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

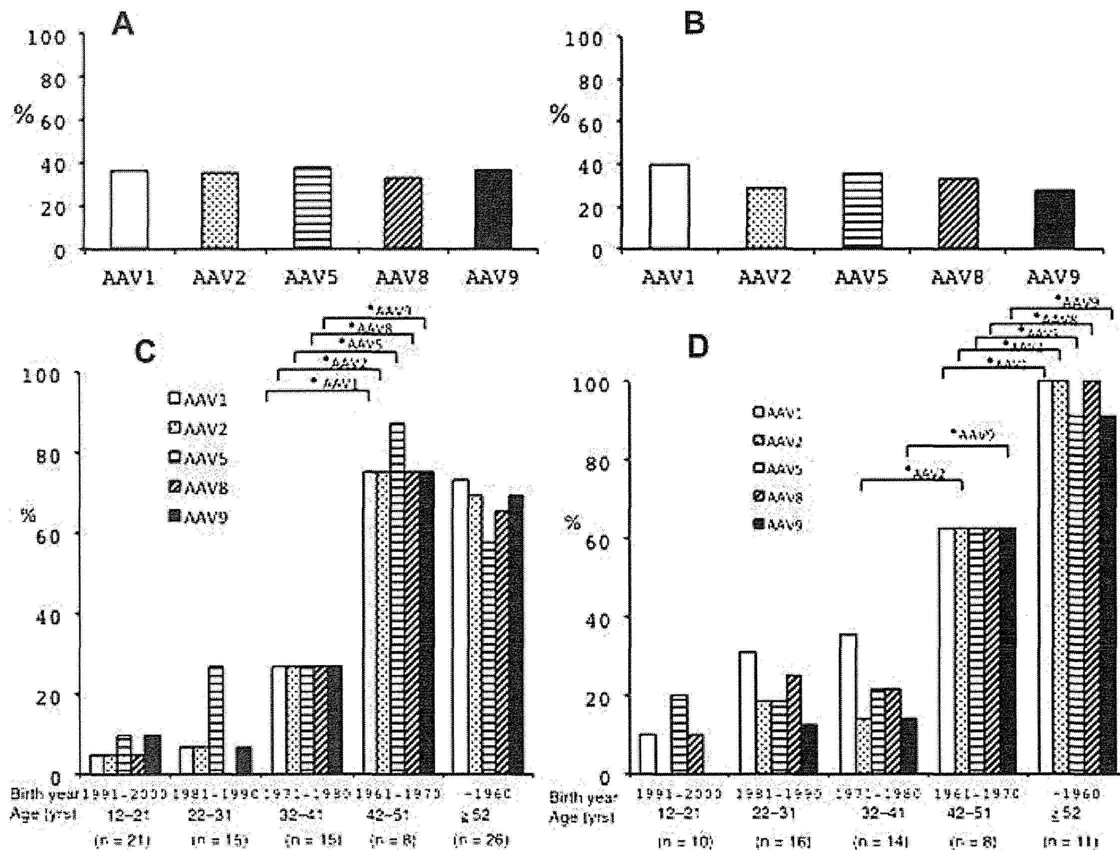

Fig. 1. Prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9. (**A**) The prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in healthy subjects. (**B**) The prevalence of neutralizing antibodies against AAV serotypes 1, 2, 5, 8, and 9 in hemophilia 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.

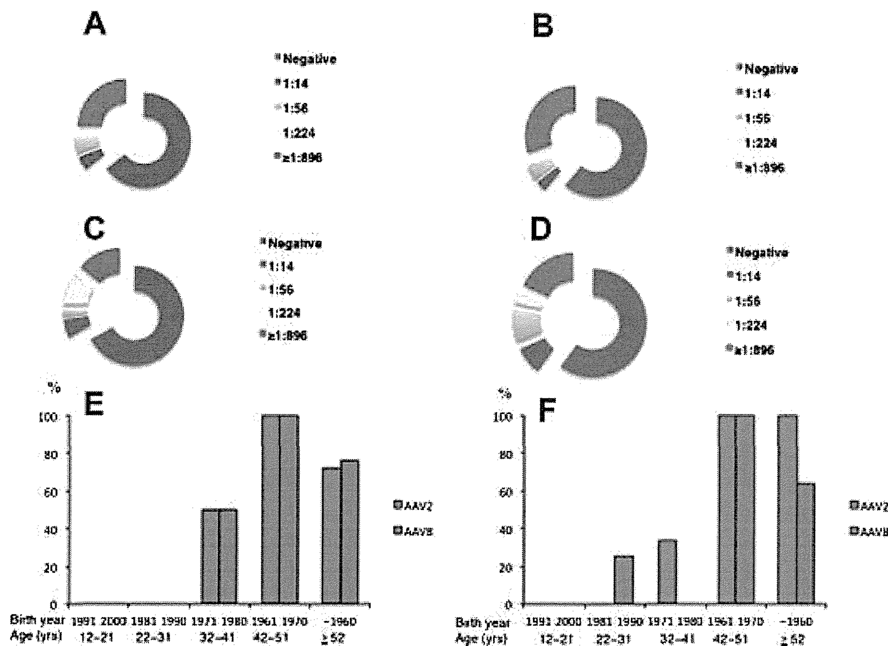

Fig. 2. Neutralizing antibody titers against AAV2 and AAV8. (**A**) Titers of neutralizing antibodies against AAV2 in healthy subjects, and (**B**) hemophilia patients. (**C**) Titers for neutralizing antibodies against AAV8 in healthy subjects, and (**D**) hemophilia patients. (**E**) Seroprevalence 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

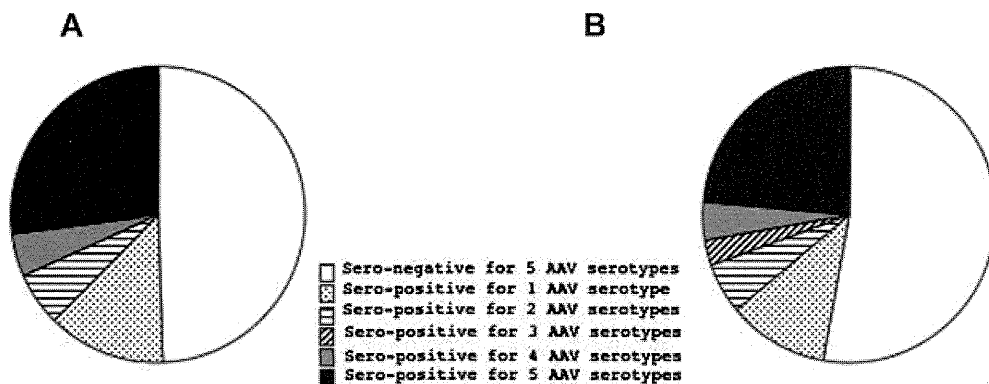

Fig. 3. Neutralizing antibodies against multiple AAV serotypes. (**A**) For healthy subjects, 49.4% were seronegative for neutralizing antibodies against all AAV serotypes tested, and 27.1% were seropositive for neutralizing antibodies against each AAV serotype tested. (**B**) For patients with hemophilia,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

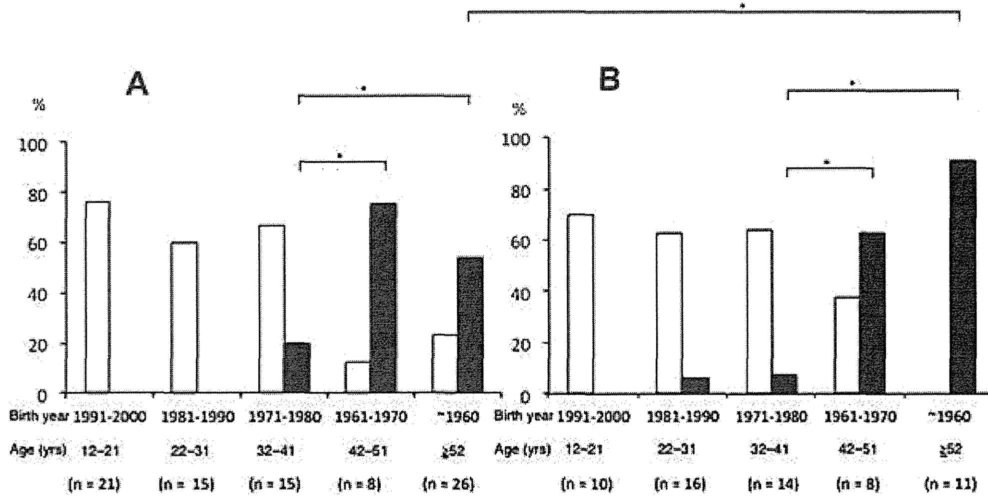

Fig. 4. Seroprevalence of neutralizing antibodies against AAV1, AAV2, AAV5, AAV8, and AAV9. (**A**) Healthy subjects that were seronegative for all AAV serotypes (white bar), and seropositive for all AAV serotypes (black bar), were sub-grouped and analyzed by generation. (**B**) Patients with hemophilia 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

