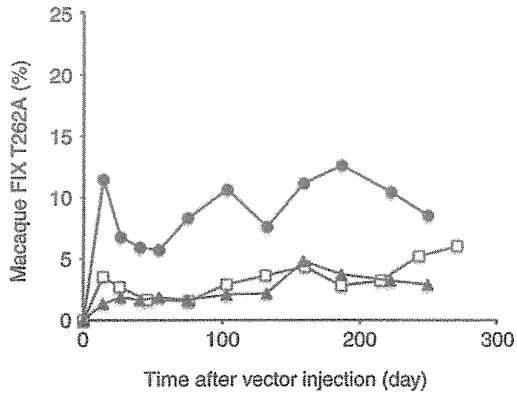

Figure 3 Expression of FIX T262A in macaques following balloon catheter-guided vector injection into portal veins. Three macaques (n = 3) were subjected to balloon catheter-guided vector injection into the portal vein. Concentrations of FIX T262A in macaque plasma samples (#37, closed circles; #38, open 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

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

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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Genetically Modified Adipose Tissue-Derived Stem/Stromal Cells, Using Simian Immunodeficiency Virus-Based Lentiviral Vectors, in the Treatment of Hemophilia B

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Abstract

Hemophilia is an X-linked bleeding disorder, and patients with hemophilia are deficient in a biologically active coagulation factor. This study was designed to combine the efficiency of lentiviral vector transduction techniques with murine adipose tissue-derived stem/stromal cells (mADSCs) as a new method to produce secreted human coagulation factor IX (hFIX) and to treat hemophilia B. mADSCs were transduced with simian immunodeficiency virus (SIV)-hFIX lentiviral vector at multiplicities of infection (MOIs) from 1 to 60, and the most effective dose was at an MOI of 10, as determined by hFIX production. hFIX protein secretion persisted over the 28-day experimental period. Cell sheets composed of lentiviral vector-transduced mADSCs were engineered to further enhance the usefulness of these cells for future therapeutic applications in transplantation modalities. These experiments demonstrated that genetically transduced ADSCs may become a valuable cell source for establishing cell-based gene therapies for plasma protein deficiencies, such as hemophilia.

Introduction

HEMOPHILIA IS a congenital bleeding disorder that is attributed predominantly to a hereditary lack of biologically active coagulation factor VIII (FVIII) or factor IX (FIX). Worldwide, 105 to 160 per million of the male population suffer from this disease (Bolton-Maggs and Pasi, 2003). Current standard therapy is generally provided after the onset of bleeding episodes and relies on the infusion of FVIII or FIX concentrates. Unfortunately, these treatments are expensive, limiting access to this type of therapy for a majority of patients with hemophilia (Pipe *et al.*, 2008). Thus, alternative molecular and cellular methods are needed for the treatment of hemophilia. Studies have shown that even a small increase in clotting activity (~1–2%) over normal levels can improve the bleeding from severe to mild-to-moderate (Bolton-Maggs and Pasi, 2003), representing a dramatic improvement in quality of life by reducing the need for immediate clotting factor injections to prevent uncontrolled bleeding.

Cell-based therapies have received a great deal of attention as a next-generation therapeutic approach for hemophilia (Oh *et al.*, 2006; Follenzi *et al.*, 2008; Kasuda *et al.*, 2008; Tatsumi *et al.*, 2008a,b; Ohashi *et al.*, 2010). There has been enormous interest in the transplantation of stem cells to produce clotting factors (Chuah *et al.*, 2004; Oh *et al.*, 2006; Coutu *et al.*, 2011). Some types of stem cells can be readily isolated from human patients with minimal invasiveness (Lin *et al.*, 2008), such as adipose tissue-derived stem/stromal cells (ADSCs, also known as adipose tissue-derived mesenchymal stem cells, AT-MSCs) (Zuk *et al.*, 2001; Li *et al.*, 2011b). ADSCs are actively proliferative *in vitro* and are multipotent, with the potential to differentiate into mesodermal, endodermal, and ectodermal lineages (Lee *et al.*, 2004; Peister *et al.*, 2004; Wang *et al.*, 2004; Seo *et al.*, 2005; Aurich *et al.*, 2007; Banas *et al.*, 2007; Liu *et al.*, 2007). These cells would be an ideal autologous source of stem cells with the potential to reduce the need for immunosuppression after reimplantation back into patients.

In terms of hemophilia, native ADSCs need to be genetically modified to produce and secrete FVIII or FIX because ADSCs do not naturally express coagulation factors. A multitude of genetic approaches (Anjos-Afonso *et al.*, 2004; Haleem-Smith *et al.*, 2005; Oh *et al.*, 2006; Talens-Visconti *et al.*, 2006; Sugii *et al.*, 2010; Coutu *et al.*, 2011; Li *et al.*,

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2011a,b) have been applied to exogenously produce clotting factors.

In this study, we used simian immunodeficiency virus (SIV) lentiviral vectors derived from SIVagmTYO-1, a simian immunodeficiency virus strain isolated from African green monkeys (Nakajima *et al.*, 2000). The SIV vector has been reported to have a different safety profile compared with other lentiviral vectors, in that this strain lacks the ability to become pathogenic in its natural host, African green monkeys, and in experimentally inoculated Asian macaques (Honjo *et al.*, 1990; Nakajima *et al.*, 2000; Kikuchi *et al.*, 2004; Ogata *et al.*, 2004). SIV also has low sequence homology to the HIV genome (Jin *et al.*, 1994). Therefore, SIV vectors are assumed to hold low or almost no risk of causing homologous recombination that generates a replication-competent virus, even in circumstances in which the SIV vector coexists with HIV in the same cells inside a patient. The SIV vector is likely safer than other vectors and maintains the inherent ability to integrate into both proliferating and non-proliferating cells (Coffin *et al.*, 1997; Walther and Stein, 2000; Li and Lu, 2009), making it an ideal vector to provide persistent expression of exogenous genes and possibly making it advantageous for application in future clinical studies.

Another approach to increasing the level of engraftment of transplanted cells at local sites is to engineer functional tissues. Our laboratory has established a cell sheet-engineering technology using temperature-responsive culture dishes that are grafted with a temperature-responsive polymer, poly (*N*-isopropylacrylamide) (PIPAAm) (Kikuchi and Okano, 2005; Yang *et al.*, 2005). This technology allows us to recover monolithic cell sheets without any enzymatic digestion and has already been applied to regenerative medicine (Nishida *et al.*, 2004; Obokata *et al.*, 2011). To establish a tissue engineering-based treatment modality with murine ADSCs (mADSCs) for hemophilia, this method was applied to create a contiguous cell sheet of vector-transduced mADSCs.

Materials and Methods

Mice

C57BL/6J male mice (8 weeks old) were purchased from a commercial vendor (CLEA Japan, Tokyo, Japan). All animal procedures were conducted in accordance with the institutional guidelines of the Animal Care Committee of Tokyo Women's Medical University (Tokyo, Japan).

Preparation of mouse ADSCs

Adipose tissues were isolated from the inguinal region in the mice, minced with forceps, and enzymatically digested with 0.1% type I collagenase (17100-017; Invitrogen/Life Technologies, Carlsbad, CA) at 37°C for 1 hr. The stromal–vascular fraction (SVF) was collected by centrifugation at $700 \times g$ for 5 min and washed twice. The SVF was resuspended with Dulbecco's modified Eagle's medium (DMEM)–F12 (11320-033; Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum (FBS, 04110101; Japan Bio-Serum, Hiroshima, Japan) and GlutaMAX-I supplement (35050-061; Invitrogen/Life Technologies). This medium is referred to in text as "basic medium." The SVF was plated on PRIMARIA tissue culture-treated dishes (35-3803; BD Biosciences, Franklin Lakes, NJ) and cultured at 37°C in a 5% CO_2 incubator. The medium was aspirated and changed 3 days after plating. Adherent proliferating cells were trypsinized for subculturing (defined as passage 1) approximately 7–8 days after plating. The subcultured cells were defined as mADSCs.

Flow cytometry

mADSCs at passage 2 were suspended and incubated with an Fc blocker (553141), followed by antibodies: fluorescein isothiocyanate-conjugated CD29 (CD29–FITC; 555005), phycoerythrin-conjugated CD44 (CD44–PE; 553134), CD90.2–PE (553005), CD31–PE (553373), CD45–PE (553081), isotype control–PE (553930), and isotype control–FITC (553960). All antibodies were obtained from BD Biosciences and the catalog numbers are shown in parentheses. The cells were analyzed with a flow cytometer (Gallios; Beckman Coulter, Brea, CA).

