rate of liver regeneration in the recipient might be higher than that of the donor. In this recipient, the monitoring of FIX activity might have been become a good indicator of the graft liver function.

The efficacy of coagulant factor administration to adult patients with hemophilia undergoing surgical interventions has been shown (1). There is no established consensus on the optimal factor levels or the duration of replacement treatment for pediatric patients with hemophilia. Recently, the management of invasive procedures in pediatric patients has been reported (14–16). We consider the target plasma FIX level to be 80–100% in surgical interventions and 60–80% in invasive procedures (e.g., liver biopsy, etc.), based on previous reports. Transplant surgeons should measure and monitor the plasma FIX level before surgical interventions or invasive procedures.

In conclusions, LDLT from donors with mild coagulation FIX deficiency could be performed effectively and safely by peri-transplant short-term FIX replacement and long-term monitoring of the plasma FIX level in the donor. The accumulation of further cases and the long-term observation of this case are needed to confirm our findings.

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Conflict of interests

None.

Authors' contributions

YS: Study design, acquisition of data, analysis and interpretation of data, and drafting of the manuscript; HS, YS, KM, NK, YK, AM, TF, AS, MH, YH, NY, NO, YI, and TU: Acquisition of data, and analysis and interpretation of data; SM, JM, KM, and YY: Analysis and interpretation of data, and critical revision of the manuscript for important intellectual content.

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

Jun Mimuro,[1]* Hiroaki Mizukami,[2] Midori Shima,[3] Tadashi Matsushita,[4] Masashi Taki,[5] Shinji Muto,[6] Satoshi Higasa,[7] Michio Sakai,[8] Tsukasa Ohmori,[1] Seiji Madoiwa,[1] Keiya Ozawa,[2] and Yoichi Sakata[1]

[1]Division of Cell and Molecular Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan
[2]Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan
[3]Department of Pediatrics, Nara Medical University, Kashihara, Japan
[4]Department of Blood Transfusion, Nagoya University Hospital, Nagoya, Japan
[5]Department of Pediatrics, St. Marianna University School of Medicine, Yokohama City Seibu Hospital, Yokohama, Japan
[6]Department of Pediatrics, St. Marianna University School of Medicine, Kawasaki, Japan
[7]Division of Hematology, Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Japan
[8]Department of Pediatrics, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan

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 ($\geq$1:224) and AAV8 ($\geq$1:224) were more evident in older individuals ($\geq$42 years old). Approximately 50% of all screened individuals were seronegative for neutralizing antibodies against each AAV tested, while approximately 25% of individuals were seropositive for each AAV serotype tested. The prevalence of seronegativity for all AAV serotypes was 67.0% (healthy subjects, 68.6%; hemophilia patients, 65.0%) and 18.6% (healthy subjects, 20.5%; hemophilia patients, 15.7%) in young (<42 years old) and older subjects ($\geq$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. 86:1990–1997, 2014.**
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KEY WORDS: AAV vector; gene therapy; epidemiology

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 neutral-izing 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
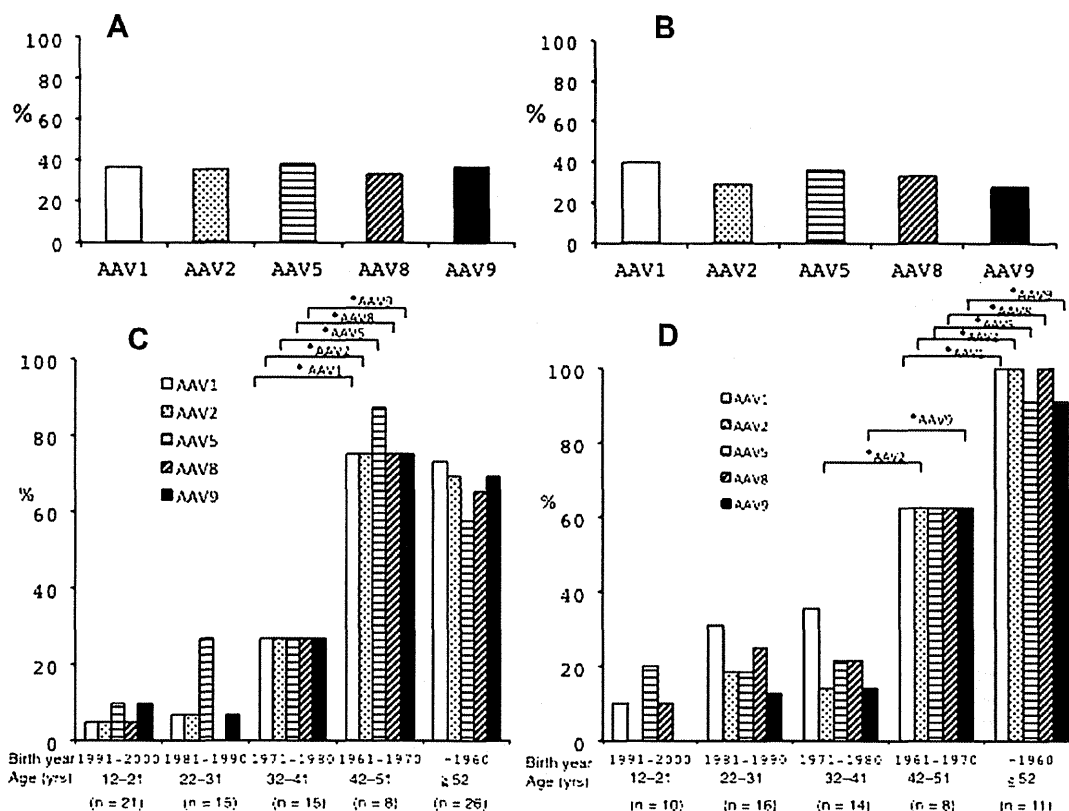
Fig. 1. Prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9. (A) The prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in healthy subjects. (B) The prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in hemophilia patients. (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.
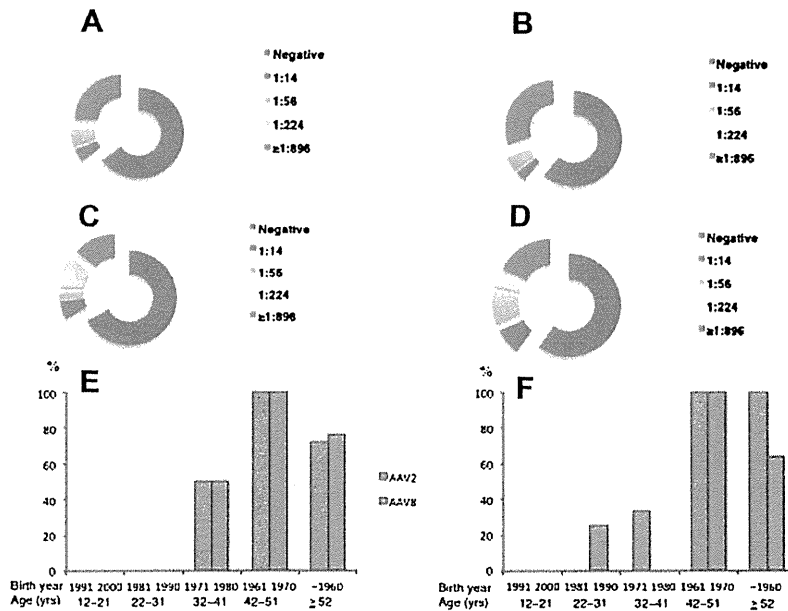
Fig. 2. Neutralizing antibody titers against AAV2 and AAV8. (A) Titers of neutralizing antibodies against AAV2 in healthy subjects, and (B) hemophilia patients. (C) Titers for neutralizing antibodies against AAV8 in healthy subjects, and (D) hemophilia patients. (E) Seroprevalence of neutralizing antibodies 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 infec-

