling the prevention of such bleeding by a sustained elevation of coagulation factor levels provides the next generation therapy for hemophilia [1–6]. Indeed, clinical trials for hemophilia gene therapy have recently been conducted, although with limited success [4–10]. Compared with gene therapy for hemophilia B, gene therapy for hemophilia A has been accompanied by difficulties involved in the efficient expression of FVIII because of the large size of the FVIII gene and the low expression of the FVIII gene in the full-length FVIII cDNA form. Recent studies have seen the development of new vectors and strategies [11–17]. Among the viral vectors, recombinant adeno-associated virus (AAV) vectors are preferred for gene therapy because they can transfer genes to nondividing cells, leading to the long-term expression of transgenes, and no pathological effects of wild-type AAV have been reported [1–7,11,18]. Because of the size limitation of genes carried on AAV vectors, the use of AAV vectors for hemophilia A gene therapy has not been as successful as that for hemophilia B. Recently, modification of the FVIII gene and the development of new AAV serotype vectors has allowed us to carry the FVIII gene on AAV vectors [15,19,20]. We previously demonstrated that canine B domain-deleted FVIII (BDDFVIII) could be expressed in skeletal muscles and liver using AAV1 vectors and AAV8 vectors, respectively, and the minimum β-actin promoter. In addition to the sustained expression of FVIII, tissue-specific expression of FVIII may also be helpful for hemophilia gene therapy to avoid adverse reactions. In the present study, we examined the possibility of liver-specific FVIII gene transfer in FVIII deficient (*fviii*$^{-/-}$) mice using the AAV serotype 8 vector carrying the canine FVIII (cFVIII) gene, which is located downstream of three different types of promoter/enhancer complex. We show that elevated liver-specific expression of this transgene can be achieved with AAV8 vectors carrying the therapeutic gene under the control of the minimum human α1-antitrypsin (HAAT) promoter in combination with the minimum hepatic control region (HCR) enhancer element *in vivo*. This can be advantageous when aiming to avoid the formation of neutralizing antibodies against the transgene product for long-term expression.

Materials and methods

Vector construction

Two DNA segments encoding the 5′ flanking region (−272 to +25; −168 to +25) of the HAAT gene were amplified by polymerase chain reaction (PCR) to obtain the 297 bp and 193 bp HAAT promoters. These DNA fragments contained the hepatic nuclear factor 1 responsive element. The minimum enhancer element (+24 to +186) of the HCR of the human apolipoprotein E gene [21–23] was also amplified by PCR. DNA fragments of the cytomegalovirus (CMV) promoter and the growth hormone intron 1 of p1.1c (Avigen Inc., Alameda, CA, USA) were replaced with the 297 bp HAAT promoter to generate p1.1HAAT. Similarly, DNA fragments of the CMV promoter and the growth hormone intron 1 of p1.1c were replaced with the minimum HCR enhancer element and the 193 bp HAAT promoter to generate p1.1HCRHAAT. Constructions of p1.1 CAG and p1.1β have been described previously [20,24]. DNA fragments encoding the canine BDDFVIII cDNA or the luciferase gene were placed downstream of the promoter sequences of p1.1HAAT or p1.1 HCRHAAT to produce plasmid vectors p1.1HAAT-cFVIII, p1.1HAAT-Luc, p1.1HCRHAAT-cFVIII and p1.1HCRHAAT-Luc, respectively. Similarly, the DNA fragment encoding the Lac Z gene was placed downstream of the promoter sequences of p1.1 HAAT to produce p1.1HAAT-Lac Z. P1.1β-Lac Z has been described previously [20]. The DNA fragment spanning the CMV promoter, the LacZ gene and the polyadenylation signal sequence of the pAAV2 CMV-Lac Z plasmid (Stratagene, La Jolla, CA, USA) was replaced by the DNA fragment spanning the HCRHAAT promoter, the cFVIII gene and the SV40 polyadenylation signal sequences of p1.1HCRHAAT-cFVIII to make pAAV2-HCRHAAT-cFVIII. Plasmids pAAV2-HCRHAAT-Luc, pAAV2-HAAT-LacZ, pAAV2-CAG-Luc and pAAV2-HAAT-cFVIII were made in a similar manner, respectively.

AAV vector production

The vector production system was kindly supplied by Avigen Inc. The AAV vectors were packaged with the AAV8 capsid by pseudotyping. 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, PA, USA) [19]. The DNA fragments harboring the cFVIII gene, the luciferase gene or the Lac Z gene located downstream of the different promoters and flanked by AAV2 inverted terminal repeats (ITRs) were packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, which were kindly supplied by Avigen Inc., with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), the adenovirus helper plasmid pHelper (Stratagene) and gene transfer plasmid vectors, as described previously [20,24]. For virus vector purification, the DNase-treated (Benzonase, Merck Japan, Tokyo, Japan) viral particle containing samples were subjected to two rounds of cesium chloride (CsCl)-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) in the presence of 25 mM ethylenediaminetetraacetic acid, at 21 °C, as previously

described [20]. Titration of recombinant AAV vectors was carried out by quantitative dot-blot hybridization using ^{32}P-labeled probes [20,24] or by quantitative PCR using a real time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan). The primer sequences used for quantification of the AAV8 vector carrying the cFVIII gene were CCGATTATTGCTCAGTACATCCG and CAACTGTTGAAGTCACAGCCCA, and the probe sequence was FAM-CAACCCATTACAGCATCCGCAGCACT. DNase in the samples was heat-inactivated before the PCR reaction.

