Table
Study Demographics.

		Thrombus Group (n = 19)	No-Thrombus Group (n = 23)	P	95% Confidence Interval
Gender	Male:Female	0:19	1:22	0.55[a]	
Ages	Years	72 (60–82)	71 (49–84)	0.76[b]	−5 to 6
Disorder distribution	Osteoarthritis:Rheumatoid arthritis	13:6	17:6	0.48[a]	
Volume of intraoperative hemorrhage	ml	46 (0–120)	32 (0–260)	0.43[b]	−22 to 49
Operation time	min	155 (122–199)	148 (106–186)	0.31[b]	−7 to 21

[a] Fisher's exact test.
[b] Unpaired t-test.

The D-dimer level showed the most significant increases at 30 s (median 0.84 μg/ml, $P = 0.01$) and 90 s (median, 0.86 μg/ml, $P = 0.01$) after tourniquet release in the thrombus groups and at 30 s (median 0.73 μg/ml, $P = 0.01$) and 90 s (median 0.7 μg/ml, $P = 0.01$) after tourniquet release in the no-thrombus group (Fig. 2).

The SFMC and e-XDP levels did not differ significantly among the various time points before and after tourniquet release in the thrombus and no-thrombus groups (Fig. 2).

Spearman's rank correlation showed that the PAI-1 levels were strongly correlated with the D-dimer levels at 30 s ($\gamma = 0.57$, $P = 0.01$) and 90 s ($\gamma = 0.6$, $P = 0.01$) after tourniquet release in the thrombus group and were not correlated with the D-dimer levels at 30 ($P = 1.00$) and 90 s ($P = 1.00$) after tourniquet release in the no-thrombus group (Fig. 3).

There was no significant difference in gender, age, volume of intraoperative hemorrhage, or operation time between the thrombus and no-thrombus groups (Table 1).

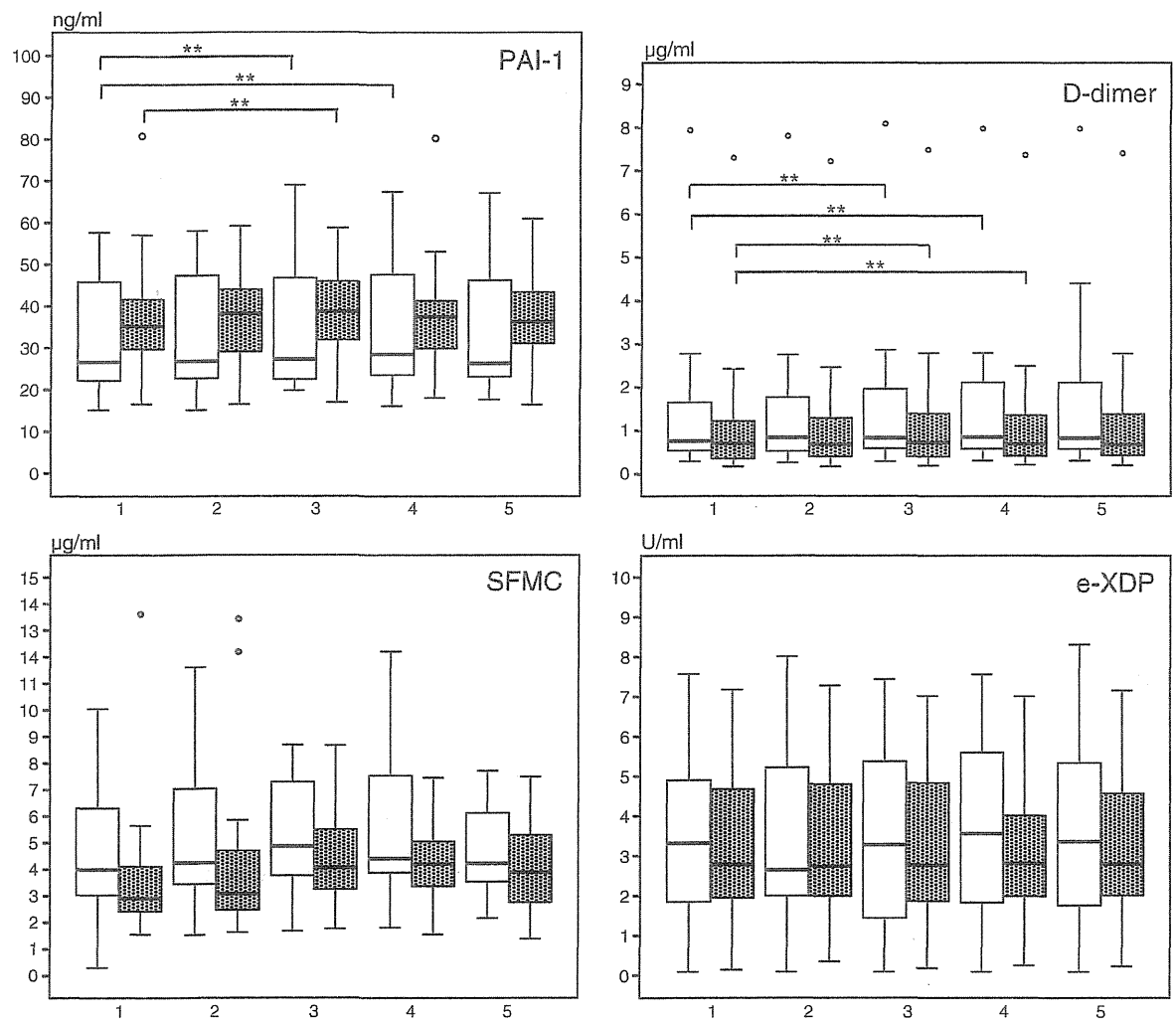

Fig. 2. Changes in the PAI-1 and SFMC, D-dimer, e-XDP levels before and after release of the pneumatic tourniquet. On the x-axis, the numbers correspond to the following: 1: immediately before release of the pneumatic tourniquet; 2: during; 3: at 30 s; 4: at 90 s; 5: at 180 s after release of the pneumatic tourniquet. White boxes, thrombus group; dot boxes, no-thrombus group. °Outlier. **$P < 0.05$ versus the preoperative level in the thrombus and the no-thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. PAI-1, plasminogen activator inhibitor-1; SFMC, soluble fibrin monomer complex; e-XDP, cross-linked fibrin degradation products by leukocyte elastase.

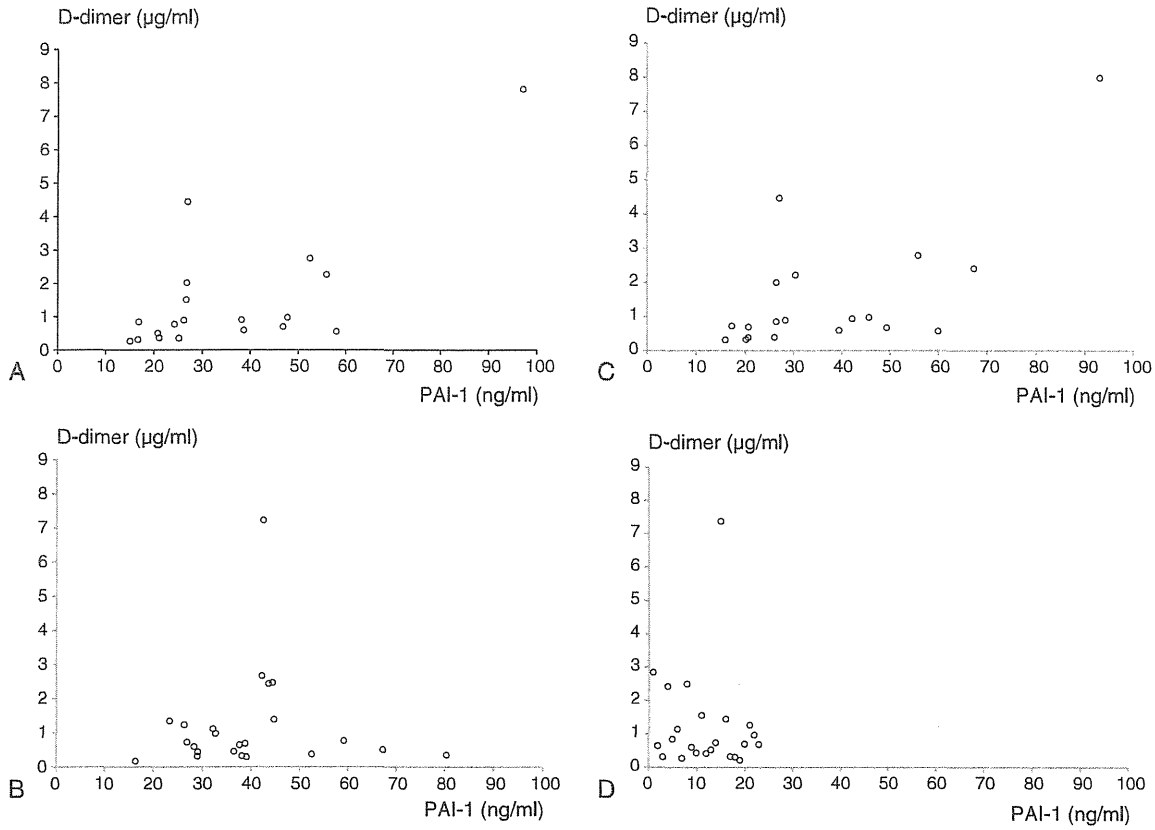

Fig. 3. Correlation between the PAI-1 and D-dimer levels at different time points. (A) At 30 s after release of the pneumatic tourniquet in the thrombus group ($P = 0.01$. $\gamma = 0.57$). (B) At 30 s after release of the pneumatic tourniquet in the no-thrombus group ($P = 1.00$). (C) At 90 s after release of the pneumatic tourniquet in the thrombus group ($P = 0.01$. $\gamma = 0.6$). (D) At 90 s after release of the pneumatic tourniquet in the no-thrombus group ($P = 1.00$). PAI-1, plasminogen activator inhibitor-1.

Discussion

Coagulation–fibrinolysis markers that may be predictive of postoperative asymptomatic DVT and VTE after TKA have been identified. Bounameaux et al [24] performed venography and D-dimer measurements on day 3 after TKA. They found that the D-dimer level was significantly elevated in patients with asymptomatic DVT and that the sensitivity and specificity were 58.8% and 73.5%, respectively, at a cutoff level of 3000 μg/ml. In 2000, Rever et al [25] performed venography after TKA and reported that the SFMC level was significantly elevated in patients with asymptomatic DVT on postoperative days 3 and 6. In 2012, Watanabe et al [26] performed MDCT after TKA and reported that the e-XDP level on postoperative day 1 and the D-dimer level on postoperative day 4 were significantly elevated in patients with asymptomatic VTE. They also noted that the sensitivity and specificity were 75% and 75%, respectively, for e-XDP levels and 59% and 63%, respectively, for D-dimer levels, with cutoff levels of 8.2 U/ml and 7.5 μg/ml, respectively.