tions 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
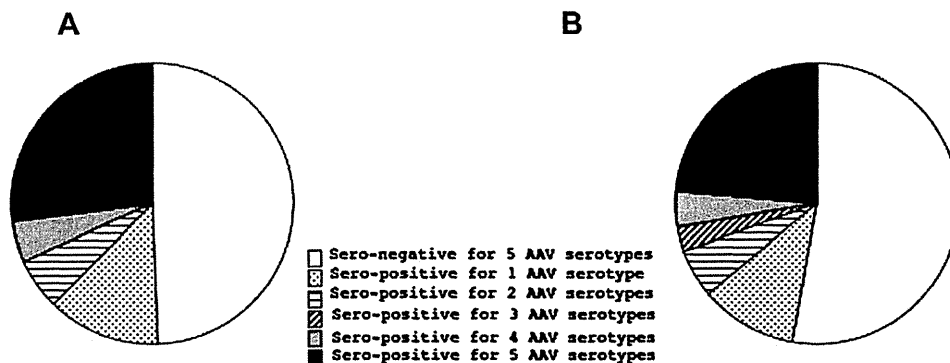

Fig. 3. Neutralizing antibodies against multiple AAV serotypes. (A) For healthy subjects, 49.4% were seronegative for neutralizing antibodies against all AAV serotypes tested, and 27.1% were seropositive for neutralizing antibodies against each AAV serotype tested. (B) For patients with hemophilia, 52.1% were seronegative for neutralizing antibodies against each AAV serotype tested, and 23.3% were seropositive for neutralizing antibodies against each AAV serotype tested.
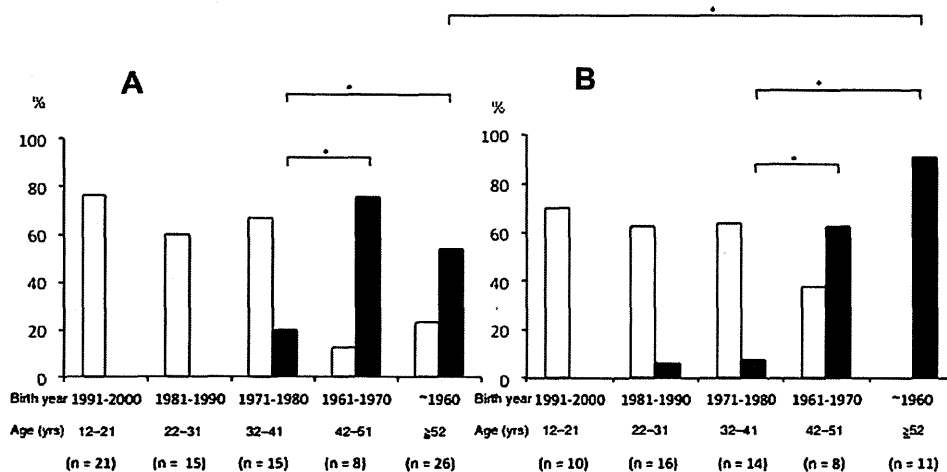
Fig. 4. Seroprevalence of neutralizing antibodies against AAV1, AAV2, AAV5, AAV8, and AAV9. (A) Healthy subjects that were seronegative for all AAV serotypes (white bar), and seropositive for all AAV serotypes (black bar), were sub-grouped and analyzed by generation. (B) Patients with hemophilia that 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 ($\geq$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
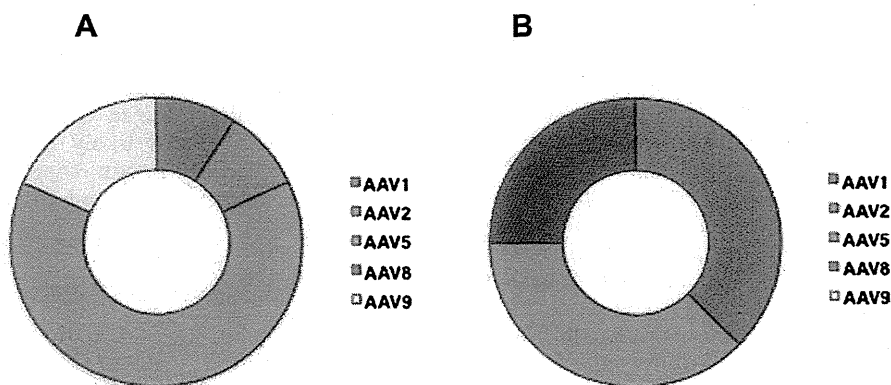

Fig. 5. Specificity of neutralizing antibodies against AAV serotypes. (A) Neutralizing antibodies against a single AAV serotype were observed in healthy subjects (n = 11), and in (B) patients with hemophilia (n = 8). Specific neutralizing antibodies against AAV5 were found in 10/19 (52.6%) individuals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

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Recommendations for performing thromboelastography/thromboelastometry in hemophilia: communication from the SSC of the ISTH

M. CHITLUR,* G. E. RIVARD,† D. LILLICRAP,‡ K. MANN,§ M. SHIMA,¶ G. YOUNG** and ON BEHALF OF THE FACTOR VIII, FACTOR IX, AND RARE COAGULATION DISORDERS SUBCOMMITTEE OF THE SCIENTIFIC AND STANDARDISATION COMMITTEE OF THE INTERNATIONAL SOCIETY ON THROMBOSIS AND HAEMOSTASIS

*Children's Hospital of Michigan, Detroit, MI, USA; †CHU Sainte-Justine, Montréal, QC; ‡Queens University, Kingston, ON, Canada; §University of Vermont, Colchester, VT, USA; ¶Nara Medical University, Kashihara, Nara, Japan; and **Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA*

Introduction

Hemostasis involves complex interactions among procoagulant proteins, their natural inhibitors, blood cells, and endothelium. Assessment of hemostasis involves plasma-based assays that screen and measure levels of factors and measurement of platelet number and function. Recently, there has been a growing interest in the use of global hemostasis assays like thrombelastography, which measures the viscoelastic changes occurring during clot formation in the study of hemophilia [1].

This report describes the recommended methodology for the use of thrombelastography in patients with hemophilia developed by the ISTH-SSC WP on standardization of thromboelastography.

Differences between thrombelastography and thromboelastometry

The two instruments that use the principles of thromboelastography are the TEG® 5000 (Haemonetics Corp., Braintree, MA, USA), referred to as thrombelastography

Correspondence: Guy Young, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, 4650 Sunset Blvd., Mail Stop #54, Los Angeles, CA 90027, USA.
Tel.: +1 323 361 5507; Fax: +1 323 361 7128.
E-mail: gyoung@chla.usc.edu

(TEG), and the ROTEM® delta (Tem International GmbH, Munich, Germany), referred to as thromboelastometry (ROTEM). The TEG consists of two heated sample cups that oscillate at ± 4° 45′ every 5 s, into which pins (one in each) are suspended by a torsion wire. In the ROTEM, the four sample cups are stationary while the pins oscillate at ±4° 45′ every 6 s. During coagulation, the forming clot results in a physical connection between each cup and pin, transferring the torque of the cup to the pin. The rate of clot formation and its elastic strength affect the magnitude of motion of the pin and its range of oscillation. For both instruments, computer software produces both quantitative parameters (see later) and a graphic representation of the phases of clot formation. Coagulation may be initiated purely by contact activation with the cup (called 'native'); however, in general, specific activators are used to reduce the time to clot formation and improve assay reliability. For the TEG, the manufacturer provides a vial coated with kaolin for activation of the intrinsic pathway, while for the extrinsic pathway, recombinant human tissue factor (TF, mostly Innovin®; Siemens Healthcare Diagnostics, Erlangen, Germany) has been used by investigators in "home-made" assays. For the ROTEM, the manufacturer provides reagents for both intrinsic activation (INTEM [partial thromboplastin phospholipid from rabbit brain]) and extrinsic activation (EXTEM [thromboplastin/recombinant tissue factor]). In addition, the ROTEM provides reagents for analysis of functional fibrinogen (FIBTEM); however, the importance of this reagent remains unclear with respect to hemophilia.