Animal experiments

C57BL/6 wild-type mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). FVIII-deficient mice (hemophilia A mice) with targeted destruction of exon 16 of the FVIIII gene were generously provided by Dr H. H. Kazazian Jr (University of Pennsylvania, PA, USA). J1 ES cells were used for targeted destruction of the FVIII gene and blastocysts derived from C57BL/6 mice were used to generate chimaeras [25]. Mice were maintained under standard lighting conditions in a clean room. All surgical procedures were carried out in accordance with the guidelines of the institutional Animal Care and Concern Committee of Jichi Medical University. AAV8 vectors were injected into the cervical vein of mice under anesthesia. Cyclophosphamide (100 μg/body/day; Sigma-Aldrich Japan, Tokyo, Japan) and tacrolimus (12.5 μg/body/day; Fujisawa Pharmaceuticals Co., Tokyo, Japan) were given (subcutaneously) for 12 weeks to AAV8-HAAT-cFVIII-injected *fviii*$^{-/-}$ mice after vector injection for immunosuppression [20]. No immunosuppresssants were administered to AAV8-HCRHAAT-cFVIII-injected *fviii*$^{-/-}$ mice.

Immunohistochemistry study

Tissues of vector-injected mice were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at 4 °C, incubated in PBS containing sucrose (10–30%), and frozen in OCT compound (Tissue-Tek, Miles Inc., Elkhart, IN, USA), in dry ice/ethanol. Sections were prepared from frozen tissues at −25 °C, and attached to polylysine-coated glass slides. For the detection of cFVIII, tissue sections were blocked with 1% casein in PBS containing 0.1% Triton-X 100 for 30 min at room temperature, and incubated with sheep polyclonal anti-human FVIII antibodies (Cedarlane Laboratories Ltd, Burlington, NC, USA) for 2 h at 37 °C. After washing in PBS, sections were incubated with biotin-conjugated rabbit anti-(sheep immunoglobulin G) antibody followed by the ABC reagent and a DAB kit (Vectastain ABC Elite kit; Vector, Burlingame, CA, USA) [20].

Analysis of the Lac Z gene expression in mouse tissues

To analyse LacZ gene expression in mice injected with AAV8 vectors carrying the Lac Z gene, mice were irrigated with saline followed by PBS containing 2% paraformaldehyde and then mouse tissues were fixed in 2% paraformaldehyde in PBS for 5 min and washed with PBS. Portions of mouse tissues were directly suspended 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. The rest of the mouse tissues were incubated further in PBS containing sucrose (10–30%), and frozen in OCT compound (Tissue-Tek) in dry ice/ethanol. Sections were prepared from frozen tissues at −25 °C, attached to polylysine-coated glass slides, 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.

Analysis of luciferase gene expression in mice

For *in vivo* bioluminescence imaging analysis, luciferin (150 μg per 100 μl/g body weight) was given to the mice injected with the AAV8 vector carrying the luciferase gene under anesthesia with isoflurane. Mice were subjected to direct imaging analysis and to quantification of photons transmitted through the mouse skin using IVIS Imaging Systems and Living Image software (Xenogen Co., Alameda, CA, USA). Photons in the area corresponding to the living mouse liver were quantified and expressed as photons/s/cm^2/sr.

Determination of cFVIII and cFVIII gene transcripts in mice

AAV8 vectors carrying the canine FVIII gene driven by the HAAT promoter or the minimum HCRHAAT enhancer/promoter complex were injected into the cervical vein plexus of 8-week-old *fviii*$^{-/-}$ mice under anesthesia. Blood was drawn from the cervical vein plexus and mixed with 1 : 10 volume of 3.8% sodium citrate periodically. Platelet-poor plasma was prepared and canine FVIII levels in mouse plasma were quantified by the activated partial thromboplastin time (APTT) method using FVIII-deficient plasma and standardized with normal canine plasma. Quantification of cFVIII transgene transcripts was performed by quantitative reverse transcriptase (RT)-PCR. RNA was isolated from mouse organs using an RNeasy Protect isolation kit (Qiagen Inc., Valencia CA, USA). DNase I-treated (Amplification grade; Invitrogen, Carlsbad, CA, USA) and heat-treated RNA samples were subjected to RT-PCR. The quantities of cFVIII transcripts were standardized against those of the GAPDH transcripts [20]. Immunohistochemistry for canine FVIII was carried out

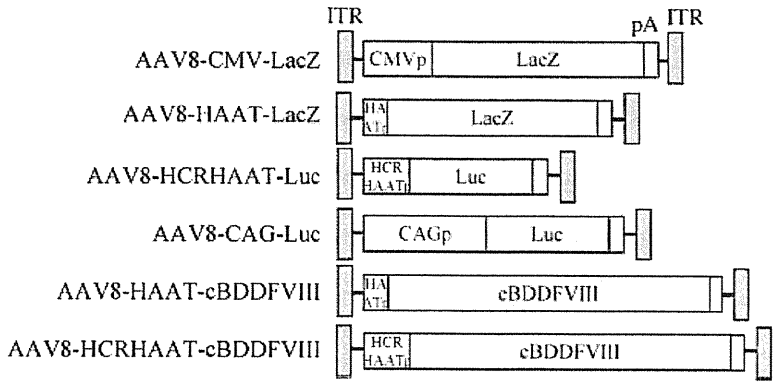

Figure 1. Schematic representation of AAV8 vectors. AAV8 vectors used in the present study are represented schematically. The promoter, the enhancer/promoter complex, or the enhancer/promoter/intron complexes, the genes for expression, and the polyadenylation signal sequence (pA) were flanked by two AAV2 ITR sequences. CMV, CMV promoter/the growth hormone gene intron 1 complex (1 kb); CAG, the CMV enhancer, β-actin promoter, and growth hormone intron 1 enhancer/promoter/intron 1 complex (1.7 kb); HAAT, the human α1 antitrypsin promoter (297 b); HCRHAAT, the hepatic control region of apolipoprotein E gene (163 b) and the human α1 antitrypsin promoter (193 b) complex; cBDDFVIII, canine B domain deleted FVIII cDNA (4.4 kb).