Osteogenic differentiation of mADSCs followed by alkaline phosphatase assay and alizarin red S staining

mADSCs (passage 2) were replated at 1×10^4 cells/cm^2 in a 6-well plate for staining with alizarin red S, and 3.3×10^4 cells/cm^2 were plated for an alkaline phosphatase (ALP) assay using minimum essential medium (MEM) α with GlutaMAX-I (32571; Invitrogen/Life Technologies) supplemented with 10% FBS. Twenty-four hours after cell plating, differentiation was initiated by incubating the cells with osteogenic differentiation medium: MEM α GlutaMAX-I with 10% FBS, containing β-glycerophosphate disodium salt hydrate (G9891; Sigma-Aldrich, St. Louis, MO) at 10 mmol/liter, ascorbic acid (323-44822; Wako, Osaka, Japan) at 50 μmol/liter, dexamethasone (Dex) (BG08A; Fuji-Seiyaku, Tokyo) at 100 nmol/liter; or commercially available osteogenic differentiation medium (hMSC osteogenic BulletKit, PT-3002; Lonza Japan, Tokyo, Japan). The osteogenic differentiation medium was changed every 3–4 days. Seven days after osteogenic induction, the ALP assay was performed on mADSCs, using a LabAssay ALP kit (291-58601; Wako) according to the manufacturer's instructions. Four weeks after osteogenic induction, mADSCs were fixed with 4% paraformaldehyde (PFA) (100412; Muto-kagaku, Tokyo, Japan), washed with purified water (Synthesis A10; Millipore, Billerica, MA), and stained at room temperature for 10 min with alizarin red S (011-01192; Wako) at 10 g/liter.

Adipogenic differentiation of mADSCs and oil red O staining

mADSCs (passage 2) were replated at 3×10^4 cells/cm^2 with DMEM–F12 supplemented with 10% FBS and GlutaMAX-I (basic medium) and cultured until confluency. The basic medium was replaced with adipogenic differentiation medium: basic medium supplemented with isobutylmethylxanthine (IBMX) (I7018; Sigma-Aldrich) at 0.5 mmol/liter, indomethacin (095-02472; Wako) at 100 μmol/liter, Dex at 500 nmol/liter, and insulin (Wako) at 10 μg/ml. Seventy-two hours later, the adipogenic differentiation medium was removed and replaced with basic medium containing insulin (10 μg/ml). This latter medium was changed every 3–4 days. Two weeks after adipogenic induction, the mADSCs were fixed with 10% formalin, washed with phosphate-buffered saline (PBS) and isopropanol (Wako), and subsequently

stained with oil red O solution (09091; Muto-kagaku) at room temperature for 10–20 min.

Preparation and titering of replication-defective SIV lentiviral vectors

Self-inactivating (SIN) simian immunodeficiency virus (SIV) vectors were used in this study as previously described (Ogata *et al.*, 2004). Enhanced green fluorescent protein (EGFP) or the hFIX minigene (hFIX cDNA containing the first intron) were cloned 3' of the cytomegalovirus (CMV) promoter. The SIV vector was pseudotyped with the G glycoprotein of the vesicular stomatitis virus (VSV-G) envelope, and the vector titers were determined as previously described (Nakajima *et al.*, 2000; Ogata *et al.*, 2004).

SIV lentiviral vector transduction of mADSCs in vitro

mADSCs were plated on a 6-well tissue culture plate at 4000 cells/cm^2 with DMEM–F12 basic medium (2 ml/well). Twenty-four hours after plating, the medium was replaced with basic medium (1 ml/well) containing lentiviral vectors (at increasing multiplicities of infection [MOIs]) in the presence of Polybrene (hexadimethrine bromide, H-9268; Sigma-Aldrich) at 8 μg/ml. Twenty-four hours after the vectors were introduced, fresh basic medium (1 ml/well) was added without Polybrene. Afterward, the transduced cells were propagated with basic medium to perform ELISAs or clotting assays as described below. To induce osteogenic differentiation or adipogenic differentiation, the medium was changed to the appropriate differentiation medium (see previously) 96 hr after vector transduction, and the cells were cultured and assessed as described previously.

ELISA

The culture medium in a 6-well tissue culture plate was replaced with fresh medium (1 ml/well) 24 hr before medium collection. The culture medium was harvested and frozen at −80°C until assessment by ELISA. hFIX ELISA was performed with an Asserachrom IX:Ag kit (630423; Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Clotting assay

The culture medium was changed to fresh medium containing menaquinone (vitamin K_2) (V-9378; Sigma-Aldrich) 24 hr before medium collection. Normal plasma (Coagtrol N, 13490; Sysmex, Hyogo, Japan) was mixed with FIX-deficient plasma (15450; Sysmex) and the clotting time was measured at 37°C to obtain a standard curve for calibration. Subsequently, the clotting time of culture medium mixed with FIX-deficient plasma was measured with a semiautomatic coagulation analyzer (KC4 Delta; Trinity Biotech, Wicklow, Ireland).

mADSC sheets

mADSCs (passage 2 or 96 hr after vector transduction) were replated on an UpCell temperature-responsive culture dish (CellSeed, Tokyo, Japan), at a density of 4.5×10^4 cells/cm^2. After 5 days of culture at 37°C in 5% CO_2, the temperature was changed from 37 to 20°C to initiate detachment of the mADSC cell sheet from the culture surface. The mADSCs were harvested as a monolayer cell sheet after an ~20-min incubation at 20°C. Fluorescence images of EGFP-transduced mADSC sheets detaching from the temperature-responsive culture dishes were captured with an OV110 imager (Olympus, Tokyo, Japan). For ELISA, hFIX-transduced mADSC sheets were harvested and reattached onto collagen type IV-coated tissue culture dishes (Asahi Glass, Tokyo, Japan). After 24 hr of culture, the medium was collected for ELISAs to detect hFIX.

Transmission electron microscopy

Transmission electron microscopy (TEM) of mADSC sheets was performed by Tokai Electron Microscopy Analysis (Aichi, Japan). mADSC sheets were fixed with a solution comprising 2% PFA, 2% glutaraldehyde (GA) in 0.1 *M* PBS, and then incubated in 2% GA in 0.1 *M* PBS (supplied by the company).

Statistical analysis

Data are presented as means ± standard deviation (SD). Group-wise comparisons were made by one-way analysis of variance (ANOVA) followed by the Tukey (HSD) post-hoc test, using SPSS statistics software (PASW statistics, version 18; SPSS, Chicago, IL). A value of $p<0.05$ was considered statistically significant.

Results

Characterization of mADSCs

Figure 1A presents an optical microphotograph of mADSCs at passage 2. The cell-surface protein profiles of mADSCs were analyzed by flow cytometry. mADSCs at passage 2 expressed the mesenchymal stem cell markers (Mitchell *et al.*, 2006) CD29 (99.7%), CD44 (95.4%), and CD90.2 (92.6%) (Fig. 1B). mADSCs were negative for CD31. A small population of the cells were CD45 positive (9.3%), but this may be due to contamination with hematopoietic cells.

The mADSCs was verified as bipotent, demonstrating the ability to differentiate into their osteogenic and adipogenic lineages. Osteogenic induction increased ALP activity (Fig. 1C) and calcium accumulation as visualized by alizarin red S staining (Fig. 1D right). No calcium deposition was found in the noninduced condition (Fig. 1D left). Adipogenic differentiation was observed by the presence of lipid droplets detected by oil red O staining (Fig. 1E right). A few lipid droplets were observed in the noninduced condition (Fig. 1E left), suggesting that some mADSCs have the ability to spontaneously differentiate along the adipogenic lineage. These results confirmed that the mADSCs isolated in this study possessed the same characteristics as those of previously reported ADSCs (Liu *et al.*, 2007).