Despite this work, there have been no reports of blood coagulation–fibrinolysis markers for predicting postoperative asymptomatic VTE in patients undergoing TKA. In the present study, the PAI-1 level at 90 s after release of the pneumatic tourniquet was significantly higher in the thrombus group than in the no-thrombus group. Furthermore, Spearman's rank correlation showed that PAI-1 was strongly correlated with D-dimer at 90 s ($\gamma = 0.6$, $P = 0.01$) after release in thrombus group and was not correlated with D-dimer at 90 s ($P = 1.00$) after release in the no-thrombus group. As there were no significant differences in gender, age, disorder distribution, volume of intraoperative hemorrhage, or operation time between the thrombus and no-thrombus groups, we can surmise that the PAI-1 level at 90 s after release of the pneumatic tourniquet may be associated with asymptomatic VTE after TKA and is a dependent marker for D-dimer. D-dimer is produced by fibrin and the presence of a thrombus. An elevation in the D-dimer level indicates fibrinolysis of the thrombus. PAI-1 inactivates fibrinolysis by acting on the plasmin in the plasminogen activator–plasmin system. Therefore, we believe that increased inactivation of fibrinolysis leads to the development of asymptomatic VTE after TKA. A recent study has also demonstrated an association between PAI-1 levels and VTE after total hip arthroplasty [27]. If PAI-1 causes asymptomatic VTE and subsequent symptomatic, fatal PE, inactivation of PAI-1 may prevent the development of symptomatic, fatal PE. This study is a single-center study. If the results of future multicenter studies are similar, it may be stated that PAI-1 is likely to cause asymptomatic VTE and, subsequently, symptomatic, fatal PE.

The D-dimer levels at 30 and 90 s and PAI-1 levels at 30 s after release of the pneumatic tourniquet were significantly elevated in both groups compared with the values before tourniquet release. Katsumata et al [14] and Nishiguchi et al [15] measured changes in coagulation–fibrinolysis markers between patients with and without the pneumatic tourniquet after TKA. They identified significantly higher D-dimer levels immediately after surgery and on the first day with the pneumatic tourniquet. In a similar study, Reikeras et al [17] also found elevated D-dimer levels immediately after release of the pneumatic tourniquet. From these clinical studies and our study, we consider that the use of the pneumatic tourniquet during TKA affects the concentrations of blood coagulation–fibrinolysis markers and may cause thrombus formation. Thus, the pneumatic tourniquet should be used at little as possible during TKA.

The PAI-1 level was strongly correlated with the D-dimer level at 30 s ($R = 0.57$, $P = 0.01$) after release in thrombus group and was

not correlated with the D-dimer level at 30 s ($P = 1.00$) after release in no-thrombus groups. The PAI-1-like products that are not associated with fibrinolysis may be in the bone marrow and may be released into the bloodstream at an early time point after release of the pneumatic tourniquet.

One limitation of our study is that we do not know whether early detection of asymptomatic VTE prevents symptomatic, fatal PE. Therefore, we have continued to follow these patients in daily clinics after completion of this study. So far, none of the patients has suffered from symptomatic, fatal PE. Another limitation of our study is that MDCT was performed 4 days preoperatively and postoperatively, and the results therefore reflect the state of asymptomatic VTE at these time points. This is because the incidence of PE after TKA was reported to be high at postoperative days 3 or 4 [16]. Furthermore, day 4 was the earliest point during the postoperative period at which the patients had less pain and could comfortably undergo MDCT. However, because MDCT was not performed between the day of surgery and postoperative day 3, it can be assumed that not all asymptomatic VTEs were detected during the perisurgical period. Thus, the incidence of postsurgical asymptomatic VTE may be underestimated, and larger studies are required to verify the changes in coagulation–fibrinolysis markers in patients with asymptomatic VTE during surgery.

In summary, we investigated changes in blood coagulation–fibrinolysis markers during TKA in thrombus and no-thrombus groups using MDCT. PAI-1 levels were highest at 30 s in both groups and at 90 s in the thrombus group. D-dimer levels were highest at 30 and 90 s in both groups. PAI-1 and D-dimer levels were strongly correlated at both time points in the thrombus group, whereas they were not correlated in the no-thrombus group. Inactivating fibrinolysis due to PAI-1 may lead to the development of asymptomatic VTE and, subsequently, to symptomatic, fatal PE after TKA. Inactivation of PAI-1 may prevent the development of symptomatic, fatal PE after TKA.

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The Prevalence of Neutralizing Antibodies Against Adeno-Associated Virus Capsids Is Reduced in Young Japanese Individuals

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Pre-existing antibodies against adeno-associated virus (AAV), caused by natural AAV infections, interfere with recombinant AAV vector-mediated gene transfer. We studied the prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in healthy subjects (n = 85) and hemophilia patients (n = 59) in a Japanese population. For healthy subjects, the prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 was 36.5%, 35.3%, 37.6%, 32.9%, and 36.5%, respectively, while that in hemophilia patients was 39.7%, 28.8%, 35.6%, 32.9%, and 27.4%, respectively. There was no difference in the prevalence of neutralizing antibody against each AAV serotype between the healthy subjects and the hemophilia patients. The prevalence of neutralizing antibodies against all AAV serotypes increased with age in both healthy subjects and hemophilia patients. High titers of neutralizing antibodies against AAV2 (≥1:224) and AAV8 (≥1:224) were more evident in older individuals (≥42 years old). Approximately 50% of all screened individuals were seronegative for neutralizing antibodies against each AAV tested, while approximately 25% of individuals were seropositive for each AAV serotype tested. The prevalence of seronegativity for all AAV serotypes was 67.0% (healthy subjects, 68.6%; hemophilia patients, 65.0%) and 18.6% (healthy subjects, 20.5%; hemophilia patients, 15.7%) in young (<42 years old) and older subjects (≥42 years old), respectively. The findings from this study suggested that young subjects are more likely to be eligible for gene therapy based on AAV vectors delivered via an intravascular route because of the low prevalence of antibodies to AAV capsids. ***J. Med. Virol.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: AAV vector; gene therapy; epidemiology

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INTRODUCTION

Adeno-associated viruses (AAVs) are members of the *Dependovirus* genus within the *Parvoviridae* family. Over the last two decades, researchers have focused on using AAVs as the backbone of recombinant viral vectors for in vivo gene transfer because of the nature of these recombinant AAV vectors [Bartel et al., 2011; Asokan et al., 2012; Chuah et al., 2012; Foster et al., 2012; Grieger and Samulski, 2012; Hufnagel et al., 2012]. AAV vectors have been used in gene therapy to treat a variety of diseases such as hemophilia and Parkinson's disease [Kay et al., 2000; High, 2007; Mingozzi and High, 2011; Asokan et al., 2012; Grieger and Samulski, 2012]. There are several distinct AAV serotypes, and each has its own tropism; therefore, it is possible to select a specific vector serotype depending on the target organ.

AAV serotype 8 (AAV8) vectors can be used to transfer therapeutic genes to the liver via peripheral injection [Gao et al., 2002; Nathwani et al., 2006; Gao et al., 2009; Nathwani et al., 2011a, 2011b]. This ability of AAV8 vectors to be delivered to the liver provides an advantage over AAV2 vectors. However, some research has shown that neutralizing antibodies against AAVs interferes with AAV vector-mediated gene transfer delivered by intravascular means. The inhibitory effect of neutralizing antibodies on AAV vector-mediated gene transfer appeared to be minimal for intramuscular AAV2 vector delivery in hemophilia patients [Kay et al., 2000; Manno et al., 2003] and intracranial AAV2 vector injection for Parkinson's disease patients [Muramatsu et al., 2010]. However, pre-existing neutralizing antibodies against AAV2 have been shown to interfere with AAV2 vector-mediated factor IX (FIX) gene transfer, via hepatic artery injection, in human subjects [Manno et al., 2006]. Interference of AAV8 vector-mediated gene transfer to the liver by neutralizing antibodies was observed in animal and human subjects [Jiang et al., 2006; Nathwani et al., 2006; Bartel et al., 2011; Chandler and Venditti, 2011; Mingozzi and High, 2011; Nathwani et al., 2011b; Mimuro et al., 2013]. Low titers of neutralizing antibodies against AAV8 significantly inhibit transduction, even when injected into the mesenteric vein branches, suggesting that a short time period is sufficient for neutralizing antibodies in the blood to neutralize AAV8 vectors [Mimuro et al., 2013]. Thus, the inhibitory effects of neutralizing antibodies markedly hamper gene expression following intravascular AAV vector delivery. Elimination of AAV vector-transduced cells by host T cells is another concern associated with AAV vector-based gene therapy [Manno et al., 2006; Mingozzi et al., 2007; Li et al., 2009; Mingozzi et al., 2009; Mingozzi and High, 2011]. However, there is no correlation between the humoral responses to AAV capsids and the T cell-mediated cellular responses to the vector capsid. Epidemiological studies on the prevalence of neutralizing antibodies against various AAV serotypes would help to determine the usefulness of AAV vector-based gene transfer, and assist in selecting the appropriate AAV serotype for clinical applications. There have been some reports on the epidemiology of AAVs across continents [Gao et al., 2004; Boutin et al., 2010; Chandler and Venditti, 2011; Li et al., 2012]. Hemophilia is thought to be an ideal target disease for gene therapy. Clinical trials of hemophilia gene therapy have recently been conducted using various vector types. For hemophilia B patients, AAV vectors carrying the FIX gene have already been used [Kay et al., 2000; Manno et al., 2003; Nathwani et al., 2011b]. However, analysis of the prevalence of AAV infection in hemophilia patients has not been widely reported. The prevalence of AAV infection in these individuals might differ significantly among continents and countries. In this study, the prevalence of neutralizing antibodies against various AAV serotypes in Japanese hemophilia patients was determined and compared with that in healthy subjects.

MATERIALS AND METHODS

Study Design

This epidemiological study was approved by the relevant Ethical Review Boards of the institutions and medical facilities that participated in this study. No therapeutic intervention was performed in this study.

Blood Collection

Blood samples were collected from healthy subjects (n = 85) and patients with hemophilia (n = 59), all of whom were Japanese. The geographic regions of the healthy subjects were not exactly the same as those of the hemophilia patients. Informed consent was given by all study participants, and samples were given new identification numbers upon blood collection with individual information removed except for birth year. Serum was prepared from blood samples and stored at −20°C until required.

AAV Vector Production

The AAV1-CMV-Lac Z vector was produced with a triple plasmid transfection of human embryonic kidney 293 (HEK293) cells (Agilent Technologies, Santa Clara, CA) using pAAV2 Rep/AAV1 Cap, pAAV2-CMV-Lac Z (Agilent Technologies), and pHelper (Agilent Technologies) [Mizukami et al., 2006]. Other AAV serotype vectors were generated in a similar manner, with the packaging plasmid comprising the AAV2 Rep gene and the appropriate Cap gene [Mizukami et al., 2006]. AAV vectors were purified with two rounds of cesium chloride density gradient ultracentrifugation, as described previously [Mizukami et al., 2006]. A quantitative polymerase chain

reaction assay was used to determine the amount of vector genome corresponding to each recombinant AAV vector.