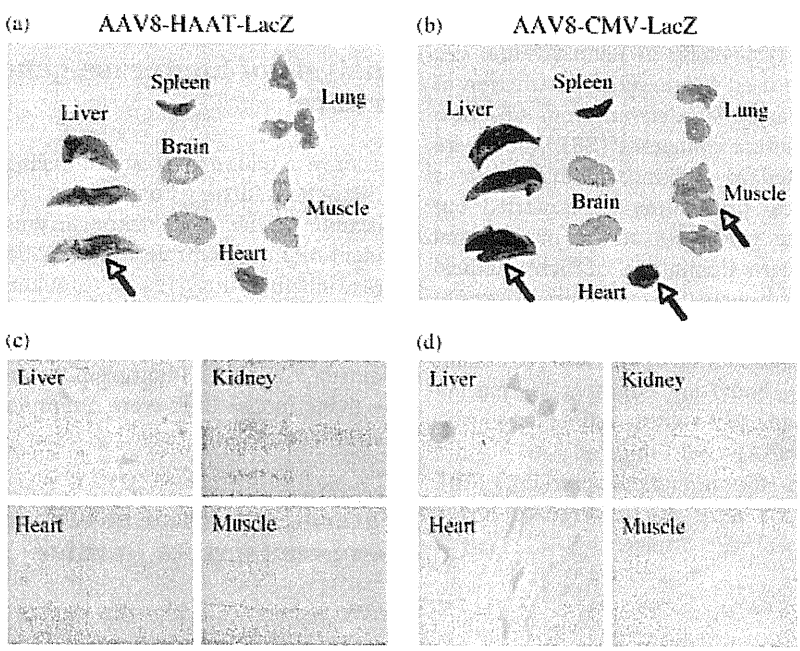

Figure 2. Lac Z gene expression in mice injected with AVV8 vectors. Expression of the Lac Z gene in vector-injected mice was analysed by X-gal staining. Macroscopic views of organs of mice injected with AAV8-HAAT-Lac Z (a) or with AAV8-CMV-Lac Z (b) and microscopic views of organs of mice injected with AAV8-HAAT-Lac Z (c) or with AAV8-CMV-Lac Z (d) are shown. Arrows indicate positive X-gal staining.

using sheep anti-(human FVIII) polyclonal antibodies, as previously described [20].

Determination of neutralizing antibody titer against cFVIII

Analysis of neutralizing antibodies against cFVIII developed in mice was performed by the Bethesda method using FVIII deficient plasma and normal canine plasma, as previously described [20].

Results

Construction of the AAV vectors

AAV8 vectors used in the present study are represented schematically (Figure 1). The lengths of the AAV8-HAAT-cFVIII and AAV8-HCRHAAT-cFVIII were 5.15 kb and 5.2 kb, respectively. The vector isolation efficiencies of AAV8-HCRHAAT-cFVIII (5.2 kb) and of AAV8-HAAT-cFVIII (5.15 kb) after purification by the two rounds density gradient ultracentrifugation of CsCl were 1.68×10^4 vector genome copies (gc)/cell and 1.87×10^4 vector

gc/cell (the average of two preparations), respectively, whereas the average vector isolation efficiency of AAV8 vectors carrying the human factor IX gene (4.3 kb) by the same procedure was 3.68×10^4 vector gc/cell (average of three experiments).

Analysis of HAAT promoter specificity with AAV8 vectors *in vivo*

To study the cell specificity of the HAAT promoter *in vivo*, AAV8 vectors carrying the Lac Z gene located downstream of the 297 b HAAT promoter (AAV8-HAAT-Lac Z) or the CMV promoter/growth hormone intron 1 (AAV8-CMV-LacZ) complex were injected into the cervical vein of C57BL/6 mice (5×10^9 gc/g body weight), and expression of the Lac Z gene was analysed by detecting β-galactosidase activity by staining mouse tissues with X-gal. Macroscopic views of organs from mice injected with AAV8-HAAT-Lac Z (Figure 2a) or AAV8-CMV-Lac Z (Figure 2b) and microscopic views of organs from mice injected with AAV8-HAAT-Lac Z (Figure 2c) or AAV8-CMV-Lac Z (Figure 2d) are shown. Arrows indicate positive X-gal staining. The β-galactosidase activity was macroscopically detected in the liver, heart and skeletal muscles of the AAV8-CMV-Lac Z-injected mice (Figure 2), whereas the β-galactosidase activity was solely detected in the liver of the AAV8-HAAT-LacZ-injected mice (Figure 2). These data in respect of β-galactosidase activity expression were confirmed by microscopic examination of these organs (Figure 2). β-galactosidase activities were microscopically detected in hepatocytes, myocardium and skeletal muscle fibers in a similar manner to the macroscopic views of organs of AAV8-CMV-Lac Z-injected mice, whereas no β-galactosidase activities were detected in the myocardium or skeletal muscle fibers in AAV8-HAAT-LacZ-injected mice (Figure 2). These data suggest that the transgene expression with AAV8 vectors preferentially occurs in the liver, but is also affected by the tissue specificity of the promoter used in the AAV8 vector, and that the hepatocyte specificity of the HAAT promoter facilitates liver-restricted transgene expression with the AAV8 vector.