The parameters measured in both systems are similar but have different nomenclature; those used in the evaluation of patients with hemophilia are:

1 Coagulation time: R on TEG and CT on ROTEM
2 Clot formation time: K on TEG and CFT on ROTEM
3 Rate of clot polymerization: α/angle on both instruments.
4 Maximum clot firmness: MA on TEG and MCF on ROTEM
5 Shear elastic modulus: G on both instruments.
6 Clot lysis at 30 and 60 min: Ly30 and Ly60 on TEG and LI30 and LI60 on ROTEM.

Activation methods

A significant area of controversy involves the activation methods, and a detailed discussion can be found in the following review [1]. For the sake of these recommendations, the major issues can be summarized as follows. Although the methodology for intrinsic factor activation is simple and straightforward and the methods for extrinsic factor activation are more complicated and controversial, a consensus to recommend intrinsic factor activation has not emerged as some investigators are concerned that this approach is not physiological, preferring extrinsic activation via TF. Activation of the extrinsic system is more physiological; however, it leads to a number of difficult problems: (i) the significant variability among different sources of TF; (ii) the lot-to-lot variability of TF due to its biologic nature; (iii) the need to dilute the TF reagent several logs (TEG only), which adds to the variability, and (iv) issues surrounding the requirement (or lack thereof) to inhibit the contact system with corn trypsin inhibitor (CTI). Although it has become routine to use CTI when extrinsic system activation is used, emerging data suggest it may not be necessary (Brit Sorensen, personal communication). Among the studies that did use CTI, several different concentrations were used and there is no consensus on the ideal concentration. It should also be noted that CTI is expensive and the currently available blood CTI collection tubes are not sterile and have no expiration date, all of which hinder clinical application of this approach. Two studies comparing intrinsic to extrinsic system activation in patients with hemophilia with and without inhibitors demonstrated that intrinsic system activation with kaolin was more sensitive than activation with TF, although it should be pointed out the CTI was not used in these studies [2,3].

Given these factors, we make the following recommendations regarding the activation methods. For current clinical application, we recommend the use of intrinsic factor activation with kaolin for the TEG and INTEM for the ROTEM (see later for details). For extrinsic factor activation, it is not currently possible to recommend it for clinical use. Thus, we recommend that additional research be performed to develop a reliable and universal (for both TEG and ROTEM) source of TF, to determine the ideal concentration of such a reagent, and to determine whether and how much CTI should be used. With respect to clinical trials, we recommend that all trials using TEG/ ROTEM should use intrinsic system activators. Investigators may also elect to use extrinsic activation methods; however, we cannot endorse any specific method for this approach at this time.

Preanalytical variables

As with any coagulation assay, proper sample procurement, processing, and storage are critical as poor technique can lead to erroneous results. Adherence to the following details will minimize such errors.

Blood collection tubes

Standard sodium citrate (3.2%) blood collection tubes should be used for both devices and activation methods. As stated, the addition and concentration of CTI when using extrinsic system activation are at the discretion of the investigator, although it appears that a concentration of 20 μg mL^{-1} is sufficient [4–6]. For manufacturers who provide collection tubes prefilled with CTI, we recommend that the tubes be sterile and have a valid expiration date and that information on the production process be available.

Application of a tourniquet for venous sampling

Venous stasis during blood collection may increase variability in coagulation test results; thus, applying a tourniquet is not recommended [7]. In children, due to the difficulty in obtaining venous blood samples, application of a light tourniquet released upon accessing the vein can be considered.

Needle size

A 21-gauge or larger needle is recommended to obtain the sample, as smaller needles have been demonstrated to result in platelet activation [7].

Multiple sampling

Repeated sampling from the same tube has been known to result in activation of platelets as well as coagulation factors and therefore should be avoided if possible [8].

Resting time

The sample may be allowed to sit at room temperature for 30 min but not longer than 2 h prior conducting the assay [9,10].

Blood count

We recommend checking a blood count when performing thromboelastography as both a low hematocrit and a low platelet count can affect the results.

Specific methodology

Recommendations for the Kaolin/INTEM method

Blood should be collected as described earlier. For the TEG, 1 mL of whole blood should be transferred into the kaolin vial and inverted gently 3–4 times, and then 340 μL should be transferred into the cup into which 20 μL of calcium chloride had been placed. For the ROTEM, the blood is transferred directly into the device for automated pipetting.

Recommendations for the tissue factor method

For investigators who wish to use TF activation in addition to intrinsic activation, we provide the following suggestions for future research. The key questions with respect to this method have been listed above. With respect to the source of TF, most of the previous research used Innovin® and used dilutions of 1:17 000, 1:42 000, and 1:50 000 [9,11,12], although the 1:17 000 dilution was not sufficiently sensitive in one study [11]. The most important requirement for the standardization of a TF method is the development of a reliable and reproducible source of TF that can be used in both the TEG and the ROTEM device. Thus far, this remains an elusive goal. Such a reagent should obviate the need for dilutions and should be as simple and easy to use as kaolin/INTEM. While data exist regarding the reliability of the EXTEM reagent in patients undergoing surgery, there is insufficient data in hemophilia and the two disorders cannot be fairly compared.

Conclusion

This WP makes the following recommendations for the use of TEG/ROTEM as it relates to the clinical management and clinical trials in patients with hemophilia:

1 Investigators and treaters may use either device (TEG or ROTEM).
2 For clinical care, we recommend the use of intrinsic pathway activation with kaolin (TEG) or INTEM (ROTEM).
3 For clinical trials, we recommend intrinsic pathway activation as the primary method. Extrinsic pathway activation can be used in an exploratory fashion, although we cannot endorse any specific method.

Last, while progress has been made over the past 10 years in the study of TEG/ROTEM in hemophilia, they are not in wide clinical use. To achieve this aim, further research directly linking laboratory results to clinical outcomes are needed. As we enter an era of personalized medicine, we believe that it will be possible in the near future to individualize approaches to the management of hemophilia with these sophisticated laboratory assays.

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Contribution of ADAMTS13 to the better cell engraftment efficacy in mouse model of bone marrow transplantation

The adhesive protein von Willebrand factor (VWF) plays an essential role in physiological hemostasis, mediating platelet adhesion and aggregation under high shear stress conditions.[1,2] The VWF-cleaving protease ADAMTS13 precisely down-regulates VWF activity to avoid pathological intravascular thrombosis in the microvasculature, including arterial capillaries, where blood flow typically creates high shear stress.[1-3] Indeed, the functional deficiency of ADAMTS13 is known to cause thrombotic thrombocytopenic purpura, a typical thrombotic occlusion of the microvasculature.[2,4,5] Thus, the proper equilibrium between VWF and ADAMTS13 is necessary for robust microcirculation *in vivo*. In this context, we hypothesized that

ADAMTS13 might contribute to better donor cell homing and engraftment in various cell therapy approaches, in which fluent blood flow in the microcirculation system could be critical. To test this hypothesis, we investigated the role of ADAMTS13 on donor cell engraftment using a bone marrow transplantation (BMT) model in ADAMTS13 gene-deleted (*Adamts13 -/-*) mice.

