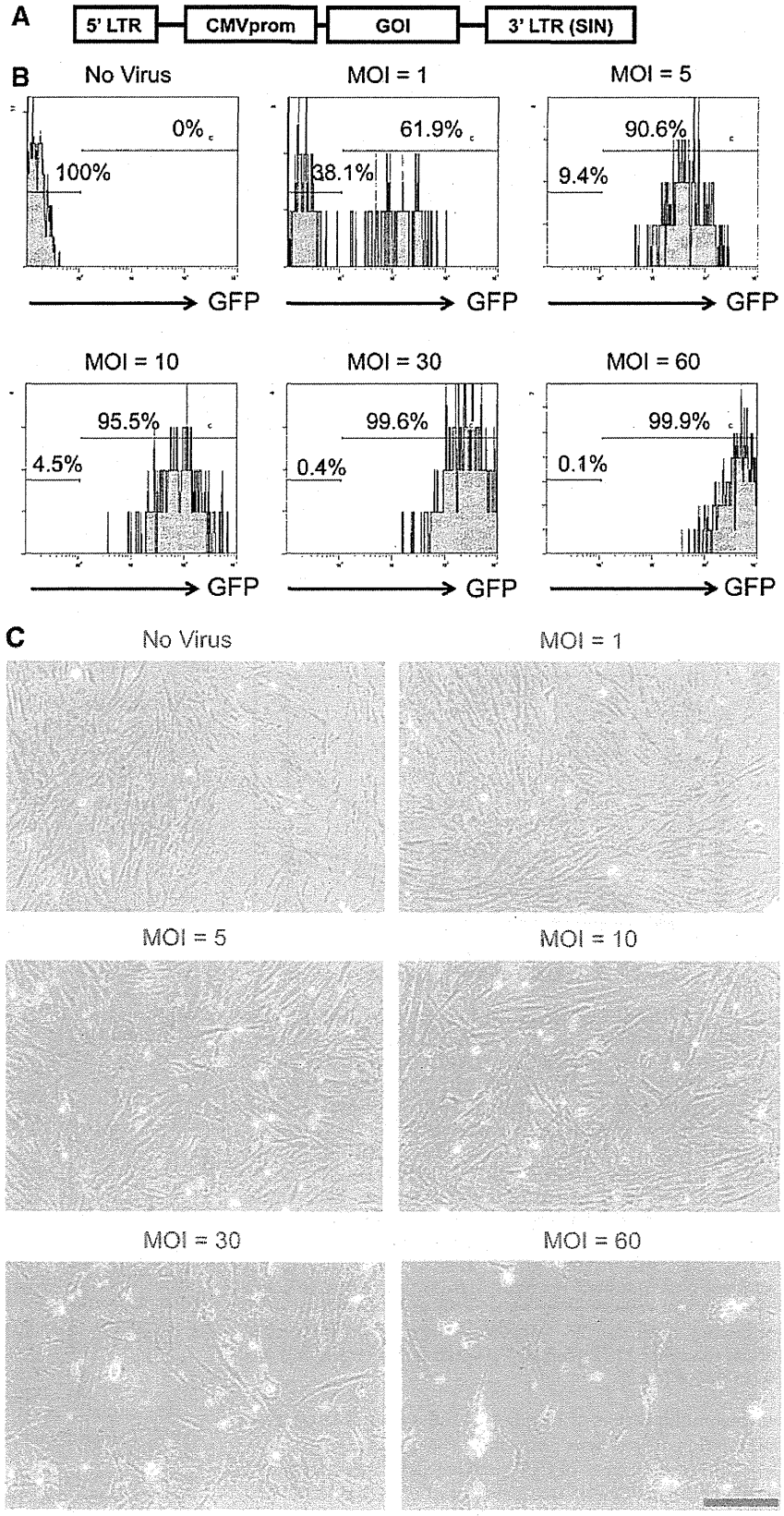

FIG. 2. SIV lentivirus transduction of mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** The SIV lentiviral vectors used in this study expressed the gene of interest (GOI) under the control of the cytomegalovirus promoter (CMVprom). 3′ LTR (SIN), self-inactivating 3′ long terminal repeat. **(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.

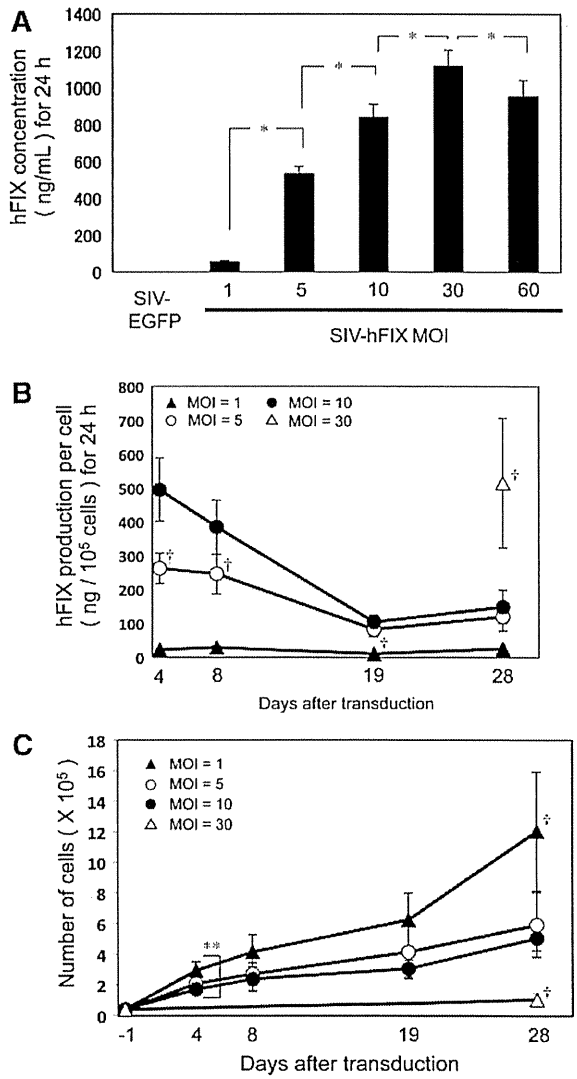

FIG. 3. SIV lentivirus-mediated human coagulation factor IX (hFIX) production from mouse adipose tissue-derived stem/stromal cells (mADSCs). mADSCs were transduced with the SIV-hFIX vector at MOIs of 1, 5, 10, 30, and 60. **(A)** The culture medium was collected 96 hr after transduction, and the amount 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

