Methods

Animal models

Haemophilia A mice (B6; $_{129}S_4$-F8^{tm1Kaz}/J) with targeted destruction of exon 16 of the FVIII gene were kindly provided by Dr H.H. Kazazian Jr (University of Pennsylvania, Philadelphia, PA, USA) [8]. All mice were housed and used in a pathogen-free facility at Jichi Medical University, Shimotsuke, Tochigi, Japan. All animal experiments were performed in accordance with regulations of the Japanese Council for Animal Care; Jichi Medical University Animal Care Committee approval all animal protocols.

iVAD system and intravenous injection of FVIII

Haemophilia A mice aged 12 weeks were anaesthetized by inhalation with 2.5% isoflurane in the anaesthesia unit (Univentor, ZTN 08, Malta). An iVAD system (SoloPort; Instech Laboratories, Plymouth Meeting, PA, USA) was placed into a pocket of back skin in the chest wall of each animal (Fig. 1). The catheter was then tunneled under the skin and introduced into the superior vena cava through a cut-down site of jugular vein under a zoom stereomicroscope (Nikon, Tokyo, Japan). The entire system was flushed with saline solution after insertion. Mice were infused with intravenous recombinant human FVIII formulated with sucrose (Kogenate FS; Bayer Healthcare, Leverkusen, Germany) through the iVAD system.

Assay for FVIII inhibitors

Inhibitory FVIII antibodies were measured according to the Bethesda assay [9]. In brief, mouse plasma was serially diluted in Owren's Veronal Buffer (Dade Behring, Deerfield, IL, USA), such that the remaining FVIII activity for each sample was between 25% and 75%, and mixed 1:1 with normal pooled human plasma at 37°C for 2 h. Residual human FVIII activity was measured by one-stage assay using 50 μL of FVIII-deficient human plasma (Kokusai-Shiyaku, Kobe, Japan) and a 50-μL sample from the previous incubation on a automated coagulometer (CA-500; Sysmex, Kobe, Japan). One BU mL^{-1} was defined as the dilution of plasma containing FVIII inhibitory activity that results in 50% inhibition of FVIII activity.

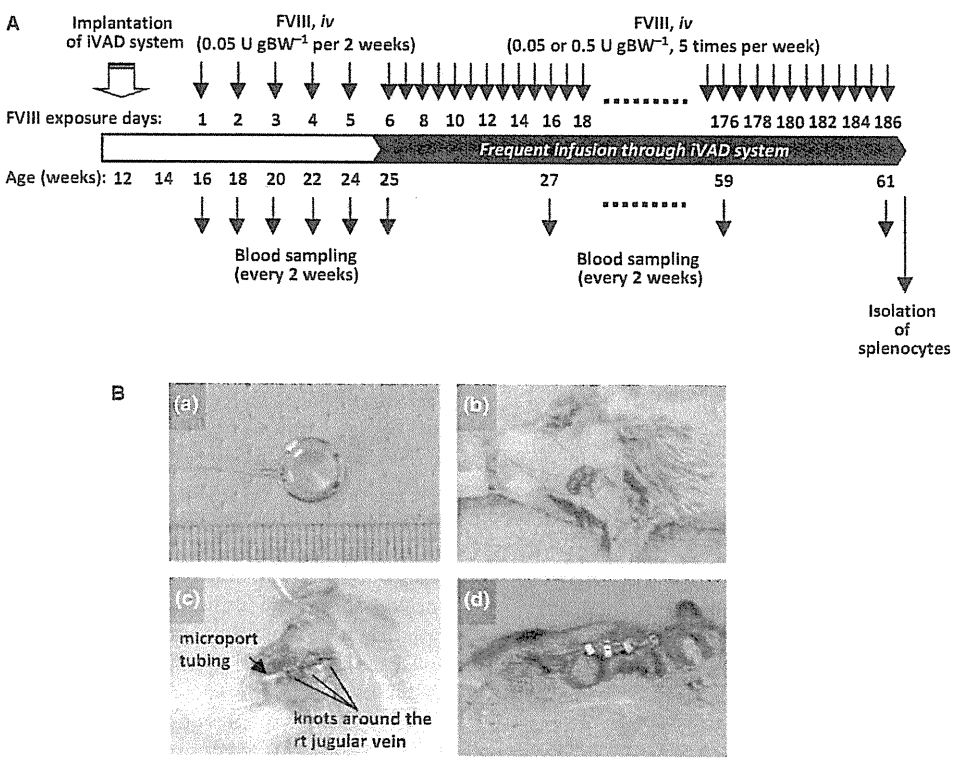

Fig. 1. Protocol for serial intravenous administration of FVIII through an implantable venous access device (iVAD) system in haemophilia A mice. A. Haemophilia A mice were implanted an iVAD system at age 12 weeks, then they were preimmunized with intravenous injection of 0.05 U g^{-1} BW recombinant human FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently administered with FVIII (0.05 or 0.5 U g^{-1} BW, five times per week) through the iVAD system. B. (a) The iVAD system consists of a stainless steel port with a molded silicon rubber and an 1.2 Fr catheter. After incision of the cervical skin in a haemophilia A mouse, the jugular vein was exposed (b), then the catheter was inserted into superior vena cava by cut-down procedure (c). The other side of the catheter was tunneled to the port that was set beneath the pocket of back skin (d).

Anti-FVIII measurement

Anti-FVIII IgG concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in microtiter wells (Nunc, Roskilde, Denmark) coated with 1 μg mL^{-1} recombinant human FVIII. After blocking with 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), serial dilutions of murine plasma were added at 4°C for 16 h. Each well was washed with 0.5% BSA in PBS containing 0.05% Tween-20. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Cappel, Aurora, OH, USA) was added at 37°C for 1 h. ABTS Microwell substrate (KPL, Gaithersburg, MD, USA) was added, and the absorbance at 405 nm was read. Anti-FVIII antibody concentrations were calculated from the linear portion of a standard curve obtained using antihuman FVIII monoclonal antibodies (kindly provided by Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan).

Determination of anti-FVIII IgG subclasses

Microtiter wells were coated with 1 μg mL^{-1} recombinant human FVIII in PBS for 16 h at 4°C. After blocking with 5% BSA in PBS, serial dilutions of murine plasma were added for 2 h at 37°C. The wells were washed with 0.5% BSA in PBS containing 0.05% Tween-20. The IgG1, IgG2a, IgG2b and IgG3 subtypes of anti-FVIII antibodies bound to immobilized human FVIII were determined by incubation with isotype-specific rabbit anti-mouse IgGs (Mouse Typer; BioRad, Hercules, CA, USA) for 1 h at 37°C. After washing with 0.5% BSA in PBS containing 0.05% Tween-20, the wells were incubated with goat anti-rabbit HRP conjugate for 1 h at 37°C. Substrate development was performed for 15 min at 25°C, using ABTS Microwell substrate as described above.

Proliferation assay with [^{3}H]-thymidine incorporation

Mice splenic $CD4^+$ T cells were prepared by depletion of non-$CD4^+$ T cells with the autoMACS cell sorting system (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Antigen-presenting cells were prepared from mice splenocytes by depletion of T cells using the magnetic sorting system with anti-CD90 (Thy1.2)-conjugated microbeads (Miltenyi Biotech) followed by irradiation with a single dose of 20 Gy (Gamma Cell; Norton International, ON, Canada), to prevent non-specific proliferative responses during the *in vitro* FVIII stimulation assay. To measure T-cell proliferation, 3×10^5 $CD4^+$ T cells per well were cultured with 0–3 nM human recombinant FVIII in the presence of antigen-presenting cells derived from FVIII-immunized mice (five times injection of 0.05 U g^{-1} BW FVIII, every 2 weeks) at 37°C for 72 h in complete RPMI-1640 (Gibco BRL, Rockville, MD, USA). [^{3}H]-Thymidine (Amersham Bioscience, Uppsala, Sweden) was added (0.037 MBq per well) at 37°C for 18 h. Then, cells were harvested, and [^{3}H]-thymidine incorporation was determined by scintillation counting (Top count; Packard, Meriden, CT, USA).

Cytokine assays

Splenocytes were incubated in 24-well plates at 1.0×10^6 cells per well in the absence or presence of 3 nM human recombinant FVIII at 37°C in 5% CO_2. Production of interleukin (IL)-2, IL-4, IL-12 and interferon (IFN)-γ by $CD4^+$ T cells derived from each mouse was analyzed at 72 h by ELISA kits (Biotrak ELISA System; Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. In addition, levels of IL-10 were measured at 96 h by ELISA system (Biotrak ELISA System).

Statistical analysis

Data are expressed as mean ± SE. Normally distributed variables were compared by Student's *t*-test. Variables not normally distributed were analyzed by two-sided Mann–Whitney U test. The data were considered statistically significant at P values < 0.05.