Efficiency of lentiviral transduction of mADSCs

Figure 2A depicts the organization of the replication-defective SIV lentiviral vector, showing the location of the cytomegalovirus promoter (CMVprom) and the gene of interest (GOI). The GOIs used in this study were EGFP (SIV-EGFP) and human FIX (SIV-hFIX). To determine the optimal dose for transducing mADSCs using SIV lentiviral vectors, mADSCs at passage 2 were infected with SIV-EGFP at

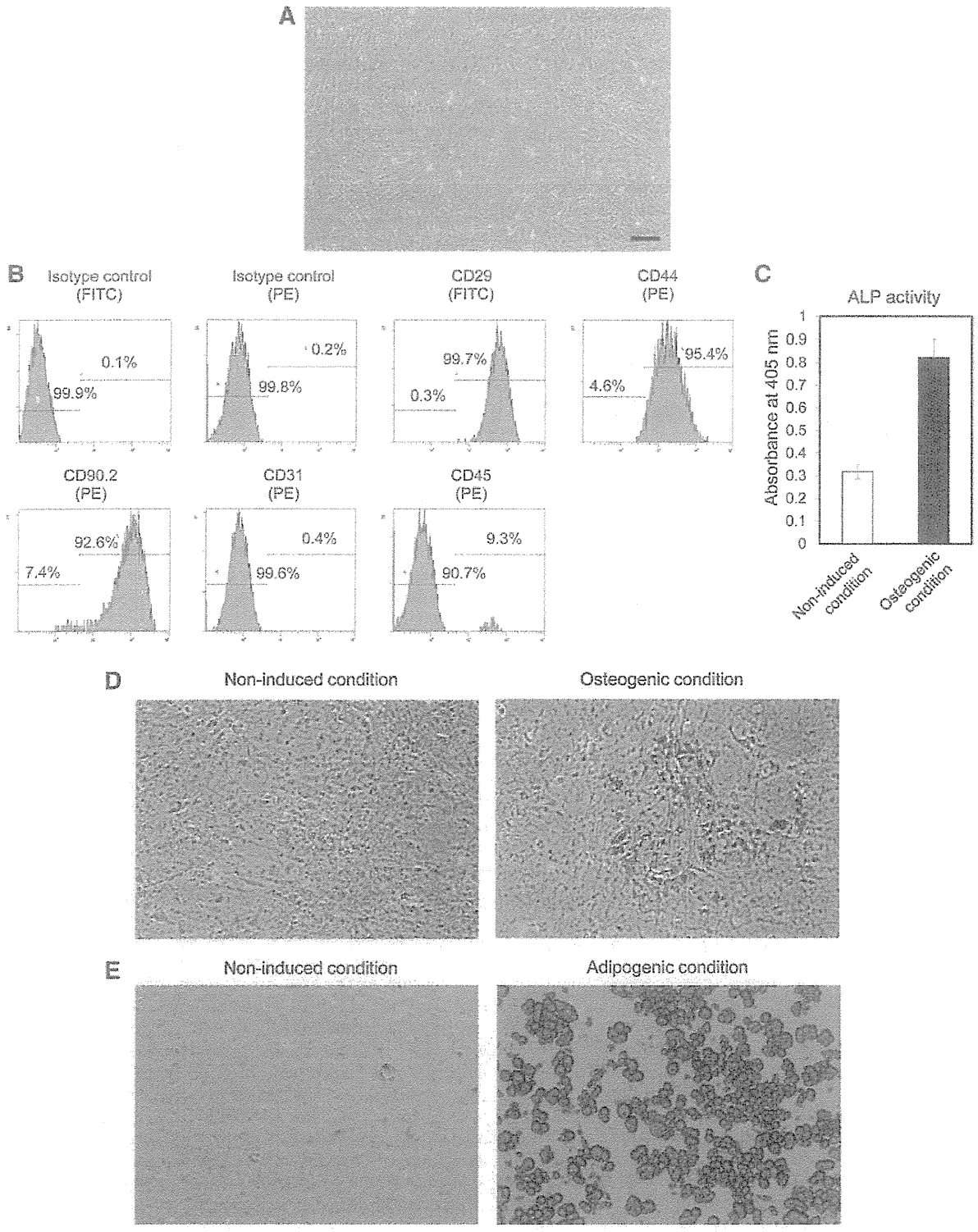

FIG. 1. Confirmation of the characteristics and bipotency of mouse adipose tissue-derived stem/stromal cells (mADSCs). (A) A microphotograph of mADSCs at passage 2, showing their spindle-like morphology. Scale bar: 50 μm. (B) Characterization of mADSCs by flow cytometry. mADSCs of passage 2 expressed the mesenchymal stem cell markers CD29, CD44, and CD90.2 and were negative for CD31 and CD45. FITC, fluorescein isothiocyanate; PE, phycoerythrin. (C–E) The osteogenic and adipogenic differentiation of mADSCs was verified by an alkaline phosphatase (ALP) assay and alizarin red S staining (markers of osteogenesis), and by oil red O staining (marker of adipogenesis), respectively. (C) The ALP assay was performed on day 7 of the culture of osteogenically differentiated mADSCs (n=3). (D) Alizarin red S staining was performed after 4 weeks of osteogenic induction culture conditions. The stain in the photograph of the induced condition indicates calcium deposition in the cells. (E) Oil red O staining was performed 2 weeks after the initiation of adipogenic induction culture conditions. Color images available online at www.liebertpub.com/hum

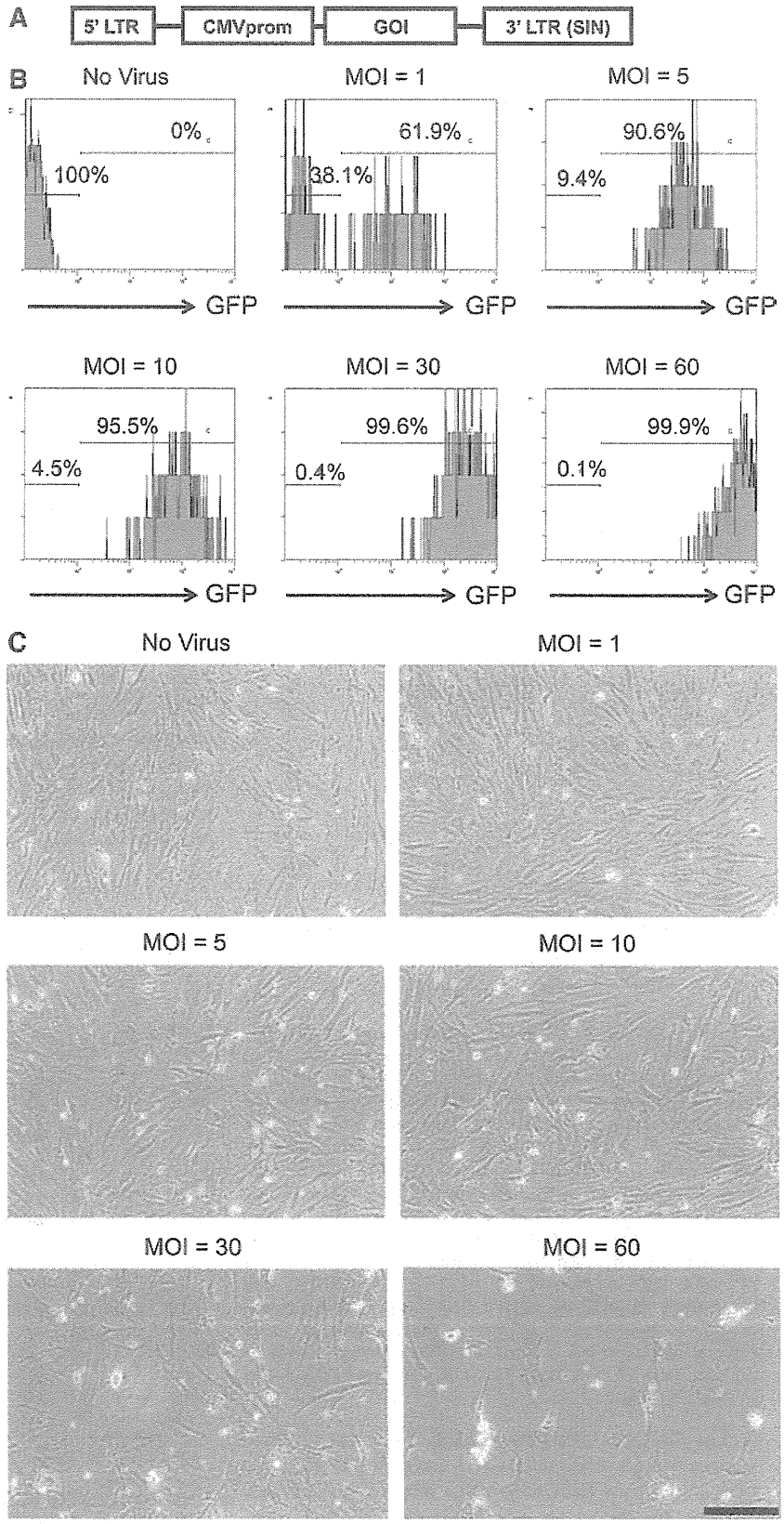

FIG. 2. SIV lentivirus transduction of mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** The SIV lentiviral vectors used in this study expressed the gene of interest (GOI) under the control of the cytomegalovirus promoter (CMVprom). 3′ LTR (SIN), self-inactivating 3′ long terminal repeat. **(B)** The efficiency of transduction was determined by measuring the proportion of EGFP fluorescence-positive cells by flow cytometry. mADSCs were transduced with SIV-EGFP at MOIs of 0, 1, 5, 10, 30, and 60. Transduction efficiencies were analyzed by flow cytometry 96 hr after transduction. **(C)** Microscopy images of mADSCs transduced with SIV-human coagulation factor IX (hFIX) at MOIs of 0, 1, 5, 10, 30, and 60. Scale bar: 50 μm. The experiments were repeated three times with similar results.