Neutralizing Antibody Assays

Assays for the detection of neutralizing antibodies against AAV serotypes were conducted as reported previously [Mimuro et al., 2013]. Briefly, 5×10^4 HEK293-derived 2V6.11 cells in 100 μl of DMEM/HAM F12, supplemented with 10% fetal bovine serum (FBS) were seeded into the wells of 96-well culture plates. Ponasterone A (10 μl) was added to induce expression of the E4 gene [Mohammadi et al., 2004]. On the day of transduction, 10 μl of serum for testing was incubated with AAV vector (10 μl) solution containing the appropriate number of vector genome copies per 2V6.11 cell (AAV1, 30; AAV2, 30; AAV5, 1,000; AAV8, 1,000; and AAV9, 1,000) for 1 hr at 37°C, and then added to culture wells. The vector doses were pre-determined to obtain a semi-saturated optical density (OD) value for each serotype. Sucrose solution (10 μl) was added to the culture medium at a final concentration of 125 mM. After a 48-hr incubation, β-galactosidase activity was quantified using ortho-nitrophenyl-β-D-galactopyranoside (Invitrogen, Carlsbad, CA) as a substrate, and a color change at 420 nm was quantified using a spectrophotometer (SpectraMax 190). If β-galactosidase activity decreased to lower than 50% of the negative control (FBS), the test sample was judged as seropositive for neutralizing antibodies. Test sera were serially diluted fourfold with FBS prior to incubation with AAV2 or AAV8 vectors. The inhibitory titers of serum samples are presented as the highest final dilution of the sample in the culture medium that demonstrated inhibitory activity.

Statistical Analysis

The χ^2 and Fisher's exact tests were used for statistical analysis of results in Statcel Version 3 for Microsoft Excel. A P-value <0.05 was considered statistically significant.

RESULTS

A subject having neutralizing antibodies against an AAV capsid at a titer of 1:14 or higher, determined by the assay, was considered to be seropositive for the AAV capsid.

AAV1, AAV2, AAV5, AAV8, and AAV9 Seropositivity Increased With Age

Overall seropositivity of neutralizing antibodies against the AAV1, AAV2, AAV5, AAV8, and AAV9 serotypes is presented in Figure 1. For healthy subjects and hemophilia patients, seropositivity was around 30% with no significant differences between the two groups. For the five aforementioned serotypes, seropositivity between the two groups was further analyzed by birth year (Fig. 1). In both healthy subjects and hemophilia patients, AAV2 neutralizing antibody seropositivity increased with age, with a similar correlation observed for other AAV serotypes (Fig. 1). There were significant differences in neutralizing antibody prevalence among all AAV serotypes for healthy subjects born between 1961 and 1970, and from 1971 to 1980 (AAV1, $P=0.026$; AAV2, $P=0.026$; AAV5, $P=0.005$; AAV8, $P=0.026$; AAV9, $P=0.026$). There were significant differences between neutralizing antibody prevalence for AAV2 and AAV9 in hemophilia patients born between 1961 and 1970, and from 1971 to 1980 (AAV2, $P=0.020$; AAV9, $P=0.020$). Significant differences were observed in neutralizing antibody seropositivity against all AAV serotypes in patients with hemophilia born prior to 1960 and for those born between 1961 and 1970 (AAV1, $P=0.017$; AAV2, $P=0.017$; AAV5, $P=0.048$; AAV8, $P=0.017$; AAV9, $P=0.048$). Additionally, neutralizing antibody seropositivity against AAV2, AAV5, and AAV8 in hemophilia patients born before 1960 was significantly higher than that in healthy subjects born prior to 1960 (AAV1, $P=0.038$; AAV2, $P=0.038$; AAV5, $P=0.048$; AAV8, $P=0.038$; AAV9, $P=0.048$). All hemophilia patients born before 1961 were seropositive for neutralizing antibodies against AAV2 and AAV8.

Neutralizing Antibody Titers Against AAV2 and AAV8 Increased With Age of Subjects

Given that the prevalence of neutralizing antibodies against AAV increased with the age of subjects, it was postulated that prevalence of AAV infection might be lower in younger Japanese subjects. Therefore, neutralizing antibody titers against AAV2 and AAV8 were quantified and the ranges of neutralizing antibody titers in age groups were analyzed (Fig. 2). The titers of neutralizing antibodies against AAV2 ($\geq$1:224) and AAV8 ($\geq$1:224) increased with increasing ages of subjects (Fig. 2E and F). More than 60% of individuals that were seropositive for AAV2 at a titer of $\geq$1:896 also had neutralizing antibodies against AAV8 at that titer (healthy subjects, 63.1%; hemophilia patients, 62.5%). Most of these individuals were seropositive for neutralizing antibodies (titer, $\geq$1:224) against AAV8 (healthy subjects, 100%; hemophilia patients, 81.3%).

Neutralizing Antibodies Against Multiple AAV Serotypes

Of the healthy subjects and patients with hemophilia, 49.4% and 52.0%, respectively, were seronegative for neutralizing antibodies against each AAV serotype tested (Fig. 3); 27.1% and 23.3% of healthy subjects and hemophilia patients, respectively, were seropositive for each AAV serotype tested (Fig. 3). Seven individuals (n = 4 healthy subjects, n = 3 patients with hemophilia) were seropositive for

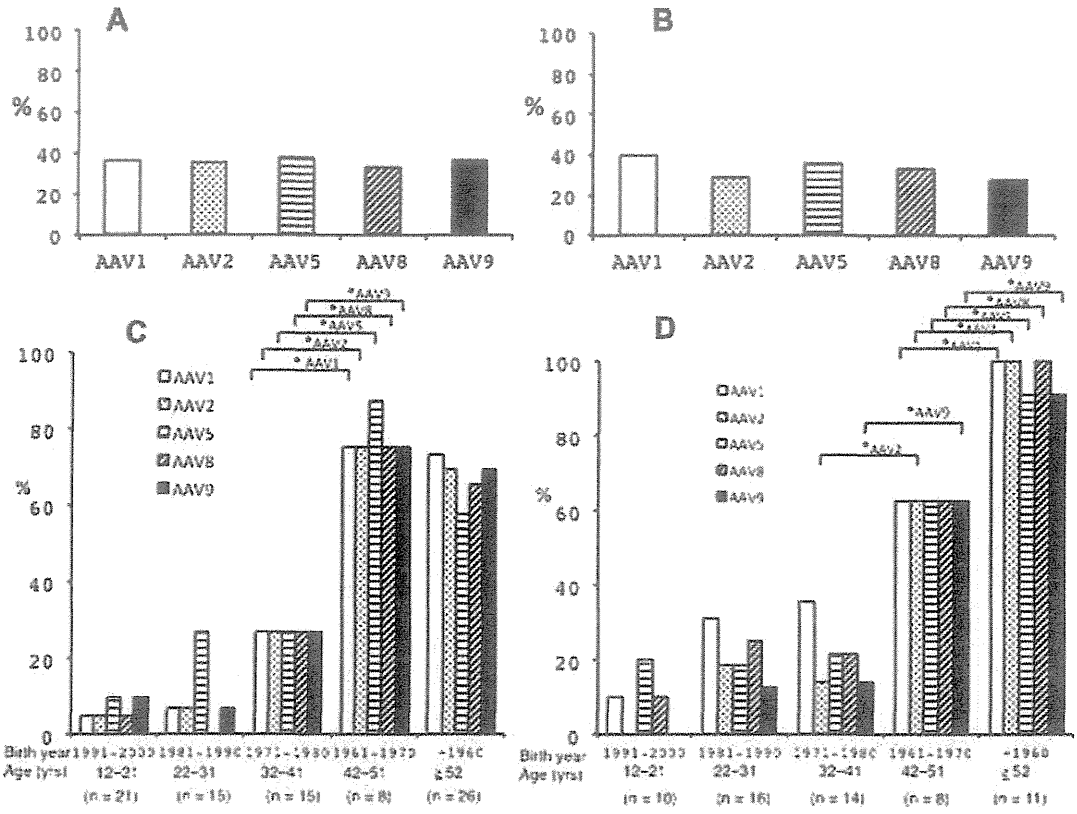

Fig. 1. Prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9. (A) The prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in healthy subjects. (B) The prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in hemophilia patients. (C) Neutralizing antibody seropositivity against AAV serotypes 1, 2, 5, 8, and 9, sub-grouped by birth years and ages for healthy subjects and (D) hemophilia patients. An asterisk (*AAV1–9) indicates a significant increase ($P < 0.05$) in seropositivity against an AAV serotype when compared among various age groups.

neutralizing antibodies against four of the five AAV serotypes, with five of the seven individuals lacking neutralizing antibodies against AAV5. Results were further analyzed by age (Fig. 4), and it was observed that the proportion of healthy subjects seropositive for neutralizing antibodies against each AAV serotype tested increased with age (32–41 years vs. 42–51 years, $P = 0.010$). This trend was more apparent in patients with hemophilia (32–41 years vs. 42–51 years, $P = 0.005$) because >90% of these patients born prior to 1961 were seropositive for neutralizing antibodies against each AAV serotype tested (32–41 years vs. ≥52 years, $P = 0.00003$). The proportion of patients with hemophilia born prior to 1960 that was also seropositive for each AAV serotype tested was significantly higher than for the same generation of healthy subjects ($P = 0.040$).

Sixty-seven percentage (healthy subjects, 68.6%; hemophilia patients, 65.0%) of the young subjects (age <42 years old) were seronegative for all AAV serotypes, whereas 18.6% (healthy subjects, 20.5%; hemophilia patients, 15.7%) of the older subjects (age ≥42 years old) were seronegative for all AAV serotypes.

Neutralizing Antibody Seropositivity Against a Single AAV Serotype

Specific neutralizing antibody seropositivity against a single AAV serotype was observed in a small number of healthy subjects (n = 11) and hemophilia patients (n = 8; Fig. 5). The prevalence of neutralizing antibodies against AAV5 was higher than that for other AAV serotypes, with a specific AAV5 neutralizing antibody found in 10 subjects (7/11 healthy subjects, and 3/8 patients with hemophilia). However, AAV5-specific seropositivity was not convergent for any particular generation. Neutralizing antibody seropositivity against a single AAV serotype, besides AAV5, was observed in four individuals for AAV1, one individual for AAV2, two individuals for AAV8, and two individuals for AAV9.