Results

Serial intravenous administration of FVIII through an iVAD system in preimmunized haemophilia A mice

We securely implanted venous access devices into haemophilia A mice at 12 weeks using a zoom microscopy, therefore, we could avoid using FVIII concentrates for haemostatic control during the procedure (Fig. 1B). After the operation-related wounds had healed, we developed immunized mice against FVIII by intravenous injection of FVIII (0.05 U g^{-1} BW) at 2-week intervals. Titers of anti-FVIII inhibitory antibodies of the mice were elevated to 100–400 BU mL^{-1} after the fifth exposure of FVIII. Thereafter, we performed serial infusion of FVIII into the preimmunized haemophilia A mice through the venous access device system. High titers (>2000 BU mL^{-1}) were developed after 10 exposure days (EDs) in mice with administration of FVIII (0.05 U g^{-1} BW five times per week) and were continued over 100–120 EDs. However, after 130–150 EDs their titers were gradually decreased despite continuing sequential stimulation of FVIII (Fig. 2a). One of the five mice was discontinued at 140 EDs because of bleeding from the site of catheter insertion (Fig. 2a; LD#3). In contrast, > 2000 BU mL^{-1} of anti-FVIII inhibitory antibodies were sustained over

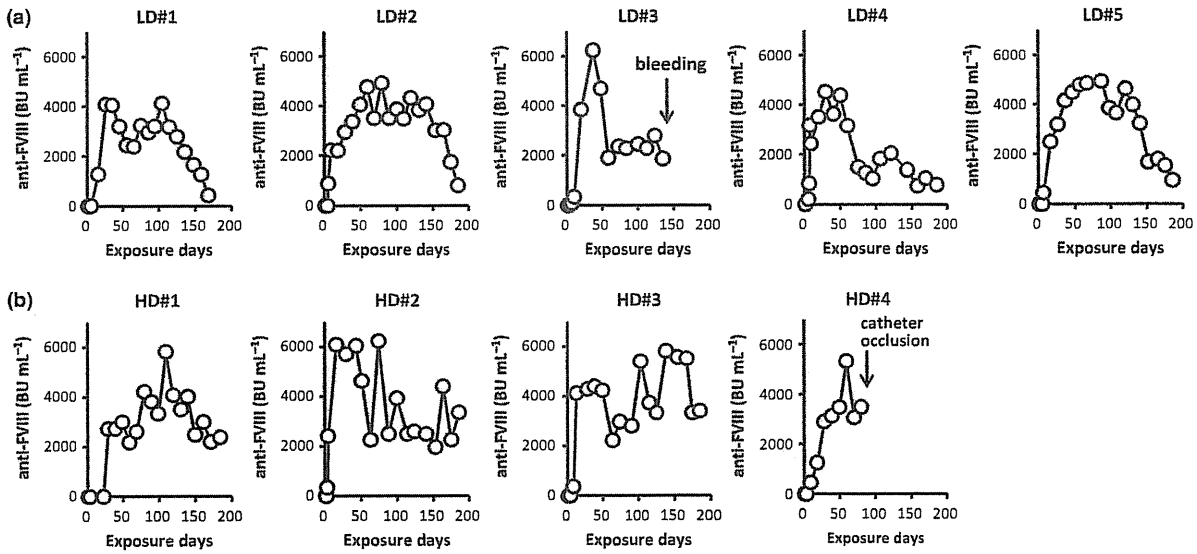

Fig. 2. Effect of serial intravenous administration of FVIII on anti-FVIII inhibitory antibody formation in preimmunized haemophilia A mice. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently administered with FVIII [(a) 0.05 U g^{-1} BW five times per week; (b) 0.5 U g^{-1} BW, five times per week] through the iVAD system. The mice were bled at every 2 weeks, and their anti-FVIII inhibitor titers were determined by Bethesda assay.

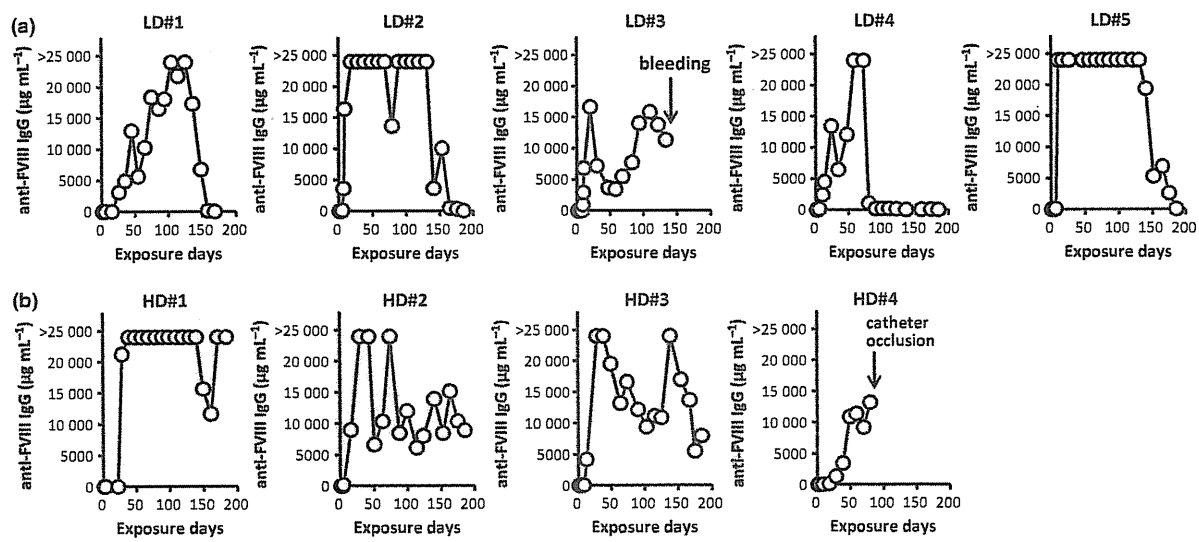

Fig. 3. Effect of repeated intravenous infusion of FVIII on FVIII-specific IgG formation in haemophilia A mice with inhibitors. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were repeatedly infused with FVIII [(a), 0.05 U g^{-1} BW, five times per week; (b) 0.5 U g^{-1} BW, 5 times per week. The mice were bled at every two weeks just before each infusion. Plasma levels of FVIII-specific IgG were measured by ELISA as described in Methods.

180 EDs in mice with serial infusion of high-dose FVIII (0.5 U g^{-1} BW five times per week) (Fig. 2b).

Effect of serial intravenous infusion of FVIII on FVIII-specific IgG and subclasses formation

Anti-FVIII IgG was detectable immediately after serial infusion of FVIII (0.05 U g^{-1} BW five times per week) in preimmunized haemophilia A mice, and were persisted for more than 80–100 EDs (Fig. 3a). Interestingly, titers against FVIII were markedly decreased after 80–150 EDs. By contrast, preimmunized mice followed by serial intravenous infusion of high-dose FVIII (0.5 U g^{-1} BW five times per week) showed high titer of anti-FVIII IgG over 150–180 EDs (Fig. 3b). One of four mice receiving sequential high-dose FVIII infusion was discontinued due to occlusion of central vein catheter at 80 EDs (Fig. 3b; HD#4). All IgG isotypes of anti-FVIII IgG

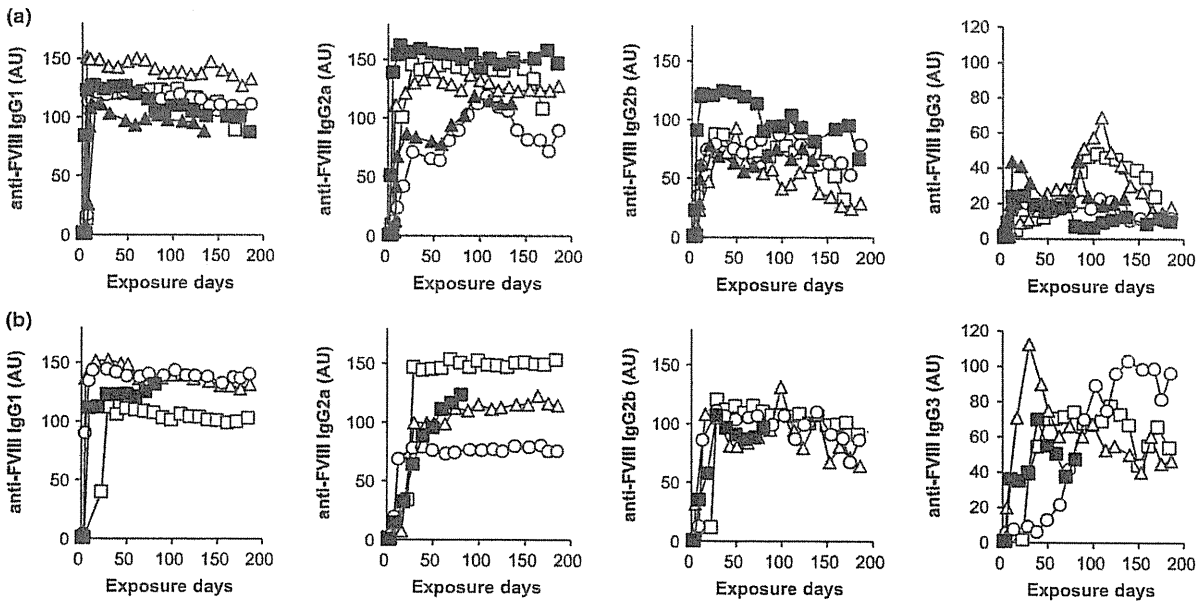

Fig. 4. Effect of serial intravenous injection on anti-FVIII IgG subclasses formation in preimmunized haemophilia A mice. (a) Haemophilia A mice (LD1, open squares; LD#2, open triangles; LD#3, closed triangles; LD#4, closed squares; LD#5, open circles) were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were repeatedly infused with FVIII (0.05 U g^{-1} BW five times per week). (b) Haemophilia A mice (HD#1, open squares; HD#2, open triangles; HD#3, open circles; HLD#4, closed squares) were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were repeatedly infused with FVIII (0.5 U g^{-1} BW five times per week). Each of the mice was bled at every two weeks just before FVIII infusion. Titers of IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) were determined by ELISA as described in Methods.

antibodies were rapidly increased after serial infusion of 0.05 U g^{-1} and 0.5 U g^{-1} BW of FVIII (Fig. 4). However, in mice with repeated administration of 0.05 U g^{-1} BW FVIII titers of IgG3 subclass antibodies were decreased after 80–100 EDs (Fig. 4b).