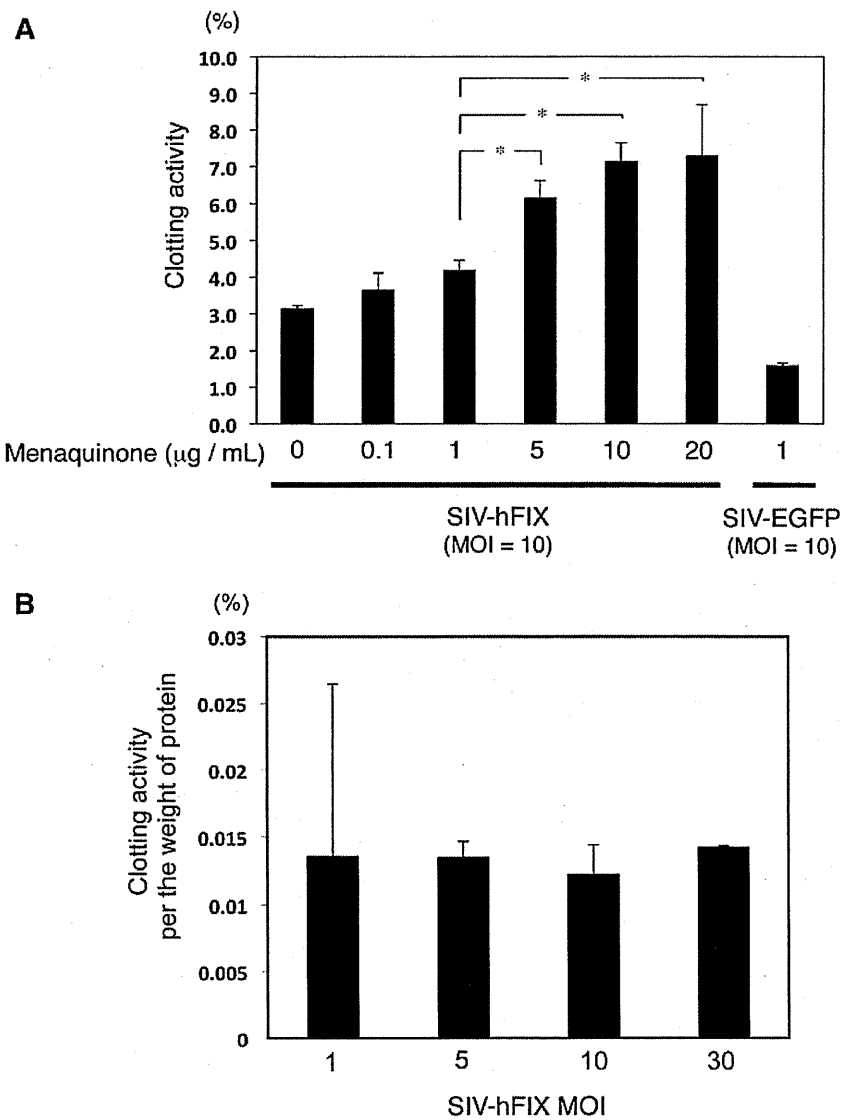

FIG. 4. Clotting activity of human coagulation factor IX (hFIX) produced by mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** mADSCs were transduced with the SIV-hFIX lentivirus vector at an MOI of 10. Seventy-two hours after vector transduction, the medium was changed to fresh medium 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

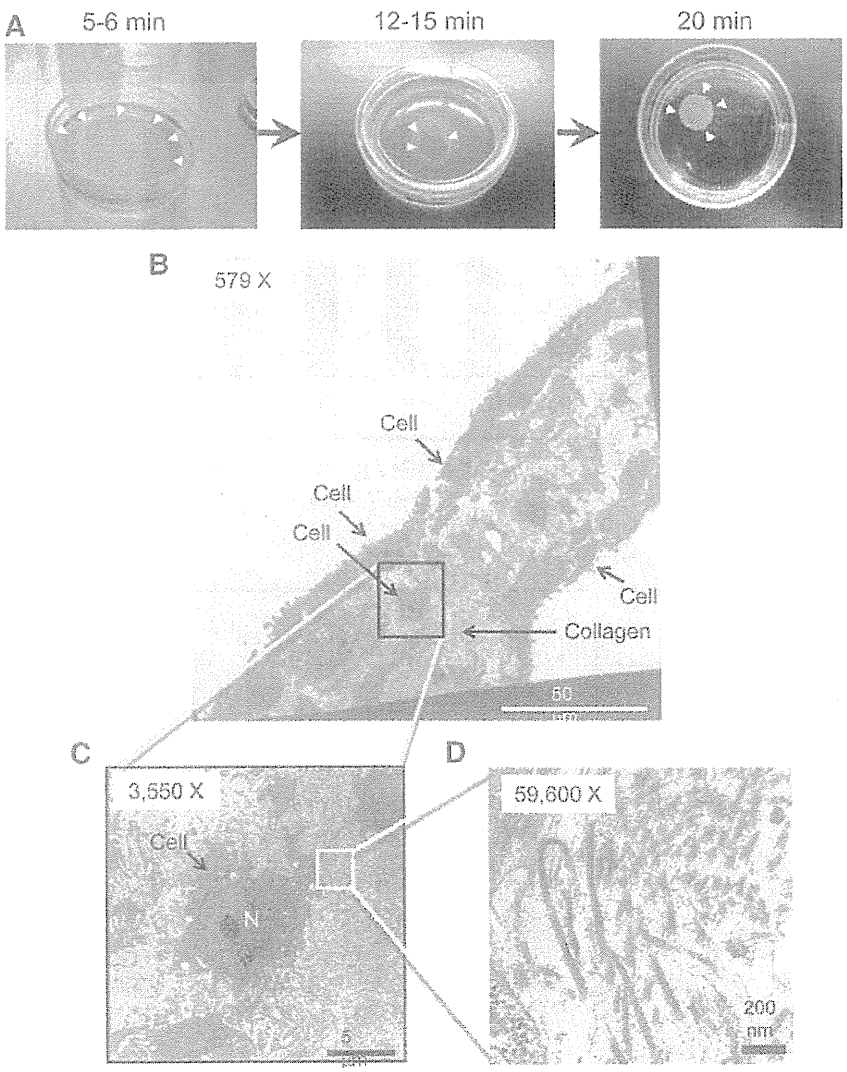

FIG. 5. Creation of cell sheets of mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** mADSCs were cultured on a temperature-responsive culture surface (UpCell 35-mm dish). Lowering the temperature to 20°C allowed mADSC sheets to detach from the edge (arrowheads, *left*, at 5–6 min), shrink 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