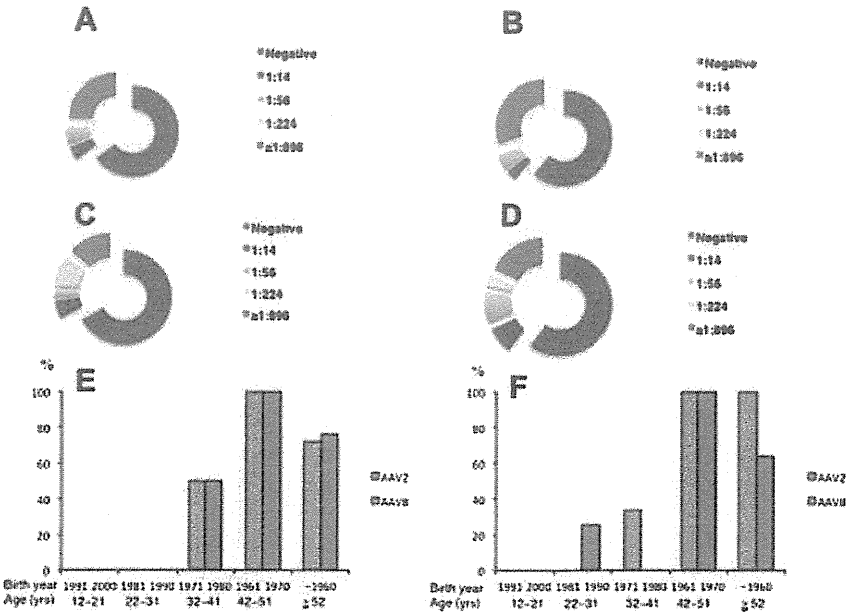

Fig. 2. Neutralizing antibody titers against AAV2 and AAV8. (A) Titers of neutralizing antibodies against AAV2 in healthy subjects, and (B) hemophilia patients. (C) Titers for neutralizing antibodies against AAV8 in healthy subjects, and (D) hemophilia patients. (E) Seroprevalence of neutralizing antibodies at high titers (≥224) sub-grouped by birth years and ages for healthy subjects and (F) hemophilia patients (AAV2, orange; AAV8, purple).

DISCUSSION

Pre-existing immunity against AAV affects the efficacy of AAV vector-mediated gene transfer administered by an intravascular route [Jiang et al., 2006; Nathwani et al., 2006, 2011b; Bartel et al., 2011; Chandler and Venditti, 2011; Mingozzi and High, 2011; Mimuro et al., 2013]. Humans can acquire immunity against AAV following natural infections; however, these infections must be confirmed by detecting antibodies against AAV.

Although there are a variety of AAV serotypes, neutralizing antibodies against a particular AAV serotype may result in cross-reactivity with other AAV serotypes because of similarities in the AAV capsid sequence [Gao et al., 2004; Calcedo et al., 2009; Boutin et al., 2010; Li et al., 2012]. The presence of neutralizing antibodies that cross-react

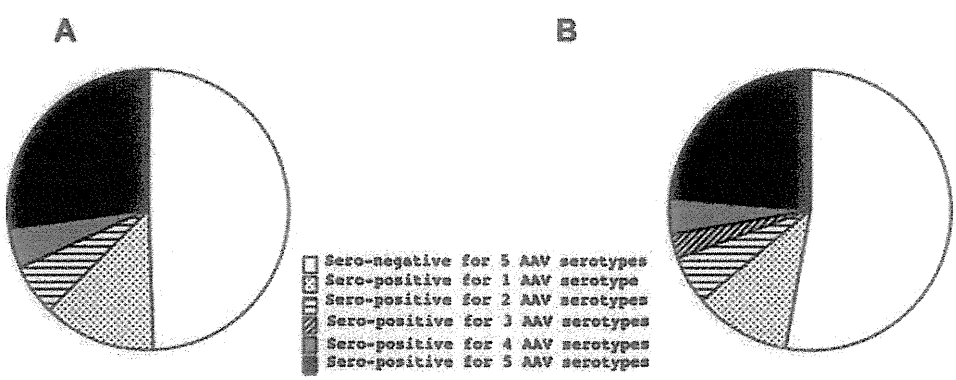

Fig. 3. Neutralizing antibodies against multiple AAV serotypes. (A) For healthy subjects, 49.4% were seronegative for neutralizing antibodies against all AAV serotypes tested, and 27.1% were seropositive for neutralizing antibodies against each AAV serotype tested. (B) For patients with hemophilia, 52.1% were seronegative for neutralizing antibodies against each AAV serotype tested, and 23.3% were seropositive for neutralizing antibodies against each AAV serotype tested.

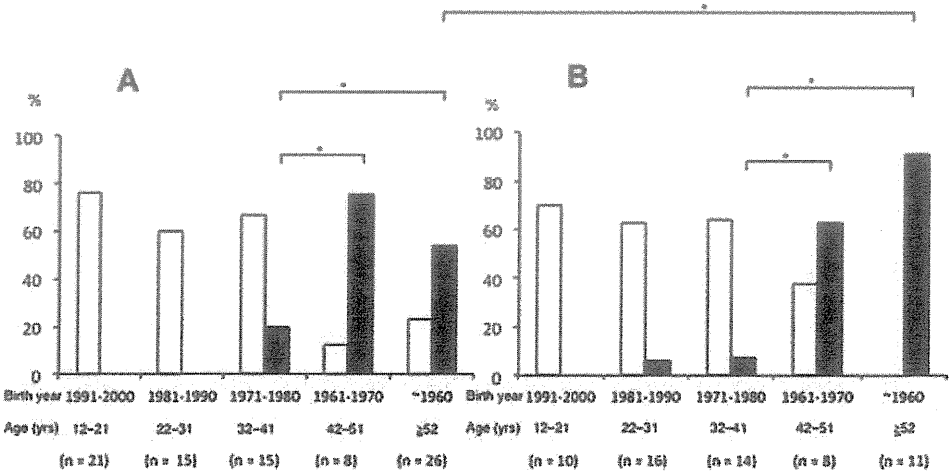

Fig. 4. Seroprevalence of neutralizing antibodies against AAV1, AAV2, AAV5, AAV8, and AAV9. (A) Healthy subjects that were seronegative for all AAV serotypes (white bar), and seropositive for all AAV serotypes (black bar), were sub-grouped and analyzed by generation. (B) Patients with hemophilia that were seronegative for all AAV serotypes (white bar), and seropositive for each AAV serotype (black bar), were sub-grouped and analyzed by generation. An asterisk (*AAV1–9) indicates a significantly higher level ($P < 0.05$) of neutralizing antibodies against every AAV serotype for compared generations.

with other AAV serotypes can prevent the spread of another wild-type AAV in the body following infection. Whether this cross-reaction prevents development of a specific neutralizing antibody against another AAV serotype remains unknown. Reports on the worldwide epidemiology of AAV infection have shown that the prevalence of neutralizing antibodies against AAV2 is approximately 50% [Gao et al., 2004; Calcedo et al., 2009; Boutin et al., 2010; Li et al., 2012]. Here we report that the prevalence of neutralizing antibodies against AAV2 (35.3% in healthy subjects and 28.8% in the hemophilia patients) were lower than those reported previously [Gao et al., 2004; Calcedo et al., 2009; Boutin et al., 2010; Li et al., 2012]. This can be somewhat accounted for by the fact that previous studies examined adult subjects, whereas in this study adolescents and elderly subjects were investigated. Another possibility affecting the results reported herein could be that the current study comprised subjects in an island country. In the present study, the prevalence of neutralizing antibodies against AAV2 in elderly subjects (≥42 years old) was high compared with young subjects. A report by Erles et al. showed that the prevalence of neutralizing antibodies against AAV2 in subjects that were

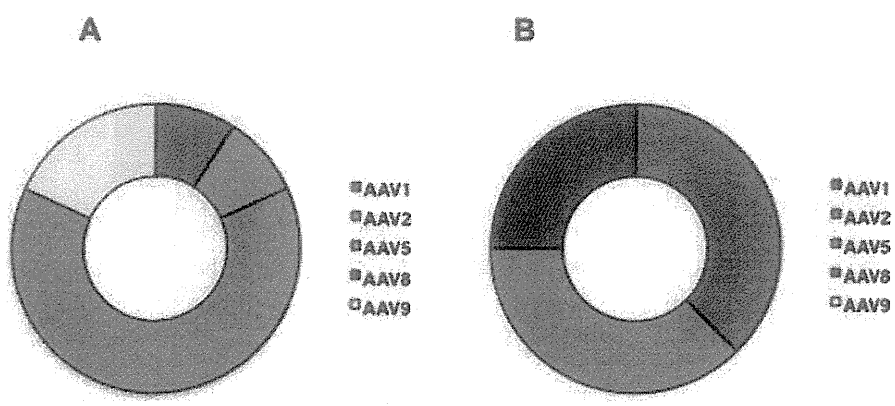

Fig. 5. Specificity of neutralizing antibodies against AAV serotypes. (A) Neutralizing antibodies against a single AAV serotype were observed in healthy subjects (n = 11), and in (B) patients with hemophilia (n = 8). Specific neutralizing antibodies against AAV5 were found in 10/19 (52.6%) individuals (7/11 healthy subjects and 3/8 hemophilia patients).

10 years old or younger was approximately 30%; this is relatively low compared with individuals older than 10 years old. This increased to approximately 60% in adolescents [Erles et al., 1999]. Analysis of neutralizing antibodies against AAV2, AAV5, and AAV8 in children with hemophilia, by Li et al. [2012], showed that their prevalence increased during early childhood. Findings from this current study clearly showed that seropositivity against AAV2 increased with age. However, the increase in prevalence of neutralizing antibodies against AAV2 was not linear but stepwise for certain generations. The level of seropositivity for neutralizing antibodies against AAV2 in adolescents reported here was lower than that previously reported by Erles et al. and exemplified a stepwise increase over two generations. Given that humans are constantly encountering AAVs over a lifetime, it was hypothesized that the level of neutralizing antibodies against AAVs would increase linearly with age. Seropositivity for AAV1 neutralizing antibodies appeared to increase linearly in hemophilia patients (there was no significant difference in the prevalence of AAV1 neutralizing antibodies between neighboring age groups), but not for other AAV serotypes in these patients. A possible explanation for the stepwise increase in neutralizing antibody seropositivity against AAV1, AAV2, AAV5, AAV8, and AAV9 in healthy subjects born between 1971 and 1980 is that there is less likelihood of younger individuals being exposed to these AAV serotypes, following on from the effects of urbanization and improvements in hygiene conditions. The prevalence of neutralizing antibodies against AAV2 at high titers in older individuals notionally supports this finding. As socioeconomic and hygiene conditions have improved, a declining trend in viral infection has also been seen for hepatitis A [Campagna et al., 2012]. Establishment of a sewage system in the late 20th century and the movement of people away from rural districts, and possible AAV reservoirs [Rapti et al., 2012], to urban areas might account for the lower AAV infection rate in younger Japanese individuals.

The prevalence of neutralizing antibodies against AAV2 at high titers increased with age (Fig. 2). The proportion of healthy subjects and hemophilia patients seropositive for neutralizing antibodies against each AAV serotype tested increased with age (Fig. 4). These data suggested that elderly subjects encountered AAV2 more frequently than younger subjects.