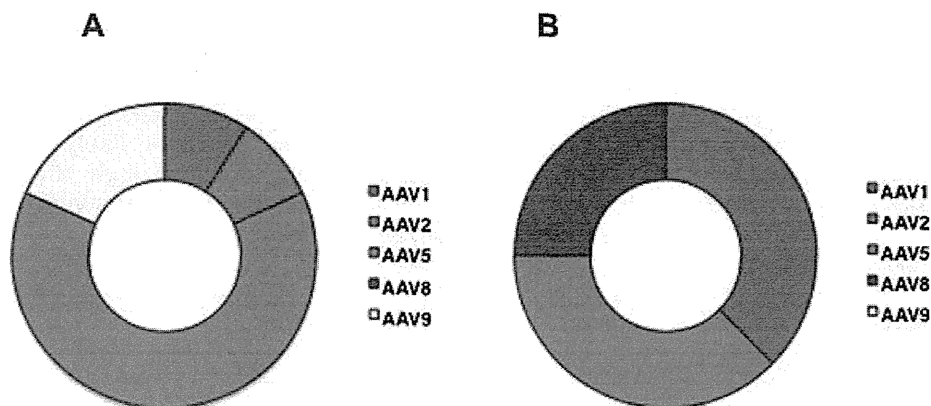

Fig. 5. Specificity of neutralizing antibodies against AAV serotypes. (**A**) Neutralizing antibodies against a single AAV serotype were observed in healthy subjects (n = 11), and in (**B**) patients with hemophilia (n = 8). Specific neutralizing antibodies against AAV5 were found in 10/19 (52.6%) individuals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

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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

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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,

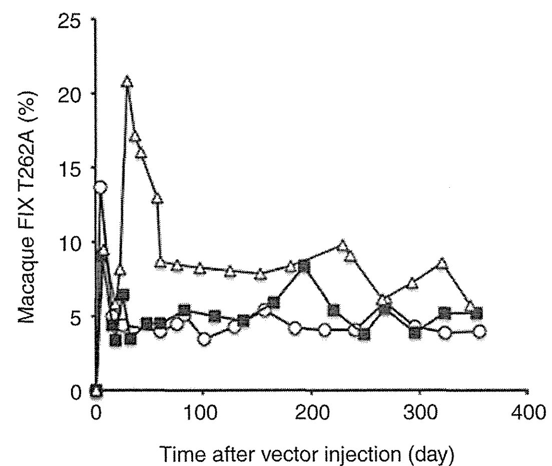

Figure 1 Expression of FIX T262A in macaques after direct vector injection into portal veins. Macaques ($n = 3$) were subjected to direct injection of AAV8 vector into the portal vein. Concentrations of FIX T262A in macaque plasma samples (macaque #26, open triangles; #27, open circles; #29, closed squares)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

#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.

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

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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),

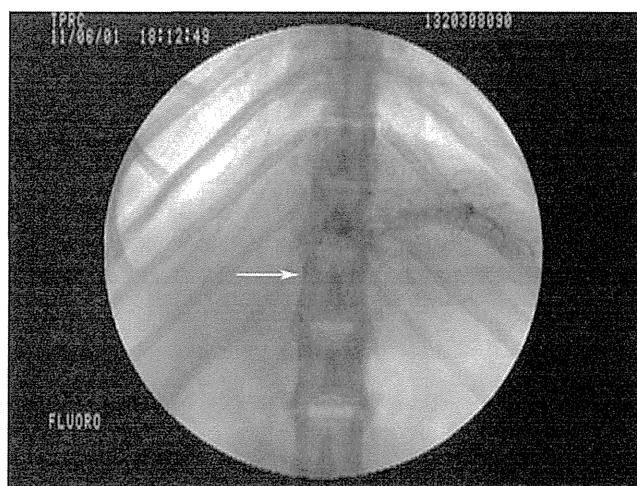

Figure 2 Fluorography in macaque #37. A balloon catheter was inserted into the portal vein of macaque #37 and contrast medium injected before vector administration. The left portal vein branches can be visualized. The arrow (white) indicates the tip of the catheter. See **Supplementary Video S1** which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

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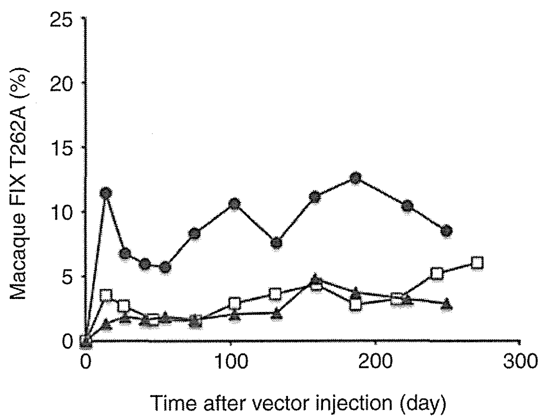

Figure 3 Expression of FIX T262A in macaques following balloon catheter-guided vector injection into portal veins. Three macaques (n = 3) were subjected to balloon catheter-guided vector injection into the portal vein. Concentrations of FIX T262A in macaque plasma samples (#37, closed circles; #38, open squares; #42, closed triangles) were measured by ELISA. ELISA, enzyme-linked immunosorbent assay; FIX, factor IX.

Table 3 Vector injection rate

Route of injection (macaque number)	Elapsed time (seconds)	Rate of injection (vg/kg/second)
Mesenteric vein (#14, #17, #24)	5	2×10^{11}–2×10^{12}
Saphenous vein (#28, #30, #31)	5	1×10^{12}
Portal vein (direct) (#26, #27, #29)	8–10	5×10^{11}
Portal vein (catheter) (#37, #38, #42)	15–22	2.3×10^{11}–3.3×10^{11}

Abbreviation: vg, vector genome.

Vector injection rates of the four different vector injection procedures are listed in **Table 3** for comparison. The vector injection rates of the portal vein-directed strategies were similar to those of bolus vector injection into the saphenous vein and the mesenteric vein. Thus, the effect of vector injection speed on the transduction efficiency of the vector was thought to be minimal.

DISCUSSION

There are many features that make recombinant AAV vectors attractive for transferring therapeutic genes into target organs, and many vectors have been tried for the treatment of various diseases.[6,7,11,15,21–23] However, lines of evidence suggest that NAbs against AAV interfere with AAV vector-mediated gene transfer by intravascular vector delivery.[7,23–26] A clinical gene therapy trial for hemophilia B using a self-complementary AAV8 vector carrying the FIX gene has been conducted and reported to be successful.[15] However, even the self-complementary AAV8 vector failed to express FIX in a subject with a relatively high anti-AAV8 antibody titer compared with other subjects with no or lower antibody titers.[15]

According to the previous reports on the prevalence of NAbs against various AAV serotypes in normal subjects, the seropositivity against AAV8 is 15–30%, which is lower than that against AAV2 (50–60%), although the technical details of the NAb assay is different.[27,28] These reports have also demonstrated that the antibody titer against AAV8 is generally lower than for AAV2. Our data suggest that a low titer of NAbs against AAV8 can interfere with transduction even if the vector is injected into the mesenteric vein. Therefore, the use of another serotype vector such as AAV5 vector could be the next approach for this type of gene therapy because of the divergence in capsid sequence of AAV5 from other AAV serotypes.[13,16] Although the prevalence of NAbs against AAV5 is much lower than those against AAV1 and AAV2, and the prevalence of NAbs against AAV5 is comparable to or even lower than that against AAV8 in humans,[25,27] it is possible that subjects of gene therapy may contain cross-reactive NAbs against various AAV serotypes.

Another approach for evading NAbs against AAV could be the use of chemically or genetically modified AAV variants. Such variants could include AAV vector mutants with amino acid substitutions, or chimeric AAV vectors made by serotype shuffling.[23] Approaches that enable evasion of NAb inhibitory effects are necessary if researchers and clinicians wish to effectively apply AAV vectors for gene therapy because of NAb cross-reactivity.