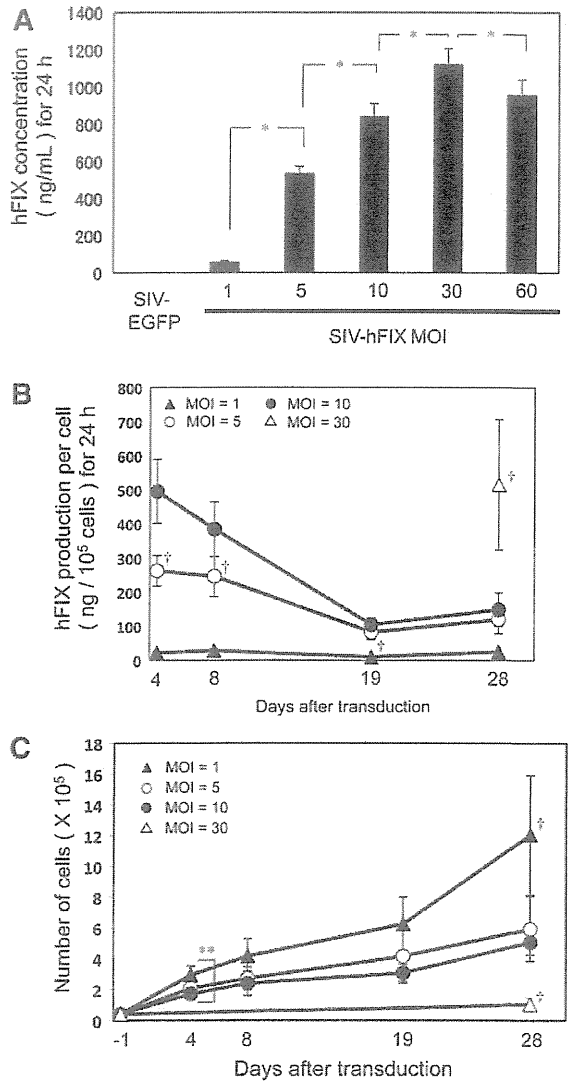

FIG. 3. SIV lentivirus-mediated human coagulation factor IX (hFIX) production from mouse adipose tissue-derived stem/stromal cells (mADSCs). mADSCs were transduced with the SIV-hFIX vector at MOIs of 1, 5, 10, 30, and 60. **(A)** The culture medium was collected 96 hr after transduction, and the amount of hFIX protein secreted over 24 hr was assessed by ELISA. mADSCs transduced with SIV-EGFP were used as the negative control. **(B)** The hFIX secretion profile from mADSCs was observed over 4 weeks. The culture medium from a 24-hr period was collected on days 4, 8, 19, and 28 after transduction. The amount of hFIX protein per 1×10^5 cells was measured by ELISA. **(C)** mADSC cell numbers were counted on days 4, 8, 19, and 28 after transduction. Cells treated at an MOI of 30 were unable to reach confluency until day 28. $^*p<0.05$ between groups. $^\dagger p<0.05$ versus the other MOI groups; $^{**}p<0.05$ between MOIs 1 and 10 on day 4 ($n=3$).

various multiplicities of infection (MOIs) from 1 to 60. Flow cytometric analysis was performed 4 days after infection to determine the number of GFP-positive cells. At an MOI of 5, ~90% of the mADSCs were transduced with SIV-EGFP (Fig. 2B). Increasing the MOI up to 60 elevated the number of GFP-positive cells to nearly 100%. Moreover, the intensity of GFP fluorescence increased in a dose-dependent manner. However, the SIV-hFIX vector induced toxicity to the mADSCs at MOIs of 30 and higher (Fig. 2C). In addition, the SIV-transduced mADSCs had the same adipogenic and osteogenic differentiation abilities as nontransduced mADSCs (Supplementary Fig. S1; supplementary data are available online at www.liebertonline.com/hum). Taken together, these data show that SIV lentiviral vectors can be used over a fairly wide range of MOIs to genetically modify mADSCs without promoting cellular injury.

hFIX production by SIV-transduced mADSCs

mADSCs at passage 2 were transduced with the SIV-hFIX lentivirus at various MOIs from 1 to 60. The culture medium was collected 96 hr after transduction, and the hFIX concentration was measured by ELISA (Fig. 3A). A dose-dependent increase in hFIX protein was observed from an MOI of 1 to 30. The amount of hFIX protein secreted at an MOI of 30 was ~1100 ng/ml (Fig. 3A). hFIX production was lower at an MOI of 60 than at an MOI of 30, which could be due to the decreased cell number at the higher dose, likely resulting from the inherent toxicity of the VSV-G pseudotype (Fig. 2C).

Sustained hFIX production in lentiviral vector-transduced mADSCs

The persistence of hFIX production and secretion from lentiviral vector-transduced mADSCs (MOI, 1 to 30) were analyzed over a 4-week period. mADSCs transduced at an MOI of 60 were not used in our studies because of their morphological deterioration attributed to the administration of a large amount of vector (Fig. 2C). On days 4, 8, 19, and 28

TABLE 1. TOTAL PRODUCTION OF hFIX PROTEIN FOR 24 HOURS

Days after transduction	*MOI*	*hFIX protein (ng) per 10^5 cells*	*Cumulative number of cells ($\times10^5$)*	*hFIX protein (ng) from cumulative cells*
4	1	21.28±5.30	2.93±0.60	60.37±3.24[a]
	5	261.57±45.23	2.08±0.33	535.49±37.69[a]
	10	494.99±93.79	1.73±0.20	843.84±65.40[a]
	30	N/A	N/A	N/A
8	1	27.81±5.08	4.15±1.15	111.63±14.45[a]
	5	245.23±59.24	2.71±0.74	641.15±80.28[a]
	10	383.78±80.05	2.40±0.80	885.83±138.03[a]
	30	N/A	N/A	N/A
19	1	9.57±2.58	6.26±1.72	57.87±11.86[a]
	5	81.26±22.14	4.15±1.77	316.31±93.21
	10	103.46±20.32	3.06±0.54	324.15±124.48
	30	N/A	N/A	N/A
28	1	24.52±13.76	12.03±3.89	270.87±128.77
	5	119.49±42.86	5.94±2.11	744.11±493.55
	10	148.97±49.51	5.05±0.78	762.32±326.91
	30	514.50±191.43	1.03±0.42	478.57±42.42

hFIX, human factor IX; MOI, multiplicity of infection; N/A, not applicable.

Note: Data are expressed as mean values±SD.

[a]$p<0.05$ versus the other groups in the same day after transduction ($n=3$).

after transduction, the culture medium was collected and cell numbers were counted by hemocytometry for cells transduced up to an MOI of 10 (Fig. 3B and Table 1). The amount of hFIX protein produced per cell at an MOI of 30 could be reliably calculated only on day 28 because the cells were not confluent at earlier time points (data not shown). hFIX production was sustained throughout the 28 days, even though the levels decreased to about one-third of the levels measured on day 4. mADSCs transduced at an MOI of 30 secreted the largest amount of hFIX per cell on day 28 (Fig. 3B and Table 1). However, considering the growth curve of the SIV-transduced mADSCs (Fig. 3C), mADSCs transduced with lower vector doses increased in cell number more rapidly than those administered the highest dose (MOI of 30). Although the hFIX production per cell was 3-fold higher for an MOI of 30 than an MOI of 10 on day 28, the cumulative amount of hFIX protein over the 28-day culture was highest for an MOI of 10 (762.32 ng) because there were 5-fold fewer cells at an MOI of 30 than at an MOI of 10 (Fig. 3C and Table 1). The cumulative amount of hFIX protein on day 8 was also highest for an MOI of 10 (885.83 ng). Taken together, the results showed that mADSC transduction at an MOI of 10 was the optimal condition to produce the greatest amount of hFIX.