Cross-reactivity of AAV2 neutralizing antibodies against other AAV serotypes has been proposed in previous epidemiological studies [Erles et al., 1999; Calcedo et al., 2009; Boutin et al., 2010; Li et al., 2012]. Li et al. [2012] demonstrated that neutralizing antibodies against AAV5 and AAV8 were detected following AAV2 infection because of partial cross reactivity of the AAV2 neutralizing antibody; the high titer of neutralizing antibodies against AAV2 was associated with seropositivity for neutralizing antibodies against AAV5 and/or AAV8. The existence of neutralizing antibodies that react with each AAV serotype was observed in approximately 25% of individuals in this current work. Seropositivity for each AAV serotype tested, converged for subjects born prior to 1971. The findings from this current work are consistent with the hypothesis that neutralizing antibodies are cross-reactive against a broad spectrum of AAV serotypes. The majority of subjects seropositive for neutralizing antibodies, at high titers, against AAV2 were also seropositive for AAV8 neutralizing antibodies at high titers, which is indicative of the cross-reactivity of AAV2 and AAV8 neutralizing antibodies. Some subjects had neutralizing antibodies against a single AAV serotype. Specific seropositivity against AAV5 would be noticeable compared with specific seropositivity against other AAV serotypes as shown in Figure 5. This may be related to the divergence of the AAV5 capsid sequence from other AAV serotype capsids. However, there may not have been an epidemic of AAV5 at a certain time period because the AAV5-specific seropositivity was not convergent for any particular generation. These data suggest that humans respond to AAV infection in a certain way, with the majority of infected individuals developing neutralizing antibodies that cross-react with a broad spectrum of AAV serotypes.

The prevalence of neutralizing antibodies against AAV serotypes in hemophilia patients was similar to that in healthy subjects, except for elderly patients born before 1961. This difference might be related to the level of care, such as blood and/or plasma-derived product transfusion, experienced by elderly patients with hemophilia. Local AAV2 infection rates might affect the prevalence of neutralizing antibodies to AAV in the healthy subjects and hemophilia patients especially in older age groups, since the geographic regions of healthy subjects were not exactly the same as those of the hemophilia patients. It was noticed that there was a decline in the prevalence of neutralizing antibodies against AAV serotypes for younger individuals in a Japanese population. These results suggested that gene therapy employing therapeutic AAV vectors, administered through a peripheral injection route, is more likely to be successful in younger populations, though there might be some difficulties in successfully applying this method to elderly patients.

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Regular Article

Overexpression of factor VII ameliorates bleeding diathesis of factor VIII-deficient mice with inhibitors

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ABSTRACT

Introduction: Factor VIII (FVIII) treatment for hemophilia A has difficulties in correcting bleeding diathesis in the presence of inhibitors.
Materials and Methods: An adeno-associated virus type 8 (AAV8) vector containing the factor VII (FVII) gene or the activated factor VII (FVIIa) gene was used to investigate the therapeutic effect of FVII or FVIIa overexpression in FVIII-deficient mice with inhibitors.
Results: Following repeated human FVIII injection, FVIII-deficient mice developed anti-human FVIII antibodies that cross-reacted with mouse FVIII. High transgene expression of murine FVII or murine FVIIa was achieved using the AAV8 vector and resulted in increased blood FVII activity greater than 800% of normal murine FVII levels in vector-injected FVIII-deficient mice. Thromboelastography analysis showed significant improvements in clotting time, clot formation time, α angle, and mean clot firmness in AAV8 vector-injected FVIII-deficient mice with inhibitors. Overexpression of FVIIa ameliorated the bleeding phenotype of FVIII-deficient mice with inhibitors and significantly increased the survival rate after tail clipping. In addition, overexpression of FVII increased the survival rate of FVIII-deficient mice with inhibitors after tail clipping though it was not as efficient as FVIIa overexpression.
Conclusions: These data suggest that FVII overexpression is an alternative strategy for the treatment of hemophilia A with inhibitors.

Introduction

Hemophilia A is an inherited X-linked bleeding disorder caused by abnormalities of the coagulation factor VIII (FVIII) gene. The current standard therapy for hemophilia A is intravenous injection of recombinant FVIII or plasma-derived FVIII concentrates. Prophylactic administration of FVIII concentrates is effective in preventing harmful bleeding. However, severe hemophilia A patients develop antibodies against FVIII (inhibitors) upon frequent infusion of FVIII concentrates. Gene therapy enables the prevention of life-threatening bleeding in the brain and harmful bleeding in joints by sustained elevation of coagulation factor levels and provides next generation therapy for hemophilia [1,2]. Indeed, clinical trials for hemophilia A and B have been conducted with a variety of gene therapy vectors [3–7]. The current strategy of gene and cell therapy for hemophilia A is the transfer of the normal FVIII gene *in vivo* or transplantation of cells expressing FVIII. However, this strategy may not work for hemophilia A with inhibitors. Thus, an alternative gene transfer approach for hemophilia A with inhibitors could be the overexpression of activated factor VII (FVIIa) [8], which is effective for reducing bleeding diathesis of hemophilia B mice and hemophilia A and B dogs [8,9]. In addition, ectopic expression of FVIIa in platelets reduced bleeding in hemophilia A mice [10]. In the current study, we investigated the forced expression of the mouse FVIIa gene or the FVII gene by an adeno-associated virus type 8 (AAV8) vector in FVIII-deficient (F8KO) mice in the presence of inhibitors against mouse FVIII to determine the therapeutic effect of overexpression of FVII using a genetic approach for hemophilia A with inhibitors.

Materials and Methods

Vector Construction

The characteristics and activity of the liver specific chimeric enhancer/promoter complex, consisting of an enhancer element of the hepatic

Abbreviations: FVII, Factor VII; FVIII, Factor VIII; AAV, adeno-associated virus; F8KO, Factor VIII knock out; HCR, hepatic control region; HAAT, human alpha-1 antitrypsin; CMV, cytomegalovirus; SV40, simian virus 40; PCR, polymerase chain reaction; PT, prothrombin time; APCC, activated prothrombin complex concentrate; FX, Factor X; FIX, Factor IX; FXII, Factor XII.

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control region (HCR, +1– + 325) of the human *Apo E/C-I* gene and the 5' flanking region of the human *α1-antitrypsin* (HAAT, −275– + 25) gene, (HCRHAAT promoter), were described previously [11,12]. The mouse FVII gene (cDNA) and mouse FVIIa gene were cloned in our laboratory as reported previously [10]. A DNA fragment encoding the murine FVII gene (cDNA) or murine FVIIa gene (cDNA) was inserted downstream of the HCRHAAT promoter of p1.1HCRHAAT [11,12] to produce p1.1HCRHAAT-mFVII or p1.1HCRHAAT-mFVIIa. A DNA fragment spanning the cytomegalovirus (CMV) promoter, the LacZ gene, and the polyadenylation signal sequence of the pAAV2 CMV-Lac Z plasmid (Strategene, La Jolla, CA, USA) was replaced by a DNA fragment spanning the HCRHAAT promoter, the FVII gene and the simian virus 40 (SV40) polyadenylation signal sequences of p1.1HCRHAAT-mFVII to produce pAAV2-HCRHAAT-mFVII. The gene transfer plasmid vector pAAV2-HCRHAAT-mFVIIa was constructed as for p1.1HCRHAAT-mFVII.

AAV Vector Production

The vector production system was kindly supplied by Avigen Inc. (San Diego, CA, USA). AAV vectors were packaged with the AAV8 capsid by pseudotyping [11,12]. The chimeric packaging plasmid for AAV8 capsid pseudotyping was synthesized as described previously [12]. DNA fragments harboring the mouse FVII gene or the mouse FVIIa gene located downstream of the HCRHAAT promoter and flanked by AAV2 ITRs were packaged by triple plasmid transfection of human embryonic kidney 293 (HEK 293) cells, kindly supplied by Avigen Inc., with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), and the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA, USA), as described previously [11–13]. For virus vector purification, DNase (Benzonase, Merck Japan, Tokyo, Japan)-treated viral particle-containing samples were subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4), in the presence of 25 mM EDTA, at 16 °C, as described previously [11,12]. Titration of recombinant AAV vectors was performed by quantitative polymerase chain reaction (PCR) using a real time PCR system (StepOnePlus, Applied Biosystems, Tokyo, Japan).

Animal Experiments

C57BL/6 wild-type mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). FVIII-deficient (F8 knock out, F8KO) mice with a targeted destruction of exon 16 of mouse *F8* were reported previously by Bi et al. [14], and generously provided by Dr. H. H. Kazazian Jr. (University of Pennsylvania, Philadelphia, PA, USA) [11,13]. 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.

Inhibitor Generation in FVIII-deficient Mice

F8KO mice were repeatedly injected with human FVIII concentrates (Kogenate FS, Bayer Yakuhin Ltd, Tokyo, Japan) according to the method described by Madoiwa [15] with modifications. Briefly, F8KO mice (16-weeks-old) were injected with 0.05 U/g of human FVIII concentrates (Kogenate) once a week, 4 times and blood was drawn after immunization. Mouse plasma samples were subjected to the FVIII inhibitor assay (Bethesda method) using human FVIII-deficient plasma (Thrombocheck Factor VIII, Sysmex, Kobe, Japan) [15]. Cross-reactivity of FVIII inhibitors raised against human FVIII to mouse FVIII was determined with normal mouse plasma and mouse FVIII-deficient plasma obtained from untreated F8KO mice. Briefly, plasma obtained from F8KO mice was mixed with plasma from wild-type mice (C57/B6) for 2 hours and remaining FVIII activity was quantified using FVIII-deficient plasma.

Determination of Mouse FVII Activity in Mice

The AAV8 vector (5×10^{13} vg/kg) carrying either the mouse FVII gene (AAV8-mFVII) or the mouse FVIIa gene (AAV8-mFVIIa) was injected to the cervical vein plexus of 20-week-old F8KO mice under anesthesia. Blood was drawn from the cervical vein plexus and mixed with 1/10 volume of 3.8% sodium citrate 4 weeks after the vector injection. Platelet-poor plasma was prepared and FVII levels in mouse plasma were quantified by the prothrombin time (PT) method using prothrombin time reagent (Thrombocheck PT, Sysmex, Kobe, Japan) and FVII-deficient plasma (Sysmex, Kobe, Japan) and standardized to normal mouse plasma. Since the plasma of hemophilia A mice has normal mouse FVII activity, the baseline FVII activity of the plasma obtained was determined before vector injection. The plasma obtained from vector-injected mice was diluted and subjected to measurement of FVII activity.

Thromboelastography Analysis

Thromboelastography analysis of mouse blood was performed using a ROTEM apparatus (Pentapharm GmbH) as previously described [10]. Briefly, blood samples containing 0.38% sodium citrate were prepared 4 weeks after vector injection and analyzed using a ROTEM apparatus with the star-TEM reagent (Pentapharm GmbH) according to the manufacturer's instructions.

Tail Clipping Test

Mice were subjected to tail clipping under anesthesia 7 weeks after vector injection. Tails of mice were excised with surgical scalpels 2 cm proximate from the tail ends. Then mice were observed under standard conditions for 24 hours to determine the rate of mortality.

Statistical Analysis

Student's *t*-test, Welch's *t*-test and Chi-square test were used for statistical analysis using software Statcel Ver.3 for Excel. *P* values less than 0.05 were considered statistically significant.

Results

Inhibitor Development in F8KO Mice After Human FVIII Injection

Human FVIII is immunogenic in mice and repeated injection of human FVIII concentrates results in antibody development. As shown in Fig. 1, F8KO mice receiving repeated injection of human FVIII concentrates developed antibodies that cross-reacted with mouse FVIII. The inhibitory titer of the antibody against mouse FVIII (inhibitors) was sufficiently high to inhibit mouse FVIII. There was no significant difference between inhibitor titers in AAV8-mFVII-injected and AAV8-mFVIIa-injected F8KO mice. Thus, these mice could be used to study genetic approaches for hemophilia A with inhibitors.