Transgene expression by the HCRHAART enhancer/promoter complex with AAV8 vectors

The DNA fragments spanning the HAAT promoter located downstream of the HCR of apolipoprotein E gene have been shown to express genes in the liver very efficiently [18,26,27]. We could also express human factor IX in mice at approximately 6–7 U/ml (18–21 μg/ml) using the AAV8 vectors carrying the human factor IX gene driven by the HCR enhancer (325 b)/HAAT promoter (297 b) complex (data not shown). However, the DNA fragments used in these studies were too large to enable the FVIII gene to be carried on AAV vectors. Thus, we utilized the minimum HCR enhancer element and the minimum HAAT promoter sequence for FVIII gene expression with AAV8 vectors. Tissue-specific expression of the luciferase gene driven by the HCRHAAT enhancer/promoter complex was quantified by analysing photons from mice under anesthesia using IVIS Imaging Systems (Xenogen Co.) and was compared with that by the CAG promoter. When the AAV8 vectors carrying the luciferase gene driven by the CAG promoter (2×10^9 gc/g) were injected to neonatal wild-type mice, luciferase gene expression was preferentially found in the liver, but also detected in the heart, tail and limbs (Figure 3). By contrast, luciferase gene expression was restricted to the liver in the neonatal mice, with injection of the AAV8 vectors carrying the luciferase gene driven by the minimum HCRHAAT enhancer/promoter complex (2×10^9 gc/g) (Figure 3). When the AAV8 vectors carrying the luciferase gene driven by the CAG promoter were injected into adult mice (2×10^9 gc/g), luciferase gene expression occurred more preferentially in the liver than in neonatal mice, but luciferase gene expression was still observed in the heart and the tail of adult mice. Again, luciferase gene expression was detected solely in the liver of mice injected with the AAV8-HCRHAAT-Luc vector (2×10^9 gc/g). These data suggest that the extrahepatic gene expression with AAV8 vectors may be relatively broad in neonatal mice compared to that in adult mice, and that the HCRHAAT enhancer/promoter complex may have liver specificity not only in adult mice, but also in neonatal mice. In addition, a comparison of the amount of photons from the mouse liver suggests that the minimum HCRHAAT enhancer/promoter complex had approximately ten-fold higher promoter activity than the CAG promoter in the mouse liver *in vivo*.

Expression of FVIII activity in *fviii*$^{-/-}$ mice with AAV vectors carrying the BDD cFVIII gene

FVIII clotting activity levels in hemophilia A mice after intravenous injection of AAV8-HAAT-cFVIII increased dose-dependently on day 28, achieving therapeutic FVIII levels (approximately 0.3 U/ml; 30% of the normal canine FVIII level) and normal FVIII levels in *fviii*$^{-/-}$ mice with the AAV8-HAAT-cFVIII at doses 5×10^9 gc/g and 5×10^{10} gc/g (Figure 4), respectively. FVIII clotting activity levels in *fviii*$^{-/-}$ mice after intravenous injection of AAV8-HCRHAAT-cFVIII were increased dose-dependently on day 28, achieving therapeutic canine FVIII levels (0.32 U/ml) and normal canine FVIII levels (1.45 U/ml) in *fviii*$^{-/-}$ mice with AAV8-HCRHAAT-cFVIII at doses of 5×10^8 gc/g and 5×10^9 gc/g, respectively (Figure 4), indicating that the high cFVIII activity level was achieved with AAV8-HCRHAAT-cFVIII at 1 : 10 of the dose of the AAV8-HAAT-cFVIII and the AAV8-β-actin-cFVIII [20].