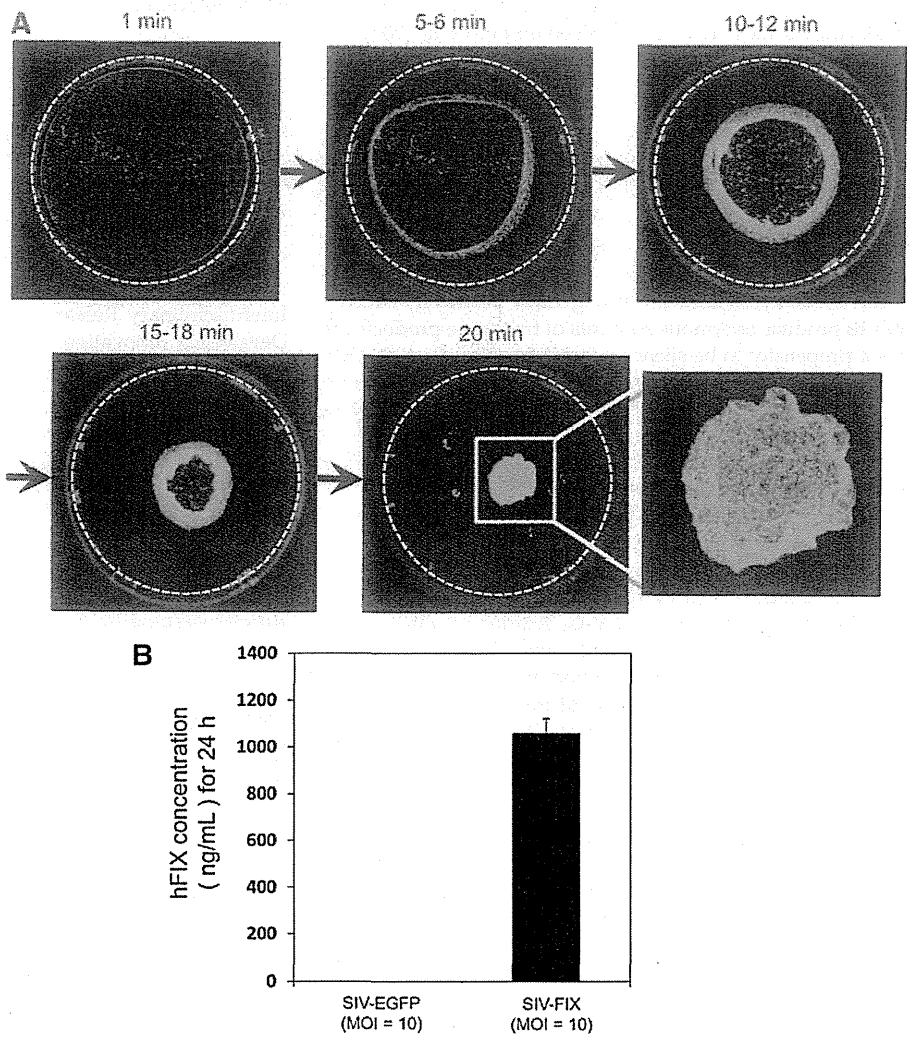

FIG. 6. Cell sheets of lentivirus-transduced mouse adipose tissue-derived stem/stromal cells (mADSCs). **(A)** mADSCs were transduced with SIV-EGFP lentiviral vector and replated on a temperature-responsive culture surface (UpCell 35-mm dish) 96 hr after transduction. After reaching confluency on day 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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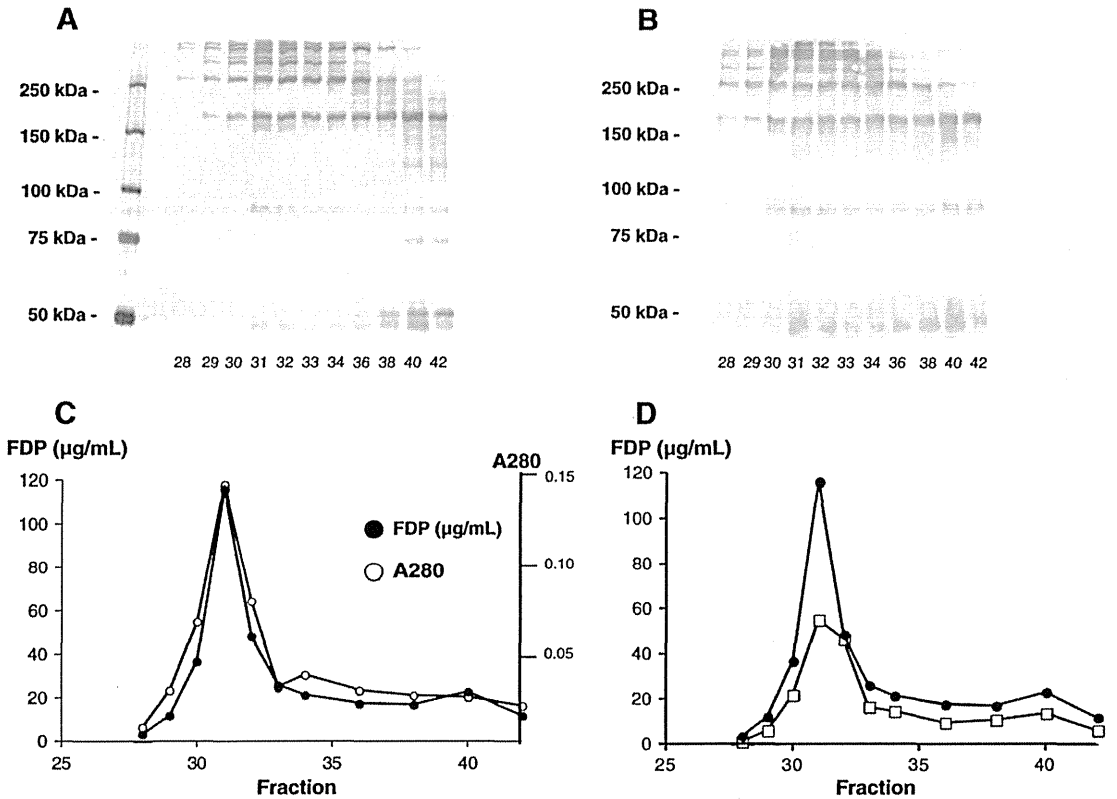