An alternative approach for overcoming the inhibitory effect of NAb against AAVs is to develop a vector injection method. In the current study, two portal vein vector delivery strategies were employed that ensured that the AAV8 vector and NAbs do not come into physical contact with each other in the blood. These strategies were investigated using macaques whether the strategies could efficiently transduce hepatocytes with the AAV8 vector in the presence of NAbs. The first approach was the direct injection of AAV8 vectors into the portal vein branch after flushing with saline to remove blood. This strategy proved to be successful for the vector expressing FIX T262A in anti-AAV8 antibody-positive macaques. Since there are safety concerns about the direct vector injection method, injection of the vector into the portal vein using a balloon catheter was investigated. The catheter-guided vector injection may be less invasive than the direct vector injection into the portal vein branch because exfoliation of hepatic hilum is not required. In addition, fine surgical skills, such as manipulation of the hepatic hilum and suturing the venotomy site of portal vein after the direct vector injection without causing stenosis, are required for the direct vector injection method into the left portal vein. Obviously, catheterization from the mesenteric vein branch is required for the balloon catheter-guided vector injection method but insertion of a catheter into the portal vein from a branch of the mesenteric vein is not difficult for a cardiologist and a radiologist familiar with angiography. In addition, suturing the venotomy site of the mesenteric vein branch is easier and safer than suturing the venotomy site of portal vein, and the ischemic effect of this procedure was expected to be less than that of the direct vector injection into the portal vein branch. Taken together, these studies suggested that both the direct vector injection into the left portal vein and the balloon catheter-guided vector injection into the left portal vein were similarly effective for hepatocyte transduction with the AAV8 vector in the presence of low titer NAbs but the balloon catheter-guided vector injection method into the left portal vein was thought to be safer than the direct vector injection into the left portal vein.

Considering that the antibody titer against AAV8 was generally lower than that against AAV2 and that NAbs at low titers could interfere with the AAV8 vector-mediated gene transfer to the liver significantly, we selected macaques with low NAb titers

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for the portal vein vector delivery strategies. However, the impact of the presence of high titer NAbs on the efficacy of these methods was not studied. Thus, the extent of AAV8 NAb titer, for that this approach is effective, needs to be investigated in the future.

In conclusion, we have provided the basis for an alternative approach for gene transfer to the liver that minimizes the deleterious effects of anti-AAV NAbs. Our result might expand the potential of the AAV vector-mediated gene delivery for medical application.

MATERIALS AND METHODS

AAV vector production. Construction of pAAV2-HCRHAAT-macFIX T262A and production of AAV8 carrying the macaque FIX T262A gene (AAV8HCRHAATmacFIXT262A) has been previously described.[17] Briefly, DNA fragments harboring the *macFIXT262A* gene located downstream of the chimeric promoter consisted of an enhancer element of HCR of the human ApoE/C-I gene and the 5′ flanking region of the human HAAT gene (HCRHAAT promoter), and the SV40 polyadenylation signal sequence flanked by AAV2 inverted terminal repeats in pAAV2-HCRHAAT-macFIX T262A. The genes were packaged by triple plasmid transfection of human embryonic kidney 293 cells (Avigen, San Diego, CA) to generate AAV8-HCRHAAT-macFIXT262A, with the chimeric packaging plasmid (AAV2 rep/AAV8 cap), and the adenovirus helper plasmid pHelper (Stratagene, La Jolla, CA), as previously described.[17] The chimeric packaging plasmid for AAV8 capsid pseudotyping[29] was constructed by inserting the synthetic AAV8 *Cap* gene (Takara Bio, Otsu, Shiga, Japan) downstream of the AAV2 Rep gene of pHelp19. For virus vector purification, the DNase (Benzonase; Merck Japan, Tokyo, Japan)-treated viral particles containing samples were subjected to two rounds of cesium chloride-density gradient ultracentrifugation in HEPES-buffered saline (pH 7.4) supplemented with 25 mmol/l EDTA at 21 °C, as previously described.[17] Titration of recombinant AAV vectors was carried out by quantitative PCR using a real-time PCR system (StepOnePlus; Applied Biosystems Japan, Tokyo, Japan).[17] AAV8HCRHAATmacFIXT262A was previously shown to express macaque FIXT262A in mice efficiently.[17] Human FIX could be expressed in macaques and detected, however, macaques developed antibody against human FIX under certain experimental conditions. Only one amino acid residue at position 262 was humanized in macaque FIX T262A for detection with the human FIX-specific monoclonal antibody.

Animals. Cynomolgus macaques were bred and maintained at the Tsukuba Primate Research Center (Ibaraki, Japan). The animal experiments using macaques were performed at the Tsukuba Primate Research Center according to the guidelines of the Institutional Animal Care and Concern Committees at Jichi Medical University and the Tsukuba Primate Research Center. The use of macaques in animal experiments was approved by the Animal Care and Concern Committees. All surgical procedures were carried out under anesthesia, with vital signs and electrocardiogram monitoring conducted in accordance with the stipulated guidelines. Male macaques with low NAb titers (<56×) were used in this study.

Vector injection from peripheral and mesenteric vein. Injection of AAV8 vector to a saphenous vein (peripheral vein) was performed under intramuscular anesthesia. Injection of the AAV8 vector into a terminal branch of the superior mesenteric vein was carried out with laparotomy under anesthesia with isoflurane and electrocardiogram monitoring.

Direct portal vein vector injection with saline flushing. Direct injection of the vector solution into the left portal vein was carried out after induction of general anesthesia with isoflurane and sterilization. A right subcostal incision (5 cm) was made through the skin and the subcutaneous tissue. The abdominal cavity was explored and the soft tissue of hepatic hilum was exfoliated surgically, then the main portal vein and its right and left branches were exposed. The main portal vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo, Tokyo, Japan), which was advanced into the left portal vein branch. The left and right portal vein branches were then clamped with vascular forceps. After flushing the left portal vein with saline, the vector solution was injected, and then a second saline solution, for flushing, was injected. Volumes of solutions used in the experiments were determined by taking a standard liver volume, a hepatic vascular bed volume, and effects of solutions on the systemic circulation into consideration.[19,30] A standard liver volume of a macaque was estimated with the formula (standard liver volume = 706.2 × body surface area + 2.4)[31] and the vascular bed volume of the liver was estimated to 25–30% of the standard liver volume.[19] A hepatic vascular bed volume can increase to 60% of the liver volume upon infusion of solutions and this may function as a reservoir and reduce the effects of the solutions on the systemic circulation.[20,32,33] The forceps were then removed immediately and the venotomy site was closed with an 8-0 prolene suture.

Catheter-guided vector injection to the portal vein with saline flushing. Balloon catheter-guided injection of the vector into the left portal vein of AAV8 antibody-positive macaques was carried out after the induction of general anesthesia. A 5-cm right paramedian incision was made through the skin and subcutaneous tissue. The abdominal cavity was carefully entered, with a part of the ileum identified and brought out through the incision. A peripheral branch of the superior mesenteric vein was cannulated with a plastic cannula type 20G needle (Surflo; Terumo). A temporary occlusion microcatheter (Iiguman 3.3F; Fuji System, Tokyo, Japan) was advanced into the left portal vein using a guide-wire (run through 0.014 (0.36 mm); Terumo) under a fluoroscope. The positions of the catheter and the balloon were confirmed by imaging with contrast medium. Blood flow in the left portal vein was occluded with a silicone balloon catheter and 40 ml of saline, followed by the AAV8 vector solution, and another 20 ml of saline was injected sequentially through the microcatheter. Volumes of solutions used in the experiments were determined as above with taking the result of the experiment of direct vector injection to the left portal vein branch into consideration. Following deflation of the balloon, the microcatheter was withdrawn and the peripheral venotomy ligated. The abdominal wall was then closed in layers.

Analysis of macaque FIX T262A expression in macaques. Macaque FIX T262A was bound to 3A6, a human FIX-specific monoclonal antibody for analyses. An enzyme-linked immunosorbent assay (ELISA) for the detection of macaque FIX T262A was carried out using 3A6, as previously described.[17,34]

NAb assay. An assay for the detection of anti-AAV8 NAbs was performed as previously reported, with some modifications.[35,36] Briefly, 5×10^4 2V6.11 cells/well were seeded in the wells of 96-well culture plates. Ponasterone A was added to the culture media the day before transduction to induce expression of the E4 gene. On the day of transduction, 10 µl of serum (undiluted, or subject to serial twofold dilutions) was incubated with the vector (AAV8-CMV-LacZ, 5×10^7 vg/10 µl) at 37 °C for 1 hour, and this mixture was added to a culture well. Sucrose was added to the culture medium such that the final concentration was 125 mmol/l. The culture medium was removed after a 48-hour incubation, and β-galactosidase activity quantified with a β-Gal assay kit (Invitrogen, Carlsbad, CA). Briefly, o-nitrophenyl-β-D-galactopyranoside was added to cell lysates, incubated for 30 minutes, and color change quantified with a microplate reader (Benchmark Plus; Bio-Rad, Hercules, CA). If β-galactosidase activity was inhibited with a test sample that contained more than 50% of control fetal bovine serum, it was judged as positive for neutralizing capacity. The inhibitory titer of the serum sample was expressed as the highest final dilution in the culture medium that showed inhibitory activity.

Quantitation of AAV8 vector DNA in macaque tissue. Quantitation of AAV8 vector DNA in macaque tissues was performed using quantitative

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PCR assays using a StepOnePlus instrument (Applied Biosystems Japan). DNA was isolated from macaque tissues using a DNeasy Blood & Tissue Kit (QIAGEN, Valencia, CA) and subjected to PCR using primers 5′-GAT AAC TGG GGT GAC CTT GG-3′ and 5′-GCC TGG TGA TTC TGC CAT GA-3′, and Cybergreen reagent (Applied Biosystems Japan).