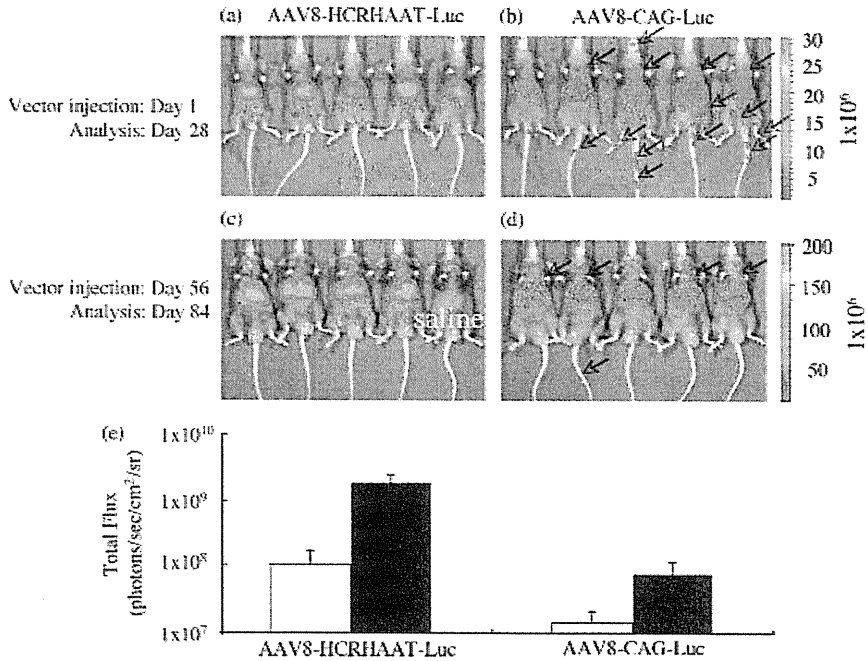

Figure 3. *In vivo* expression of luciferase gene driven by the HCRHAAT enhancer/promoter complex. Wild-type mice injected with AAV8-HCRHAAT-Luc or the AAV8-CAG-Luc on day 1 after birth (a, b) or on day 56 after birth (c, d) were analysed for expression of the luciferase gene using an *in vivo* imaging system on day 28 or on day 84, respectively. Photons detected through the mouse skin were visualized (a–d). Significant luminescence was detected at positions corresponding to the liver. Arrows indicate extrahepatic luminescence. No luminescence signal was detected in the nonvector injected mouse (saline, saline-injected mice, control). Photons transmitted through the skin of mice transduced with AAV8-HCRHAAT-Luc or with AAV8-CAG-Luc on day 1 (open square) or on day 56 (closed square) after birth were quantified 28 days after vector injection (e).

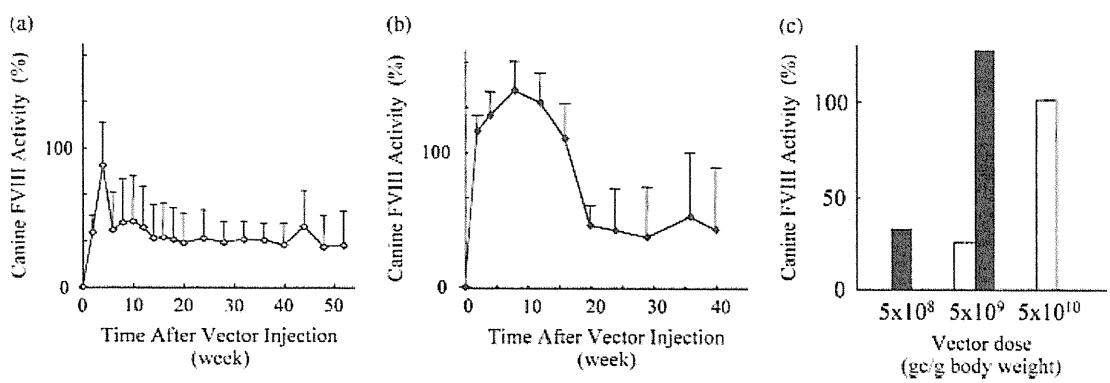

Figure 4. Expression of canine FVIII in *fviii*$^{-/-}$ mice injected with AAV8 vectors carrying the canine FVIII gene. The canine FVIII levels in *fviii*$^{-/-}$ mice injected with 5×10^{10} gc/g body weight of AAV8-HAAT-cFVIII (a) or 5×10^{9} gc/g body weight of AAV8-HCRHAAT-cFVIII (b) are shown. Values represent the mean ± SD. The canine FVIII levels in *fviii*$^{-/-}$ mice injected with AAV8-HCRHAAT-cFVIII (black bars; doses of 5×10^{8} gc/g body weight or 5×10^{9} gc/g body weight) or AAV8-HAAT-cFVIII (white bars; doses of 5×10^{9} gc/g body weight or 5×10^{10} gc/g body weight) on day 28 after vector injection are shown (c). FVIII activities were determined by the one-step APTT method using FVIII deficient human plasma and were standardized with normal canine plasma. One unit canine FVIII/ml represents 100% canine FVIII clotting activity.

Analysis of transcripts of canine FVIII transgene in organs of *fviii*$^{-/-}$ mice injected with AAV8 vectors carrying the canine FVIII gene

Analysis of cFVIII transcripts in vector-injected mice suggests that the cFVIII gene was specifically expressed in the liver (Figure 5) and no significant amount of cFVIII transcripts were detected by RT-PCR or quantitative RT-PCR in other organs of mice injected with AAV8-HCRHAAT-cFVIII or AAV8-HAAT-cFVIII. These data confirm that the expression of the cFVIII gene by the HAAT promoter or the HCRHAAT enhancer/promoter complex was liver specific. Transcript levels of the cFVIII transgene in the liver of AAV8-HCRHAAT-cFVIII injected mice were approximately ten-fold higher than in AAV8-HAAT-cFVIII-injected mice at the same vector dose. These data are in accordance with the cFVIII levels in the vector-injected

DOI: 10.1002/jgm

mice, suggesting that the HCRHAAT enhancer/promoter complex had ten-fold higher transgene expression activity than the HAAT promoter *in vivo*.

Immunohistochemistry of canine FVIII in the liver of mice injected with AAV8 vectors carrying the canine FVIII gene

Immunohistochemistry analysis confirmed that cFVIII was efficiently expressed in hepatocytes of mice injected with a low dose of AAV8 vectors carrying the cFVIII gene under the control of the HAAT promoter or the HCRHAAT enhancer/promoter complex (Figure 6).