Adamts13 -/- (KO) mice were back-crossed for more than 15 generations to the C57BL/6 background, as described.[6] Wild-type (WT) mice (C57BL/6-background) were purchased from Japan SLC (Shizuoka, Japan). All mice used in this study were 8-12 weeks old with body weights of 25-30 grams. Mouse experiments were performed in accordance with protocols approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University. In the BMT experiment, recipient WT or KO mice were conditioned for cellular transplantation with lethal total body irradiation (TBI: 5.5 × 2 = total 11 Gray) using a cesium irradiator (MBR-1520, Hitachi, Tokyo,
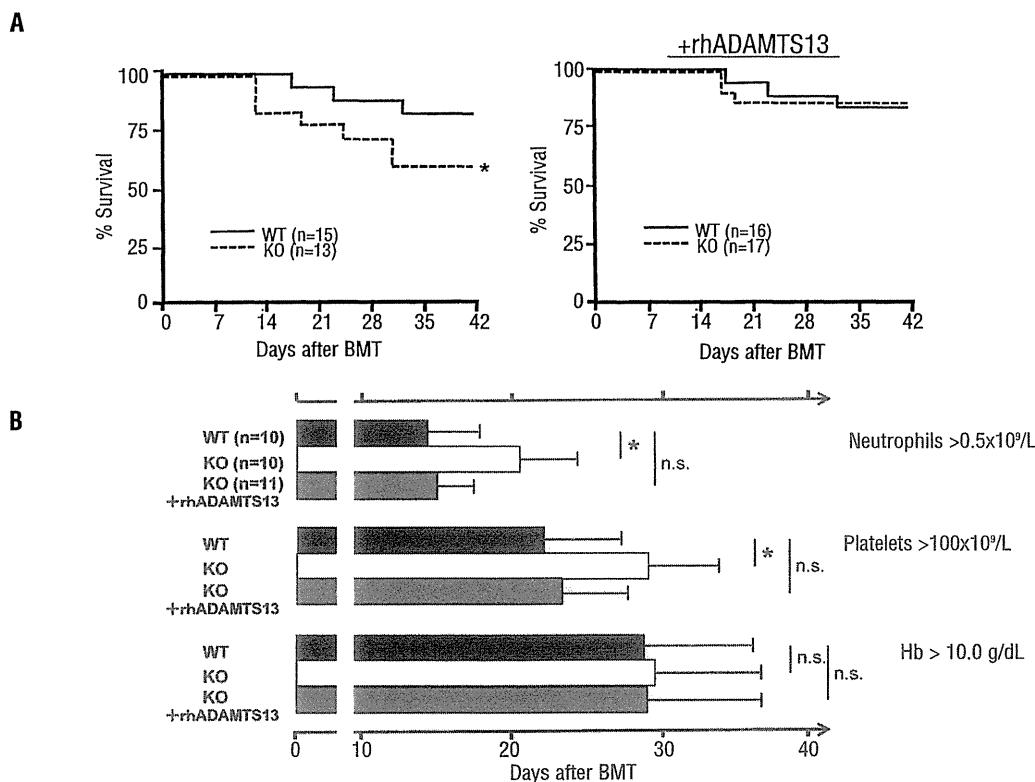
A

B

Figure 1. Survival rates and bone marrow suppression of wild-type (WT) or *Adamts13 -/-* (KO) mice receiving TBI and subsequent BMT. (A) Kaplan-Meier analysis of survival rates of WT or KO mice receiving TBI and BMT. GFP-positive donor bone marrow cells (5 × 10⁶/mouse) were transplanted to sex-matched WT (n=15) or KO (n=13) mice within 6 hours after TBI (total dose of 11 Gray/mouse) via tail vein. In some indicated experiments (right panel), recombinant human ADAMTS13 (rhADAMTS13; 5 μg/mouse, equivalent to 200 U/kg) was added to the donor bone marrow cell suspension prior to cellular transplantation. Significance of survival studies was quantified using Kaplan-Meier analysis and log rank tests. Note that the survival rate of KO mice began declining significantly (*P<0.05) at Day 14 of BMT, as compared to the WT mice (left panel; WT: 100% vs. KO: 76.9%). Following bolus administration of rhADAMTS13, this impaired survival rate in KO mice (WT: 81.0% vs. KO: 61.5% at Day 35) improved and became nearly indistinguishable from WT mice (see right panel). (B) Sequential peripheral blood analysis of WT or KO mice after TBI and BMT. Recipient mice were anesthetized using isoflurane inhalation, and 70 μL of blood was collected from the saphenous vein. Complete blood counts of recipient WT (n=10) or KO (n=10) were determined with an automatic blood cell counter (pocH®-100i: Sysmex, Kobe, Japan) every three days following BMT. Each bar represents the mean ± standard deviation (SD) duration that neutrophils counts were > 0.5x10⁹/L, platelet tcounts were > 100x10⁹/L, or hemoglobin values (Hb) were > 10.0 g/dL. Differences between groups were evaluated by Student's t-test. Note that nadir periods of KO mice are significantly (*P<0.05) longer than those of WT mice with regard to neutrophil and platelet counts (WT: 14.4 ± 3.3 and 22.4 ± 3.5 days vs. KO: 20.2±3.8 and 28.5±4.8 days, respectively), while no differences between these 2 groups are seen in Hb (WT: 28.2 ± 7.8 days vs. KO: 29.7 ± 7.5 days). These nadir period prolongations were improved by rhADAMTS13 (n=11) to an extent comparable to those of WT (n.s.: not significant).

Japan). Bone marrow cells to be transplanted were collected from femurs and tibias of donor green fluorescence protein (GFP) mice[7] (purchased from Japan SLC: C57BL/6-background), as described.[8] After removing the red blood cells by lysing with Tris-buffered ammonium chloride, suspended donor bone marrow mononuclear cells were transplanted into irradiated sex-matched recipient mice via tail vein. In some indicated experiments, recombinant human ADAMTS13 (rhADAMTS13), which was prepared as previously described,[9] was added to the donor bone marrow cell suspension prior to cellular transplantation. The VWF-cleaving activity of rhADAMTS13 was determined by *in vitro* FRETS-VWF73 assay.[10]

Kaplan-Meier analysis showed that the mean survival rate of KO mice receiving TBI and subsequent BMT was significantly lower than that of WT mice starting at Day 14 after BMT, and recombinant ADAMTS13 restored the survival rate of KO mice to that of WT mice (Figure 1A). Since

all WT and KO mice that underwent TBI without BMT died within 21 days (*results not shown*), the mortality rates under our experimental conditions most likely depended upon the cell engraftment efficacy during BMT and indicate an important contribution of ADAMTS13 in this regard. Indeed, peripheral blood analysis following BMT revealed the longer nadir period in KO mice with regard to neutrophils and platelets (Figure 1B), which was shortened significantly by recombinant ADAMTS13, with the resulting nadir periods comparable to those of WT mice (Figure 1B).