Expression of Mouse FVII and Mouse FVIIa in F8KO Mice with Inhibitors

The AAV8 vector (5×10^{13} vg/kg) carrying either the mouse FVII gene or the mouse FVIIa gene was injected to F8KO mice with inhibitors against mouse FVIII, to investigate whether the vector could express high levels of FVII or FVIIa in mice. The mean level of FVII activity increased to more than 800% of the baseline in F8KO mice receiving AAV8-mFVII (Fig. 2). The levels of FVII activity in AAV8-mFVIIa injected mouse plasma were similar to those for AAV8-mFVII-injected F8KO mice. There was no significant difference between the FVII activity levels of AAV8-mFVII-injected F8KO mice and AAV8-mFVIIa-injected F8KO mice.

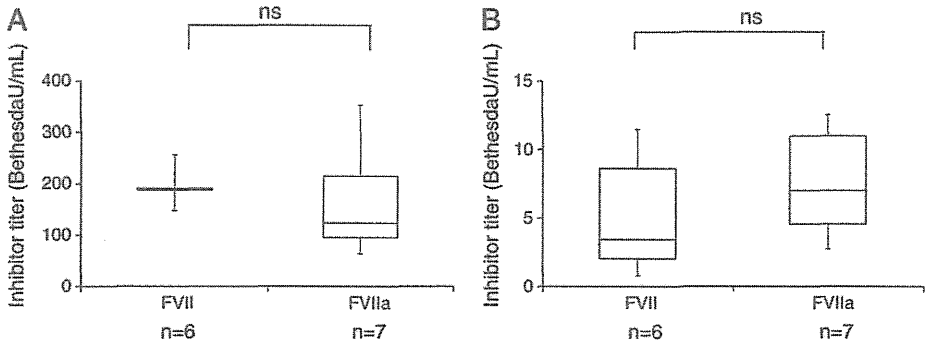

Fig. 1. Inhibitory titers of anti-FVIII antibodies. Inhibitory activity against human FVIII (A) and mouse FVIII (B) in F8KO mice are shown. Plasma samples were diluted in buffer, incubated with normal human plasma or wild-type mouse plasma for 2 hours and subjected to the APTT assay using FVIII-deficient plasma. Inhibitory titers were calculated according to the Bethesda method. Values are shown as Whisker Box plots. There were no significant differences between the FVII levels of AAV8-mFVII-injected mice (n = 6) and AAV8-mFVIIa-injected mice (n = 7) (Student's *t*-test). ns: not significant.

Treatment Efficacy of Overexpressed FVII or FVIIa

Treatment efficacy of overexpressed FVII and FVIIa by the respective AAV8 vectors was studied in F8KO mice with inhibitors by thromboelastography analysis and a tail-clipping test (Figs. 3, 4 and Table 1). The representative thromboelastograms of AAV8-mFVIIa-injected mice were comparable to wild-type mice except for the clotting time (Fig. 3). Thromboelastography analysis of blood from AAV8-mFVII-injected mice showed that the parameters and thromboelastogram improved but that the changes were to a lesser extent compared with AAV8-mFVIIa-injected mice. Thromboelastography analysis showed that the clot formation time, maximum clot firmness and α angle improved in F8KO mice with AAV8-mFVIIa injection and were similar to those of wild-type mice. All thromboelastography parameters except for the clotting time of FVIIa-overexpressed F8KO mice were similar to those of wild-type mice. Although the clotting time was shortened in FVIIa overexpressed F8KO mice, it was still prolonged relative to wild-type mice. The thromboelastography parameters of FVII-overexpressed F8KO mice were inferior to those of FVIIa-overexpressed mice. The improvement of the thromboelastography parameters from FVII-overexpressed F8KO mice was apparent. However, the *in vivo* effect of FVII or FVIIa overexpression might be less than expected from the thromboelastography analysis. Therefore, these mice were subjected to tail clipping challenge 7 weeks after vector injection to investigate whether overexpression of FVII or FVIIa could ameliorate the bleeding diathesis of F8KO mice. As reported previously, all F8KO mice that did not receive vector injection (n = 7) died within 24 hours of tail clipping regardless of the presence of inhibitors against mouse FVIII. The survival rate of AAV8-mFVIIa-injected mice (n = 7) was 85.7% and 50.0% for AAV8-mFVII-injected mice (n = 6), suggesting that overexpression of FVII and of FVIIa significantly ameliorated bleeding diathesis of F8KO mice with inhibitors.

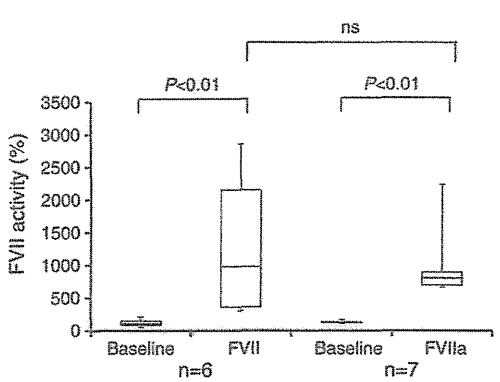

Fig. 2. Mouse FVII and mouse FVIIa expression in F8KO mice after injection of AAV8 vectors. FVII activity levels in plasma from F8KO mice 4 weeks after vector injection (FVII, AAV8-mFVII-injected mice (n = 6); FVIIa, AAV8-mFVIIa-injected mice (n = 7) were quantified. The baseline FVII activity levels (baseline) of these mice were also measured simultaneously. Values are shown as Whisker Box plots. The differences between values were analyzed by Student's *t*-test and *p* values are shown. ns: not significant.

The FVII activities in AAV8-mFVII-injected F8KO mice and AAV8-mFVIIa-injected mice were 515% (18 weeks after vector injection) and 488% (29 weeks after vector injection), respectively.

Discussion

Inhibitor (antibody against infused FVIII) development in hemophilia A patients interferes with FVIII treatment. Thus, the next generation therapy for hemophilia A using a genetic approach to force expression of the normal FVIII gene or transplanting cells expressing FVIII may be applicable to hemophilia A patients without inhibitors, but may not be effective in correcting bleeding diathesis in hemophilia A patients with inhibitors. The incidence of inhibitor development in hemophilia A is much higher than for hemophilia B, suggesting that inhibitor development in hemophilia A patients is a serious problem that interferes with therapy [16]. In the medical practice setting, bleeding events of hemophilia A patients with inhibitors can be treated with activated prothrombin complex concentrate (APCC) or recombinant FVIIa. Therefore, forced expression of FVIIa is an alternative approach for hemophilia gene therapy when the recipients have inhibitors as described previously [8]. Treatment with overexpression of FVIIa in hemophilia B mice using an AAV2 vector was effective for ameliorating the bleeding phenotype of hemophilia B mice [8]. This was also shown in hemophilia A and B dogs treated with AAV8 vectors carrying the canine FVIIa gene [9]. In the current study, we explored the possibility of ameliorating bleeding diathesis of hemophilia A mice with inhibitors by overexpressing FVII or FVIIa using an AAV8 vector carrying the respective gene.

Inhibitors against human FVIII develop by repeated infusion of human FVIII concentrates to FVIII-deficient mice. This inhibitor is thought to be an antibody against the xenoantigen (human FVIII) but it also cross-reacts with mouse FVIII (Supplementary Fig. S1). Therefore, it could act as an alloantibody against mouse FVIII in F8KO (hemophilia A) mice. Titers of the inhibitor against mouse FVIII were approximately 1/40–1/20 of that against human FVIII by the Bethesda assay but high enough to inhibit mouse FVIII activity. Thereby, hemophilia A mice with inhibitors generated by repeated injection of human FVIII concentrates could be used as a model of hemophilia A with inhibitors.

We overexpressed FVII or FVIIa in hemophilia A mice with inhibitors against mouse FVIII, using an AAV8 vector. Levels of FVIIa in mice are thought to be comparable to the therapeutic levels of

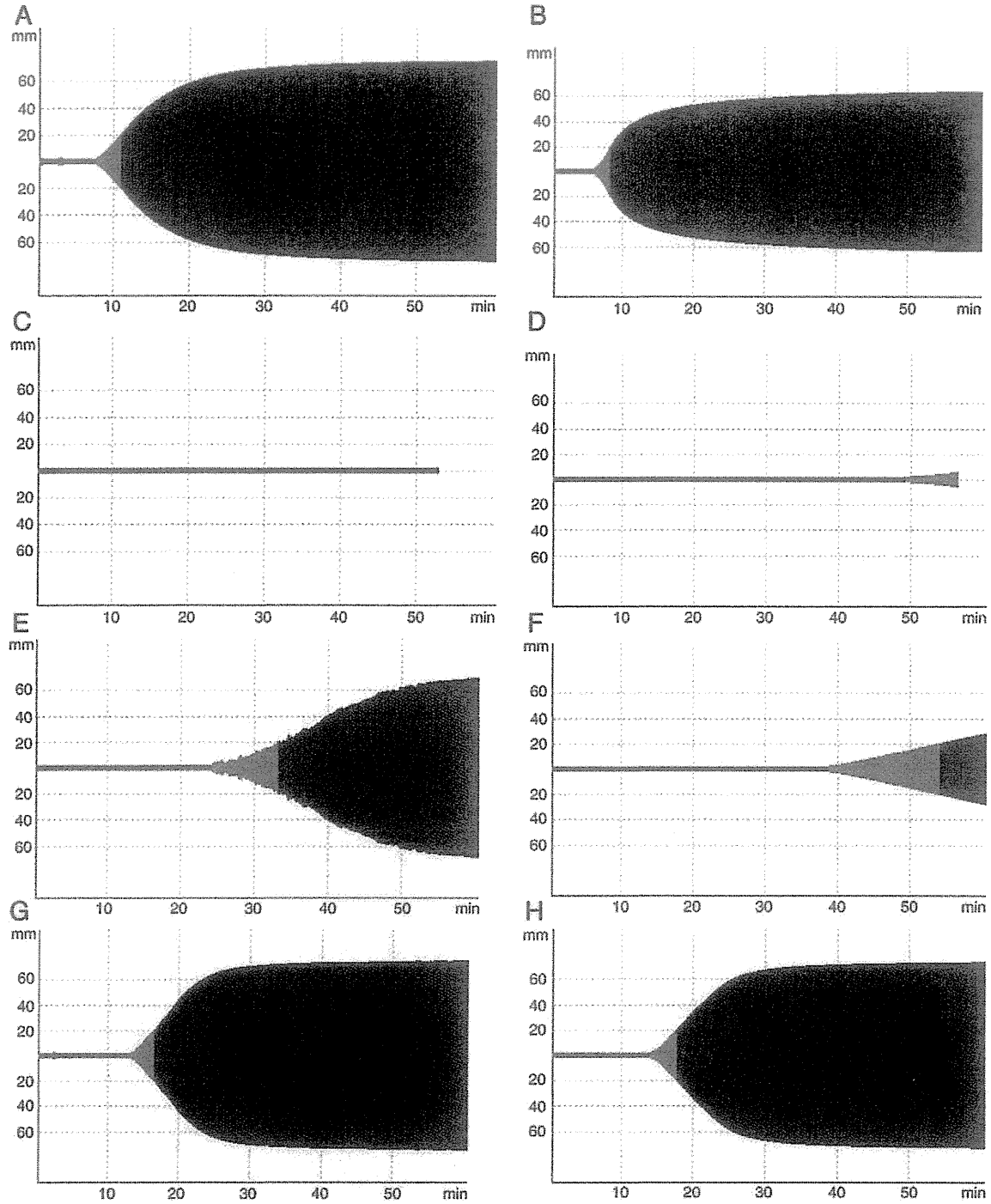

Fig. 3. Thromboelastography analysis. Mouse blood obtained from wild-type mice (A, B), F8KO mice (C, D), AAV8-mFVII-injected F8KO mice with inhibitors (E, F), or AAV8-mFVIIa-injected mice with inhibitors (G, H) was analyzed using a ROTEM delta. The representative thromboelastograms of these mice are shown.