Clotting assay

Because FIX proteins are intracellularly modified with the quinone vitamin K_1 (phylloquinone) or vitamin K_2 (menaquinone) (Blostein *et al.*, 2008; Napolitano *et al.*, 2010), the medium was supplemented with menaquinone (0–20 μg/ml) 72 hr after vector transduction. Twenty-four hours after the medium change, culture supernatant was collected and the clotting activity of hFIX secreted from the mADSCs (MOI, 10) was assessed. The clotting activity of the hFIX-containing medium depended on the menaquinone concentration (0 to 10 μg/ml) (Fig. 4A). The highest clotting activity, ~7% of

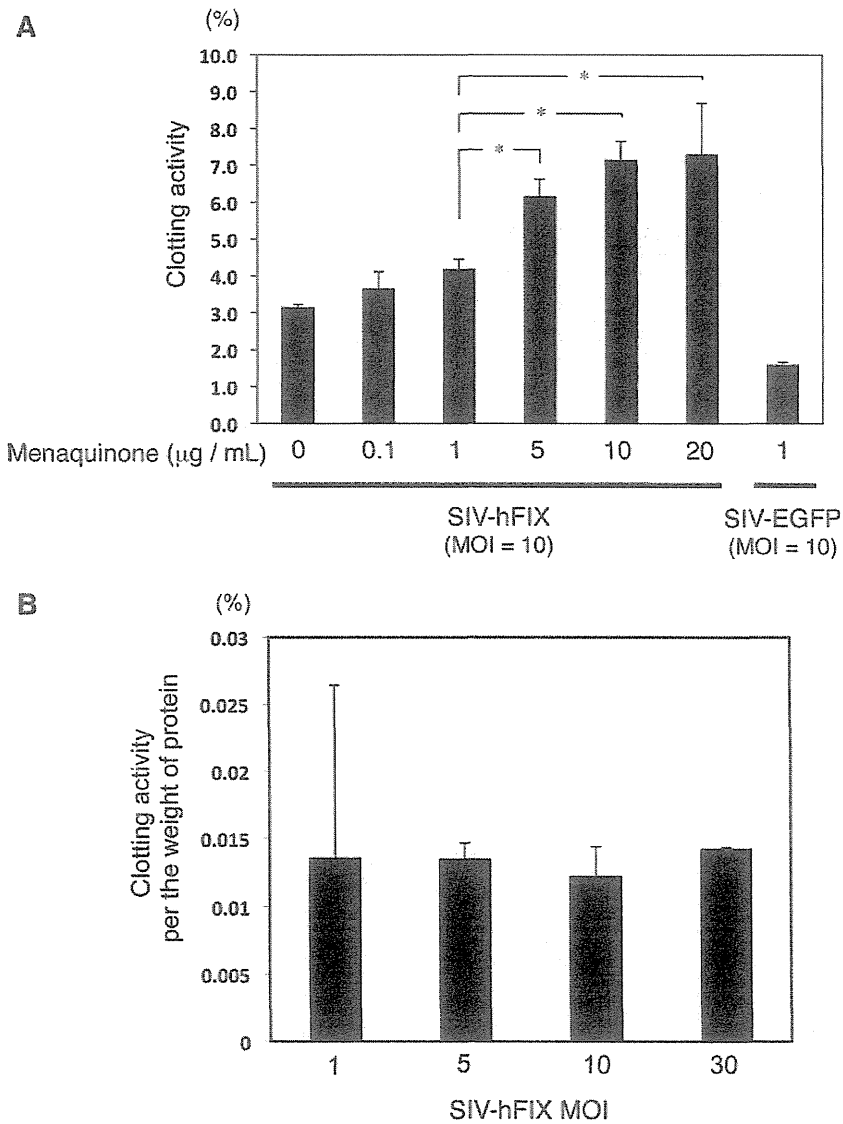

FIG. 4. Clotting activity of human coagulation factor IX (hFIX) produced by mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** mADSCs were transduced with the SIV-hFIX lentivirus vector at an MOI of 10. Seventy-two hours after vector transduction, the medium was changed to fresh medium containing menaquinone (vitamin K_2) at various concentrations (0, 0.1, 1, 5, 10, and 20 μg/ml). Twenty-four hours later, the culture supernatant was analyzed by a clotting assay. **(B)** The clotting activities of hFIX from mADSCs transduced at MOIs of 1, 5, 10, and 30 were analyzed 28 days after vector transduction. The medium was changed to fresh medium containing menaquinone (10 μg/ml) 24 hr before medium collection. *$p < 0.05$ between groups ($n = 3$).

normal human plasma, was observed at a menaquinone concentration between 10 and 20 μg/ml. As menaquinone is dissolved in an organic solvent (i.e., 100% ethanol), which is harmful to most cultured cells, the remaining studies used menaquinone at 10 μg/ml. Figure 4B shows that the clotting ability per amount of hFIX protein did not depend on the vector dose.

mADSC sheets

To bioengineer cell sheets, we used our standard protocol developed with other cell types (Miyahara *et al.*, 2006; Ohashi *et al.*, 2007) to assess whether mADSCs (passage 2) were capable of forming cell sheets. Reducing the culture temperature from 37 to 20°C for 20 min successfully released mADSCs cultured on temperature-responsive culture dishes as an intact cell sheet (Fig. 5A). Transmission electron microscopy (TEM) analysis revealed that (1) mADSC sheets consisted of two or three cell layers and (2) the spaces between cells were filled with collagen fibers (Fig. 5B–D), suggesting that mADSCs cultured on a temperature-responsive culture dish proliferate and produce extracellular matrix. Low-power fluorescence microscopy analysis was used to visualize the process of detachment of EGFP-transduced mADSCs from the temperature-responsive culture dish as a cell sheet (Fig. 6A). Furthermore, hFIX-transduced mADSC sheets were harvested and reattached on a plastic dish coated with collagen type IV. The transferred hFIX-transduced mADSC sheets produced significant levels of hFIX (Fig. 6B). These results suggest that cell sheets of gene-transduced mADSCs are functional materials that could improve the clotting ability of patients with hemophilia after transplantation.

Discussion

This study demonstrates a relatively simple and efficient protocol to genetically modify mADSCs, using lentiviral vectors derived from SIV. As previously reported, coagulation factor IX (FIX) gains its full biological function after intracellular modification by a group of enzymes including γ-glutamyl carboxylase, vitamin K epoxide reductase, quinone