In addition to the above long-term observation experiment, some recipient mice were sacrificed at Days 1, 7, and 14 after BMT to check the extent of donor cell engraftment to the bone marrow and to assess the pathohistological conditions of major organs. After removing the red blood cells, the recipients' bone marrow was collected from the femurs and tibias and used to assess donor cell engraftment efficacy based on the percentage of GFP-positive cells rela-
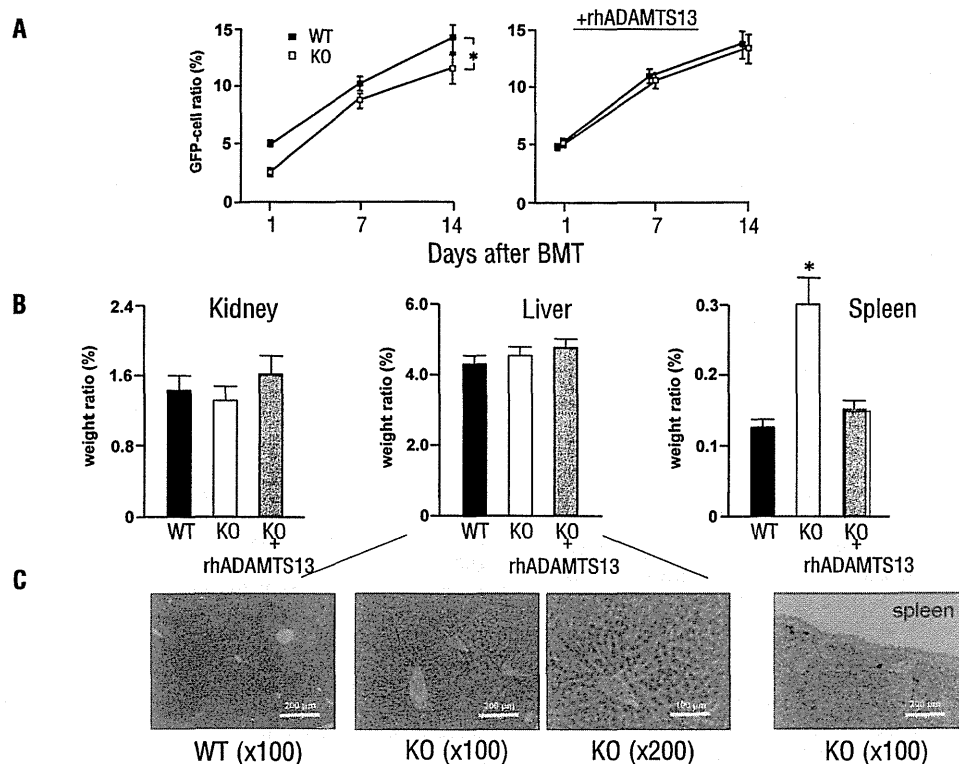

Figure 2. Bone marrow analysis and pathohistological studies in WT or KO mice that received TBI and subsequent BMT. These series of experiments, in which the recipient mice were sacrificed at Days 1, 7, and 14 after BMT (n=5 each), were performed independently of the long-term observation experiments in Figure 1. (A) Flow cytometric analysis of bone marrow cells from WT or KO mice that received TBI and BMT. Each data point represents the average ± SD of "GFP-cell ratio," the percentage of GFP-positive cells relative to total mononuclear cells in bone marrow. Note that a significant (*P < 0.05) reduction of donor GFP-positive cells in KO mice is already seen at Day 1 and continues throughout the observation period. In terms of cell propagation in KO mouse marrow, GFP-cells gradually increased in a time-dependent manner similar to that of WT (left panel). This GFP-cell reduction in KO mice was eliminated by rhADAMTS13 (see right panel). (B) Macroscopic findings of major organs in mice sacrificed at Day 7. Each bar represents the average ± SD of "weight ratio," the percentage of each organ weight (kidney, liver, or spleen) relative to total mouse body weight. In terms of macroscopic appearance, no particular differences were seen between WT and KO mice, except for a larger spleen in KO mice (*results not shown*). In fact, the calculated weight ratio confirmed the significant (*P < 0.05) splenomegaly in KO mice, which was eliminated by rhADAMTS13 administration. Mild splenomegaly, the extent of which was improved, remained in the corresponding Day 14 samples of KO mice (*results not shown*). (C) Microscopic findings of liver or spleen in mice sacrificed at Day 7. Images displayed are representative of 5 independent mouse samples. The liver samples with hematoxylin-eosin staining (×100 or ×200: original magnification) demonstrate slight dilation of the portal and central veins as well as mild sinusoidal congestion in both WT and KO mice, albeit less pronounced in WT mice. KO mouse livers do not exhibit either typical thrombotic legions in micro-vessels or SOS-lesions. As consistent with macroscopic splenomegaly, mild congestion and external capsule hypertrophy are observed in spleen of KO mice. These microscopic findings are basically similar to the corresponding Day 14 samples (*results not shown*).

tive to total mononuclear cells using flow cytometer (BD LSR-II: Nippon Becton Dickinson Company Ltd., Tokyo, Japan). Consistent with the findings in the peripheral blood, flow cytometric analysis of recipient bone marrow revealed the reduction of donor GFP-positive cells in KO mice that was already significant at Day 1 after BMT (Figure 2A). The population of GFP-positive cells in the bone marrow of KO mice expands gradually in a time-dependent manner similar to that of WT mice (Figure 2A), suggesting that ADAMTS13 is likely to play a role in the initial donor cell homing rather than cell propagation in the bone marrow cell engraftment. Thus, our results could verify the initial hypothesis that ADAMTS13 may contribute to better donor cell homing to the target recipient marrow, a process that requires fluent blood flow in the microvasculature including arterial capillaries.

Thrombotic microangiopathy (TMA) is a well-recognized serious complication of BMT, especially in the liver in the form of sinusoidal obstruction syndrome (SOS), and is known to be associated with functional ADAMTS13 deficiency.[11] Our histological studies, however, have only confirmed mild congestion and sinusoidal dilatation in the liver as well as significant splenic enlargement and congestion in KO mice, without typical thrombotic or SOS lesions of microvessels (Figure 2B and C). These histological findings may be consistent with possible portal hypertension, perhaps reflecting transient occlusion of the microvasculature by enhanced leukocyte plugging or platelet micro-aggregate formation that may occur in systemic microcirculation. Thus, the reduced local microcirculation could result in the poor donor cell homing to bone marrow that was observed in KO mice. Indeed, some clinical symptoms of TMA with functional deficiency of ADAMTS13 are known to be labile and variable,[5] suggesting the existence of transient microvasculature occlusion that cannot be reproducibly demonstrated in final tissue sample sections.

Recent mouse model studies by us and others demonstrated that proper functional regulation of VWF by ADAMTS13 significantly ameliorates the severity of fatal arterial thrombosis in conditions such as cerebrovascular accident or myocardial infarction.[12-15] ADAMTS13 reduces VWF-dependent platelet microaggregate formation as well as inflammatory responses such as leukocyte accumulation at ischemic sites, both of which may result in local microvasculature occlusion.[5] Thus, this property of ADAMTS13 can protect against impaired microcirculation in vivo, and may also contribute to better donor cell homing and engraftment in various cell therapy approaches that require fluent blood flow in the microvasculature.

In conclusion, our results illustrate that the regulation of VWF-mediated thrombotic or inflammatory responses by ADAMTS13 may contribute to the improved systemic microcirculation critical for efficient donor cell homing and engraftment in BMT, suggesting a clinical therapeutic potential of ADAMTS13 in cell therapy approaches.