Fig. 1. Analysis of FDP generated in vitro. Fractions of gel filtration column chromatography on Sephacryl S-300 were analyzed by SDS-PAGE with silver staining (A). The same samples were analyzed by Western blotting with a rabbit polyclonal antibody against human fibrinogen (B). Most of the protein bands,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

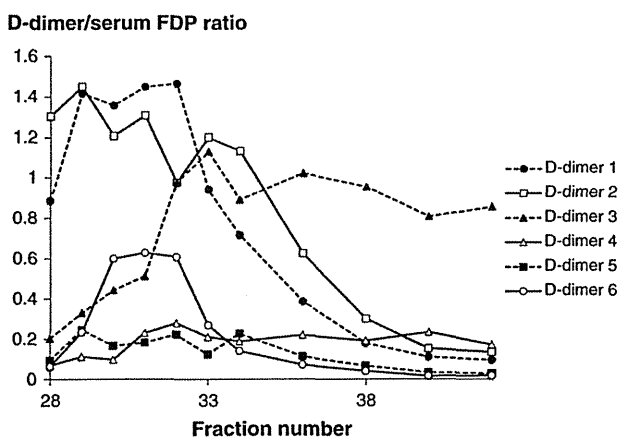

Fig. 2. Relative D-dimer values against serum FDP-2 values in the gel filtration column chromatography fractions. Fractions #28–42 of gel chromatography were further analyzed with 6 D-dimer reagents (D-dimer-1, D-dimer-2, D-dimer-3, D-dimer-4, D-dimer-5, and D-dimer-6) for clinical laboratory testing.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

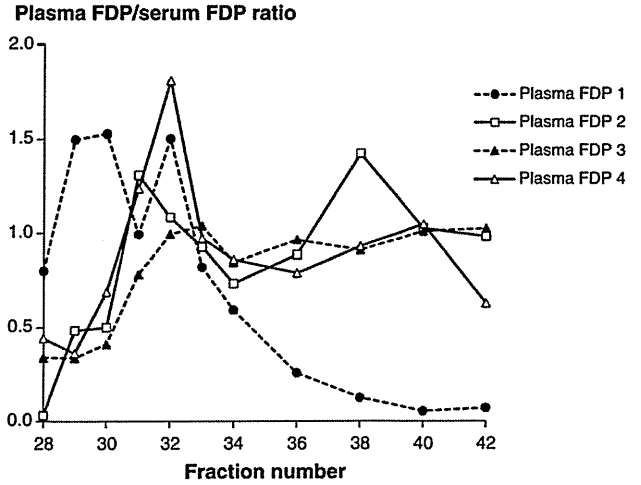

Fig. 3. Relative plasma FDP values against serum FDP-2 values in the gel filtration column chromatography fractions. Fractions #28–42 of gel chromatography were quantified with four plasma FDP reagents (plasma FDP-1, plasma FDP-2, plasma FDP-3, and plasma FDP-4) in a similar manner to Fig. 2. The FDP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

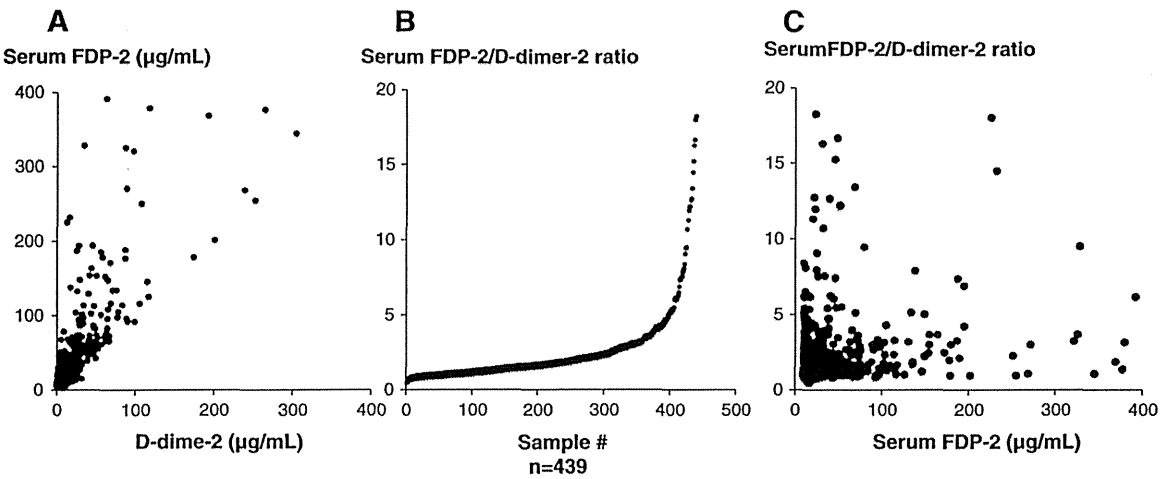

Fig. 4. Distribution of serum FDP/D-dimer ratios in clinical samples. Serum FDP-2 levels (10–400 μg/mL) of clinical samples (n = 439) were plotted against the D-dimer-2 level of the same sample (Fig. 4A). The ratios (<20) of the serum FDP-2/D-dimer-2 of clinical samples were sorted and plotted (B). Serum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