SUPPLEMENTARY MATERIAL

Figure S1. Changes in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in macaques.
Table S1. Direct vector injection into the portal vein of macaques.
Table S2. Balloon catheter-guided vector injection into macaques.
Video S1.

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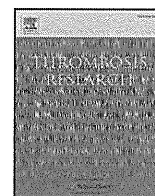

Regular Article

Distinct reactivity of the commercially available monoclonal antibodies of D-dimer and plasma FDP testing to the molecular variants of fibrin degradation products

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ABSTRACT

Fibrin degradation products (FDP) are an important marker of coagulopathy. We assessed the reactivity of the monoclonal antibodies used in clinical laboratory testing (6 D-dimer reagents, D-dimer-1–6; 4 plasma FDP reagents, plasma FDP-1–4) to quantify FDP using *in vitro*-generated FDP as well as FDP in clinical samples. The monoclonal antibodies used in D-dimer-1, -2, -5, and -6 reacted poorly to the low molecular weight forms of *in vitro*-generated FDP. The monoclonal antibodies used in D-dimer-3 and -4 had better reactivity to the low molecular weight forms of *in vitro*-generated FDP. The monoclonal antibodies used in plasma FDP-2, -3, and -4 reacted well to the high and low molecular weight FDP forms, while the monoclonal antibody in plasma FDP-1 reacted poorly to the low molecular weight FDP forms. Analysis of clinical samples revealed deviations in FDP molecular weight forms in DIC samples. The reactivity of the monoclonal antibodies of laboratory FDP testing to FDP variants in clinical samples was similar to that of *in vitro*-generated FDP. In conclusion, the monoclonal antibodies used in clinical laboratories to detect FDP have distinct reactivity to the molecular variants of FDP generated *in vitro* as well as those present in clinical samples. Our findings support the consensus for the standardization of D-dimer and plasma FDP testing.

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Introduction

Fibrin degradation products (FDP) are an important marker of coagulopathy such as disseminated intravascular coagulation (DIC) and venous thromboembolism (VTE) [1–8]. Historically, serum FDP assays using polyclonal antibodies against fibrinogen have been used to detect FDP in serum samples [4,5]. FDP can be detected by immunoassays using monoclonal antibodies as well as polyclonal antibodies for standard serum FDP assays. Many D-dimer reagents using the respective monoclonal antibody against cross-linked fibrin have been developed and used for more than 20 years [6–13]. These monoclonal antibodies bind to cross-linked fibrin-derived FDP, but not to fibrinogen or fibrinogen degradation products, which allows them to detect cross-linked fibrin-derived FDP in plasma samples. In addition, FDP assays utilizing monoclonal antibodies, those that can detect both FDP and fibrinogen degradation products (FgDP) in plasma samples, are available as plasma FDP reagents. Various molecular forms of FDP may be present in the blood of patients; however the reactivity of the monoclonal antibodies used in conventionally available reagents for laboratory testing (D-dimer testing, plasma FDP testing, assay for FDP and FgDP in plasma samples) may be distinct to different FDP variants. In other words, laboratory reagents may be different from each other. Therefore, there is a concern that a deviation in the FDP variants of clinical samples may be present and that these may not be quantified accurately because of the distinct monoclonal antibody specificity. Such deviations may also vary depending upon the nature of the underlying disease.

The aims of this study were to compare the reactivity of monoclonal antibodies used in clinical FDP assays to various molecular forms of FDP made *in vitro* and those present in the plasma samples of patients, and provide a basis for understanding the characteristics of FDP assays used in clinical laboratory testing.

Materials and Methods

In vitro generation of fibrin degradation products

Normal pooled citrated platelet-poor plasma (8 mL) obtained from 6 healthy subjects was reconstituted with tissue plasminogen activator

Abbreviations: FDP, fibrin degradation product; FgDP, fibrinogen degradation product; DIC, disseminated intravascular coagulation; VTE, venous thromboembolism.

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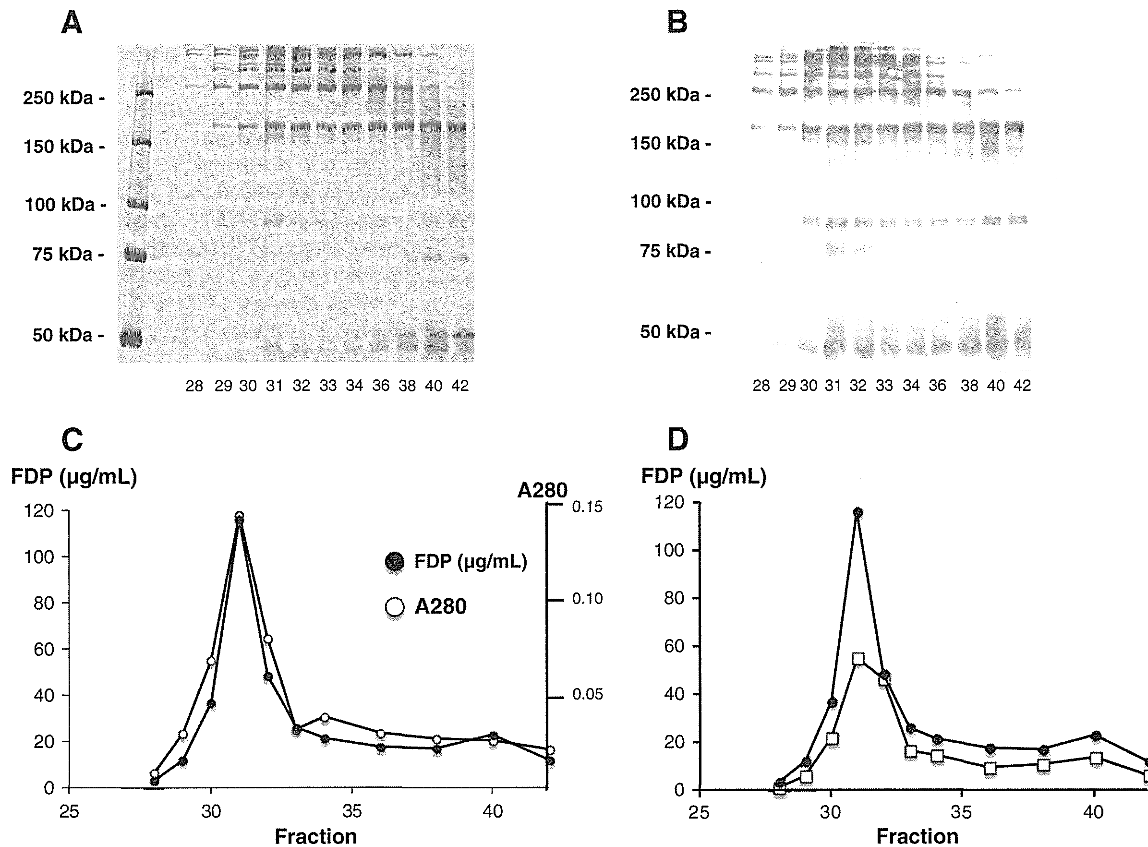

Fig. 1. Analysis of FDP generated in vitro. Fractions of gel filtration column chromatography on Sephacryl S-300 were analyzed by SDS-PAGE with silver staining (A). The same samples were analyzed by Western blotting with a rabbit polyclonal antibody against human fibrinogen (B). Most of the protein bands, except for a protein that migrated approximately 50 kDa, seen in the silver stained gel were bound to the anti-fibrinogen antibody. FDP concentrations (closed circle) determined with serum FDP-2 and A280 (open circle) were plotted (C). FDP concentrations of the fractions determined with serum FDP-2 (closed circle) and serum FDP-1 (open square) were shown (D).

(tPA) at a concentration of 10 ng/mL and mixed with 2 U/mL thrombin and CaCl2 (25 mM) in glass tubes (2 mL/tube). After incubation at 37 °C for 30 min, fibrin clots were squeezed and washed with Tris buffered saline (20 mL Tris, 150 mM NaCl pH 7.4) three times to remove plasma proteins and were then incubated in 5 mL of Tris buffered saline at 37 °C for 96 h. All the buffers, tubes, and pipet chips were sterilized before use. FDP released into the buffer was harvested, incubated with phenylmethylsulfonyl fluoride (1 mM) for the inactivation of protease activity in the samples, and analyzed. During the incubation of plasma with thrombin in the presence of calcium ions, γ dimer formation and α polymer formation by factor XIIIa were completed [14].