Hideto Matsui,[1*] Maiko Takeda,[2] Kenji Soejima,[3]
Yasunori Matsunari,[1] Shogo Kasuda,[4] Shiro Ono,[5]
Kenji Nishio,[5] Midori Shima,[6] Fumiaki Banno,[7]
Toshiyuki Miyata,[7] and Mitsuhiko Sugimoto[1*]

[1]Department of Regulatory Medicine for Thrombosis; [2]department of Pathology; [4]department of Legal Medicine; [5]department of General Medicine; [6]department of Pediatrics, Nara Medical University, Kashihara; [3]The Chemo-Sero-Therapeutic Research Institute,Kumamoto; [7]Department of Molecular Pathogenesis, National Cerebral and Cardiovascular Center, Suita, Japan

Correspondence: sugi-ped@naramed-u.ac.jp

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Delivery of Full-Length Factor VIII Using a *piggyBac* Transposon Vector to Correct a Mouse Model of Hemophilia A

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Hideto Matsui[1]*, Naoko Fujimoto[2,3], Noriko Sasakawa[2], Yasuhide Ohinata[4,5], Midori Shima[6], Shinya Yamanaka[2,3,7], Mitsuhiko Sugimoto[1], Akitsu Hotta[2,3,5]*

1 Department of Regulatory Medicine for Thrombosis, Nara Medical University, Kashihara, Nara, Japan, 2 Department of Reprogramming Science, Center for iPS Cell Research and Application (CiRA), Kyoto University, Sakyo-ku, Kyoto, Japan, 3 iCeMS, Kyoto University, Kyoto, Japan, 4 Life Science Experimental Facility, Department of Biotechnology, Faculty of life and Environmental Sciences, University of Yamanashi, 4-4-37 Takeda, Kofu, Yamanashi, Japan, 5 PRESTO, Japan Science and Technology Agency (JST), Saitama, Japan, 6 Pediatrcs, Nara Medical University, Kashihara, Nara, Japan, 7 Gladstone Institute of Cardiovascular Disease, San Francisco, California, United States of America

Abstract

Viral vectors have been used for hemophilia A gene therapy. However, due to its large size, full-length Factor VIII (FVIII) cDNA has not been successfully delivered using conventional viral vectors. Moreover, viral vectors may pose safety risks, e.g., adverse immunological reactions or virus-mediated cytotoxicity. Here, we took advantages of the non-viral vector gene delivery system based on *piggyBac* DNA transposon to transfer the full-length FVIII cDNA, for the purpose of treating hemophilia A. We tested the efficiency of this new vector system in human 293T cells and iPS cells, and confirmed the expression of the full-length FVIII in culture media using activity-sensitive coagulation assays. Hydrodynamic injection of the *piggyBac* vectors into hemophilia A mice temporally treated with an immunosuppressant resulted in stable production of circulating FVIII for over 300 days without development of anti-FVIII antibodies. Furthermore, tail-clip assay revealed significant improvement of blood coagulation time in the treated mice.*piggyBac* transposon vectors can facilitate the long-term expression of therapeutic transgenes *in vitro* and *in vivo*. This novel gene transfer strategy should provide safe and efficient delivery of FVIII.

Introduction

Hemophilia A is a congenital bleeding disorder caused by a deficiency of procoagulation Factor VIII (FVIII). Repeated intravenous injection of recombinant FVIII protein can prevent bleeding events, but alternative treatments could potentially improve patients' quality of life; therefore, hemophilia A is an attractive target disease for the application of gene therapy. Significant advances in both preclinical animal models and phase I/II human clinical trials have been reported [1].

Most preclinical studies of hemophilia A gene therapy have focused on the use of viral vectors, such as adenovirus [2,3], adeno-associated virus (AAV) [4–6], gammaretrovirus [7], and lentivirus [8–12]. However, concerns still remain over the risk of adverse immunological reactions against viral proteins [13] and vector-mediated cytotoxicity [14].

Due to the large size of human FVIII cDNA (7.0 kb in total), it cannot be effectively delivered by most existing viral vectors; this technical issue is one of the biggest obstacles to the gene augmentation approach. Consequently, most gene therapy studies, including those aimed at clinical applications, use truncated FVIII that lacks the non-essential B-domain, so called BDD FVIII. However, the B-domain contains several glycosylation sites and is involved in intracellular interactions that regulate protein quality control and secretion; in particular, this domain is presumed to be involved in the clearance of FVIII from plasma [15]. Whether the B-domain is beneficial for FVIII expression in the context of gene therapy remains to be determined.

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Recently, non-viral gene-transfer technologies, such as the site-directed integration approach, have been significantly improved [16,17]. Although conventional plasmid vector systems are inefficient at stably integrating into the target genome, transposon vectors have emerged as attractive gene-delivery tools because of their ability to stably integrate into the genome and achieve efficient and prolonged transgene expression both *in vitro* and *in vivo*. In fact, the Tc1/*mariner* family member *Sleeping Beauty* has been utilized to deliver BDD FVIII into the hemophilia A mouse model [18].

More recently, the *piggyBac* DNA transposon vector was derived from cabbage looper *Trichoplusia ni*. This vector is active not only in insect cells [19], but also in mammalian cells [20]. In HEK293 cells and human primary T cells, *piggyBac* vectors have higher transposition activity than other widely used transposon vector systems, such as *Sleeping Beauty* or *Tol2* [21,22]. Importantly, the *piggyBac* transposon has a larger cargo size, and can deliver up to 9.1 kb of foreign sequence without significant loss of integration efficiency [20]. Moreover, a recent paper showed that a 100-kb DNA fragment from the *HPRT* gene locus could be inserted into mouse ES cells using *piggyBac*, although the efficiency was low and selective pressure was required [23]. In addition, other groups have shown that *piggyBac* vectors can deliver a reporter gene, such as *lacZ* or luciferase, into mice via hydrodynamic tail-vein injection [24], suggesting that *piggyBac* vectors could be utilized as a non-viral vector for *in vivo* gene therapy applications.

In this study, we investigated the ability of a *piggyBac* vector to deliver full-length FVIII, both in cultured cells and in a mouse model. Injection of *piggyBac* vector into hemophilia A mice resulted in stable and sustained expression of full-length FVIII and improvement of the bleeding phenotype.

Materials and Methods

piggyBac vector construction

A schematic representation of the *piggyBac* transposon vectors used in this study is shown in Figure 1. In brief, to construct the PB-EF1a-EiP vector, the 5' and 3' terminal repeat (TR) regions were derived from the PB-MSCII plasmid, based on *Trichoplusia ni* IFP2 *piggyBac* transposon [kind gift from Dr. Knut Woltjen]. Human EF1α promoter was derived from PL-sin-EF1a-EiP [25] as a HindIII–NcoI fragment, and a DNA fragment containing an internal ribosome entry site (IRES) derived from encephalomyocarditis virus and the puromycin resistance gene was obtained by *NcoI-ClaI* digestion from the PL-sin-EF1a-EiP plasmid. For construction of PB-EF1a-GWiP, the NcoI (blunted with Klenow)–XbaI fragment of the EF1α promoter and the SacII (blunted by Klenow)–HindIII fragment of the Gateway destination cassette (attR1-Cm^R-ccdB-attR2) were introduced into the XbaI–HindIII sites of the PB-EF1a-EiP plasmid. For construction of PBaseII, we digested the pCyL-43 plasmid [kind gift from Dr. Knut Woltjen] with SalI and KpnI, blunted it with Klenow, and self-ligated to remove the PGK/puromycin-resistance cassette. The 1.8 kb fragment of HyAcPBase gene [26] with optimized codon usage to human was chemically synthesized (GeneScript). To generate pCAG-HyAcPBase, the gene fragment was cloned into the SacI-NheI site of pCAGGS vector [27].

To construct PB-EF1a-hF8(BDD), BDD FVIII [28] cDNA in the HSQ-MXABS-ReNeo plasmid [29], together with the Adenoviral Type2 promoter and the SV40 poly A site, was digested with NcoI and MfeI and ligated into the NcoI–EcoRI site of PB-EF1a-EiP. Then, the additional SV40 pA site was removed by replacing the HpaI–ClaI SV40 pA-IRES-Puro fragment with

the EcoRI (blunted with Klenow)–ClaI fragment of IRES-Puro from PB-EF1a-EiP. Subsequently, the Adenoviral Type2 promoter was removed by replacing the NcoI–MluI fragment with a 1.3-kb PCR fragment amplified from HSQ-MXABS-ReNeo using the following primers (hSQ-FVIII-start-NcoI: 5'-AGCccatggCATG-CAAATAGAGCTCTCC-3'; hSQ-FVIII/MluI: 5'-CacgcgtCT-TAAAGGTTTCA-3'). To remove the additional ATG start codon, the NcoI site was destroyed by S1 nuclease treatment after NcoI digestion.