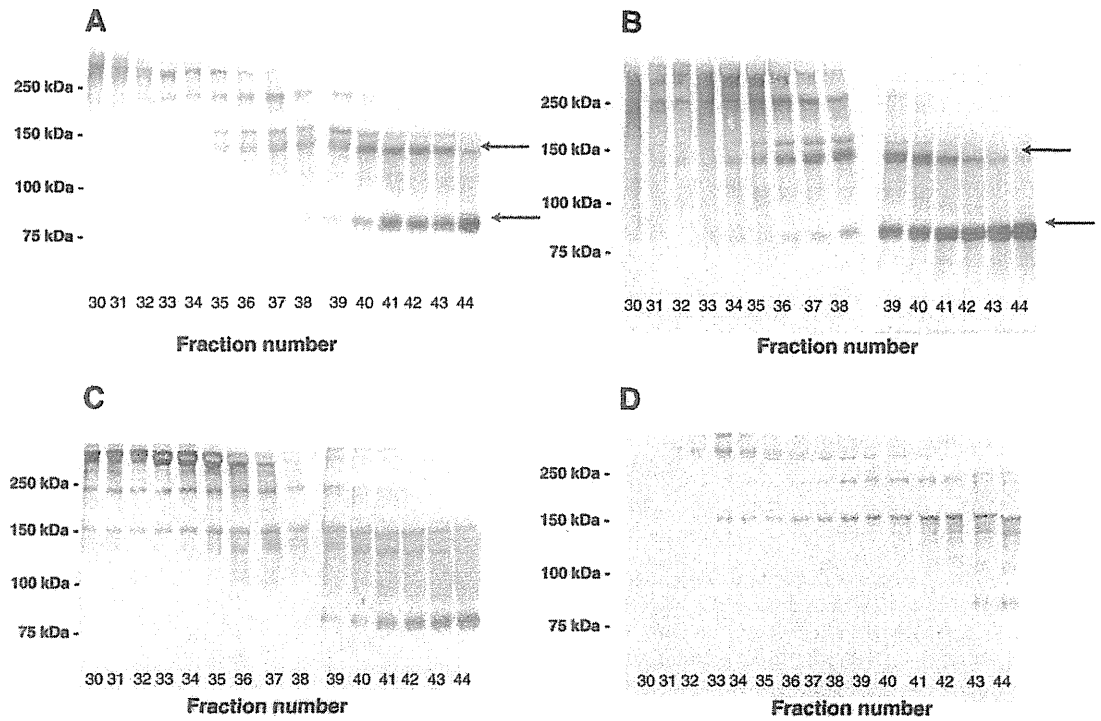

Fig. 5. Molecular variants of FDP in clinical samples. Four representative clinical DIC sample FDP levels (high FDP (>100 μg/mL) and high serum FDP-2/D-dimer-2 ratios (A, 18.0; B, 9.5); high FDP levels (>100 μg/mL) and low serum FDP-2/D-dimer-2 ratios (C, 1.0; D, 1.0) were subjected to gel filtration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

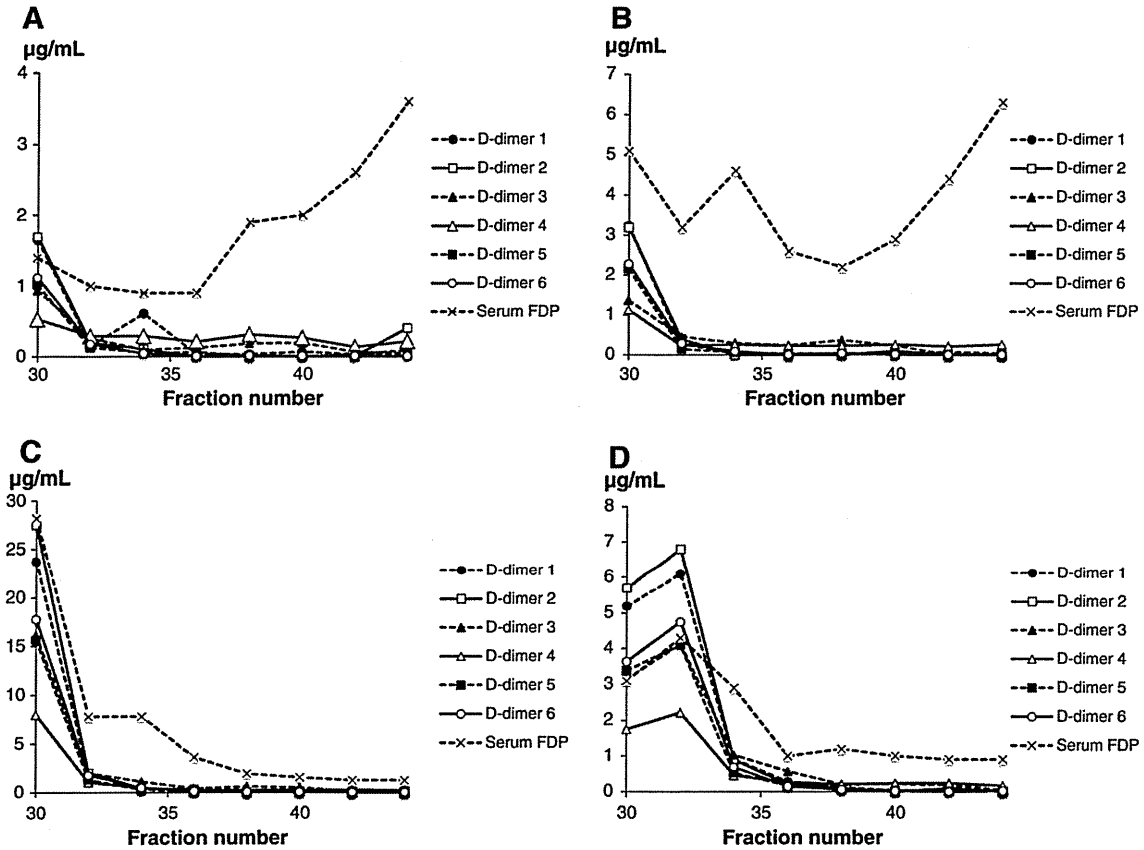

Fig. 6. Reactivity of the monoclonal antibodies used in laboratory D-dimer reagents to the molecular variants of FDP in clinical samples. Gel filtration column chromatography fractions of clinical samples A (A), B (B), C (C), and D (D) were subjected to quantification with D-dimer reagents. FDP concentrations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