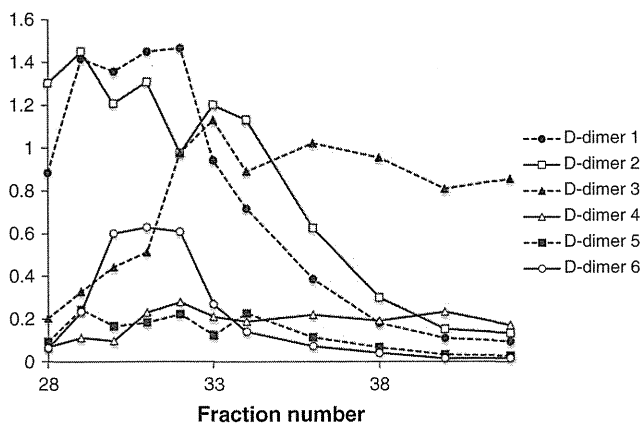

Fig. 2. Relative D-dimer values against serum FDP-2 values in the gel filtration column chromatography fractions. Fractions #28–42 of gel chromatography were further analyzed with 6 D-dimer reagents (D-dimer-1, D-dimer-2, D-dimer-3, D-dimer-4, D-dimer-5, and D-dimer-6) for clinical laboratory testing. The D-dimer value of each fraction was divided by the respective serum FDP-2 value and the ratios were plotted. If the D-dimer value was the same as the serum FDP-2 value, the ratio was 1.0.

Gel filtration column chromatography

Gel filtration column chromatography of the FDP samples on Sephacryl S-300 (1.6 × 100 cm, GE Healthcare Japan, Tokyo, Japan) equilibrated with 20 mM Tris 500 mM and NaCl at pH 7.4 was carried out at 4 °C. Fractions (2 mL) of gel filtration column chromatography were collected and analyzed. Samples containing FDP were analyzed by SDS-PAGE followed by Western blotting with rabbit anti human fibrinogen (DAKO, Carpinteria, CA), and FDP in these fractions was quantified with immunoassays using latex particles (latex immunoassay, LIA).

Immunoassay

An immunoassay was carried out with equipment optimized for the respective reagent. LIA reagents and laboratory equipment used in the present study were as follows; LPIA-NV7 for LPIA-ACE D-dimer II (D-dimer-1), LPIA FDP-P (plasma FDP-1), LPIA FDP (serum FDP-1) (Mitsubishi Chemical Medience Corp, Tokyo, Japan); CS-2000i (Sysmex Corp, Kobe, Japan) for Latextest BL-2 FDP (serum FDP-2), Latextest BL-2 P-FDP (plasma FDP-2), and LIAS AUTO D-dimer NEO (D-dimer-2) (Sysmex Corp, Kobe, Japan) [10,15–17]; Coapresta2000 for Nanopia D-dimer reagent (D-dimer-3) and Nanopia FDP reagent (plasma FDP-3) (SEKISUI MEDICAL Co. Ltd., Tokyo, Japan); STA-R (Roche Diagnostics Japan, Tokyo, Japan) for Hexamate P-FDP (plasma FDP-4, MBL, Nagoya,

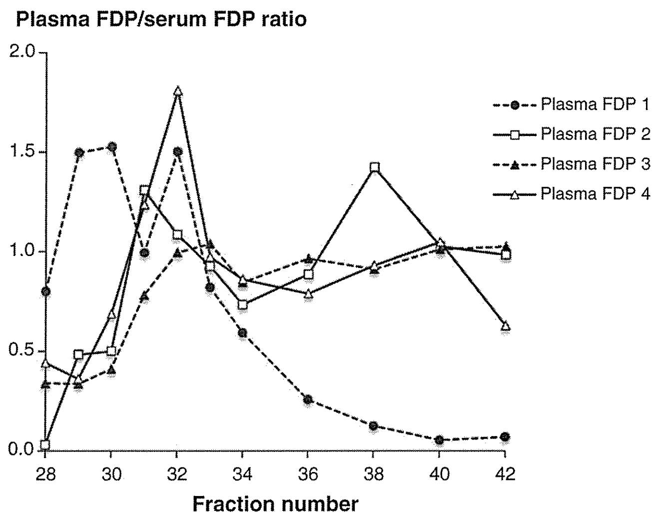

Fig. 3. Relative plasma FDP values against serum FDP-2 values in the gel filtration column chromatography fractions. Fractions #28–42 of gel chromatography were quantified with four plasma FDP reagents (plasma FDP-1, plasma FDP-2, plasma FDP-3, and plasma FDP-4) in a similar manner to Fig. 2. The FDP value of each fraction determined with a plasma FDP reagent was divided by the respective serum FDP-2 value of the fraction. If the plasma FDP value was the same as the serum FDP-2 value, the ratio was 1.0.

Japan), Hexamate D-dimer (D-dimer-4, MBL, Nagoya, Japan), TA Liatest D-Di (D-dimer-6, Diagnostica STAGO, Parsippany-Troy Hills, NJ); Hitachi 7180 for Tinaquant D-Dimer (D-dimer-5, Roche Diagnostics Japan, Tokyo, Japan). None of the reagents shared the same monoclonal antibody.

Clinical sample analysis

This study was carried out according to the Declaration of Helsinki and was approved by the Ethical Committee of Jichi Medical University. Clinical diagnosis including underlying diseases and laboratory data (coagulation assays and the biomarkers of blood coagulation) were extracted from the clinical records of patients. Residual plasma and serum of routine laboratory reagents was used for the chromatography analysis. Gel filtration column chromatography of patient serum on Sephacryl S-300 (1.6 × 120 cm) equilibrated with 20 mM Tris and 500 mM NaCl at pH 7.4 was carried out at 4 °C. Fractions (2 mL) of gel filtration column chromatography were collected and analyzed by SDS-PAGE followed by Western blotting and immunoassays for FDP as described above.

Statistical analysis

Pearson's correlation coefficient was calculated to investigate the relationship between serum FDP and D-dimer levels in clinical plasma samples using Statcel Version 3 for Microsoft Excel.

Results

Analysis of FDP generated in vitro

Cross-linked FDP generated *in vitro* was fractionated by gel filtration column chromatography on Sephacryl-S300 and analyzed by SDS-PAGE and Western blotting with a polyclonal antibody against human fibrinogen (Fig. 1A, B). These samples were subjected to the quantification of absorbance at 280 nm (A280, DU 730, Beckman Coulter) and of FDP concentrations by a serum FDP test (Fig. 1C). Most of the protein bands detected by SDS-PAGE (Fig. 1A, silver stained gel) were bound to the polyclonal antibody against fibrinogen (Fig. 1B, Western blot). A protein that migrated in the low molecular weight region (approximately 50 kDa, fractions 40 and 42) was not bound to the anti-fibrinogen antibody; however, the amount of this protein was small. A comparison of A280 values with the FDP concentrations of the fractions (serum FDP-2) revealed a correlation between changes in the A280 values and FDP concentrations of these samples (correlation coefficient, $r = 0.978$; Fig. 1C). These results suggest that the FDP sample generated *in vitro* mostly consisted of cross-linked FDP and that the serum FDP test (serum FDP-2) accurately quantified the various FDP molecular forms. FDP concentrations in the fractions of gel chromatography were quantified with two laboratory serum FDP reagents (serum FDP-1, serum FDP-2). There were differences in these values, but the relative ratios of these differences were mostly constant (1.73 ± 0.29) and correlated well (correlation coefficient, $r = 0.921$) (Fig. 1D). These results suggest that the reactivity of each antibody in serum FDP-1 and serum FDP-2 to FDP was similar. Therefore, serum FDP-2 was used as the reference to evaluate D-dimer reagents and plasma FDP reagents. The results also suggest that there are differences in the net values between these reagents. However, the standardization of these reagents can be performed using the same purified fibrinogen material as the standard.

Relative D-dimer values against serum FDP values in the gel filtration column chromatography fractions

FDP concentrations in fractions #28–42 of gel chromatography were further analyzed with the six D-dimer reagents (D-dimer-1, D-dimer-2, D-dimer-3, D-dimer-4, D-dimer-5, and D-dimer-6) used for clinical laboratory testing. The D-dimer value of each fraction was divided by the respective serum FDP value to investigate the relative reactivity of the monoclonal antibodies of respective tests to the polyclonal antibody used in serum FDP-2 (Fig. 2). If the specificity of the monoclonal antibody used in the D-dimer test to various FDP molecular forms was similar to the polyclonal antibody used in the serum FDP-2, the ratio of the D-dimer value divided by the serum FDP value would be constant throughout the fractions. Fig. 2 shows the relative ratios of D-dimer values to serum FDP-2. The D-dimer-1/serum FDP-2 and D-dimer-2/serum FDP-2 ratios of fractions #28–34, those containing very high and high molecular weight FDP molecules, were 1.0–1.5; however, these values declined to 0.1–0.6 in fractions #36–42, which contained the low molecular weight FDP forms, suggesting that the reaction of the antibody used in D-dimer-1 and D-dimer-2 to low molecular weight FDP molecules was poorer than that to high molecular weight FDP molecules. Similar changes in D-dimer/serum FDP ratios were observed in the ratios of D-dimer-5/serum FDP-2 and D-dimer-6/serum FDP-2. The D-dimer-3/serum FDP-2 ratios in fractions #28–31 containing very high molecular weight FDP molecules were lower than 0.5; however, these ratios were 0.8–1.1 in fractions #32–42, which indicated that the antibody used in D-dimer-3 reacted to very high molecular weight FDP molecules weaker than to low molecular weight FDP molecules. This reactivity of the antibody used in D-dimer-3 to various FDP molecular forms was opposite to that of the antibody used in D-dimer-1, D-dimer-2, D-dimer-5, and D-dimer-6. Though the D-dimer-4/serum FDP-2 ratios were relatively low, changes in the

Table 1
Reactivity of FDP reagents to FDP molecular variants.