Full-length human FVIII cDNA in pENTR 223.1 (pENTR-hF8-full, ID 100066426) was obtained from DNAFORM (Japan), and the entire cDNA sequence was confirmed by Sanger sequencing. Full-length FVIII cDNA in pENTR-hF8-full was subcloned into PB-EF1a-GWiP by Gateway LR recombination.

piggyBac vector transfection *in vitro*

Human embryonic kidney 293T cells were maintained in DMEM media supplemented with 10% fetal bovine serum (FBS), ampicillin, and streptomycin. Lipofectamine 2000 (Life Technologies, CA, USA) was used to transfect 293T cells as described in the manufacturer's protocol. Human iPS cells (201B7 [30]) were maintained in ReproCELL Primate ES Cell media (ReproCELL, Japan) or Knockout-DMEM media (Life Technologies) supplemented with 15% Knockout serum replacement, 2 mM Gluta-MAX, 2 mM MEM non-essential amino acids, 0.1 mM 2-mercaptoethanol, and 10 ng/ml recombinant human basic-FGF. Puromycin- and hygromycin-resistant SNL cells (SNL-PH) treated with mitomycin C were used as a feeder layer for human iPS cell cultures. To transfect human iPS cells, we treated the cells with ROCK inhibitor Y-27632 (final 10 μM) for 1 hour, and then dissociated them with CTK solution (0.25% trypsin, 0.1 mg/ml collagenase IV, 1 mM CaCl$_2$, and 20% KSR) to remove feeder cells. Human iPS cell colonies were dissociated into single cells using Trypsin–EDTA solution, and then washed twice with Opti-MEM media. We used either the Neon system (Life Technologies) with pulse voltage = 1200 V, pulse width = 40 ms, and 1 pulse, or the NEPA21 electroporation system (NepaGene Inc., Japan) with poring pulse voltage = 125 V and poring pulse width = 5 ms. Transfection efficiencies of the *piggyBac* vectors with fluorescence reporter were monitored by flow cytometry as previously described [25].

DNA or RNA extraction and qRT-PCR

For genomic DNA extraction, cell pellets were lysed with 200 μg/ml Proteinase K solution (50 mM Tris-HCl, 20 mM EDTA, 100 mM NaCl, 1%w/v SDS) at 55°C for 3–18 hours, and genomic DNA was extracted by phenol-chloroform-isoamyl extraction method. For RNA, cell samples were lysed using the Trizol reagent (Life Technologies), and total RNA was extracted as described in the manufacturer's protocol. One microgram of RNA was reverse transcribed into cDNA using random primers (9 mer) and Oligo dT (20 mer) with ReverTraAce (Toyobo Inc., Japan).

Real-time quantitative PCR was performed on a StepOnePlus thermal cycler (Life Technologies) with SYBR Select Master Mix (Life Technologies). For genomic copy number analysis, genomic DNA with 3 copies of *piggyBac* vector insertion sites was used as a standard curve, and amount of input genomic DNA was normalized with Ct value by the primers to specifically amplify the NANOG gene region. For cDNA quantification, diluted plasmid DNA (PB-EF1a-EiP) was used to establish a standard curve for the IRES primers, and cDNA input was normalized with the GAPDH primers. Primer sequences are listed in Table S1. Data are presented as means ± standard deviation. The statistical
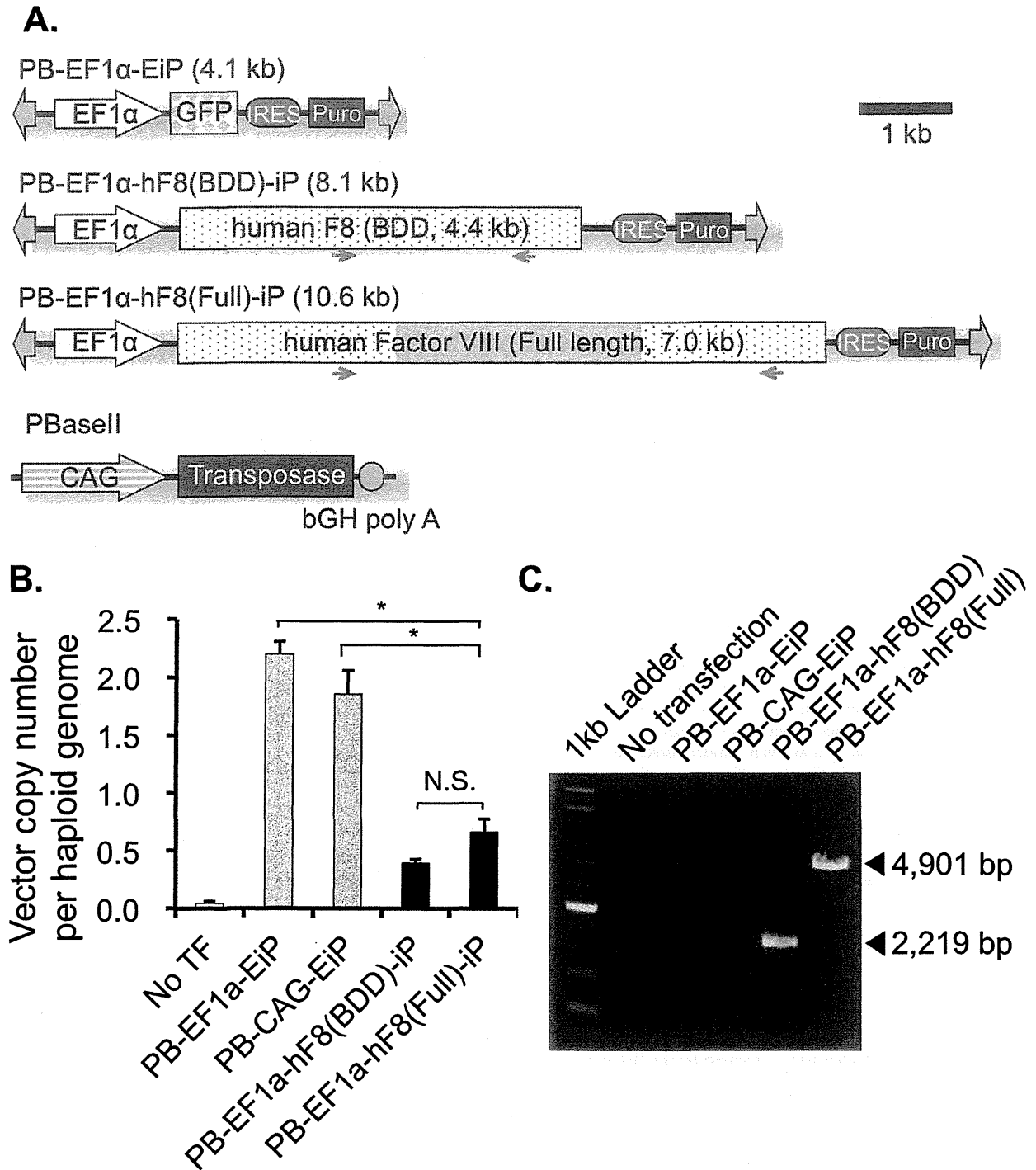
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A.

B.

C.