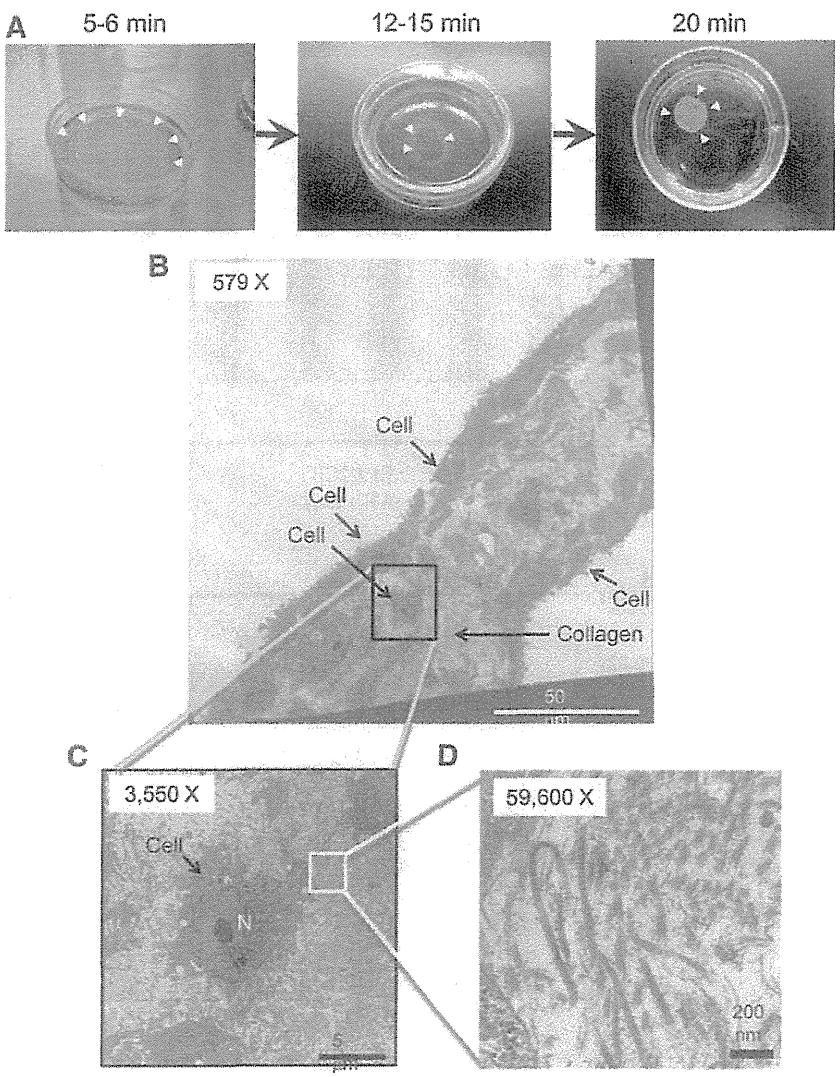

FIG. 5. Creation of cell sheets of mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** mADSCs were cultured on a temperature-responsive culture surface (UpCell 35-mm dish). Lowering the temperature to 20°C allowed mADSC sheets to detach from the edge (arrowheads, *left*, at 5–6 min), shrink in size (arrowheads, *middle*, at 12–15 min), and float to the top of the medium (arrowheads, *right*, at 20 min). **(B)** Transmission electron microscopy (TEM) image showing two or three layers of mADSCs and collagen fibers in a cell sheet (original magnification, ×579). **(C)** Magnified TEM image of the outlined region shown in **(B)** (original magnification, ×3550). N, nucleus. **(D)** Magnified TEM image of the outlined region in **(C)** (original magnification, ×59,600). Numerous striped fibers were observed, the typical feature of collagen. Scale bars: **(B)** 50 μm; **(C)** 5 μm; **(D)** 200 nm. Color images available online at www.liebertpub.com/hum

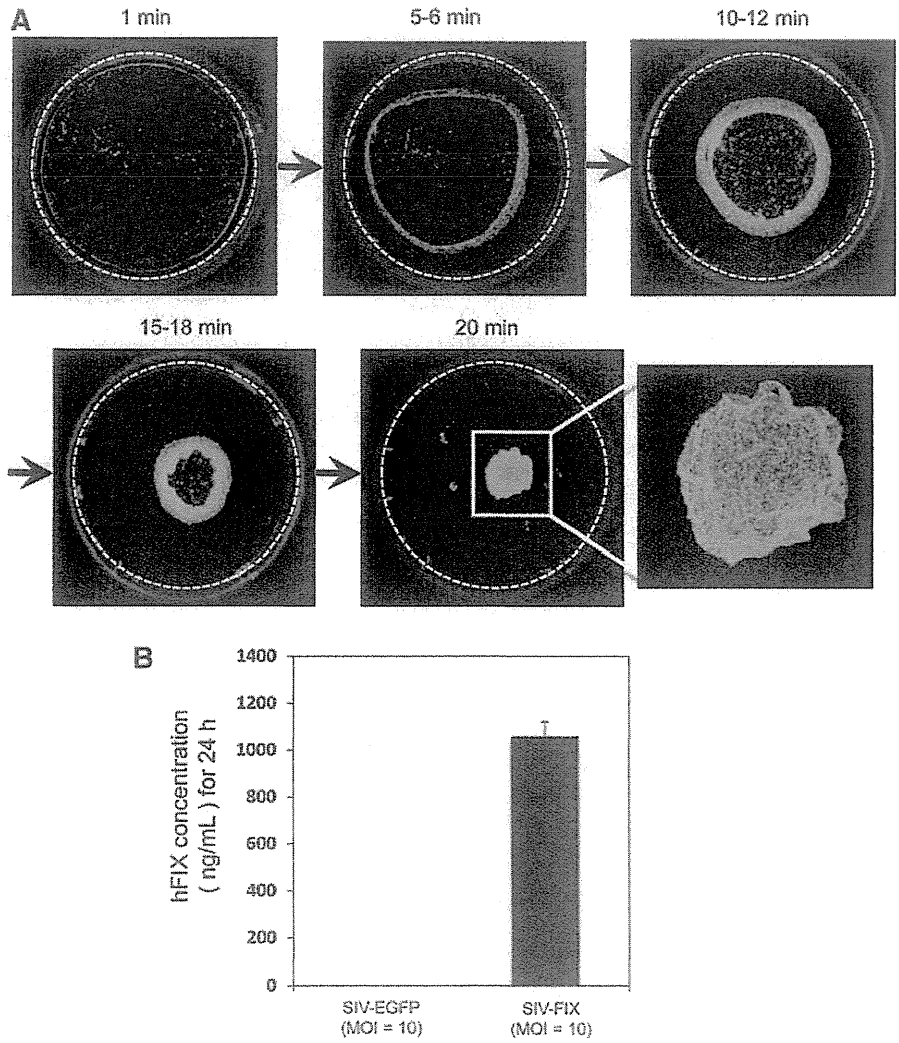

FIG. 6. Cell sheets of lentivirus-transduced mouse adipose tissue-derived stem/stromal cells (mADSCs). (A) mADSCs were transduced with SIV-EGFP lentiviral vector and replated on a temperature-responsive culture surface (UpCell 35-mm dish) 96 hr after transduction. After reaching confluency on day 4 of culture, the culture temperature was lowered to 20°C to observe cell sheet harvesting. EGFP-transduced mADSCs started to detach from the edge as a cell sheet, shrank in size, detached, and floated in the medium after 20 min. *Bottom right:* High-magnification image of a floating EGFP-transduced cell sheet. The dashed circle represents the edge of the temperature-responsive culture dish (diameter, 35 mm). (B) SIV-hFIX-transduced mADSCs were replated on a temperature-responsive culture dish 96 hr after transduction. After 4 days of culture, the hFIX-transduced mADSCs were harvested as a cell sheet by lowering the culture temperature to 20°C and were reattached onto another culture dish. After 24 hr of culture, the medium was collected to measure hFIX protein levels by ELISA.

reductase, and paired basic amino acid cleaving enzyme (PACE)/furin (Blostein *et al.*, 2008; Napolitano *et al.*, 2010; Tie *et al.*, 2011). Our results showed that the clotting activity of *de novo*-synthesized hFIX was modulated by menaquinone (vitamin K_2) in a dose-dependent manner. These results provide evidence that mouse ADSCs possess the posttranslational modification mechanisms required to produce biologically active FIX. Furthermore, mADSCs could form cell sheets that may be applicable for use in transplantation therapy.

The liver produces the majority of coagulation factors in humans. Although liver organ transplantation has cured patients with hemophilia A and B (Gordon *et al.*, 1998), shortages of available livers have greatly limited the ability of liver transplantation to become a standard treatment. In earlier proof-of-concept studies, our laboratory has demonstrated the therapeutic value of cell-based approaches for hemophilia, including hepatocyte transplantation and liver tissue engineering (Tatsumi *et al.*, 2008b; Ohashi *et al.*, 2010). These successful experiments prompted us to generate FIX-producing cells from an autologous tissue origin. Autologous cells would present advantages to patients including (1) the reduction or complete avoidance of immunosuppressive regimens, and (2) the minimization of ethical problems by using the patient's own cells. Regardless of the cell type used for transplantation, the native cells that differentiate after implantation are generally insufficient to recapitulate normal function. For this reason, genetic methods are required to safely transport genes of interest into the cells to mass produce intracellular or secreted proteins or to inhibit the transcription/translation of deleterious genes. Earlier studies have shown some promise in the use of genetically modified bone marrow-derived mesenchymal stem cells (BM-MSCs) as a possible cell-based treatment modality for hemophilia (Oh *et al.*, 2006; Coutu *et al.*, 2011). Compared with BM-MSCs, ADSCs may have an advantage in that they are abundant and can be obtained by less invasive procedures (Kern *et al.*, 2006). Various vectors have been used in investigations of methods to modify cells to express genes of interest, including nonviral systems such as nucleic acid transfection and viral vectors based on adenoviruses, adeno-associated viruses (AAVs), Sendai virus, or simple and

complex retroviruses (Coffin *et al.*, 1997; Walther and Stein, 2000; Anjos-Afonso *et al.*, 2004; Haleem-Smith *et al.*, 2005; Oh *et al.*, 2006; Talens-Visconti *et al.*, 2006; Zaragosi *et al.*, 2007; Li and Lu, 2009; Sugii *et al.*, 2010; Coutu *et al.*, 2011; Kim *et al.*, 2011; Li *et al.*, 2011b).

In our study, we used SIV-based lentiviral vectors to genetically manipulate mADSCs. Although the SIV vectors were able to transduce mADSC cells to produce hFIX, the level of expression tended to decrease over the duration of the experiment. It is not clear from our studies why this occurred, but the main reason may be the choice of promoter in our vector system. We used the strong CMV promoter, which is able to produce enormous amounts of transgene products but has a propensity to be silenced over time (Prosch *et al.*, 1996; Mehta *et al.*, 2009; Duan *et al.*, 2012). Thus, some small molecules such as DNA methyltransferase inhibitors or histone deacetylase inhibitors might be able to sustain hFIX gene expression. In addition, other mammalian promoters need to be studied in the context of these SIV vectors.