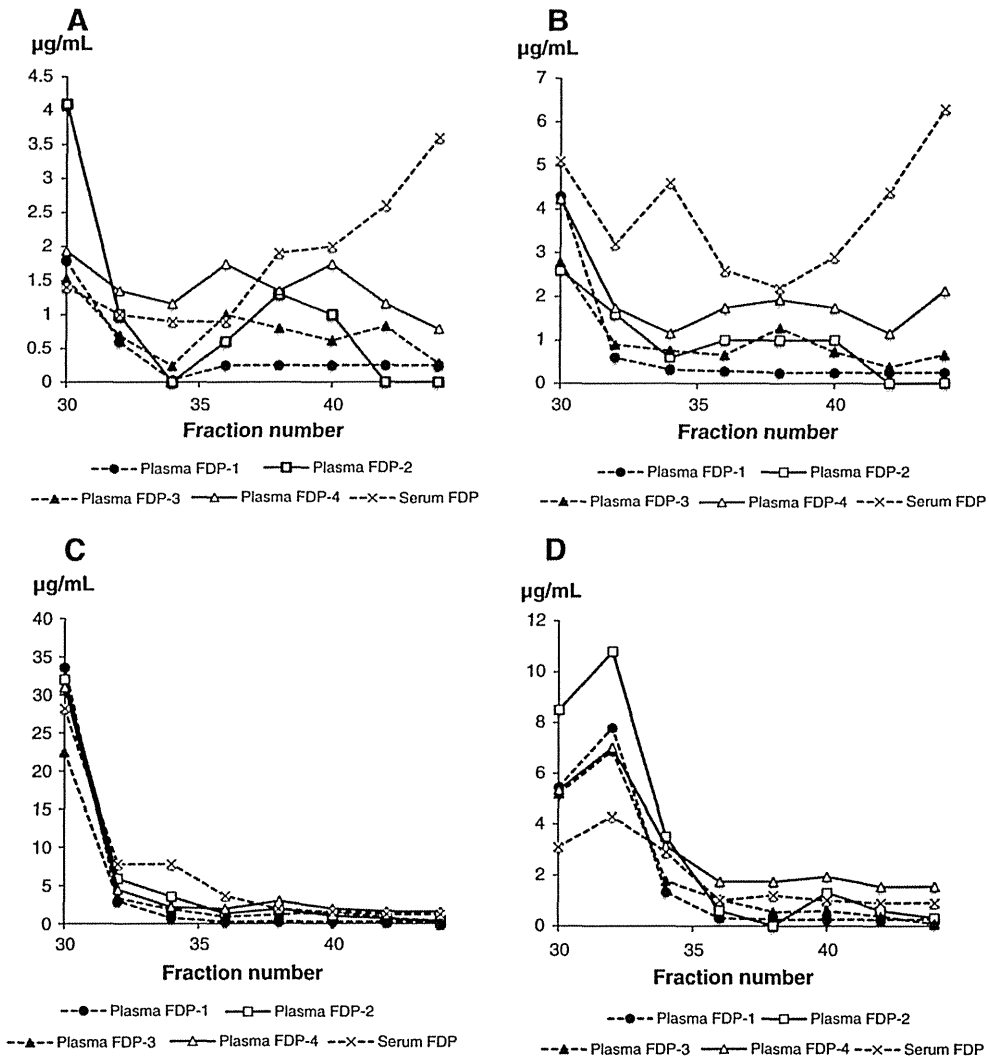

Fig. 7. Reactivity of the monoclonal antibodies used in laboratory plasma FDP reagents to the molecular variants of FDP in clinical samples. Gel filtration column chromatography fractions of clinical samples A (A), B (B), C (C), and D (D) were subjected to quantification with plasma FDP reagents. FDP 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.

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Evaluation of hemostatic biomarker abnormalities that precede platelet count decline in critically ill patients with sepsis☆,☆☆

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Early diagnosis;
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Abstract

Purpose: The hemostatic biomarkers for early diagnosis of sepsis-associated coagulopathy have not been identified. The purpose of this study was to evaluate hemostatic biomarker abnormalities preceding a decrease in platelet count, which is a surrogate indicator of overt coagulopathy in sepsis.

Materials and Methods: Seventy-five septic patients with a platelet count more than $80 \times 10^3/\mu L$ were retrospectively analyzed. Hemostatic biomarkers at intensive care unit admission were compared between patients with and patients without a subsequent decrease in platelet count ($\geq 30\%$ within 5 days), and the ability of biomarkers to predict a decrease in platelet count was evaluated.

Results: Forty-two patients (56.0%) developed a subsequent decrease in platelet count. Severity of illness, incidence of organ dysfunction, and 28-day mortality rate were higher in patients with a subsequent decrease in platelet count. There were significant differences between patients with and patients without a subsequent decrease in platelet count in prothrombin time–international normalized ratio, fibrinogen, thrombin-antithrombin complex, antithrombin, protein C (PC), plasminogen, and α_2-plasmin inhibitor (α_2-PI). Receiver operating characteristic curve analysis showed that PC (area under the curve, 0.869; 95% confidence interval, 0.699-0.951) and α_2-PI (area under the curve, 0.885; 95% confidence interval, 0.714-0.959) were strong predictors of a subsequent decrease in platelet count.

☆ Authors' contributions: K.K. conceived and designed the study. K.K. and S.T. prepared the data for analysis. K.K. conducted the data analysis. S.M. assisted with interpretation of the results. Y.S., J.M., and S.N. supervised the study. K.K. and S.M. drafted the article. All authors read and approved the manuscript. K.K. and S.M. take responsibility for the manuscript as a whole.

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Conclusions: Decreased PC and α_2-PI activity preceded a decrease in platelet count in intensive care unit patients with sepsis.

1. Introduction

Coagulation and fibrinolytic abnormalities are observed in most patients with sepsis [1]. Severe inflammation in sepsis is associated with tissue factor–mediated activation of coagulation, which leads to thrombin generation and results in widespread fibrin deposition. The severity of coagulopathy in sepsis ranges from subclinical abnormalities, which are detectable by a mild increase in fibrin degradation products (FDPs) and prolongation of global clotting times, to fulminant disseminated intravascular coagulation (DIC), characterized by widespread microvascular thrombosis [2]. A number of studies have reported on the association between DIC and organ failure and found that DIC is an independent risk factor for mortality in patients with sepsis [2-5]. Early diagnosis and treatment may therefore improve outcomes in septic patients with DIC [6].