Effect of serial administration of FVIII on anti-factor VIII CD4⁺ T cells proliferation

Next, we evaluated whether serial infusion of FVIII exerts a suppressive effect on FVIII-specific T cells, $CD4^+$ T cells obtained after the final injection were assayed for a T-cell proliferative response to FVIII. We observed a dose-dependent $CD4^+$ T-cell proliferative response to FVIII in preimmunized mice (five times injection of FVIII every two weeks, Fig. 5). In the group with sequential infusion of 0.5 U g^{-1} BW FVIII the T cells significantly proliferated in response to FVIII stimulation. By contrast, no response was observed at any FVIII dose in $CD4^+$ T cells from the mice after serial infusion of 0.05 U g^{-1} BW FVIII.

Effect of serial infusion of FVIII on cytokine response

Mice that were immunized with FVIII every two weeks developed splenocytes, which proliferated and produced

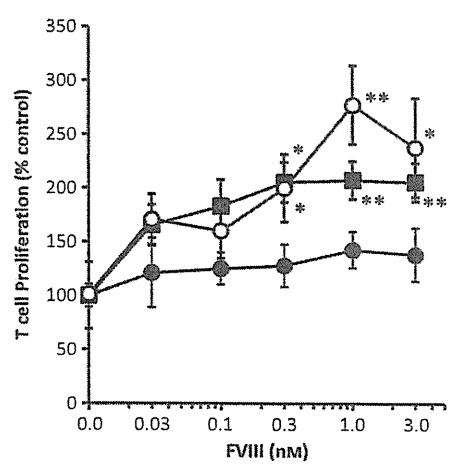

Fig. 5. Effect of repeated administration of FVIII on anti-factor VIII T-cell proliferation of haemophilia A mice. Haemophilia A mice were given intravenous injection of 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory-antibodies titers, preimmunized mice were frequently infused with FVIII through the iVAD system. $CD4^+$ T cells of preimmunized mice (n = 5; open circles), mice with infusion of FVIII (0.05 U g^{-1} BW, five times per week; n = 4; closed circles), and mice with injection of FVIII (0.5 U g^{-1} BW, five times per week; n = 3; closed squares) were obtained three days after final immunization. The amount of ^{3}H-thymidine incorporation was measured under *in vitro* stimulation with FVIII (0–3 nM) in the presence of the FVIII-immunized mice-derived antigen-presenting cells by scintillation counting as described in the Methods. Data are means ± SD. $^*P < 0.05$; $^{**}P < 0.03$ when compared with the proliferation in the absence of FVIII.

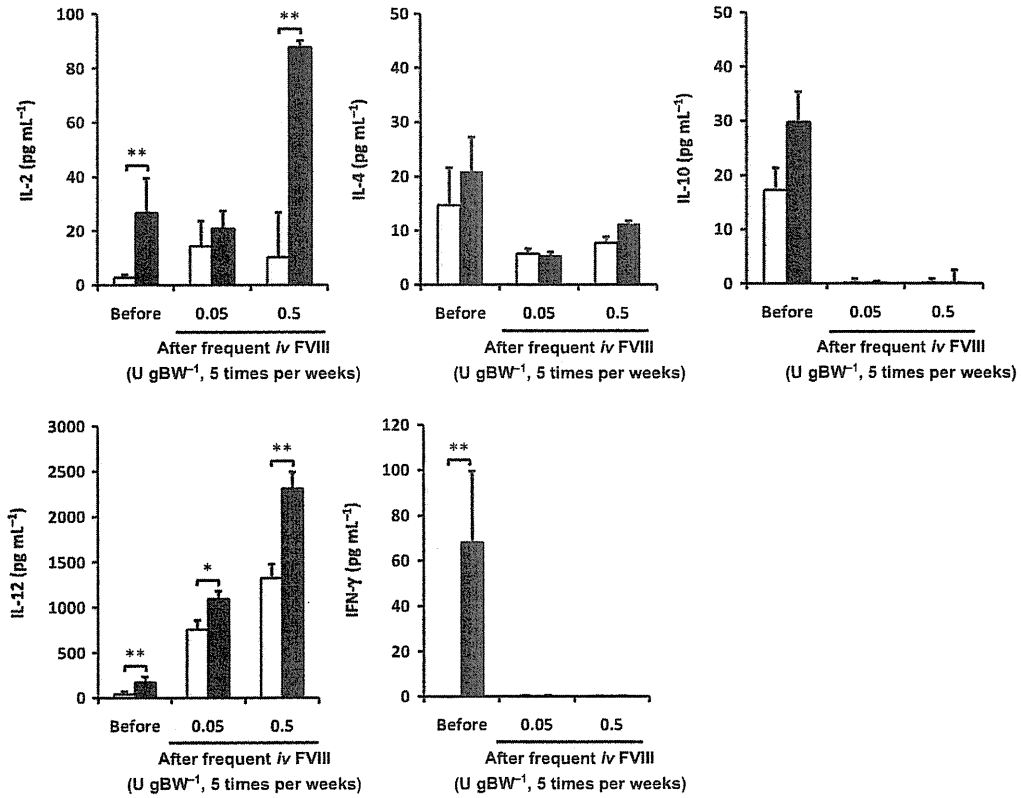

Fig. 6. Effect of serial infusion of FVIII on cytokine response of pre-immunized haemophilia A mice. Haemophilia A mice were intravenously immunized with 0.05 U g^{-1} BW FVIII at 16, 18, 20, 22 and 24 weeks. After measurement of anti-FVIII inhibitory antibodies titers, preimmunized mice were frequently administered with FVIII (0.05 or 0.5 U g^{-1} BW five times per week) through iVAD system. Splenocytes from pre-immunized (before, n = 5), frequently FVIII-infused [0.05 (n = 4) or 0.5 (n = 3) U g^{-1} BW five times per week] mice were cultured in the absence (open bars) or presence (closed bars) of 3 nM FVIII, and their cytokine production (IL-2, IL-4, IL-10, IL-12 and IFN-γ) were analyzed by ELISA as described in Methods. Values (pg mL^{-1}) are means ± SD. $^{*}P < 0.05$; $^{**}P < 0.03$.

IL-2, IL-12 and IFN-γ in response to *in vitro* FVIII stimulation (Fig. 6). In contrast, splenocytes deriving from mice with serial infusion of 0.05 U g^{-1} BW FVIII did not increase their production of IL-2 and IFN-γ, although they could secrete IL-12 after addition of FVIII. Moreover, 0.5 U g^{-1} BW FVIII-repeated administrated mice produced significant amounts of IL-2 and IL-12, but did not change IFN-γ levels even after stimulation of FVIII.

Discussion

Haemophilia A patients with inhibitors are infused daily FVIII according to immune tolerance protocols with the aim of eradicating the antibody [10,11]. Central VADs have been used to facilitate repeated administration of clotting factor concentrates in haemophiliac children requiring ITI [12,13]. We here established a method to implant a VAD into haemophilia A mice (Fig. 1). We could prevent exposure to FVIII antigen in the mice during the surgical procedure, because it is known that the innate immune system is activated by endogenous 'danger signals' such as tissue damage that involves necrotic cell death [14]. Indeed, titers of anti-FVIII inhibitory antibodies of the mice were elevated up to 400 BU mL^{-1} after the fifth intermittent stimulation of FVIII, in good agreement with previous findings [9]. Central VADs are associated with infectious and thrombotic complications necessitating the removal [6], although recent data from the international-ITI study showed that infectious episodes during ITI may not influence treatment outcome [15]. In our animal models, one mouse that had been frequently administered 0.05 U g^{-1} BW of FVIII exhibited a catheter-related bleeding (Fig. 2; LD#3), whereas another one with 0.5 U g^{-1} BW of FVIII had occlusion of iVAD system (Fig. 2, HD#4). Nonetheless, the iVAD would be a useful tool to evaluate immune response against sequential infusion of FVIII antigen in haemophilia A mice because they could be repeatedly infused more than 180 times over 50 weeks.

Recent study showed that port systems are suitable for inhibitor-expressing children with good predictors of ITI success [16,17]. In our murine model, high titers against FVIII (>2000 BU mL^{-1} during 50–100 EDs) were decreased to <500 BU mL^{-1} after 130–150 EDs in all

mice with serial infusion of 0.05 U g^{-1} BW of FVIII, even though they were continually exposed to FVIII antigen (Fig. 2). In contrast, mice administered high-dose 0.5 U g^{-1} BW FVIII five times a week had high titers of anti-FVIII inhibitory antibodies over 180 EDs, suggesting that dose of FVIII antigen might be crucial for the immune response in haemophilia A mice. We could not induce immune tolerance in any adult haemophilia A mouse with sequential infusion of FVIII antigen, according to the international consensus in which successful immune tolerance induction in haemophilia A is currently defined as both an undetectable inhibitor titer (less than or equal to 0.6 BU mL^{-1}) and normalized FVIII pharmacokinetics [18]. However, anti-FVIII IgG titers were markedly decreased to undetectable levels after 80–180 EDs in mice with serial infusion of 0.05 U mL^{-1} FVIII (Fig. 3). The discrepancy between anti-FVIII inhibitory titers and anti-FVIII IgG titers may be dependent on assay methods in which the former was one-stage APTT measurement and the latter was ELISA using an anti-FVIII monoclonal antibody as standard.