	Very High MW FDP	High MW FDP	Medium FDP	Low MW FDP
D-dimer-1	high	high	good–low	low
D-dimer-2	high	high	good	low
D-dimer-3	low	low–good	good	good
D-dimer-4	low	good	good	good
D-dimer-5	low	good	good	low
D-dimer-6	low	high	good	low
Plasma FDP-1	high	high	good–low	low
Plasma FDP-2	low	good	good	good
Plasma FDP-3	low	good	good	good
Plasma FDP-4	low	good	good	good

MW: molecular weight.

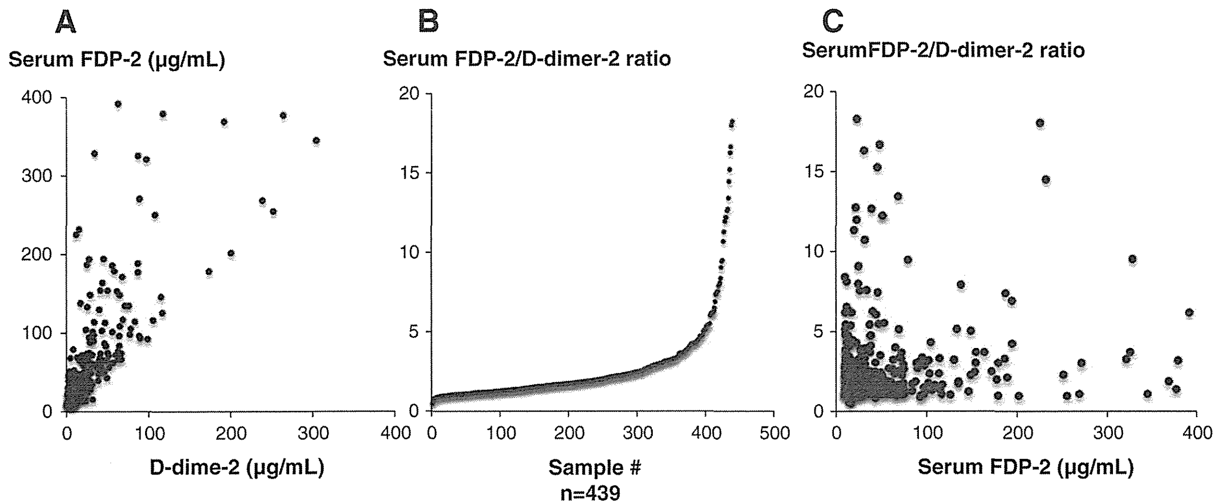

Fig. 4. Distribution of serum FDP/D-dimer ratios in clinical samples. Serum FDP-2 levels (10–400 µg/mL) of clinical samples (n = 439) were plotted against the D-dimer-2 level of the same sample (Fig. 4A). The ratios (<20) of the serum FDP-2/D-dimer-2 of clinical samples were sorted and plotted (B). Serum FDP-2/D-dimer-2 ratios were plotted against serum FDP-2 (C). There was no correlation between these values.

D-dimer/serum FDP-2 ratios in the fractions measured with D-dimer-4 were similar to those of D-dimer-3. These results suggest that the reactivity of the monoclonal antibody used in these 6 reagents was not the same, and that the reactivity of the monoclonal antibodies used in D-dimer-1, D-dimer-2, D-dimer-5, and D-dimer-6 to various molecular weight FDP molecules was similar. The reactivity of the monoclonal antibodies used in D-dimer-3 and D-dimer-4 may be similar. FDP values in the fractions determined with D-dimer-3, D-dimer-4, D-dimer-5, and D-dimer-6 to molecular weight FDP molecules were lower than those determined with serum FDP-2 (ratio: D-dimer-3, 0.16 ± 0.16; D-dimer-4, 0.18 ± 0.06; D-dimer-5, 0.14 ± 0.08; D-dimer-6, 0.24 ± 0.25).

Relative plasma FDP values against serum FDP values in the fractions of gel filtration column chromatography

FDP concentrations in fractions #28–42 of gel chromatography were further analyzed using the four plasma FDP reagents (plasma FDP-1, plasma FDP-2, plasma FDP-3, and plasma FDP-4) used in clinical laboratories. The plasma FDP value of each fraction was divided by the

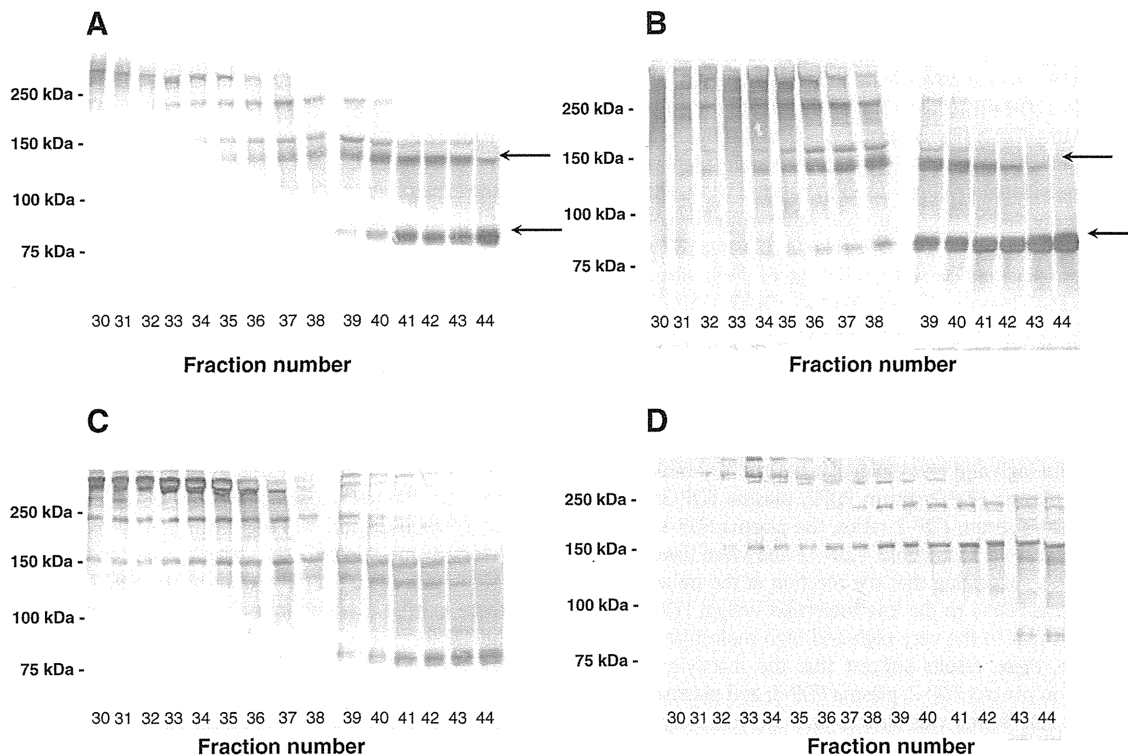

Fig. 5. Molecular variants of FDP in clinical samples. Four representative clinical DIC sample FDP levels (high FDP (>100 µg/mL) and high serum FDP-2/D-dimer-2 ratios (A, 18.0; B, 9.5); high FDP levels (>100 µg/mL) and low serum FDP-2/D-dimer-2 ratios (C, 1.0; D, 1.0) were subjected to gel filtration column chromatography on Sephacryl S-300. Fractions were analyzed by SDS-PAGE followed by Western blotting with the anti-fibrinogen polyclonal antibody. Arrows indicate the presence of protein fragments was higher in samples A and B than in samples C and D.