The International Society on Thrombosis and Haemostasis (ISTH) criteria are currently widely used for diagnosing DIC [7] and are a strong independent predictor of mortality in patients with severe sepsis [8]. Although the ISTH criteria for overt DIC are simple and clinically useful, they have some limitations to be applied for early stage of DIC. The ISTH criteria define nonovert DIC as the stage before overt DIC, for the purpose of early diagnosis [7]. However, previous studies have shown that few patients with nonovert DIC progress to overt DIC and that mortality rates are similar between patients with nonovert DIC and patients with overt DIC [8], suggesting that septic coagulopathy diagnosed according to the ISTH criteria for nonovert DIC may not necessarily be an early stage of overt DIC.

Previous studies have evaluated a number of hemostatic biomarkers including D-dimer, antithrombin (AT), thrombin-AT complex (TAT), plasmin–α_2-plasmin inhibitor complex (PIC), and plasminogen activator inhibitor-1 (PAI-1); however, no single marker that can effectively diagnose early stage of DIC has been identified [9,10]. It is therefore important to develop clinical markers that can detect progression of septic coagulopathy in initial phase, so that early intervention can be instituted.

The objective of this study was to evaluate the ability of hemostatic biomarkers for predicting progression of coagulopathy in septic patients admitted to the intensive care unit (ICU). We used a decrease in platelet count as a marker for overt stage of septic coagulopathy. Platelet activation, consumption, and destruction may occur at the endothelial cell surface as a result of thrombin generation and fibrin meshwork formation secondary to coagulation activation. Platelet count decreases over a few days after the development of sepsis [5], which may indicate ongoing activation of coagulation [11]. Thrombocytopenia may reflect the advanced stage of DIC, which is associated with late death in patients with severe sepsis [5,12]. We therefore considered that a decreasing platelet count could be an indicator of disease progression in sepsis-induced coagulopathy.

2. Methods

2.1. Patients

The medical records of all patients admitted to the ICU at Jichi Medical University Hospital from September 2010 to December 2011 were retrospectively reviewed. Patients with a diagnosis of sepsis and a platelet count of more than $80 \times 10^3/\mu L$ on the day of ICU admission were included in the study. Sepsis was defined as fulfillment of at least 2 of the 4 criteria for systemic inflammatory response syndrome [13] and proven or suspected infection. Exclusion criteria were as follows: age younger than 18 years, prior hematologic disorder including platelet disorder, liver cirrhosis or failure, chronic renal failure with dialysis, history of chemotherapy, anticoagulation therapy with or without AT substitution, and blood transfusion during the preceding 4 weeks. This study was approved by the Institutional Research Ethics Committee of Jichi Medical University, which did not consider informed consent to be necessary because of the study design.

Our facility provides 24-hour coverage of attending ICU physicians. Management of patients followed the Surviving Sepsis Campaign Guideline [14], with the goal of initial resuscitation and infection control. Treatment for DIC was at the discretion of the responsible ICU physicians. The basic approach to treatment was anticoagulation therapy using gabexate mesilate (a serine protease inhibitor) [15,16], with or without AT substitution therapy. Some patients with a bleeding risk, or with complications, were transfused with platelet concentrate or fresh-frozen plasma at the discretion of the treating physicians.

2.2. Data collection

Descriptive data including demographic data, diagnoses, sources of infection, and clinical data were collected from the electronic medical records of all eligible patients. Acute Physiology and Chronic Health Evaluation (APACHE) II [17] and Simplified Acute Physiology (SAPS) II [18] scores were calculated to estimate the severity of disease within the

first 24 hours of ICU admission. The Sequential Organ Failure Assessment (SOFA) scoring system [19] was used to evaluate organ dysfunction during the first 7 days of ICU stay or until ICU discharge. The ISTH criteria were used to diagnose overt and nonovert DIC, with scores calculated for each of the dates tested. Prognosis was evaluated by ICU-free days during the first 28 days [20] and all-cause 28-day mortality.

2.3. Biomarker measurements

Platelet counts were measured on the day of ICU admission (day 1) and on each of days 2 to 5. Prothrombin time–international normalized ratio (PT-INR), activated partial thromboplastin time (APTT), fibrinogen, FDP, AT, protein C (PC), plasminogen, α_2-plasmin inhibitor (α_2-PI), TAT, and PIC were measured on day 1. Assays of hemostatic parameters were performed using the CS-2100i automatic coagulation analyzer (Sysmex, Hyogo, Japan). Antithrombin, PC, plasminogen, and α_2-PI were measured using Berichrom assays (Siemens Healthcare Diagnostics, Tokyo, Japan). Thrombin-AT complex and PIC were quantitated using the TAT test F and PIC test F enzyme immunoassay, respectively (Sysmex).

2.4. Data analysis

We defined a subsequent decrease in platelet count as a decrease of 30% or more within 5 days of ICU admission. The study population was grouped according to the presence or absence of a subsequent decrease in platelet count. Differences between groups were analyzed using the Student *t* test for normally distributed variables and the Mann-Whitney *U* test for nonnormally distributed variables. Categorical data were compared using the χ^2 test or Fisher exact test.

Receiver operating characteristic (ROC) curve analysis was performed to calculate the area under the curve (AUC) for coagulation and fibrinolytic biomarkers, and the AUCs were compared to evaluate their ability to predict a subsequent decrease in platelet count. Cutoff values were calculated by maximizing the sum of sensitivity and specificity.

All *P* values were 2 tailed, and $P < .05$ was considered statistically significant. Data were analyzed using JMP version 10 (SAS Institute, Tokyo, Japan).

3. Results

3.1. Characteristics of the 75 eligible patients with sepsis

Of the 1343 patients admitted to the ICU during the study period, 108 had a diagnosis of sepsis on the day of ICU admission. Thirty-three patients were excluded according to the study criteria, and the remaining 75 patients were included in the study. Forty-two patients developed a subsequent decrease in platelet count within 5 days of ICU admission (Fig. 1).