In haemophilia A patients, several researchers reported that IgG4 is the major component of anti-FVIII antibodies, although all IgG subclasses have been found [19,20]. In murine models, we showed that kinetics of anti-FVIII IgG1, IgG2a and IgG2b titers of haemophilia A mice with serial infusion of 0.05 U g^{-1} BW FVIII were similar to those administered 0.5 U g^{-1} BW FVIII (Fig. 4). In contrast, titers of anti-FVIII IgG3 subclass were decreased after 50–100 EDs in mice with serial infusion of 0.05 U g^{-1} BW FVIII. The Th1 immune response is believed predominant in patients with inhibitors in the long term [21], and was also the predominant response in mice that developed antibodies after challenge in adulthood [22,23]. We demonstrated a dose-dependent $CD4^+$ T-cell proliferative response to FVIII in preimmunized mice (five times injection of FVIII every 2 weeks), which is compatible with previous studies demonstrating that human FVIII is highly immunogenic in haemophiliac mice (Fig. 5) [24]. Interestingly, we observed that haemophilia A mice with sequential infusion of 0.05 U g^{-1} BW FVIII after 180 EDs failed to develop $CD4^+$ T-cell proliferative response to *in vitro* stimulation of FVIII antigen (Fig. 5). These T cells could not produce any IL-2, IL-4, IL-10, nor IFN-γ (Fig. 6), whereas those from mice immunized with five-times infusion of 0.5 U g^{-1} BW FVIII were able to secrete significant amounts of IL-2, IL-12 and IFN-γ. It is known that Th1 cells are initiators of antibody responses, and that they participate in class switching by releasing IFN-γ, which preferentially induces IgG2a and IgG3 in mouse [25]. Consequently, the FVIII-specific Th1 cytokine response may be partially suppressed by serial administration of FVIII in haemophilia A mice with inhibitors.

Several potential mechanisms of ITI have been identified [26]. These include clonal deletion (i.e. removal of immune-response cells through programmed cell death or apoptosis), anergy (failure of immune cells to respond to the FVIII molecule), or ignorance (i.e. the immune-response cells are 'blind' to the presence of FVIII). Our data suggest that sequential exposure of FVIII antigen could partially block anti-FVIII inhibitory antibody production, inducing T-cell anergy in haemophilia A mice with inhibitor, although our murine ITI model against heteroantibodies is fundamentally different from human ITI therapy against alloantibodies. However, further evaluation using completely continuous infusion system for the exposure of FVIII antigen will be necessary to confirm its efficacy in inducing immune tolerance [27]. Furthermore, understanding of the underlying mechanisms of immune tolerance induced by serial administration of FVIII is essential for the development of this strategy for haemophilia A patients with inhibitors.

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Author contributions

SM designed and performed the research, analyzed data and wrote the paper; EK, YK, AY and AS performed experiments; SM, TO, JM and YS analyzed data and revised the paper.

Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

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Regular Article

Predictive blood coagulation markers for early diagnosis of venous thromboembolism after total knee joint replacement

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Venous thromboembolism

ABSTRACT

Pulmonary embolism development may be prevented if asymptomatic venous thromboembolism (VTE) can be predicted and treated preoperatively or soon after total knee arthroplasty (TKA). The purpose of this study was to evaluate whether asymptomatic VTE can be predicted by blood coagulation markers preoperatively or early after TKA. This prospective single-centre study enrolled 68 patients (6 men, 62 women; mean age: 71 years) who underwent TKA between September 2004 and August 2009. Sixteen-row multidetector computed tomography was performed 4 days before and after surgery for diagnosis of asymptomatic VTE. Blood samples were taken to measure the plasma levels of soluble fibrin monomer complex (SFMC), D-dimer and cross-linked fibrin degradation products by leukocyte elastase (e-XDP) at 4 days preoperatively, and at 1 hour, 1 day and 4 days postoperatively. The preoperative SFMC, D-dimer and e-XDP levels did not differ significantly between the thrombus ($n=36$) and no-thrombus ($n=32$) groups. D-dimer and e-XDP levels showed the most significant increases at days 4 and 1, respectively, after surgery in the thrombus group. With cut-off points of 7.5 μg/ml for D-dimer and 8.2 U/ml for e-XDP, the sensitivities were 75% and 75%, and the specificities were 63% and 59%, respectively. By multiple logistic regression analysis, D-dimer at day 4 and e-XDP at day 1 postoperatively were independent markers for early diagnosis of VTE (odds ratio = 1.61 and 1.19, $P=0.01$ and 0.04, respectively). The postoperative occurrence of new asymptomatic VTE may be predicted by D-dimer at day 4 and e-XDP at day 1 after TKA.

Introduction

After arthroplasty, it is extremely important to prevent the development of postoperative venous thromboembolism (VTE), particularly symptomatic and fatal pulmonary embolism (PE), in orthopaedic surgery [1]. Since the 1990s, antithrombotic therapies using agents such as unfractionated and low molecular weight heparin have been administered to patients after surgery. However, despite the implementation of aggressive antithrombotic protocols, including those mandated by the American College of Chest Physicians (ACCP) [2], the incidence of fatal PE remains at 0.15% [3] and that of symptomatic PE remains at 0.41% [4] after total knee arthroplasty (TKA), with no changes since the 1990s. Pellergrini et al. [5] reported that 17% of patients with untreated deep vein thrombosis (DVT) experienced symptomatic PE after total hip arthroplasty (THA). While it is thought that prophylactic antithrombotic treatments are necessary to prevent postoperative fatal and symptomatic PE, previous reports found no differences in the incidences of fatal or symptomatic PE, regardless of whether or not prophylactic antithrombotic therapy was given [3,4,6], and that the infection rate was increased owing to haematoma caused by haemorrhage [7–9] and coagulation abnormalities [10] associated with prophylactic antithrombotic therapy early after surgery. It is also important for orthopaedic surgeons to avoid these complications, because such infections can last a lifetime or the patients can have a relapsing course if they achieve remission from the infection. The routine administration of prophylactic antithrombotic treatment is not recommended in the Japanese Guideline for Prevention of Venous Thromboembolism [11].

Abbreviations: ACCP, American College of Chest Physicians; e-XDP, Cross-linked fibrin degradation products by leukocyte elastase; DVT, deep vein thrombosis; MDCT, multidetector-row computed tomography; PE, pulmonary embolism; SFMC, Soluble fibrin monomer complex; THA, Total hip arthroplasty; TKA, total knee arthroplasty; VTE, Venous thromboembolism.

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Based on these observations, it is clinically important to detect asymptomatic VTE that may cause fatal or symptomatic PE before or shortly after surgery without prophylactic antithrombotic treatments to reduce postoperative infections in low-risk patients, and to start antithrombotic therapy only in those patients who need it [12].

The purpose of this study was to determine whether the postoperative occurrence of new asymptomatic VTE can be predicted by preoperative and postoperative measurements of blood coagulation markers in patients undergoing TKA, and to identify independent markers that will facilitate early diagnosis of asymptomatic VTE. We performed a prospective study using multidetector-row computed tomography (MDCT), which can detect PE and DVT simultaneously, to evaluate the predictive accuracy of various blood coagulation markers as indicators for postoperative asymptomatic VTE.

Materials and methods

Patients

The study protocol was approved by the Ethics Review Board of our university. This prospective single-centre study enrolled patients who underwent TKA at our institution between September 2004 and August 2009 and gave consent to participate in the study (Fig. 1). The necessary sample size was calculated for an alpha of 0.05 and a power of 0.90 using the statistical software 'G*Power 3' [13,14], and found to be 67. For exclusion criteria, patients with a past history of symptomatic VTE, cerebral haemorrhage, cerebral infarction, cardiac infarction and drug allergy to a contrast medium were excluded from the study. In addition, patients with liver disease, renal disease and congenital clotting factor deficiencies and those undergoing antithrombotic therapy or haemodialysis were excluded from the study. Patients with asymptomatic VTE by preoperative MDCT were also excluded from the study (Fig. 1). However, patients with hypertension, diabetes mellitus and rheumatoid arthritis were included in this study.

We finally enrolled 68 patients with low risk factors who underwent TKA for osteoarthritis (45 knees) or rheumatoid arthritis (23 knees). The patients comprised 6 men and 62 women, with a mean age of 71 years (range, 49–84 years). TKA was performed under general anaesthesia in all patients and a pneumatic tourniquet was used. During and after the surgery, the patients wore elastic stockings and used an intermittent pneumatic compression device until the initiation of walking training, in accordance with the Japanese Guideline for Prevention of Venous Thromboembolism [11]. No postoperative prophylactic antithrombotic therapy was administered. If the patients developed symptomatic VTE and if VTE was detected by MDCT, the study was discontinued and aggressive antithrombotic therapy was initiated.

MDCT

For diagnosis of VTE, 16-row MDCT was performed at 4 days preoperatively (day of admission) and 4 days postoperatively (Fig. 2), the point at which the incidences of PE and VTE are reported to be high [15] and the earliest point at which the patients could comfortably undergo MDCT during the postoperative period. The slice thicknesses were 2 mm in the thoracic region and 5 mm from the abdomen to the lower limbs. The window levels were 40–60 and 40–50 and the window widths were 400–500 and 200–400, respectively. A single radiologist (M.D.) evaluated the MDCT images in a blinded manner before and after the surgery. The incidence of postoperative new asymptomatic VTE was calculated.

Patients with asymptomatic VTE ($n=7$) by preoperative MDCT were excluded from the study. Preoperative MDCT revealed asymptomatic PE of the pulmonary segmental artery in one patient, proximal asymptomatic DVT in one patient and distal asymptomatic DVT in five patients (Fig. 1). These patients did not show D-dimer abnormalities. Preoperative MDCT revealed no asymptomatic VTE in the 68 patients included in the study. For the patients with proximal asymptomatic DVT and asymptomatic PE, antithrombotic therapy was initiated because the occurrence of fatal or symptomatic PE was considered likely [16].