Analysis of anti-cFVIII neutralizing antibody in *fviii*$^{-/-}$ mice

In our previous study, we showed that the expression of the cFVIII gene with the AAV8 vector has an advantage over AAV1 vector-mediated cFVIII gene transfer to the skeletal muscles in terms of the immune reaction to the transgene product [20]. No neutralizing antibody development was observed until 12 weeks after vector injection of AAV8-β-actin-cFVIII under immunosuppression [20]. However, neutralizing antibodies against cFVIII developed in four out of four mice at 12 weeks after termination of immunosuppression (i.e. week 24 after vector injection). When no immunosuppressants were given throughout the course, antibodies against cFVIII were formed in six of eight mice 4–20 (mean 12.8) weeks after AAV8-β-actin-cFVIII vector injection (Table 1). By contrast to the AAV8-β-actin-cFVIII-injected *fviii*$^{-/-}$ mice, neutralizing antibodies were found in only one out of six mice with AAV8-HAAT-cFVIII injection under the same immunosuppression. Interestingly, the level of neutralizing antibody against cFVIII in that mouse gradually decreased and became undetectable by week 8 after termination of immunosuppression (i.e. week 20 after vector injection), and the cFVIII activity in the mouse started to increase from week 12 after termination of immunosuppression (i.e. week 24 after vector injection) and reached a plateau of 0.45 U/ml (45% of the normal canine FVIII level) by week 24 after termination of immunosuppression. Therapeutic levels of cFVIII in other AAV8-HAAT-cFVIII-injected *fviii*$^{-/-}$ mice were sustained for more than 40 weeks without immunosuppression (i.e. week 52 after vector injection) (Figure 4 and Table 1). These data lead us to speculate that the extrahepatic expression of cFVIII gene might correlate with the development of neutralizing antibodies. On the basis of this notion, the AAV8-HCRHAAT-cFVIII vector was injected to *fviii*$^{-/-}$ mice without any immunosuppression and cFVIII expression and neutralizing antibody formation was investigated. High cFVIII gene expression and an elevation of blood cFVIII levels (Figures 4 and 5) without neutralizing antibody formation were achieved in AAV8-HCRHAAT-cFVIII-injected *fviii*$^{-/-}$ mice (Table 1). These data suggest that liver-restricted expression of cFVIII with AAV8 vector and the liver-specific promoter facilitates the prevention of inhibitor formation to cFVIII in *fviii*$^{-/-}$ mice. Although the liver-restricted cFVIII gene transfer facilitated hypo-responsiveness to transgene-derived cFVIII, antibody formation against AAV8 capsid developed in the mice with AAV8-HCRHAAT-cFVIII injection in a similar manner to that in mice receiving other AAV8 vectors (data not shown).

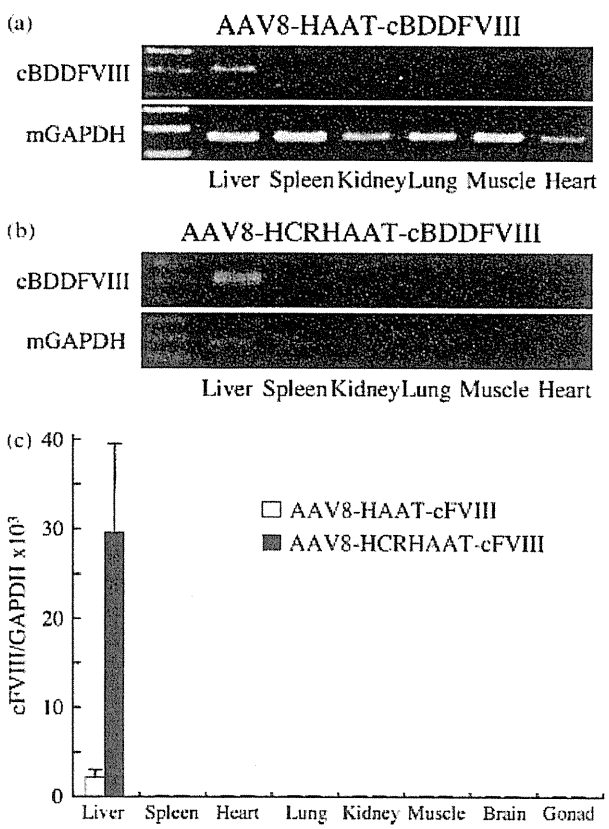

Figure 5. Analysis of transcripts of the canine FVIII transgene in organs of mice injected with AAV8 vectors carrying the canine FVIII gene. The transcripts of canine FVIII transgene in organs of fviii$^{-/-}$ mice injected with 5×10^9 gc/g body weight AAV8-HAAT-cFVIII (a) or 5×10^9 gc/g body weight AAV8-HCRHAAT-cFVIII (b) were detected by RT-PCR and were quantified using real-time PCR. The quantity of canine FVIII transgene transcripts was standardized with GAPDH transcripts (c) (AAV8-HAAT-cFVIII, white bar; AAV8-HCRHAAT-cFVIII, black bar).

Discussion

Various serotypes of AAV vectors have been developed, and each AAV serotype has its own tropism [11]. However, the tropism of an AAV serotype is not completely specific for a certain type of cell and transgene expression in target cells and organs may also be affected by the tissue specificity of the promoter used in the AAV vectors. Ubiquitous promoters, such as the CMV promoter and the CAG promoter, have been used in early studies of gene therapy; however, the use of a tissue-specific promoter for

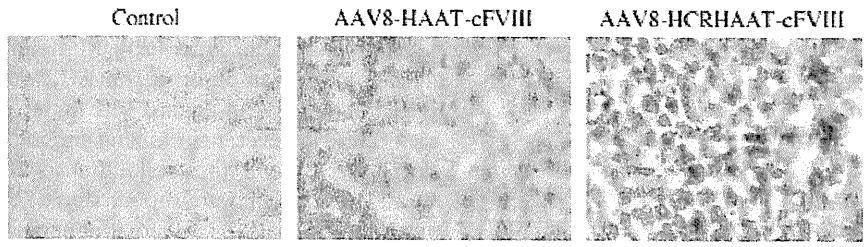

Figure 6. Immunohistochemistry of canine FVIII in the liver of mice injected with AAV8 vectors carrying the canine FVIII gene. Canine FVIII in the liver of *fviii*$^{-/-}$ mice injected with 5 x 10^9 gc/g body weight of AAV8-HAAT-cFVIII or 5 x 10^8 gc/g body weight AAV8-HCRHAAT-cFVIII was detected by immunohistochemistry with sheep anti-human FVIII polyclonal antibodies, as described in the Materials and methods. Positive staining is brown. As a control, liver sections obtained from *fviii*$^{-/-}$ mice without vector injection were simultaneously processed with the same antibody solution as the control.