recombinant human FVIIa concentrates in hemophilia A patients with inhibitors to stop bleeding. The therapeutic effect of overexpression of FVII or FVIIa shown in the thromboelastography analysis was confirmed by tail clipping challenge *in vivo*. The overexpression of FVIIa by an AAV8 vector significantly increased the survival rate of hemophilia A mice with inhibitors. The therapeutic effect of FVII overexpression was also significant in the tail clipping challenge in hemophilia A mice with inhibitors. The survival rate of FVII-overexpressed F8KO mice after tail clipping was higher than untreated F8KO mice and lower than FVIIa-overexpressed F8KO mice. Although the survival rate was lower than that for mice with FVIIa overexpression, the difference between these survival rates was not statistically significant.

When hemophilia patients were administered the anti-virus drug ribavirin for the treatment of hepatitis C, these patients bled less frequently [17]. Increased levels of FVII in these patients might account for reduced bleeding diathesis [18], suggesting that high expression levels of FVII in hemophilia patients with or without inhibitors

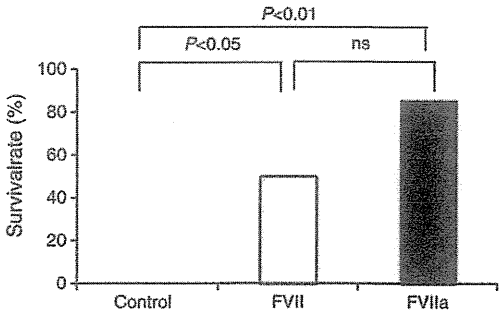

Fig. 4. Survival rates of F8KO mice after tail clipping. The survival rates of F8KO mice are shown. All wild-type mice survived challenge by tail clipping (data not shown), while all F8KO mice (n = 7) died after tail clipping regardless of the presence of inhibitors against FVIII. Significant numbers of mouse FVII overexpressing F8KO mice with inhibitors (n = 6) and mouse FVIIa overexpressing F8KO mice with inhibitors (n = 7) survived challenge by tail clipping. The differences between values were analyzed by Chi-square test and *p* values are shown. ns: not significant.

might ameliorate bleeding. Our results were consistent with this previous report. The therapeutic effect of forced FVII expression using an AAV vector was previously studied in FVII deficient (F7KO) mice [19]. In this animal model, forced FVII expression protected postnatal hemorrhage and improved the survival of vector-injected F7KO mice. The mechanism of therapeutic effect of forced FVII expression in F7KO mice was similar to that of forced FVIII expression in F8KO mice. The current study showed a therapeutic effect of FVII overexpression on bleeding diathesis in F8KO mice.

The hypothetical mechanism of ameliorating bleeding diathesis of F8KO mice by FVII overexpression could be explained as follows. FVII is converted to FVIIa mainly by activated factor X (FXa), and possibly by activated factor IX (FIXa), activated factor XII (FXIIa), thrombin, and FVIIa [20–23]. The physiological role of FVII activating protease in blood coagulation is still unknown [24]. When bleeding occurs, FVIIa binds to tissue factor and activates FX, FIX, and possibly FVII [25]. When FVII is present at a high concentration due to AAV8 vector-induced FVII overexpression, subsequently generated FXa, FIXa, and FVIIa activate FVII and produce a large amount of FVIIa that could in turn bind to tissue factor at the bleeding site and accelerate coagulation. However, this may be less efficient than the amplification of the coagulation cascade by the FIXa/FVIIIa pathway. Enhanced hemostasis with FVII overexpression might differ from FVIIa overexpression. When FVIIa is overexpressed, the FVIIa concentration in the circulation is high and FVIIa in complex with tissue factor activates FX and FIX. Compared with FVIIa overexpression, the FVIIa concentration in the circulation may not be such high when FVII is overexpressed, but the local FVIIa concentration at the bleeding site might be high due to amplification effects as described above. This might account for the increased survival of F8KO mice with FVII overexpression after tail clipping although the improvement of ROTEM parameters with FVII was modest.

Studies to test the safety of overexpression of FVIIa demonstrated it was related to the early death of mice [26,27]. This was apparent in mice with overexpression of an FVIIa variant that had a higher coagulation activity [26]. This adverse event was attributed to the thrombogenicity of the overexpressed FVIIa or its variant. Therefore, continuous overexpression of FVIIa might have a higher risk of adverse events than intermittent administration of FVIIa. The safety of overexpression of FVIIa in dogs was previously shown using molecular markers [9]. However, no pathological examination was conducted, so the thrombogenicity of FVIIa overexpression in the canine model is not clear. Overexpression of human FVII with an AAV8 vector was studied in monkeys and human FVII overexpression continued up to 28 weeks after vector injection without toxicity [19]. Although further studies are required to determine the safety of continuous FVII overexpression regarding thrombogenicity, we demonstrated a therapeutic effect of FVII overexpression in a mouse model of hemophilia A with inhibitors. Our future studies will investigate the safety of FVII overexpression in hemophilia mice and wild type mice in comparison with FVIIa overexpression. In addition to the genetic approach, FVII variants with a longer plasma half-life or drugs that enhance FVII gene expression might be an alternative therapeutic approach for hemophilia patients with inhibitors in clinical settings.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2013.03.007.

Table 1
Changes in thromboelastography parameters following the over expression of mFVII and mFVIIa.

	CT	CFT	MCF	α angle
Wild type	255 ± 91.8 (n = 4)	175.5 ± 25.1 (n = 4)	64.3 ± 1.0 (n = 4)	60.5 ± 2.1 (n = 4)
F8KO	2726 ± 409.7 (n = 8)	ND	ND	ND
FVII	1698 ± 403.0 (n = 4)	1132 ± 572.0 (n = 5)	33 ± 35.9 (n = 5)	28.5 ± 10.1 (n = 4)
FVIIa	751.6 ± 139.4 (n = 5)	183 ± 36.0 (n = 5)	74.4 ± 1.7 (n = 5)	59.2 ± 4.6 (n = 5)

ND: values were not determined because CFT, α angle, and MCF could not be measured in most control F8KO mouse blood samples.
CT: clotting time, CFT: clot formation time, MCF: maximum clot firmness.
Wild-type: wild-type mice, F8KO: F8 knock out (FVIII-deficient) mice, FVII: FVII over expressing F8KO mice, FVIIa: FVIIa over expressing F8KO mice.

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Conflict of Interest Statement

The authors confirm no conflicts of interest to declare.

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Minimizing the Inhibitory Effect of Neutralizing Antibody for Efficient Gene Expression in the Liver With Adeno-associated Virus 8 Vectors

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Neutralizing antibodies (NAbs) against adeno-associated viruses (AAVs) are known to interfere with AAV vector-mediated gene transfer by intravascular delivery. Evading the inhibitory effects of antibodies against AAV vectors is necessary for efficient transfer of therapeutic genes clinically. For this purpose, we tested the efficacy of saline flushing in order to avoid contact of vectors with NAbs present in blood. Direct injection of the AAV8 vector carrying the *factor IX* (*FIX*) gene into the portal vein of macaques using saline flushing achieved transgene-derived FIX expression (4.7 ± 2.10–10.1 ± 5.45% of normal human FIX concentration) in the presence of NAbs. Expression was as efficient as that (5.43 ± 2.59–12.68 ± 4.83%) in macaques lacking NAbs. We next tested the efficacy of saline flushing using less invasive balloon catheter-guided injection. This approach also resulted in efficient expression of transgene-derived FIX (2.5 ± 1.06–9.0 ± 2.37%) in the presence of NAbs (14–56× dilutions). NAbs at this range of titers reduced the efficiency of transduction in the macaque liver by 100-fold when the same vector was injected into mesenteric veins without balloon catheters. Our results suggest that portal vein-directed vector delivery strategies with flushing to remove blood are efficacious for minimizing the inhibitory effect of anti-AAV antibodies.

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INTRODUCTION

Gene and cell therapies are expected to be the next generation of therapies for a variety of inherited diseases. Hemophilia is thought to be an ideal target disease for these approaches as it is caused by a genetic abnormality in the factor VIII gene for hemophilia A, or the factor IX (FIX) gene for hemophilia B.[1–7] The current strategy of hemophilia gene therapy involves inducing expression of the normal coagulation factor gene or transplanting cells expressing the respective coagulation factor. The liver is normally the primary target of gene transfer for coagulation factors since the majority of these coagulation factors are synthesized in the liver with appropriate post-translation modifications before secretion into the circulatory system.

Substantial effort has been applied to express coagulation factor genes using various vector types. Among the viral vectors, recombinant adeno-associated virus (AAV) vectors are preferred for therapeutic gene transfer *in vivo* because they reside in the episome and rarely integrate into genomes. However, retrovirus vectors including lentivirus vectors require integration into the host cell genome.[6,7] In addition, AAV vectors can transfer genes to nondividing cells and allow long-term expression of transgenes in these cells.

Clinical trials for hemophilia gene therapy have recently been conducted using various types of vectors.[4–11] These trials were designed based upon data obtained from mouse models of hemophilia and hemophiliac dogs and proved to be more efficient in these models than for humans. Species differences between humans and these other animal models might partially account for the results observed. Therefore, gene transfer studies in non-\ human primates may well predict the efficacy of gene transfer in humans. Indeed, *FIX* gene transfer studies using a new type of vector have been conducted in rhesus macaques.[12,13] The results from these studies provided the basis for recent hemophilia B gene therapy clinical trials employing an AAV8 vector.[13–16] Gene transfer in mice using AAV vectors results in excellent transduction efficiency. This is especially so for AAV8 vector-mediated gene transfer in the mouse liver;[12–14,17] however, the efficacy of AAV8 vectors is modest in macaques.[13]

There are also difficulties associated with *FIX* gene expression when using AAV8 vectors in nonhuman primates. Growing evidence suggests that the presence of neutralizing antibodies (NAbs) against AAV8, due to previous natural infection by wild-type AAV, significantly inhibits transduction in the macaque liver. It is likely that antibodies against one serotype of AAV cross-react with other AAV serotypes.[18] A hemophilia B gene therapy

J.M., H.M., S.H., and T.I. contributed equally to this work.