The thrombus group was defined as patients with new asymptomatic VTE detected by MDCT after the surgery, and the no-thrombus group was defined as patients without asymptomatic VTE by MDCT after the surgery.

Blood coagulation markers

Blood samples were taken to measure the plasma levels of soluble fibrin monomer complex (SFMC), D-dimer and cross-linked fibrin

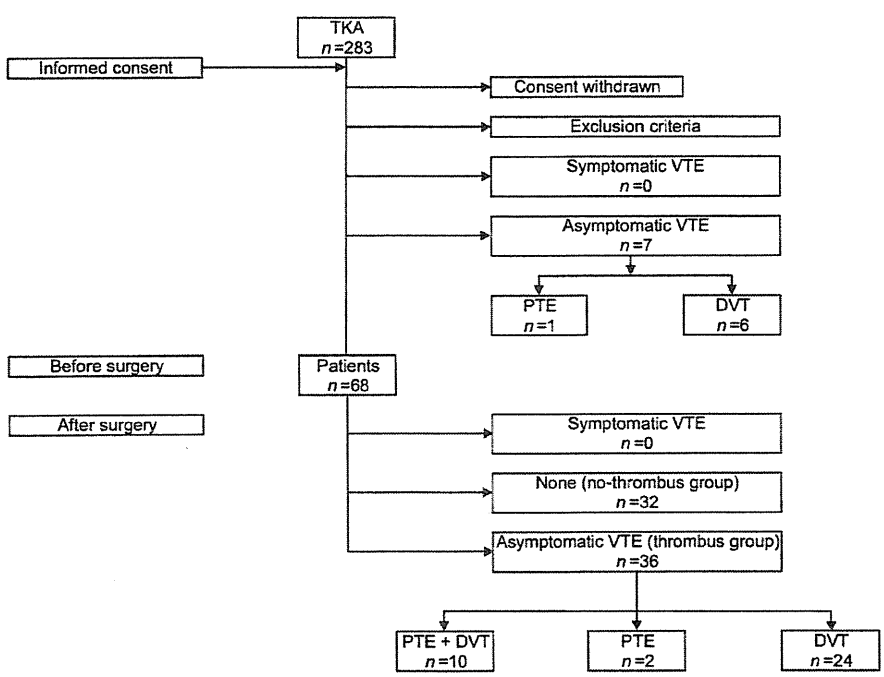

Fig. 1. Flowchart of the 283 patients undergoing TKA during the study period. *n*, number of patients.

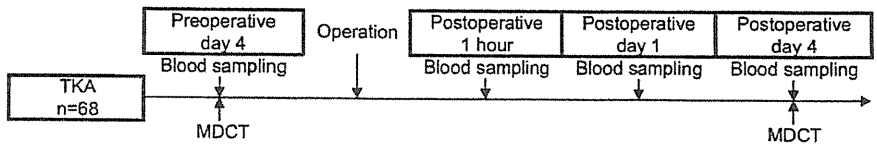

Fig. 2. Study protocol.

degradation products by leukocyte elastase (e-XDP) at 4 days preoperatively, and then at 1 hour, 1 day and 4 days postoperatively (Fig. 2). Citrated plasma samples were stored at −80 °C until analysis. The plasma SFMC levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) using the monoclonal antibody IF-43 [17]. Plasma D-dimer levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody JIF-23 [18]. Plasma e-XDP levels were measured by a latex immunoagglutination assay (Mitsubishi Chemical Medience Corporation) using the monoclonal antibody IF-123 [19].

Statistical analysis

Statistical analyses were performed using SPSS for Windows version 11.0 software (SPSS, Chicago, IL, USA). SFMC, D-dimer and e-XDP levels were analysed by the Shapiro–Wilk test if they did not fit a normal distribution. SFMC, D-dimer and e-XDP levels were compared at 4 days preoperatively and at 1 hour, 1 day and 4 days preoperatively using the Friedman test. If a significant difference was noted, the data were compared using the Wilcoxon signed rank test and corrected using Bonferroni's inequality. SFMC, D-dimer and e-XDP levels were compared between the thrombus and no-thrombus groups using the Mann–Whitney *U* test. Sex and diabetes mellitus distributions were compared between the thrombus and no-thrombus groups using Fisher's exact test, while hypertension distributions were compared between the thrombus and no-thrombus groups using the chi-square test. Age, volume of intraoperative haemorrhage, operation time, other presurgical factors and blood markers were compared using an unpaired *t*-test. Multiple logistic regression analyses were used to determine whether blood coagulation markers were independent predictors of the postoperative occurrence of new asymptomatic VTE or were affected by other factors. The level of statistical significance was set at $P<0.05$ for all tests.

Results

No patients developed symptomatic VTE after TKA in this study (Fig. 1). Postoperative MDCT revealed asymptomatic VTE in 36 patients (thrombus group) and no VTE in 32 patients (no-thrombus group) (Fig. 1). Aggressive antithrombotic therapy was initiated in the 36 patients in whom new asymptomatic VTE was detected on postoperative MDCT.

Preoperative blood coagulation markers

There were no significant differences in preoperative SFMC, D-dimer and e-XDP levels between the thrombus and no-thrombus groups ($P=0.13$, $P=0.18$ and $P=0.15$, respectively; Fig. 3).

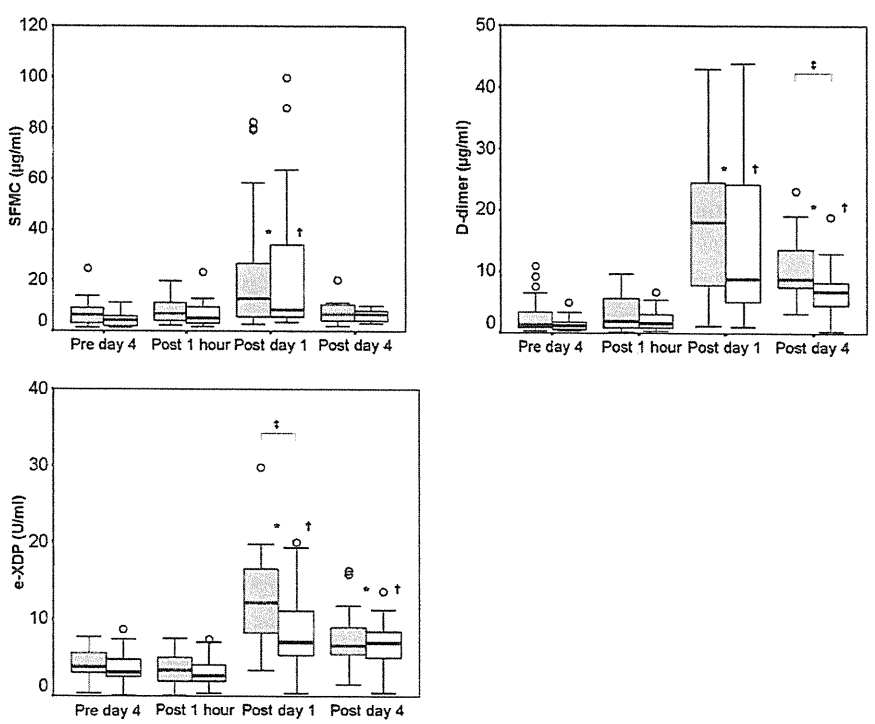

Fig. 3. Preoperative and postoperative SFMC, D-dimer and e-XDP levels. Gray boxes, thrombus group; white boxes, no-thrombus group. The circles are outliers. *$P<0.05$ versus the preoperative level in the thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. †$P<0.05$ versus the preoperative level in the no-thrombus group by the Wilcoxon signed-rank test with correction by Bonferroni's inequality. ‡$P<0.05$, thrombus group versus no-thrombus group by the Mann–Whitney *U* test.

Postoperative blood coagulation markers

SFMC

SFMC levels differed significantly at day 1 postoperatively in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, a significant increase in the SFMC level was observed at postoperative day 1 (median: 12.8 μg/ml; interquartile range: 5.3 to 27.2) compared with the preoperative value (median: 6.2 μg/ml; interquartile range: 3.1 to 9.3; $P=0.01$). Similarly, in the no-thrombus group, a significant increase in the SFMC level was observed at postoperative day 1 (median: 8.5 μg/ml; interquartile range: 5.6 to 34.9) compared with the preoperative value (median: 4.2 μg/ml; interquartile range: 2.2 to 6.4; $P=0.01$). However, postoperative changes in the SFMC level at postoperative day 1 did not differ significantly between the thrombus and no-thrombus groups ($P=0.85$; Fig. 3). SFMC levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour (median: 6.9 μg/ml; interquartile range: 4.3 to 11.4; $P=0.10$; and median: 5.3 μg/ml; interquartile range: 3.1 to 9.8; $P=0.07$, respectively) and 4 days (median: 6.8 μg/ml; interquartile range: 4.1 to 10.6; $P=0.27$; and median: 6.6 μg/ml; interquartile range: 4.2 to 8.2; $P=0.06$, respectively) postoperatively (Fig. 3).