Table 1. Neutralizing antibody against cFVIII in vector injected *fviii*$^{-/-}$ mice

Vector	AAV8-β-cFVIII	AAV8-β-cFVIII	AAV8-HAAT-cFVIII	AAV8-HCRHAAT-cFVIII
Immunosuppression*	Yes, 12 weeks	No	Yes, 12 weeks	No
CFVIII activity on day 56 after vector injection (%)	77.6 ± 21.3 (mean ± SD)	79.8 ± 81.8 (mean ± SD)	87.5 ± 30.6 (mean ± SD)	127.0 ± 17.1 (mean ± SD)
Neutralizing antibody formation (*n*)	4/4	6/8	1/6	0/6
Inhibitor titer (Bethesda U/ml)	10.7 ± 0.5 (mean ± SD)	18.5 ± 13.6 (mean ± SD)	4.0	Not detected
Spontaneous regression of neutralizing antibody	No	No	Yes	Not applicable

*Cyclophosphamide and tacrolimus were injected to mice after vector injection for 12 weeks.

cell-specific expression of a transgene is required to avoid undesirable effects. One such side-effect is the formation of antibody against the transgene product. In particular, a serious concern in hemophilia A gene therapy is the formation of antibody against transgene-derived FVIII. Liver-specific expression of transgene products upon gene transfer is attractive with regard to immune tolerance induction to the transgene products [18,28–30]. Indeed, AAV vector-mediated gene transfer to the liver has been shown to have a reduced pro-inflammatory risk compared to lentivirus vector-medicated gene transfer [18,31]. In addition, AAV8 vectors and AAV9 vectors do not express transgenes in the spleen [18]. On the basis of these notions, we developed an AAV8 vector carrying the cFVIII gene driven by the HAAT promoter or the HCRHAAT enhancer/promoter complex, and investigated the expression of cFVIII in *fviii*$^{-/-}$ mice.

Canine FVIII is a xenoantigen to mice; therefore, mice might develop neutralizing antibodies to cFVIII if cFVIII is expressed in mice. Indeed, *fviii*$^{-/-}$ mice developed neutralizing antibodies against cFVIII, even under immunosuppression, when the cFVIII gene driven by the β-actin promoter was expressed in skeletal muscles using the AAV1 vector [20]. However, when the cFVIII gene, driven by the same promoter, was transduced to *fviii*$^{-/-}$ mice using the AAV8 vector, no neutralizing antibodies against cFVIII developed in vector-injected *fviii*$^{-/-}$ mice under the same immunosuppression, suggesting that AAV8 vector-mediated FVIII gene transfer to the liver was advantageous over AAV1 vector-mediated gene transfer to the skeletal muscle in terms of neutralizing antibody formation against the transgene product cFVIII. However, the AAV8 vector-mediated cFVIII gene transfer with the β-actin promoter was not sufficient to prevent neutralizing antibody formation against transgene-derived cFVIII, as shown in the present study (Table 1). The present study demonstrated that extrahepatic expression of the transgene might function to develop neutralizing antibodies to cFVIII in *fviii*$^{-/-}$ mice. The minimum β-actin promoter, a part of the CAG promoter, had a significant promoter activity in HEK293 cells and was approximately one-half to one-third of that of the CAG promoter [20]. By contrast, the activities of the HAAT promoter and the HCRHAAT enhancer/promoter complex used in the present study were almost the same as the promoter-less control vector in HEK293 cells (not shown), suggesting that leaky gene expression of the HAAT promoter and the HCRHAAT enhancer/promoter complex in nonhepatocyte cells can be minimized. In addition, the leaky expression of the Lac Z gene driven by the HAAT promoter or of the luciferase gene driven by the HCR/HAAT promoter was not apparent *in vivo* (Figures 2 and 3). On the basis of this notion, we attempted to express cFVIII with AAV8-HCRHAAT-cFVIII in *fviii*$^{-/-}$ mice without immunosuppression to determine whether liver-restricted expression of cFVIII is sufficient for hypo-responsiveness of inhibitor (antibody) formation to cFVIII. In this experiment, none of the mice injected with the AAV8-HCRHAAT-cFVIII developed neutralizing antibodies against canine FVIII for up to 10 months without immunosuppression. Taken together, these data suggest that the liver-restricted transgene expression would be effective to reduce the immune reaction to transgene-derived canine FVIII. Immune tolerance induction to the transgene product is one of the key issues of gene therapy for genetic disease caused by a

single gene abnormality and has been extensively studied in a mouse hemophilia B model by expressing factor IX with viral vectors [18,28–30]. Hypo-responsiveness to transgene product FVIII including immune tolerance induction may be more important for hemophilia A gene therapy than for hemophilia B gene therapy because approximately 21–32% of severe hemophilia A patients develop inhibitors (alloantibody) against therapeutically injected FVIII, whereas inhibitors against factor IX form in approximately 9% of severe hemophilia B patients upon factor IX infusion. A variety of approaches for induction of hypo-responsiveness to FVIII including immune tolerance have been shown to be effective [32–34]. In this regard, liver-restricted expression of FVIII using the AAV8 vector together with the liver-specific promoter might be an alternative gene transfer approach for this purpose.