Another issue with the SIV vectors is the toxicity to mADSCs observed at MOIs greater than 30. It is likely that the pseudotype envelope protein VSV-G was the root cause of this problem, as observed in previous studies using HIV-based vectors coated with VSV-G. Additional effort could be made to minimize the toxicity and improve the safety profile of this vector. One method would be to perform "spinfection," in which the cells of interest are centrifuged with the vector medium to improve the efficiency of viral infection (Li and Lu, 2009). Another possibility would be to replace the wild-type FIX cDNA with a hyperactive form of FIX. Researchers have described that the R338L mutation resulted in 8-fold higher biological activity, whereas V86A, E277A, and R338A resulted in 13-fold higher biological activity than wild-type FIX (Simioni *et al.*, 2009; Lin *et al.*, 2010).

For genetically modified ADSCs to become valuable as a therapy for hemophilia, further optimization is required to achieve efficient cell engraftment. Intravenous infusion of ADSCs has been associated with negative events including thromboembolism (Yukawa *et al.*, 2009). To circumvent this issue, site-specific engraftment would be preferable to avoid these effects from intravenous administration. As a proof of principle, Coutu and colleagues (2011) created a transplantable cellular composite for hemophilia consisting of genetically modified BM-MSCs seeded on a biodegradable polymer scaffold. The cell sheet tissue-engineering technology developed in our laboratory (Shimizu *et al.*, 2003; Kikuchi and Okano, 2005; Yang *et al.*, 2005; Yamato *et al.*, 2007) was successfully used in the current study to create mADSC sheets that should be sufficiently durable and viable for transplantation applications, similar to previous studies in which cell sheets secreted α_1-antitrypsin or insulin (Ohashi *et al.*, 2007; Shimizu *et al.*, 2009).

In conclusion, this study demonstrated that SIV lentiviral vectors can efficiently transduce mADSCs and that hFIX expressed *de novo* from mADSCs is posttranslationally modified and able to form clots. Moreover, the genetically modified mADSCs were engineered as a cell sheet, indicating that they could become a valuable cell source for genetically engineered cell therapy to treat hemophilia, and possibly other genetic and nongenetic diseases in which patients lack secreted proteins, such as in diabetes.

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Author Disclosure Statement

Teruo Okano is an investor in CellSeed (JAPAN) and an inventor/developer designated on patents for the temperature-responsive culture surfaces described in this paper (patent nos. JP1972502, US5284766, FR0382214, NL0382214, DE0382214, GB0382214, SE0382214, CH0382214, and CH0382214).

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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.

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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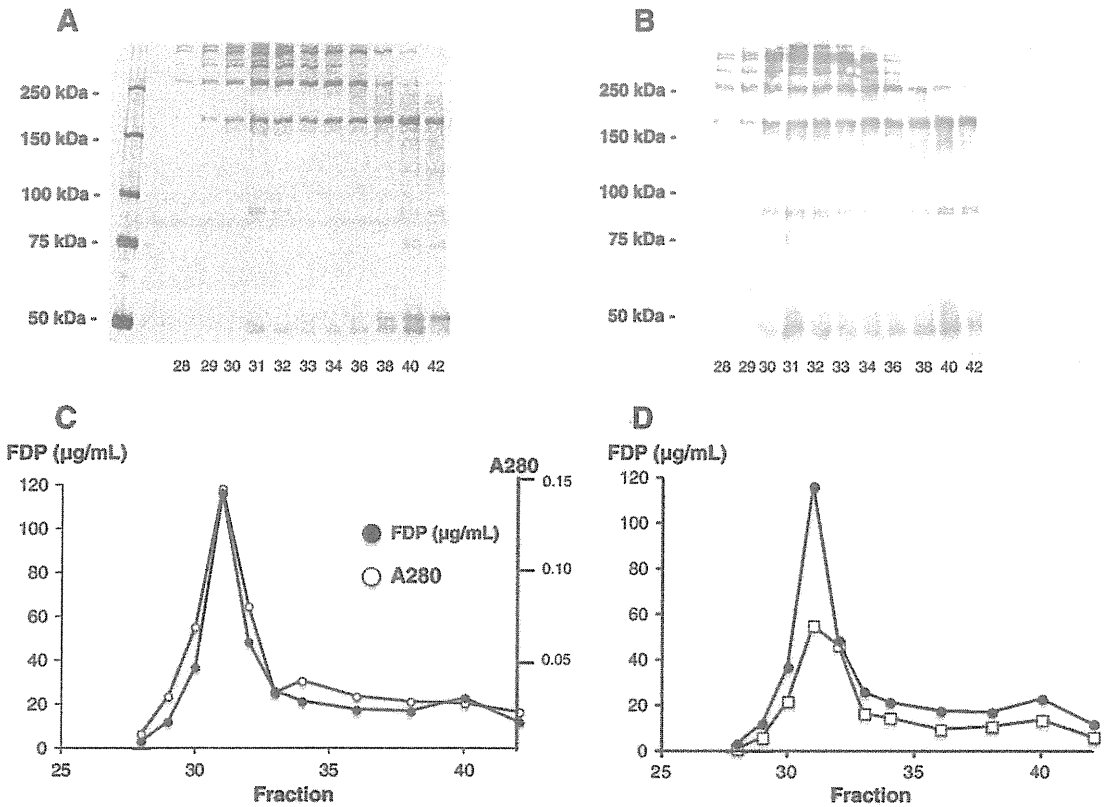

Fig. 1. Analysis of FDP generated in vitro. Fractions of gel filtration column chromatography on Sephacryl S-300 were analyzed by SDS-PAGE with silver staining (A). The same samples were analyzed by Western blotting with a rabbit polyclonal antibody against human fibrinogen (B). Most of the protein bands, 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].

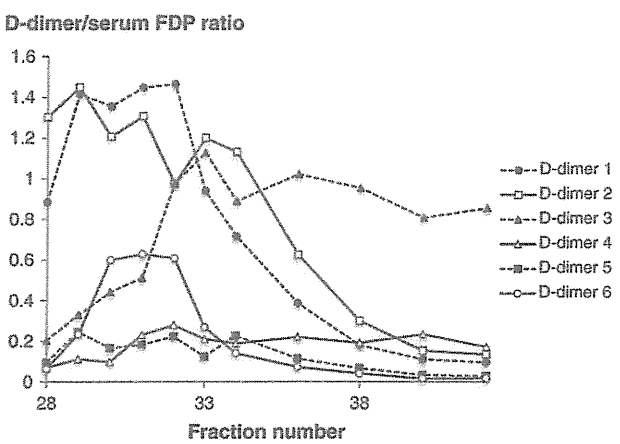

Fig. 2. Relative D-dimer values against serum FDP-2 values in the gel filtration column chromatography fractions. Fractions #28–42 of gel chromatography were further analyzed with 6 D-dimer reagents (D-dimer-1, D-dimer-2, D-dimer-3, D-dimer-4, D-dimer-5, and D-dimer-6) for clinical laboratory testing. 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,

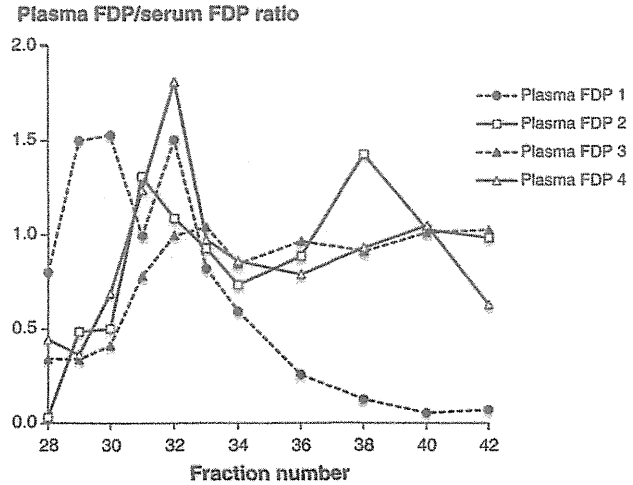

Fig. 3. Relative plasma FDP values against serum FDP-2 values in the gel filtration column chromatography fractions. Fractions #28–42 of gel chromatography were quantified with four plasma FDP reagents (plasma FDP-1, plasma FDP-2, plasma FDP-3, and plasma FDP-4) in a similar manner to Fig. 2. The FDP 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.