D-dimer

D-dimer levels differed significantly at postoperative days 1 and 4 in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, significant increases in D-dimer levels were observed at postoperative day 1 (median: 18.1 μg/ml; interquartile range: 7.3 to 25.8) and day 4 (median: 8.8 μg/ml; interquartile range: 7.4 to 13.7) compared with the preoperative value (median: 1.3 μg/ml; interquartile range: 0.8 to 3.9; $P=0.01$ for both time points). In the no-thrombus group, significant increases in D-dimer levels were observed at postoperative day 1 (median: 8.8 μg/ml; interquartile range: 4.8 to 25.2) and day 4 (median: 6.8 μg/ml; interquartile range: 4.3 to 8.6) compared with the preoperative value (median: 1.2 μg/ml; interquartile range: 0.6 to 1.8; $P=0.01$ for both time points). With regard to the postoperative changes, the D-dimer level at postoperative day 4 was significantly higher in the thrombus group than that in the no-thrombus group ($P=0.01$; Fig. 3). With a cut-off D-dimer level of 7.5 μg/ml, the sensitivity was 75%, the specificity was 63% and the likelihood ratio (sensitivity/1−specificity) was 2.03 for predicting postoperative asymptomatic VTE (Fig. 4). D-dimer levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour postoperatively (median: 1.9 μg/ml; interquartile range: 0.9 to 5.6; $P=0.39$; and median: 1.5 μg/ml; interquartile range: 0.9 to 3.4; $P=0.09$, respectively) (Fig. 3).

e-XDP

The e-XDP levels differed significantly at postoperative days 1 and 4 in the thrombus and no-thrombus groups (Fig. 3). In the thrombus group, significant increases in e-XDP levels were observed at postoperative day 1 (median: 12.2 μg/ml; interquartile range: 7.9 to 18.2) and day 4 (median: 6.7 μg/ml; interquartile range: 5.3 to 9.0) compared with the preoperative value (median: 3.8 μg/ml; interquartile range: 3.0 to 5.6; $P=0.01$ for both time points). In the no-thrombus group, significant increases in e-XDP levels were observed at postoperative day 1 (median: 7.0 μg/ml; interquartile range: 5.3 to 11.1) and day 4 (median: 7.0 μg/ml; interquartile range: 4.6 to 8.4) compared with the preoperative value (median: 3.1 μg/ml; interquartile range: 2.6 to 4.7; $P=0.01$ for both time points). With regard to the postoperative changes, the e-XDP level at postoperative day 1 was significantly higher in the thrombus group than that in the no-thrombus group ($P=0.01$; Fig. 3). With a cut-off e-XDP level of 8.2 U/ml, the sensitivity was 75%, the specificity was 59% and the likelihood ratio was 1.84 for predicting postoperative asymptomatic VTE (Fig. 4). The e-XDP levels did not differ significantly from the preoperative values in the thrombus and no-thrombus groups at 1 hour postoperatively (median: 3.3 U/ml; interquartile range: 2.0 to 5.1; $P=0.33$; and median: 2.7 U/ml; interquartile range: 2.0 to 4.4; $P=0.08$, respectively) (Fig. 3).

There was a significant difference in the minimum blood pressure, but no significant differences in age, sex, volume of intraoperative haemorrhage, operation time or other presurgical factors between the thrombus and no-thrombus groups (Table 1). Multiple logistic regression analyses revealed that the D-dimer level at postoperative day 4 differed significantly among the other factors and was an independent marker of postoperative new asymptomatic VTE by MDCT (odds ratio = 1.61, $P=0.01$; Table 2). Likewise, the e-XDP level at postoperative day 1 differed significantly among the other factors and was an independent marker of postoperative new asymptomatic VTE (odds ratio = 1.19, $P=0.04$; Table 2).

Discussion

Recently, MDCT has been used as a technique to identify VTE. In addition, MDCT is able to diagnose PE to the level of the subsegmental pulmonary arteries and can provide rapid and objective detection and

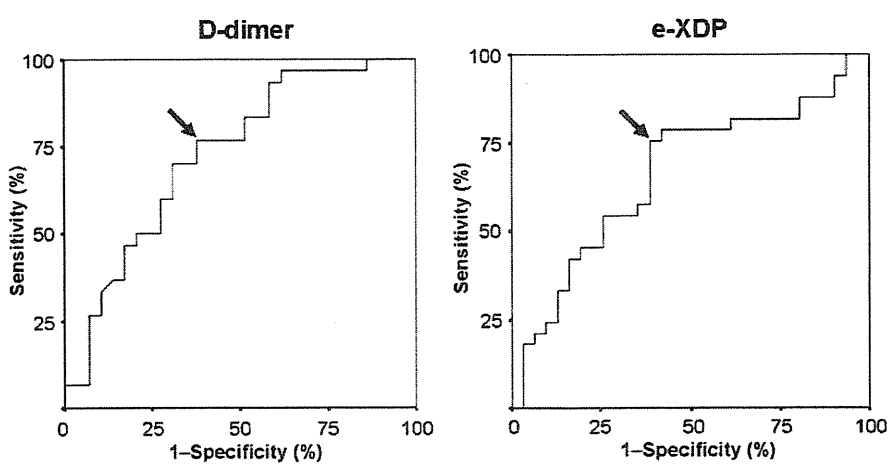

Fig. 4. Receiver-operator characteristic curves. Left: At a cut-off point of 7.5 μg/ml for the D-dimer level at postoperative day 4, the sensitivity is 75% (95% confidence interval: 73 to 77), the specificity is 63% (95% confidence interval: 60 to 66) and the likelihood ratio is 2.03. Right: At a cut-off point of 8.2 U/ml for the e-XDP level at postoperative day 1, the sensitivity is 75% (95% confidence interval: 73 to 77), the specificity is 59% (95% confidence interval, 56 to 62) and the likelihood ratio is 1.84.

Table 1
Patient characteristics.

		Thrombus group (n=36)	No-thrombus group (n=32)	P		95% Confidence interval
					(Odds ratio)	
Sex, male:female		1:35	5:27	0.09*	0.15§	0.02 to 1.40
Hypertension:normotension		18:18	14:18	0.61†	1.29¶	0.50 to 3.35
Diabetes mellitus:normoglycaemia		2:34	4:28	0.41*	0.41#	0.07 to 2.42
					*(Difference**)*	
Age	years	71 (68 to 73)††	72 (68 to 75)††	0.82‡	-0.5	-5 to 4
Intraoperative haemorrhage	ml	167 (0 to 344)††	175 (4 to 346)††	0.95‡	-7	-252 to 236
Operation time	min	161 (147 to 174)††	150 (140 to 160)††	0.23‡	10	-7 to 27
Before surgery						
Height	cm	148 (146 to 151)††	150 (148 to 153)††	0.25‡	-2	-6 to 1
Weight	kg	57 (54 to 60)††	57 (52 to 61)††	0.91‡	0	-5 to 5
Body mass index	kg/m^2	26 (25 to 27)††	25 (23 to 27)††	0.51‡	1	-2 to 3
Blood pressure (maximum)	mmHg	128 (124 to 133)††	127 (121 to 132)††	0.61‡	2	-5 to 9
Blood pressure (minimum)	mmHg	74 (71 to 77)††	67 (63 to 70)††	0.01‡	8	4 to 12
Pulse rate	/min	73 (70 to 76)††	71 (68 to 73)††	0.18‡	3	-1 to 7
Temperature	°C	36.5 (36.4 to 36.6)††	36.5 (36.4 to 36.7)††	0.58‡	-0.1	-0.2 to 0.1

*Fisher's exact test; †chi-square test; ‡unpaired t-test.
§Male/female; ¶hypertension/normotension; #diabetes mellitus/normoglycaemia; **thrombus-no thrombus; ††mean (95% confidence interval).

measurement of thrombi in both PE and DVT [20–24]. In patients in whom symptomatic PE is suspected, the usefulness of this modality is supported by its high sensitivity and specificity, which have been reported to be 100% and 89% for PE using pulmonary angiography as the reference standard [20], 100% and 96.6% for proximal DVT using doppler sonography as the reference standard [21] and 93% and 97% for distal and proximal DVT using doppler sonography and venography as the reference standard [22], respectively. Additionally, sensitivity and specificity have been reported to be 100% and 97% for distal DVT, 100% and 97% for proximal DVT, respectively, using conventional venography as the reference standard in patients in whom symptomatic DVT was suspected [23]. However, it is unknown how the sensitivity and specificity for only distal DVT of MDCT can be estimated, because there are few studies that have estimated sensitivity and specificity; overall, the sensitivity (ranges from 71—100%) and specificity (ranges from 93—100%) of MDCT are high [24]. A highly sensitive and specific imaging examination is necessary for early detection of VTE. However, there are problems with this approach that limit its practicality, such as exposure to radiation [25,26], invasive administration of contrast medium, potential for drug allergy, cost of equipment and the frequent imaging required to detect VTE, the occurrence of which is unpredictable. Therefore, initial evaluation of the presence of VTE using blood markers is preferable, with only high-risk patients examined by imaging techniques, to reduce the potential risks associated with radiation or contrast exposure and to improve the cost-effectiveness.

There were no blood coagulation markers for predicting early postoperative asymptomatic DVT after TKA until 1997 [27,28]. In 2000, Rever at al. [29] performed venography after TKA and reported that the SFMC level was significantly elevated in patients with asymptomatic DVT at postoperative days 3 and 6. However, they concluded that there was no clinically significant cut-off point. Similarly, the present study could not establish that the SFMC level was associated with asymptomatic VTE after TKA or an independent marker for predicting the postoperative occurrence of new asymptomatic VTE.