The vector doses required for the increase of the cFVIII activity level to 0.4–1.2 U/ml in *fviii*$^{-/-}$ mice suggested that the AAV8-HCRHAAT-cFVIII vector was approximately ten-fold more potent than both the AAV8-HAAT-cFVIII and the AAV8-β-actin-cFVIII vectors. Expression of the transgene may be mainly driven by the internal promoter used in the AAV vector; however, it is still possible that transgene expression is affected by the presence of the ITR and the A/D sequences because these elements may function as cis-acting elements in human cells, thereby interfering with the regulated downstream gene expression cassette [35,36]. In the context of minimizing nonspecific transgene expression with AAV vectors, a reduction of vector doses for gene transfer is also important and can be achieved using the AAV8 vector carrying the therapeutic gene driven by the HCRHAAT enhancer/promoter complex to avoid an undesirable immune reaction to the transgene product. This efficient cFVIII expression in FVIII deficient mice could be achieved by the use of this enhancer promoter complex [21,22,27], the removal of the DNA segment coding the FVIII B domain from the FVIII gene [37,38], and the high liver transduction efficiency of the AAV8 vector [11,15,39].

The site of extrahepatic expression of canine FVIII contributing to inhibitor formation has not been determined. One possibility is the expression of FVIII in skeletal muscles [40]. This remains the subject of future studies.

Acknowledgements

This study was supported by Grants-in-Aid for Scientific Research (20591155, 21591249 and 21790920) and Support Program for Strategic Research Infrastructure from the Japanese Ministry of Education and Science, and Health Labour and Science Research Grants for Research on HIV/AIDS and Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare.

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Published online on 3 September 2009 Ann Clin Biochem, doi: 10.1258/acb.2009.009077

Short Report

A convenient enzyme-linked immunosorbent assay for rapid screening of anti-adeno-associated virus neutralizing antibodies

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Abstract

Background: Recombinant adeno-associated virus vectors based on serotype 2 (AAV-2) have become leading vehicles for gene therapy. Most humans in the general population have anti-AAV-2 antibodies as a result of naturally acquired infections. Pre-existing immunity to AAV-2 might affect the functional and safety consequences of AAV-2 vector-mediated gene transfer in clinical applications.

Methods: An enzyme-linked immunosorbent assay (ELISA) method was developed using microwell plates coated with intact particles of recombinant AAV-2 vectors, and horseradish peroxidase-conjugated anti-human immunoglobulin G (HRP-IgG). Neutralizing antibody titres were analysed by assessing the ability of serum antibody to inhibit transduction into HEK293 cells of AAV vectors that express β-galactosidase.

Results: Anti-AAV-2 antibodies were detected by ELISA in two of 20 healthy subjects. The positivity criterion (optical density >0.5) in ELISA corresponded to the cut-off value (320-fold dilution of serum) in the AAV-2 neutralization assay. Influences of interfering substances were not observed.

Conclusion: This ELISA method may be useful for rapid screening of anti-AAV-2 neutralizing antibodies in candidates for gene therapy.

Ann Clin Biochem 2009: 1–3. DOI: 10.1258/acb.2009.009077

Introduction

Adeno-associated virus (AAV) is a small single-stranded DNA virus within the parvovirus family.[1,2] Among more than 100 genotypes of primate AAV, serotype 2 (AAV-2) is the most studied and was the first to be engineered for vector development. Recombinant AAV-2 vectors efficiently transduce both dividing and non-dividing cells and provide long-term gene expression without significant toxicity. Growing numbers of clinical trials have been conducted using AAV-2 vectors to combat various diseases. However, one major problem is the high prevalence of anti-AAV-2 antibodies in the human population. More than 90% of adults demonstrate antibodies that cross-react with one or more AAV serotypes, although markedly fewer (18–32%) show neutralizing antibodies (nAb).[3,4] Pre-existing immunity to AAV-2 may block transduction and intensify the innate response to vector administration, leading to a poor outcome of gene therapy. Thus, measurement of the anti-AAV-2 nAb titre is necessary.

Methods

Recombinant AAV-2 vectors were produced by the triple transduction method as described previously.[5] In brief, HEK293 cells were transfected with the following three plasmids: pAAV2-Rep/vp (containing the AAV-2 *rep* and *cap* genes), pAd (containing the adenovirus genome) and pW1 (containing the β-galactosidase-expression cassette). After three days of incubation, the transfected cells were frozen and thawed, and the recombinant AAV-2 vector particles that were released were purified by two sequential CsCl density gradient centrifugations.

Serum samples from healthy adults were purchased from Advanced BioServices LLC, (Reseda, CA, USA). AAV-specific antibodies were detected using an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtitre plates (Invitrogen, Carlsbad, CA, USA) were coated with 0.5 μg (1.4×10^8 vector genomes [vg]) of AAV-2 vector particles per well. After blocking with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), the plates were washed with 2% sucrose. Serum samples diluted at 1:1000 with PBS/0.1% BSA were added to each well (100 μL/well). The plates were incubated for 1 h at room temperature (RT) and washed three times with PBS/0.05% Tween 20. A solution containing 1 μg/mL horseradish peroxidase-conjugated anti-human immunoglobulin G (HRP-IgG; self-prepared using a heterobifunctional