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Table 1 Expression of macaque T262A in nonhuman primates with AAV8-HCRHAAT-macFIXT262A

Macaque number	Age	Vector dose (vg/kg)	Route of vector injection	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#14	5.7	1×10^{12}	Mesenteric vein	0.02 ± 0.019	0.1	56×
#17	5.8	1×10^{13}	Mesenteric vein	0.13 ± 0.081	0.4	56×
#24	6.6	1×10^{12}	Mesenteric vein	0.09 ± 0.048	0.5	14×
#28	7.8	5×10^{12}	Saphenous vein	12.68 ± 4.83	38.2	Negative
#30	2.9	5×10^{12}	Saphenous vein	5.43 ± 2.59	48.2	Negative
#31	2.9	5×10^{12}	Saphenous vein	7.64 ± 2.32	49.6	Negative

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region.
The concentration of macaque FIX T262A is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 neutralizing antibody (NAb) titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

clinical study using an AAV8 vector was successfully conducted in hemophilia B patients negative for pre-existing antibodies against AAV8.[15] Because of the high prevalence of AAV infection in humans,[18] evading NAbs against this virus is an important hurdle to overcome before AAV8 vectors can be routinely and effectively employed for therapies.

The aim of our study was to develop an administration method of AAV8 vectors that assisted in minimizing the inhibitory effect of NAbs against AAV in macaques that were already seropositive for AAV8 antibodies.

RESULTS

The AAV8 vector carrying the macaque *FIX T262A* gene located downstream of the liver-specific chimeric promoter consisted of an enhancer element of hepatic control region (HCR) of the *ApoE/C-I* gene and the 5′ flanking region of the *α1-antitrypsin* (HAAT) gene (AAV8-HCRHAAT-macFIXT262A). This vector was used to express mutant macaque FIX containing a single amino acid substitution of Thr to Ala at the position 262 (macaque FIX T262A) in the following experiments. Macaque FIX T262A but not wild-type macaque FIX could be bound to human FIX-specific monoclonal antibody 3A6, thereby macaque FIX T262A expressed in macaques with AAV8-HCRHAAT-macFIXT262A could be precisely quantified by an enzyme immunoassay with 3A6.[17] The amino acid sequence of macaque FIX is highly homologous to the human FIX amino acid sequence. Twelve amino acid residues of human FIX are different at corresponding positions of macaque FIX, while only one amino acid of macaque FIX T262A is different from wild-type macaque FIX. Expression of macFIX T262A in a macaque would mimic a situation where normal human FIX is expressed in a hemophilia B patient with a missense mutation in the *FIX* gene.

Results corresponding to the expression of macaque FIX T262A following injection of AAV8HCRHAATmacFIXT262A can be seen in Table 1. When AAV8HCRHAATmacFIXT262A (5×10^{12} vector genome copies (vg)/kg) was injected into the saphenous veins of three AAV8 NAb-negative macaques (#28, #30, #31), expression of macFIX T262A in the therapeutic range (>5% of normal FIX concentration) was achieved. However, injection of the same vector (1×10^{12}–1×10^{13} vg/kg) into the mesenteric vein branches of AAV8 NAb-positive macaques (#14, #17, #24; inhibitory titers: 14–56×) resulted in subtherapeutic levels (<0.2%) of macFIX T262A expression. The amount of vector DNA in the liver of AAV8 NAb-positive macaques was ~1% of that seen in AAV8 NAb-negative macaques (Table 1). These data suggest that low titers of NAbs against AAV8 significantly inhibit transduction even when the vector is injected into the mesenteric vein branches. In addition, only short period of time may be required for NAbs in the blood to neutralize the AAV8 vector since the blood of the mesenteric vein rapidly goes to the liver through the portal vein after gathering with the blood from other viscera.

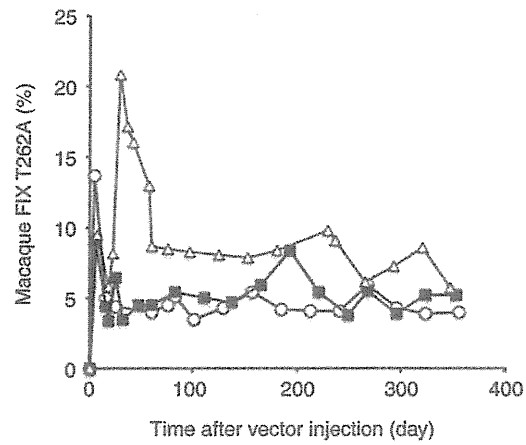

Figure 1 Expression of FIX T262A in macaques after direct vector injection into portal veins. Macaques (n = 3) were subjected to direct injection of AAV8 vector into the portal vein. Concentrations of FIX T262A in macaque plasma samples (macaque #26, open triangles; #27, open circles; #29, closed squares) were measured by ELISA. AAV, adeno-associated virus; ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Evading AAV8 NAbs could be achieved by ensuring the AAV8 vector and NAbs do not come into physical contact with each other in the blood. Blood enters the liver from the hepatic artery and portal vein. The hepatic artery accounts for ~20–30% of blood flow, while the portal vein supplies the remaining blood flow to hepatocytes.[19,20] Blood from the portal vein and hepatic artery are eventually mixed in the sinusoids of the liver; however, the blood from the portal vein mainly supplies hepatocytes. Therefore, direct injection of AAV8 vectors into the portal vein branch was

Table 2 Expression of macaque T262A in nonhuman primates with direct, and balloon catheter-guided vector (AAV8-HCRHAAT-macFIXT262A) injection into the portal vein

Macaque ID	Age	Vector dose (vg/kg)	Injection method to portal vein branch	FIX T262A concentration (%)	Vector genome copies in liver tissue (vg/diploid genome)	Anti-AAV8 NAb titer
#26	10.1	5×10^{12}	Direct	4.7 ± 2.10	77.9	28×
#27	7.4	5×10^{12}	Direct	10.1 ± 5.45	28.5	14×
#29	11.0	5×10^{12}	Direct	5.3 ± 1.40	64.3	14×
#37	7.5	5×10^{12}	Catheter-guided	9.0 ± 2.37	61.1	14×
#38	10.7	5×10^{12}	Catheter-guided	3.2 ± 1.21	13	56×
#42	7.7	5×10^{12}	Catheter-guided	2.5 ± 1.06	15.3	14×

Abbreviations: AAV, adeno-associated virus; FIX, factor IX; HAAT, α1-antitrypsin; HCR, hepatic control region; Nab, neutralizing antibody.
FIX T262A concentration is expressed as a percentage of normal human plasma FIX concentration; anti-AAV8 NAb titer is expressed as the final dilution of the test serum in the assay; vector genome (vg) copies in liver cells were determined by quantitative PCR and expressed as copy numbers per cell.

investigated to determine whether saline flushing to remove blood from the portal vein just before injection of the vector would diminish the inhibitory effects of anti-AAV8 NAbs. Three macaques (#26, #27, #29; inhibitory titers: 14–28×) were directly injected with vector (5×10^{12} vg/kg) into the left portal vein after flushing saline to remove blood (**Supplementary Table S1**). Expression of transgene-derived FIX (macaque FIX T262A) increased to therapeutic levels with the AAV8 vector carrying the macaque *FIX T262A* gene and persisted for greater than 1 year in the three macaques (Figure 1). Average FIX and vector genome levels in macaque liver tissues are presented in Table 2. Compared with the results of vector injection to the mesenteric vein of NAb-positive macaques #14, #17, and #24 (Table 1), the levels of macaque FIX T262A in the circulation of the macaques #26, #27, and #29 that received vector injection directly to the left portal vein with flushing to remove blood, were increased to therapeutic levels with significant amounts of vector genome detected (Table 2).

Blood chemistry analysis and liver biopsies were conducted following administration of the vector to determine whether there were any adverse effects induced by the injection. Moderate increases in liver enzymes, such as transaminases, were observed just after injection of the vector (**Supplementary Figure S1**). However, no significant pathological changes were seen in liver biopsy samples taken on days 14, 28 or 48 (data not shown). We did not observe an increase in the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)-positive hepatocytes in the liver biopsy specimens (data not shown).

The direct injection of the AAV8 vector into the left portal vein branch with saline flushing to remove blood from the portal vein just before injection of the vector was effective to minimize the inhibitory effects of anti-AAV8 NAbs. Therefore, we explored the possibility of utilizing a balloon catheter to perform the vector injection into the portal vein branch with saline flushing to remove blood, taking the concern about the safety of the procedures into consideration. Using a microballoon catheter, we injected the vector into the left portal vein of three anti-AAV8 antibody-positive macaques (#37, # 38, #42; inhibitory titers: 14–56×) (Table 2, **Supplementary Table S2**). Fluorography in macaque #37 representing angiography of the portal vein branch is shown in Figure 2 and **Supplementary Video S1**. Increase of FIX T262A to therapeutic levels was achieved in the three macaques (#37, # 38, #42),

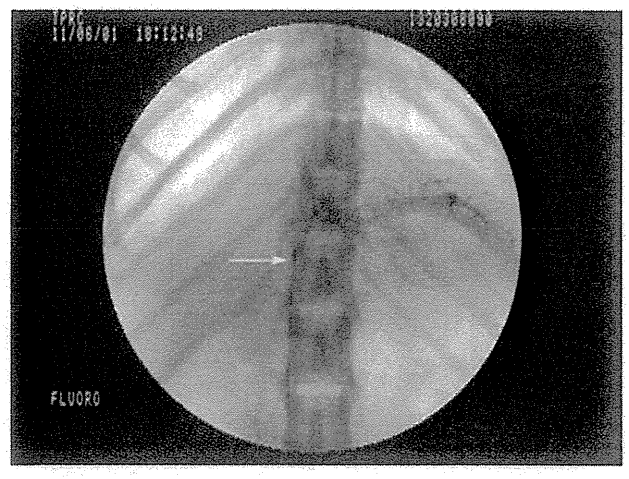

Figure 2 Fluorography in macaque #37. A balloon catheter was inserted into the portal vein of macaque #37 and contrast medium injected before vector administration. The left portal vein branches can be visualized. The arrow (white) indicates the tip of the catheter. See **Supplementary Video S1** which also recorded inflation of the balloon before the vector administration and deflation of the balloon after the administration.

and macaque FIX T262A expression in the circulation persisted (Figure 3, Table 2). The two portal vein vector delivery methods were successful in expressing macaque FIX T262A with the AAV8 vector in NAb-positive macaques (Table 2). The data suggest that the gene transfer efficiency using the catheter-guided vector injection method is similar to that of the direct vector injection into the portal vein branch with flushing.

Blood chemistry analysis and liver biopsies were conducted following injection of the vector. Increases in the levels of liver enzymes just after injection of the vector were not observed, suggesting that the ischemic effect of the temporary occlusion of the left portal vein branch was minimum compared with that of the direct vector injection procedure. Moderate increases in transaminases were observed following the vector injection, but did not persist (**Supplementary Figure S1**). Although the cause of the changes in the liver enzymes was not elucidated, no animals showed pathological changes, including histology of liver biopsy samples (data not shown).