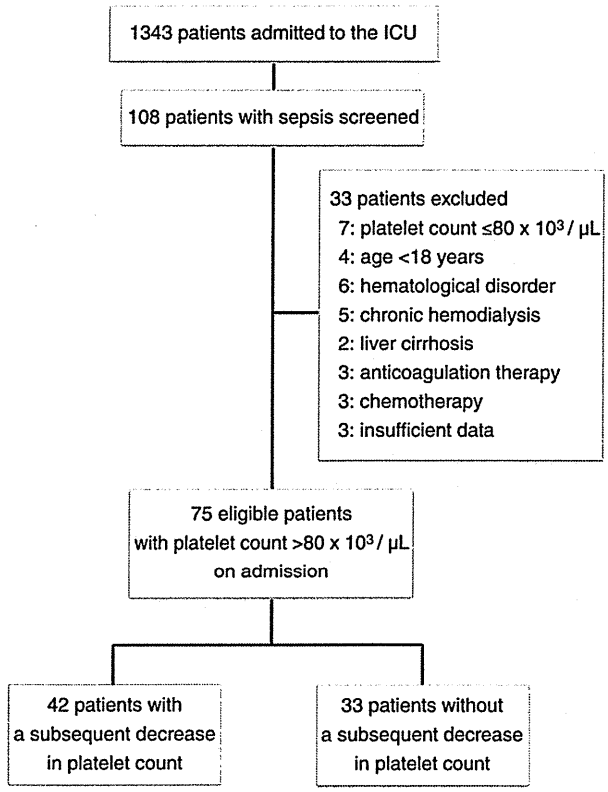

Fig. 1 Flowchart of the study patients. Sepsis was defined as fulfillment of at least 2 criteria for systemic inflammatory response syndrome and a source of infection. Eligible patients were grouped according to the presence or absence of a decrease of in platelet count 30% or more within 5 days of ICU admission.

Table 1 shows the baseline characteristics of the 75 patients. The most common cause of sepsis was abdominal infection, including 26 patients (34.7%) with lower intestinal perforation, 9 (12.0%) with acute cholangitis or cholecystitis, 5 (6.7%) with intra-abdominal abscess, and 3 (4.0%) with liver abscess (Table 1). Fifty (66.7%) of the 75 patients were surgical, and 25 (33.3%) were medical. The mean APACHE II score was 23.2 ± 7.8, and the mean SAPS II score was 49.8 ± 15.5. The all-cause 28-day mortality rate was 8.0%, with all deaths occurring during the ICU stay (Table 2).

Platelet counts over time (days 1-5) in patients with and without a subsequent decrease in platelet count are shown in Fig. 2. On the day of ICU admission, platelet counts were not significantly different between patients with and patients without a subsequent decrease in platelet count. The nadir of platelet count was on day 4 in patients with a subsequent decrease in platelet count and on day 2 in patients without a subsequent decrease in platelet count. During the first 7 days

Table 1 Baseline characteristics of the 75 patients with sepsis

	All patients (n = 75)	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	P^a
Age (y)	70.0 ± 11.7	70.1 ± 11.9	70.0 ± 11.5	.98
Male	41 (54.7)	18 (42.9)	23 (69.7)	.035
Sepsis				
Pneumonia	15 (20.0)	7 (16.7)	8 (24.2)	.56
Abdominal infection	46 (61.3)	30 (71.4)	16 (48.5)	.057
Urinary tract infection	5 (6.7)	3 (7.1)	2 (6.1)	1.00
Soft tissue infection	9 (12.0)	2 (4.8)	7 (21.2)	.038
Comorbidity				
IHD	5 (6.7)	2 (4.8)	3 (9.1)	.65
CHF	5 (6.7)	2 (4.8)	3 (9.1)	.65
COPD	4 (5.3)	2 (4.8)	2 (6.1)	1.00
CVD	2 (2.7)	0 (0.0)	2 (6.1)	.19
CKD	5 (6.7)	4 (9.5)	1 (3.0)	.38
Severity of illness				
APACHE II score	23.2 ± 7.8	25.1 ± 8.2	20.8 ± 6.8	.021
SAPS II score	49.8 ± 15.5	55.5 ± 16.1	42.9 ± 11.8	.0004
DIC score on admission				
ISTH overt	2.6 ± 1.3	2.7 ± 1.5	2.5 ± 1.1	.51
ISTH nonovert	5.0 ± 1.8	5.2 ± 1.9	4.8 ± 1.5	.33

Data are expressed as mean ± SD or number (%).
IHD indicates ischemic heart disease; CHF, chronic heart failure; COPD, chronic obstructive pulmonary disease; CVD, cerebrovascular disease; CKD, chronic kidney disease.
[a] Comparison of groups with and without a subsequent decrease in platelet count.

of ICU stay, circulatory failure and renal dysfunction were more frequent in patients with than without a subsequent decrease in platelet count (Table 2). Patients with a subsequent decrease in platelet count had a higher mortality rate than did patients without a subsequent decrease in platelet count (Table 2).

Table 2 Interventions, organ dysfunction, and prognosis in 75 patients with sepsis

	All patients (n = 75)	Subsequent decrease in platelet count (n = 42)	No subsequent decrease in platelet count (n = 33)	P^a
Transfusion (days 1-7)				
Red blood cell	18 (24.0)	11 (26.2)	7 (21.2)	.62
Platelet	9 (12.0)	9 (21.4)	0 (0.0)	.0039
Fresh-frozen plasma	16 (21.3)	15 (35.7)	1 (3.0)	.0005
Treatment for DIC (days 1-7)				
Gabexate mesilate	29 (38.7)	28 (66.7)	1 (3.0)	<.0001
Antithrombin	21 (28.0)	21 (50.0)	0 (0.0)	<.0001
Organ dysfunction (days 1-7)				
CVS (SOFA ≥ 3)	39 (52.0)	30 (71.4)	9 (27.3)	.0002
Lung (SOFA ≥ 3)	26 (48.0)	23 (54.8)	13 (39.4)	.25
Renal (SOFA ≥ 2)	16 (21.3)	14 (33.3)	2 (6.1)	.0045
Liver (SOFA ≥ 2)	28 (37.3)	18 (42.9)	10 (30.3)	.34
DIC				
ISTH overt	23 (30.7)	21 (50.0)	2 (6.1)	<.0001
ISTH nonovert	54 (72.0)	38 (90.5)	16 (48.5)	<.0001
Prognosis				
ICU-free days	19 (11.5-23)	18 (7–21)	22 (15–23.5)	.027
ICU mortality	6 (8.0)	6 (14.6)	0 (0.0)	.031
28-d mortality	6 (8.0)	6 (14.6)	0 (0.0)	.031

Data are expressed as median (interquartile range) or number (%).
CVS indicates cardiovascular system.
[a] Comparison of groups with and without a subsequent decrease in platelet count.