◄ 4,901 bp

◄ 2,219 bp

Figure 1. *piggyBac* **vectors to express Factor VIII cDNAs.** (A) Schematic diagram of *piggyBac* vectors expressing EGFP, B-domain–deleted human FVIII, and full-length human FVIII under the control of the human EF1α promoter. The PBasell vector expresses *piggyBac* transposase under the control of the CAG promoter. IRES: internal ribosomal entry site. (B) Copy number of genomic *piggyBac* vectors. Indicated *piggyBac* vectors were transfected into 293T cells and selected with puromycin resistance. Approximately three months after transduction, genomic DNAs were extracted, and *piggyBac* vector copy numbers were assessed by real-time PCR using the *piggyBac* 5' TR primers. The data are normalized to a haploid genome calculated from the copy number of NANOG gene. *: $P<0.05$ by two-sided Student's t test (n = 3). N.S.: Not significant. (C) The sizes of inserted FVIII cDNA (2,219 bp for BDD and 4,901 bp for full-length FVIII) were confirmed by genomic PCR using the hF8insertC primers flanking the B-domain (indicated as small arrows in Figure 1A).
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difference was determined by two-sided Student's t test, and $P <$ 0.05 was considered as significant.

FVIII activity measured by activated partial thromboplastin time (aPTT) assay

To confirm FVIII secretion *in vitro*, supernatant from cell cultures (seeded at 2×10^5 cells per one well of 12-well plate for 293T cells) was collected 3 days after medium change, centrifuged at $11,000 \times g$ for 5 minutes to remove cell debris, and then stored at $-80°C$ until being subjected to the aPTT assay as previously reported [10–12]. In brief, samples were diluted 10-fold by Owren's Veronal buffer (Sysmex Corporation, Japan) and mixed with FVIII-deficient plasma and aPTT reagent (Sysmex Corporation, Japan). After 3 minutes of incubation at 37°C, the coagulation reaction was activated by addition of 0.02 M CaCl$_2$ solution. Coagulation time was measured using a STart4 hemostasis analyzer (Roche Diagnostics). Recombinant human FVIII (Kogenate-FS, Bayer HealthCare Pharmaceuticals, NJ, USA) was used to prepare the standard curve.

FVIII gene transfer studies in hemophilia A mice

All animal procedures were reviewed and approved by the Nara Medical University Animal Care Committee (Permit Number: 10728). Hemophilia A knockout mice, in which the *FVIII* gene is disrupted by insertion of the *neomycin* gene into Exon 16, were purchased from the Jackson Laboratory (stock number 004424) and back-crossed with C57BL/6 mice. Mice were used at 6–8 weeks of age. Plasmid DNA was diluted in a 10% body-weight volume of lactated Ringer solution and injected via the tail vein within 7 seconds [31]. Because human FVIII is inherently immunogenic in hemophilia A mice, some of the hemophilia A mice also received intraperitoneal injection of cyclophosphamide (20 mg/kg per injection) on the day of vector injection, and then biweekly for 4 weeks. Mouse blood was first collected 1 week after injection from the saphenous vein under isofluorane/oxygen anesthesia, using 10% buffered citrate as an anticoagulant. To generate platelet-poor plasma, collected blood samples were centrifuged at $11,000 \times g$ for 5 minutes and stored at $-80°C$ prior to testing.

Chromogenic assay for FVIII activity, FVIII antigen ELISA, and detection of anti-FVIII antibodies

A chromogenic assay was used to measure plasma FVIII functional activity (FVIII: C) as previously described [10–12]. Normal pooled human plasma was used to establish the FVIII standard curve; the sensitivity of this assay is 10 mU/mL. In some mouse plasma samples, FVIII antigen (FVIII:Ag) was determined by ELISA (Affinity Biologicals Inc., Ancaster, ON, Canada). Anti-FVIII inhibitory antibodies were measured by the Bethesda assay as previously described [11].

Tail-clip challenge test

At the termination of the experiments, phenotypic correction was tested in both untreated and vector-treated hemophilia A mice, as previously reported [12]. In brief, mice were anesthetized with isoflurane, and their tails were clipped off at a position near the end of the tail where the cross-sectional diameter was 2.0 mm. The mice were then observed to determine the bleeding time.

Immunohistochemical analysis of FVIII in multiple organs

Immunohistochemical analysis of FVIII expression in both untreated and vector-treated hemophilia A mice were performed as previously reported [11,12] with minor modifications. In brief,

after tail-clip challenge tests, the mice were sacrificed, multiple organs liver, spleen, kidney, heart, and lung) were harvested, fixed with 10% formalin, embedded in paraffin and sectioned for staining. To assess transgene expression in tissues, specimens were stained with Cy3-labeled human anti-FVIII IgG [32] using the FluoroLink Ab labeling kit (Amersham Biosciences, Buckinghamshire, UK). Staining images were captured with FV300 confocal laser scanning microscope (Olympus Co., Tokyo, Japan).

Results

Stable expression of FVIII by *piggyBac* vector in multiple cell types

In order to achieve high-level transgene expression, we first optimized the internal promoter of our *piggyBac* vector. We constructed EGFP-expressing *piggyBac* vectors containing various promoters including those of PGK, CAG, and Elongation factor 1α (EF1α or EEF1A1). The EF1α promoter was the strongest among those we tested (Figure S1). Next, we optimized the amount of the *piggyBac* vector DNA and transposase-expressing plasmid. In agreement with previous reports [33], we found that a higher amount of *piggyBac* vector and a lower amount of transposase (400 µg/µl and 50 µg/µl, respectively) were optimal for transduction of our *piggyBac* vectors (Figure S2). We also tested a hyperactive version of the *piggyBac* transposase [24,26], but observed no significant improvement in transduction efficiency relative to the wild-type transposase (data not shown). To examine the effect of vector size on transduction efficiency, we constructed mCherry-expressing *piggyBac* vectors containing cDNAs of various sizes. Although the transduction efficiencies were reciprocally correlated with the vector sizes, the full-length FVIII cDNA (10.6 kb) could be transduced as efficiently as the BDD FVIII cDNA (Figure S3). In fact, when we transfected *piggyBac* vectors expressing EGFP, BDD FVIII, or full-length FVIII (Figure 1A), we observed that full-length FVIII vector could integrate into host chromosomes as efficiently as BDD FVIII vector (Figure 1B).

Next, to evaluate the expression of FVIII cDNAs by *piggyBac* vectors, we transfected these vectors into 293T cells and human iPS cells (clone 201B7); the former cell type is easy to transfect, whereas the latter type retains a normal karyotype while retaining unlimited self-renewal capacity and pluripotency [34]. We then extracted total RNA from these cells and quantitated transcription levels from *piggyBac* vectors by qRT-PCR using IRES primers. Even though the transcription levels of the FVIII vectors were lower than those of the of EGFP control vectors, comparable levels of transcription were detected from BDD and full-length FVIII vectors (Figure 2A,B).

To assess the level of secreted and functional FVIII protein, we measured the FVIII coagulation activity in the culture supernatant by activated partial thromboplastin time (aPTT) assay. We observed higher coagulation activity with full-length FVIII than BDD FVIII (Figure 2C,D). We also observed lower secretion of FVIII protein in undifferentiated iPS cells than in 293T cells, partly due to the lower transduction efficiency of iPS cells. The undifferentiated state of iPS cells may be associated with immaturity of the secretory pathways involved in FVIII production.

Successful long-term phenotypic correction of hemophilia A mice by hydrodynamic injection with *piggyBac* vectors

As a proof-of-concept that *piggyBac* vectors are applicable to gene therapy applications, we injected FVIII-expressing *piggyBac* vectors into hemophilia A model mice. Because FVIII is mainly