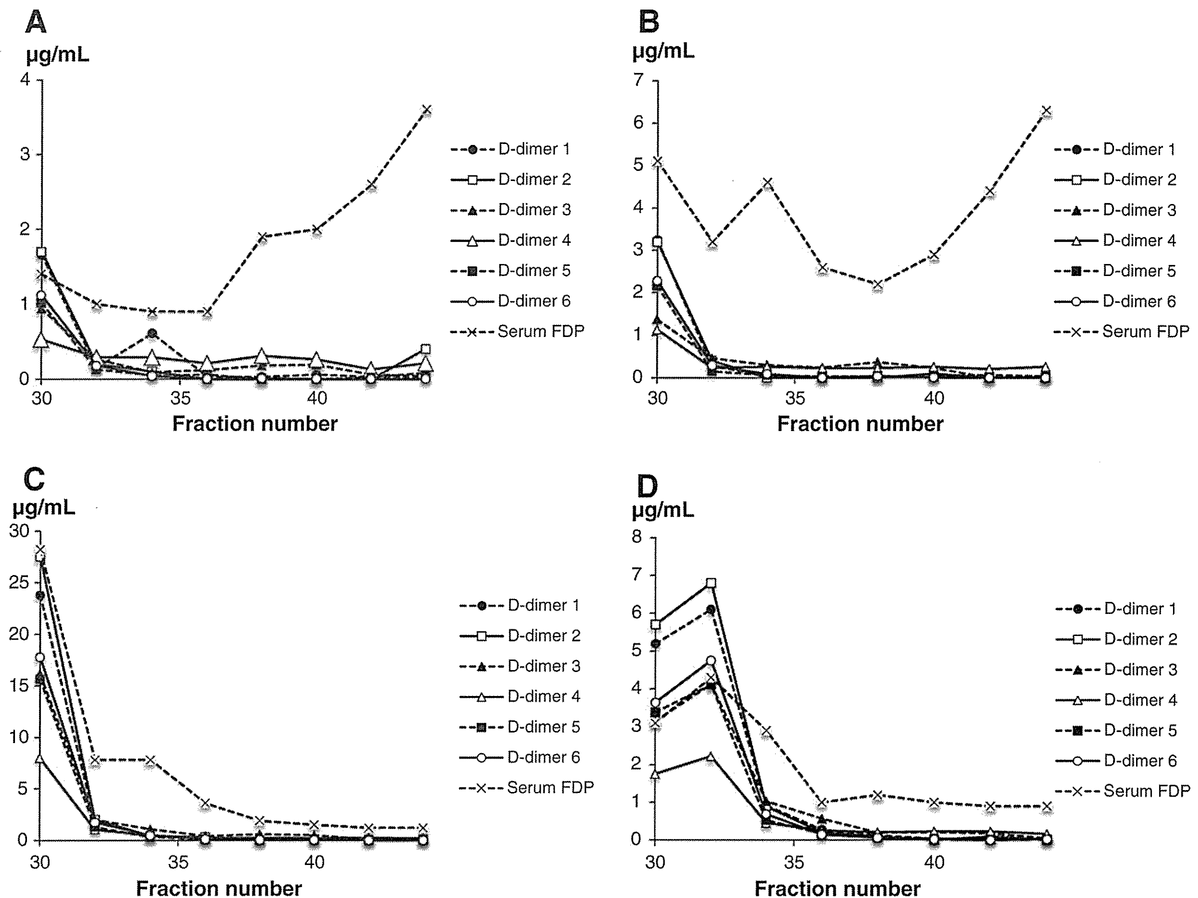

Fig. 6. Reactivity of the monoclonal antibodies used in laboratory D-dimer reagents to the molecular variants of FDP in clinical samples. Gel filtration column chromatography fractions of clinical samples A (A), B (B), C (C), and D (D) were subjected to quantification with D-dimer reagents. FDP concentrations of the fractions were determined with serum FDP-2 (cross), D-dimer-1 (closed circle), D-dimer-2 (open square), D-dimer-3 (closed triangle), D-dimer-4 (open triangle), D-dimer-5 (closed square), and D-dimer-6 (open circle).

respective serum FDP-2 value to investigate the relative reactivity of the monoclonal antibody of the respective plasma FDP test to the polyclonal antibody used in serum FDP-2 (Fig. 3). If the specificity of the monoclonal antibody used in a plasma FDP reagent to FDP molecular variants was similar to the polyclonal antibody used in serum FDP-2, the ratio of the D-dimer value divided by the serum FDP value would be constant throughout the fractions. Fig. 3 shows the relative plasma FDP values to serum FDP values (plasma FDP/serum FDP) quantified with serum FDP-2. The plasma FDP-2/serum FDP-2, plasma FDP-3/serum FDP-2, and plasma FDP-4/serum FDP-2 ratios in fractions #28–30 containing high molecular weight FDP molecules were around 0.5; however, these values were around 1.0 in fractions #32–42, which suggested that the reaction of the antibody used in plasma FDP-2, plasma FDP-3, and plasma FDP-4 to very high molecular weight FDP molecules was poorer than that to the high and low molecular weight FDP molecules. Compared with the plasma FDP-2/serum FDP2, plasma FDP-3/serum FDP-2, and plasma FDP-4/serum FDP-2 ratios, the plasma FDP-1/serum FDP-2 ratios were around 1.5 in fractions #29–32 and were lower than 0.6 in fractions #34–42, suggesting that the reaction of the monoclonal antibody used in plasma FDP-1 to the low molecular weight FDP molecules was poorer than that to the very high and high molecular weight FDP forms. Therefore, these results suggest that the reactivity of the monoclonal antibody in plasma FDP-2, plasma FDP-3, and plasma FDP-4 to various molecular FDP forms was similar and the reactivity of the monoclonal antibody in plasma FDP-1 was distinct from the monoclonal antibody in the three other reagents.

The reactivity of D-dimer and plasma FDP reagents to various molecular variants of FDP was summarized in Table 1 for a quick comparison of the reagents.

Relative ratios of serum FDP to D-dimer in clinical samples

Serum FDP and D-dimer are markers used to diagnose DIC and venous thromboembolism. Therefore, these values were extracted from the clinical laboratory data of patients with coagulopathy admitted to the Jichi Medical University Hospital. Serum FDP-2 and D-dimer-2 have been used to quantify serum FDP and D-dimer levels in these patients in the clinical laboratory of the hospital. Since D-dimer-2 detects low molecular weight FDP more poorly than very high and high molecular weight FDP forms, a high ratio of serum FDP-2/D-dimer-2 may indicate that the concentration of low molecular weight FDP is relatively high. Based on this hypothesis, the FDP levels (10–400 μg/mL) of clinical samples were plotted against the D-dimer levels of the same samples (Fig. 4). Although the FDP and D-dimer values were correlated in these samples, the correlation coefficient values were $r = 0.788$ for FDP (10–400 μg/ml) samples ($r = 0.628$ for FDP 100–400 μg/mL samples). When the ratios of the serum FDP/D-dimer of clinical samples were sorted and plotted, the serum FDP-2/D-dimer-2 ratios in the clinical samples were distributed from 1.0 to 92.1. These results suggest that there may be a deviation in the concentration of the low molecular weight FDP forms in clinical samples. There was no correlation between serum FDP-2/D-dimer-2 ratios and serum FDP-2 values (Fig. 4).

Molecular variants of FDP in clinical samples

Four representative DIC samples were selected based upon laboratory reagents. The sera of two patients (A, B) with high FDP levels (>100 μg/mL) and high serum FDP-2/D-dimer-2 ratios (A, 18.0; B, 9.5), and the sera of two patients (C, D) with high FDP levels (>100 μg/mL)