In 1998, Bounameaux et al. [30] performed venography and D-dimer measurements at day 3 after TKA. They found that the D-dimer level was significantly elevated in patients with asymptomatic DVT and that the sensitivity and specificity were 58.8% and 73.5%, respectively, at a cut-off level of 3000 μg/ml. In the present study, the D-dimer level at postoperative day 4 was significantly higher in the thrombus group than that in the no-thrombus group. The sensitivity, specificity and likelihood ratio of the D-dimer level at postoperative day 4 using a cut-off point of 7.5 μg/ml were 75%, 63% and 2.03, respectively, for predicting postoperative asymptomatic VTE. Furthermore, by multiple logistic regression analysis, the D-dimer level at postoperative day 4 was an independent marker for predicting postoperative asymptomatic VTE, whereas, except for the minimal blood pressure, there were no significant differences in age, sex, volume of intraoperative haemorrhage, operation time and presurgical factors or blood markers between the thrombus and no-thrombus groups.

Table 2
Multiple logistic regression analyses of blood coagulation markers and other factors.

		Postoperative day 1			Postoperative day 4		
		Odds ratio	95% Confidence interval	P	Odds ratio	95% Confidence interval	P
SMFC	μg/ml	1.00	0.98 to 1.02	0.84	1.00	0.97 to 1.04	0.88
D-dimer	μg/ml	0.97	0.93 to 1.02	0.29	1.61	1.12 to 2.20	0.01
e-XDP	U/ml	1.19	1.01 to 1.40	0.04	0.75	0.54 to 1.05	0.10
WBC	/μl	1.00	1.00 to 1.00	0.67	1.00	1.00 to 1.00	0.93
RBC	$\times 10^4/\mu l$	1.00	0.97 to 1.03	0.96	1.03	0.98 to 1.08	0.26
Hct	%	0.37	0.09 to 1.55	0.18	0.47	0.12 to 1.84	0.28
Hb	g/dl	13.49	0.30 to 604.51	0.18	3.34	0.06 to 239.21	0.52
Plt	$\times 10^4/\mu l$	1.05	0.94 to 1.17	0.40	1.16	0.97 to 1.38	0.10
Blood pressure (maximum)	mmHg	0.55	0.30 to 1.00	0.51	0.55	0.25 to 1.22	0.14
Blood pressure (minimum)	mmHg	2.31	0.84 to 6.40	0.11	2.73	0.93 to 8.00	0.07
Pulse rate	/min	0.91	0.42 to 2.00	0.82	0.75	0.29 to 1.94	0.55
Temperature	°C	1.2	0.46 to 3.08	0.72	12.47	0.88 to 177.10	0.06

WBC=white blood cell count; RBC=red blood count; Hct=haematocrit; Hb=haemoglobin; Plt, platelet count.

It has been reported that the level of e-XDP, which are the fibrin degradation products by leukocyte elastase released from activated leukocytes [31], is useful for the diagnosis and prognosis of disseminated intravascular coagulation [32–34] and the diagnosis of symptomatic DVT [35]. However, no previous studies have measured the e-XDP level before and after orthopaedic surgery to evaluate its usefulness for diagnosis of postoperative VTE. In this study, the e-XDP level at postoperative day 1 was significantly higher in the thrombus group than that in the no-thrombus group. The diagnostic sensitivity, specificity and likelihood ratio of the e-XDP level at postoperative day 1 using a cut-off point of 8.2 U/ml were 75%, 59% and 1.84, respectively, for predicting postoperative asymptomatic VTE. Therefore, the e-XDP level at postoperative day 1 is estimated to be a blood marker for early prediction of postoperative asymptomatic VTE. Furthermore, by multiple logistic regression analysis, the e-XDP level at postoperative day 1 differed significantly among the other factors and there were no significant differences in age, sex, volume of intraoperative haemorrhage, operation time or other presurgical factors, except for the minimal blood pressure, or blood markers between the thrombus and no-thrombus groups. Therefore, this study has established that the e-XDP level at postoperative day 1 and the D-dimer level at postoperative day 4 are associated with asymptomatic VTE after TKA and are independent markers for predicting the postoperative occurrence of new asymptomatic VTE. Recent studies have demonstrated that leukocyte elastase-mediated fibrinolysis is activated to varying degrees depending on the amount of systemic inflammation, such as a major surgical procedure, and sepsis as an alternative pathway to the plasminogen activator-plasmin system [32–36]. We consider that leukocyte elastase-mediated fibrinolysis may have been mainly activated in the early phase as an alternative pathway, and then plasmin may have been mainly activated in the late phase as the plasminogen activator-plasmin system in patients who developed asymptomatic VTE after TKA. If leukocyte elastase causes asymptomatic VTE and subsequently symptomatic and fatal PE, inactivation of leukocyte elastase may prevent the development of symptomatic and fatal PE.

The postoperative occurrence of new asymptomatic VTE could not be predicted from the preoperative SFMC, D-dimer or e-XDP levels. Dunn et al. [37], who performed venography after TKA or THA, and Bounameaux et al. [30], who employed venography after TKA, compared the preoperative D-dimer levels between patients who did and did not develop DVT postoperatively and reported no significant differences. Our results are consistent with these previous reports, and we could not establish that preoperative blood coagulation markers are associated with VTE after TKA.

In this study, the early diagnosis of new asymptomatic VTE that can be predicted by the D-dimer level at postoperative day 4 and the e-XDP level at postoperative day 1 after TKA may prevent PE development after TKA. Therefore, in daily clinical practice, we consider that measurements of e-XDP at postoperative day 1 and D-dimer at postoperative day 4 are necessary for early detection of asymptomatic VTE in patients who have low risk factors because they are independent markers. However, we need to further verify these measurements in larger studies to determine the adequate cut-off points, sensitivities and specificities of e-XDP at postoperative day 1 and D-dimer at postoperative day 4 for preventing symptomatic and fatal PE in the perisurgical period. One limitation of our study is that we do not know whether early detection of asymptomatic VTE prevents symptomatic and fatal PE. Therefore, we have continued to follow up the patients in daily clinics after completing this study, and no patients have suffered from symptomatic and fatal PE to date. Another limitation of our study is that MDCT was performed 4 days preoperatively and postoperatively, and the results therefore reflect the state of asymptomatic VTE at these time points. We believe that almost all asymptomatic VTEs may disappear spontaneously within 2 or 3 days postoperatively and that an asymptomatic VTE that does not disappear spontaneously within 2 or 3 days postoperatively can cause symptomatic and fatal PE in low-risk patients after TKA. This is because the incidence of PE was reported to be high at days 3 or 4 postoperatively after TKA [15]. Furthermore, that is the earliest point at which the patients had less pain and could comfortably undergo MDCT during the postoperative period. However, since MDCT was not performed from the operative day to postoperative day 3, it can be assumed that not all asymptomatic VTE were detected in the perisurgical period. Therefore, the incidence of postsurgical asymptomatic VTE may be underestimated, and the roles of e-XDP and D-dimer levels in predicting asymptomatic VTE in the perisurgical period must be further verified in larger studies.

Conflict of interest

The authors state that they have no conflict of interest.

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第72回日本血液学会学術集会
シンポジウム4
Recent Advance in Thrombosis, Hemostatis, and Vascular Biology: From a Clinical Aspect

血友病に対する遺伝子細胞治療—最近の進歩

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Key words: Hemophilia, Gene therapy, Adeno-associated virus vector, Lentivirus vector

1. はじめに

現在行われている血友病の治療は，出血時に出血時に凝固因子製剤を投与するオンデマンド治療が大部分で，定期補充療法は一部の症例に行われているにすぎない。凝固因子の定期補充療法は出血リスクを減らすが週2ないし3回の凝固因子製剤の静脈注射を行う必要がある。凝固因子を体内で産生し治癒にも繋がる血友病遺伝子治療も精力的に研究され，臨床治験もいくつか行われてきた。ほとんどの臨床治験は十分な効果が得られていなかったが，最近始められた血友病B遺伝子治療では有効であるとの報告がされていることから，遺伝子治療は血友病の次世代治療法として期待されている。

しかし，遺伝子治療が完全に安全であるということではない。種々の疾患に対する次世代治療法として遺伝子治療が研究されてきた。レトロウイルスベクターをもちいγc遺伝子を導入する重症免疫不全症X-SCIDの臨床治験は最も成功した遺伝子治療であるが，T細胞白血病様の病態がおこるという重大な副作用も生じた[1~3]。この事象は血友病遺伝子治療には当てはまらないと考えられているが，遺伝子治療に附随する安全性の問題は常に検証されなくてはならないことである[4]。

2. 血友病遺伝子治療のクリニカルエンドポイント

血友病は重症（欠乏する凝固因子活性1%未満），中等症（欠乏する凝固因子活性1~5%未満），軽症（欠乏する凝固因子活性5%以上）に分けられ，重症では日常生活においても出血が起こるが，中等症では重症出血の頻度は低く，軽症例では日常生活ではほとんど出血しない。これらの臨床経験を踏まえて，血友病遺伝子治療の対象は重症例であり，エンドポイントは欠乏する凝固因子活性を1%以上に，理想的には5%以上に上昇させることである。このように，血友病治療として恒常的に発現させる目標凝固因子レベルの治療域が広く遺伝子治療の良い適応といえる。

3. 血友病遺伝子細胞治療の戦略

血友病はX染色体上の第VIII因子遺伝子あるいは第IX因子遺伝子の異常によりこれらの凝固因子欠乏が起こるため，現在の血友病の遺伝子治療研究は正常凝固因子遺伝子を導入し正常凝固因子を産生させることに主眼がおかれている。もちろん将来的には遺伝子異常の是正のような究極のストラテジーも期待出来る。

正常凝固因子を生体内で発現させる方法は，凝固因子遺伝子を搭載したベクターを直接生体に投与する体内法と，凝固因子を発現する細胞を移植する体外法に大別できる（図1）。