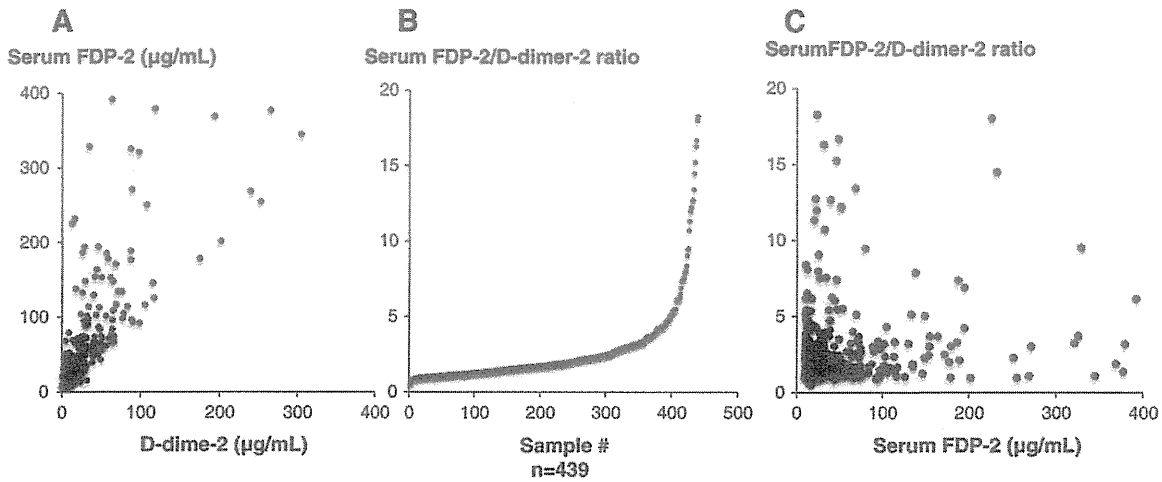

Fig. 4. Distribution of serum FDP/D-dimer ratios in clinical samples. Serum FDP-2 levels (10–400 μg/mL) of clinical samples (n = 439) were plotted against the D-dimer-2 level of the same sample (Fig. 4A). The ratios (<20) of the serum FDP-2/D-dimer-2 of clinical samples were sorted and plotted (B). Serum 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

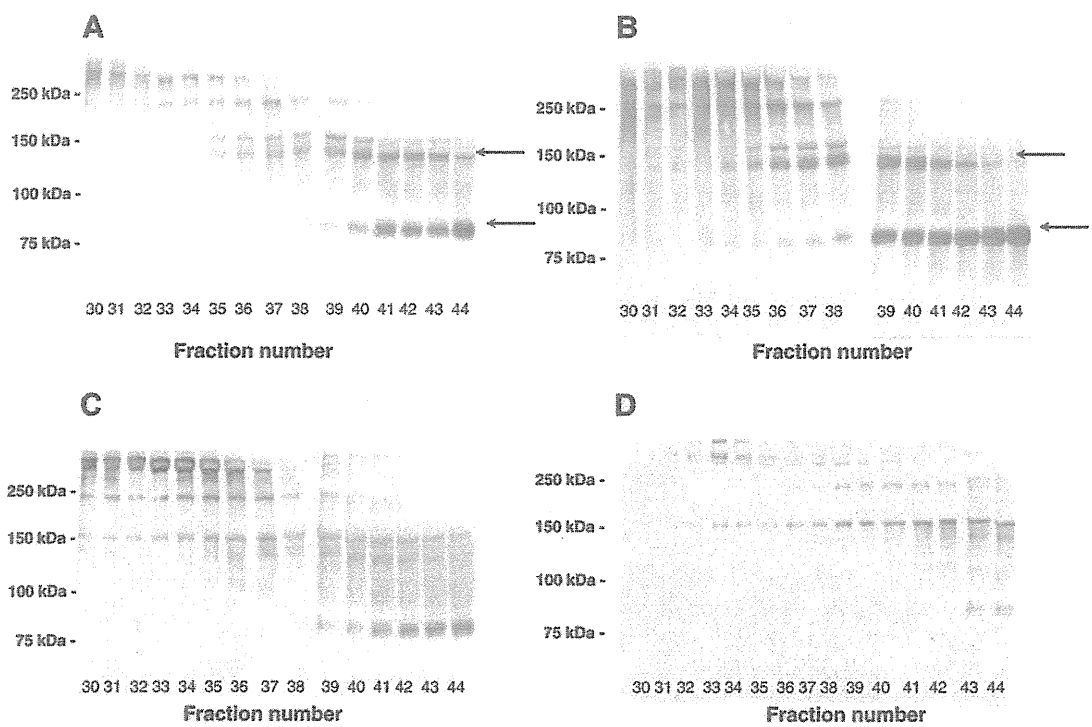

Fig. 5. Molecular variants of FDP in clinical samples. Four representative clinical DIC sample FDP levels (high FDP (>100 μg/mL) and high serum FDP-2/D-dimer-2 ratios (A, 18.0; B, 9.5); high FDP levels (>100 μg/mL) and low serum FDP-2/D-dimer-2 ratios (C, 1.0; D, 1.0) were subjected to gel filtration 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.

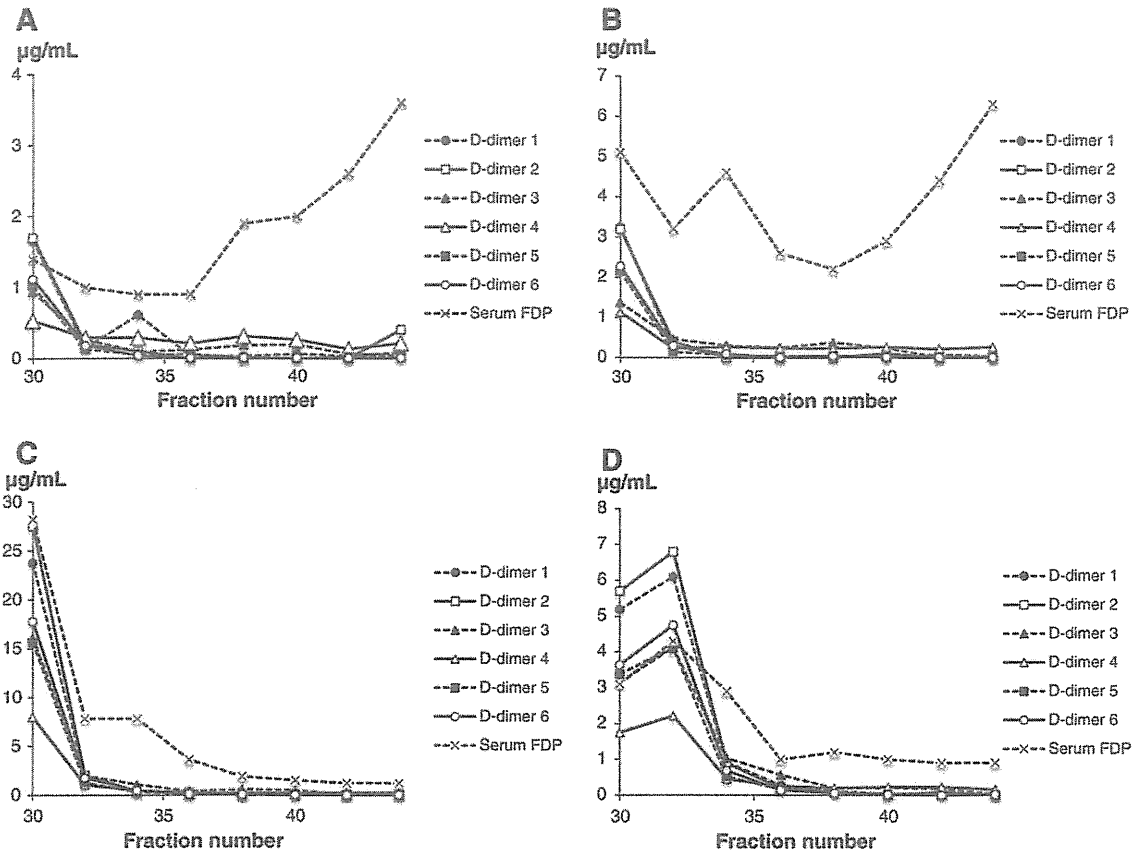

Fig. 6. Reactivity of the monoclonal antibodies used in laboratory D-dimer reagents to the molecular variants of FDP in clinical samples. Gel filtration column chromatography fractions of clinical samples A (A), B (B), C (C), and D (D) were subjected to quantification with D-dimer reagents. FDP concentrations 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)