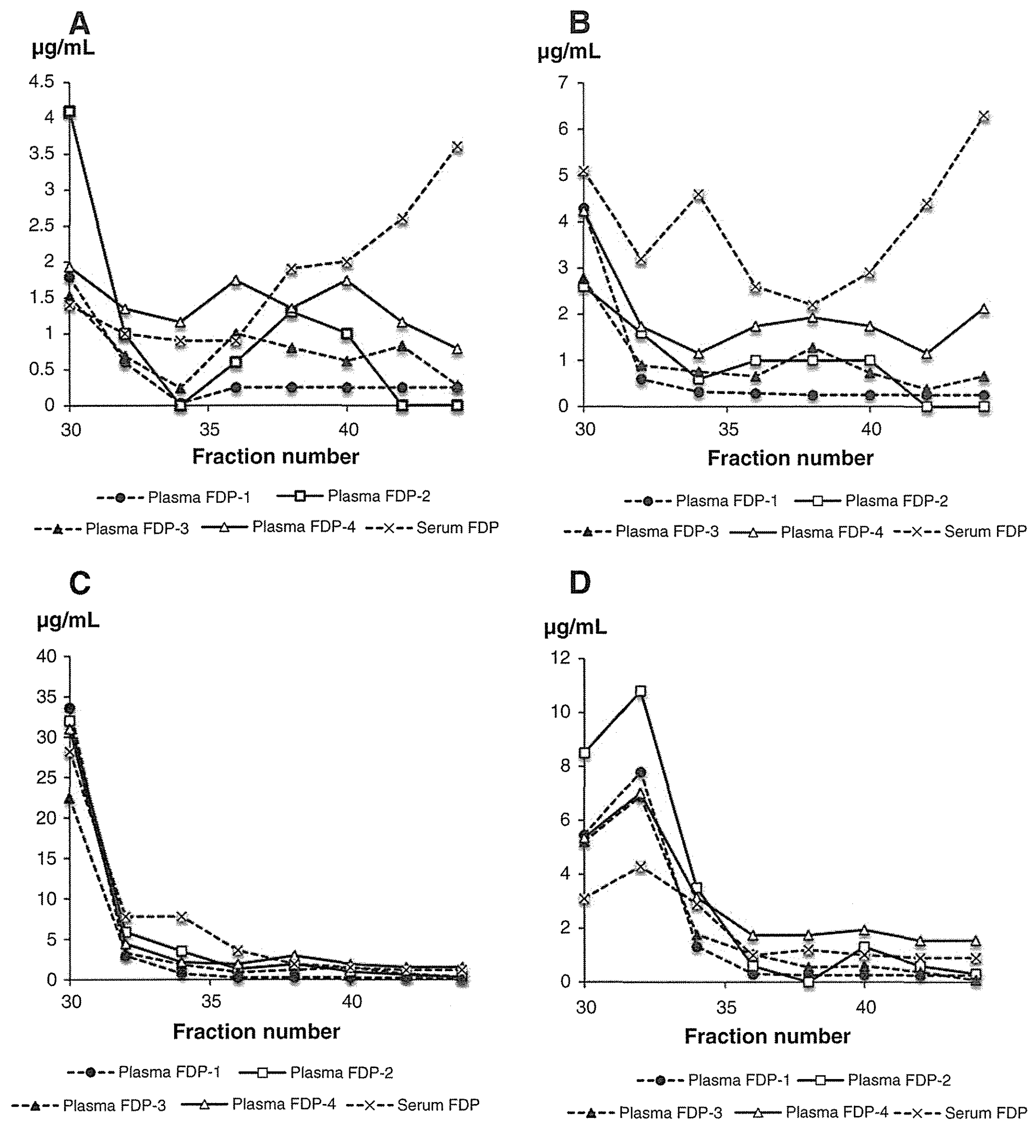

Fig. 7. Reactivity of the monoclonal antibodies used in laboratory plasma FDP reagents to the molecular variants of FDP in clinical samples. Gel filtration column chromatography fractions of clinical samples A (A), B (B), C (C), and D (D) were subjected to quantification with plasma FDP reagents. FDP concentrations of the fractions were determined with serum FDP-2 (cross), plasma FDP-1 (closed circle), plasma FDP-2 (open square), plasma FDP-3 (closed triangle), and plasma FDP-4 (open triangle).

and low serum FDP-2/D-dimer-2 ratios (C, 1.0; D, 1.0) were subjected to gel filtration column chromatography on Sephacryl S-300. The underlying diseases of the four patients were lung cancer (A), prostate cancer (B), sepsis (C), and pneumonia (D). Fractions of column chromatography were analyzed by Western blotting with the polyclonal antibody against human fibrinogen (Fig. 5), and D-dimer levels (Fig. 6) and plasma FDP levels (Fig. 7) were quantified as above. Western blot analysis with the anti-fibrinogen polyclonal antibody of the fractions (Fig. 5) revealed that the protein bands recognized by the antibody migrating approximately 130 kDa and 90 kDa (arrows) was higher in the fractions of samples A and B than that in samples C and D.

Reactivity of the monoclonal antibodies used in laboratory FDP reagents to the molecular variants of FDP in clinical samples

Various FDP forms in samples A and B (Fig. 6A, B) were quantified in fractions #30–44 with serum FDP-2. However, none of the D-dimer reagents could detect the FDP (D-dimer) well in fractions #32–44 of these samples. The FDP of samples C and D (Fig. 6C, D) was higher in fractions #30–34 than in fractions #36–44. D-dimer-1– -6 could detect the FDP of these samples in fractions 30 and 32. These results suggest that a wide variety of molecular forms of FDP were present in samples A and B (high FDP level and high serum FDP-2/D-dimer-2 ratio), and low molecular weight FDP molecular forms were not accurately quantified with D-dimer-1–6. The nature of FDP in clinical samples A and B may be different from that of *in vitro*-generated FDP. The presence of low molecular FDP forms indicated by arrows in Western blotting (Fig. 5) may partly account for this difference. These FDP forms may be FgDP or very degraded FDP missing monoclonal antibody epitopes. The results of analysis of clinical samples C and D also suggest that the high molecular forms of FDP may be the main variants of FDP molecules in samples C and D (high FDP level and low serum FDP/D-dimer ratio), and that only the high molecular FDP variants could be similarly quantified with D-dimer-1, D-dimer-2, D-dimer-3, D-dimer-4, D-dimer-5, and D-dimer-6, as with serum FDP-2.

Various FDP forms in samples A and B (high FDP level and high serum FDP/D-dimer ratio) (Fig. 7A, B) were detected in fractions #30–44. Plasma FDP-2, -3, and -4 could also detect the FDP variants of

these samples in fractions #32–44. However, plasma FDP-1 failed to detect the low weight FDP forms of these samples in fractions #32–44 (Fig. 7A, B). The FDP variants in samples C and D were detected with plasma FDP-1– plasma FDP-4, similar to that with the serum FDP reagent. Though the relative values were different, plasma FDP-2–plasma FDP-4 could quantify the various FDP that formed in samples C and D in a similar manner to that with serum FDP-2. The reactivity of plasma FDP-1 to the low molecular FDP forms may be similar to D-dimer-1, D-dimer-2, D-dimer-5, and D-dimer-6.

It is possible that FgDP may be present in clinical samples A and B and may be detected by serum FDP-2 in the gel filtration column chromatography fractions. This was suggested because D-dimer-3 could not detect significant D-dimer signals in samples A and B in fractions #34–44, whereas plasma FDP-2, plasma FDP-3, and plasma FDP-4 could. Therefore, the presence of FgDP in the gel filtration column chromatography fractions of samples A and B remains to be determined.

Discussion

Various biomarkers have been developed to diagnose coagulopathy and are commercially available in clinical laboratories. Though serum FDP testing has been the standard assay used to detect thrombolysis following thrombus formation, many D-dimer reagents and plasma FDP reagents have been developed and are being widely used without standardization in clinical laboratory testing [6–8,10,11]. D-dimer reagents are considered to be specific to cross-linked fibrin derived FDP [6–8,10,11,17]. This characteristic provides the basis for the advantage of D-dimer reagents over serum FDP reagents. Plasma FDP testing enables the detection of not only cross-linked fibrin derived FDP, but also FgDP. These reagents are able to detect fibrin-derived fragments in plasma without using special test tubes for FDP. However, most of these reagents have so far not been evaluated simultaneously using the same materials and clinical samples, and have also not yet been standardized. The DIC diagnosis criteria of the Japanese Ministry of Health, Labour, and Welfare established in 1988 utilized serum FDP as a diagnostic score for DIC with cut-off values [4,18]. The DIC diagnosis criteria for acute medicine established by the Japanese Association for Acute Medicine in 2005 allows for the conversion of D-dimer values to FDP values by applying respective coefficients [1,19]. D-dimer testing has been used as a negative predictive value to rule out the presence of VTE [11–13]. Because of the importance of this test, these reagents should be standardized for the accurate diagnosis of coagulopathy such as DIC and VTE. The present study evaluated 6 D-dimer reagents and 4 plasma FDP reagents simultaneously with the same *in vitro*-generated cross-linked FDP and clinical samples to elucidate the nature of the monoclonal antibodies used in these reagents.

The results of the present study showed that the reactivity of the monoclonal antibodies used in D-dimer reagents was distinct. At least four (D-dimer-1, D-dimer-2, D-dimer-5, D-dimer-6) of six D-dimer reagents may have lower affinity for the low molecular forms of cross-linked FDP than for the very high and high molecular weight molecular forms of FDP (Table 1). This feature differs from the other two D-dimer reagents (D-dimer-3 and D-dimer-4). Three (plasma FDP-2, plasma FDP-3, and plasma FDP-4) of the four plasma FDP reagents had similar reactivity for *in vitro*-generated cross-linked FDP, while reactivity for the *in vitro*-generated FDP of plasma FDP-1 was distinct.

Analysis of clinical samples in the present study (Fig. 4) showed that the amount of low molecular weight FDP forms relative to that of the high molecular weight forms may deviate based upon the underlying disease. Analysis of the four representative clinical samples suggested that the reactivity of the D-dimer reagents for the high and low molecular weight forms of FDP was virtually consistent with that of *in vitro*-generated FDP molecules. The results of the present study also suggest that the reactivity of plasma FDP reagents for the high molecular weight and low molecular weight forms of FDP was nearly consistent with that for *in vitro*-generated FDP molecules.

In conclusion, the present study revealed that the reactivity of the monoclonal antibodies used in clinical laboratory testing to quantify FDP and D-dimer to various FDP molecular forms is distinct, and that FDP and D-dimer values in clinical samples may differ when measured with two different reagents. Although the results of our study also suggest that the development of a "universal" calibrator may be very difficult, standardization of D-dimer reagents and plasma FDP reagents is required for the accurate diagnosis of coagulopathy. One possible alternative approach would be the use of two monoclonal antibodies, with one having good affinity to very high to high molecular weight FDP variants and the other having good affinity to middle to low molecular weight FDP variants.

Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgments

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