1）体外法におけるベクターの選択

ベクターはウイルスベクターと非ウイルスベクターに大別できる。ウイルスベクターは，遺伝子導入効率は優れるが免疫系との確執がある。すなわち，ヒトは病原体として認識するウイルスを排除するように，また次の感染に対処するように反応する。一方，ウイルスは宿主に感染し自己増殖する。このためウイルスベクターは細胞へ感染し遺伝子導入をおこなう効率が高いが免疫系を活性化する。一方，非ウイルスベクターは免疫系との反応は少ないと考えられているが遺伝子導入効率は低く，現在の遺伝子治療研究はウイルスベクターを用いる方法が主流である。

生体に直接投与可能なベクターには，(1) 遺伝子導入効率が良い (2) 染色体に組み込まれない (3) 非分裂細胞にも遺伝子導入可能である (4) 病原性がない，など

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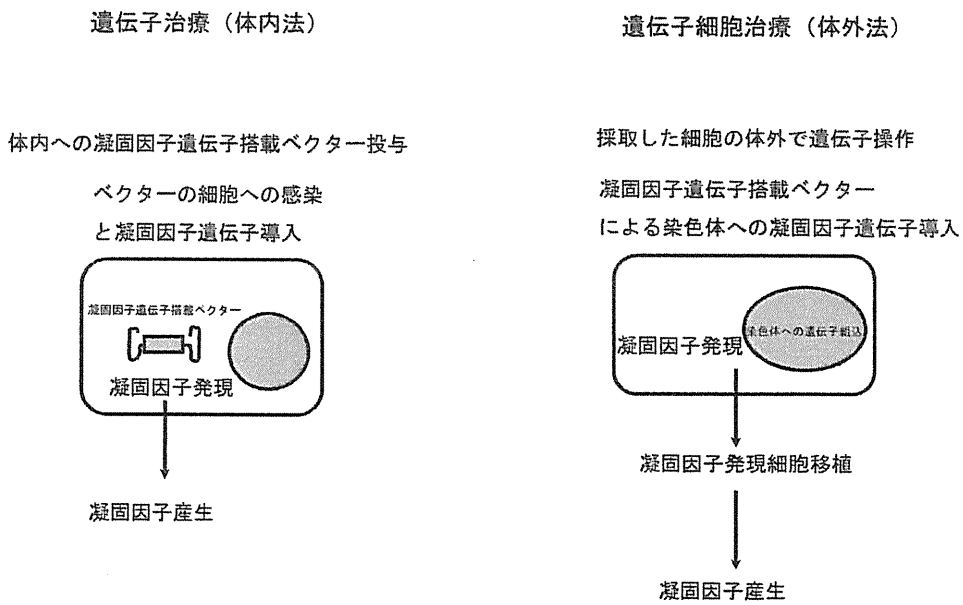


図 1　遺伝子・細胞治療
治療遺伝子搭載ベクターを直接体内に投与する体内法と，遺伝子操作を加えた細胞を移植する体外法を模式的に示す。

の性質が要求される。遺伝子治療研究の初期に用いられていたアデノウイルスベクターは病原性，血液障害性，さらに免疫系への影響が強く，レトロウイルスベクターとレンチウイルスベクターは染色体への組込が必至である。これらのベクターと異なり，アデノ随伴ウイルス（adeno-associated virus, AAV）ベクター（図 2）は上記条件を満たすことから遺伝子治療研究と臨床治験に用いられている。パーキンソン病遺伝子治療を始めとするAAV ベクターを用いた臨床治験が行われ，成果が得られている。ベクター開発が進み，血友病遺伝子治療研究では実験動物レベルで凝固因子の正常域からそれ以上までの発現も可能となり，最近の血友病 B 遺伝子治療臨床治験でも成果が得られつつある。

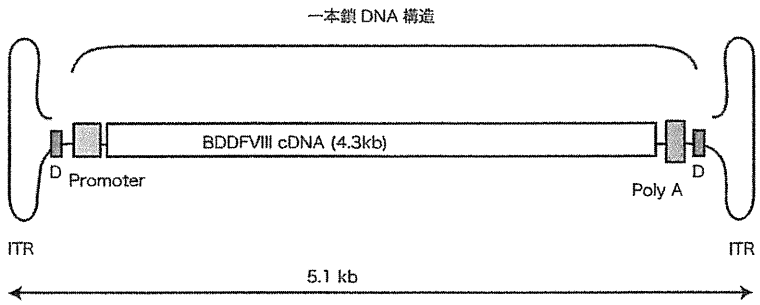
(1) アデノ随伴ウイルス（Adeno-associated virus, AAV）ベクター

非病原性のパルボウイルスの AAV に由来する AAV ベクターは，両端に Inverted terminal repeat（ITR）配列を残すのみで，他の大部分のウイルス由来遺伝子が除去されプロモーター・治療遺伝子・poly A 付加配列に置換されている（図 1）。自己複製能はなく，染色体への組込がほとんど起こらないため安全性が高く，さらに非分裂細胞にも遺伝子導入可能で導入遺伝子の長期発現が期待できるなど，遺伝子治療のベクターとしての適性を備えている。また，AAV 血清型（serotype）により細胞・臓器特異性が異なり，標的臓器に適した血清型 AAV ベクターを選択することが出来る。AAV serotype 2 (AAV2) ベクターは，血友病 B 遺伝子治療の臨床治験にも用いられてきたが，肝臓を遺伝子導入のターゲットとするには AAV5 ベクターや AAV8 ベクターが適していると考えられる。しかし，搭載可能な遺伝子サイズには依然として全長で約 5 kb の制限がある。FVIII 遺伝子を分割して AAV ベクターに搭載する dual vector system では同一細胞内で FVIII 重鎖と FVIII 軽鎖が発現し結合しなくてはならず，効率が悪い。極短い塩基長のミニマムプロモーターと BDDFVIII cDNA を用いた single vector system などの試みがなされ（図 2），肝臓への遺伝子導入効率が高い AAV8 ベクターと発現効率の高いミニマムプロモーターを用いることで高いレベルの第 VIII 因子発現が期待できる[5,6]。

(2) レンチウイルスベクター

従来用いられてきた MLV レトロウイルスベクターと比較し，分裂細胞，非分裂細胞のどちらにも効率の良い遺伝子導入が可能である。染色体への組み込みがおこり，分裂細胞へ治療遺伝子が組み込まれれば安定した治療遺伝子の発現が期待できる。レンチウイルスベクターとして human immunodeficiency virus（HIV）由来のベクターが詳細に検討され，第三世代化されほとんどのウイルス由来の遺伝子は除かれ LTR も改変され自己不活化（SIN vector）されている。central poly purine tract や WPRE などを組み込むことで遺伝子導入効率や発現効率が著しく改善し，さらにインシュレーターを組み込むことで安全性を高めている。第三世代化 HIV ベク

A 第 VIII 因子遺伝子 (BDDFVIII) 搭載 AAV vector



B 第 IX 因子遺伝子 (FIX minigene) 搭載 AAV vector

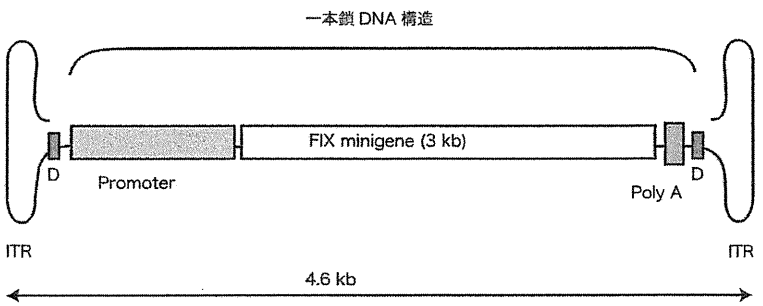


図 2 AAV ベクター

AAV は本来一本鎖 DNA ウイルスで，一般的に AAV ベクターといえば一本鎖 AAV ベクター single strand AAV vector を指す。inverted terminal repeat（ITR）配列に挟まれるように promoter-治療遺伝子-polyA 付加配列と並んでいる。AAV は複製の過程で二量体構造をとるが，AAV Rep の作用により一本鎖 DNA となり Capsid 内にパッケージされ AAV ベクターとなる。

ベクターが感染した細胞内で一本鎖 DNA として放出されるが，ベクター同士が結合あるいは相補鎖が合成されることで二本鎖となり効率のよい遺伝子発現が得られる。

A: 第 VIII 因子遺伝子搭載 AAV ベクター

AAV ベクターは搭載遺伝子長が約 5 kb に限られ，BDDFVIII cDNA は 4.3 kb あるため，0.3 kb までの極短い塩基長プロモーターしか用いることが出来ない。

B: 第 IX 因子遺伝子搭載 AAV ベクター

FIX 遺伝子搭載 AAV ベクターは FIX 遺伝子（イントロン配列を含む minigene）が約 3 kb であるため 1 kb 以上のプロモーターを用いることが出来る。

ターがサラセミア遺伝子治療臨床治験に用いられ治療効果が得られている[7~9]。しかし，遺伝子治療による HMGA2 遺伝子の活性化も起こっている。これ自体は治療に結びつくものであり白血病を引き起こすことに繋がっていないが，いわゆる挿入変異に関連する事象と考えられる。HIV ベクター以外では simian immunodeficiency virus（SIV）由来のベクターなどが検討されている。我々も SIV ベクターを利用して，CD34 陽性細胞への効率の良い遺伝子導入[10]と高い第 VIII 因子発現を得ることが出来た（図 3）。この方法を発展させ，血小板へ第 VIII 因子あるいは活性型第 VII 因子を，血小板プロモーターを用いて特異的に発現させることで血友病 A

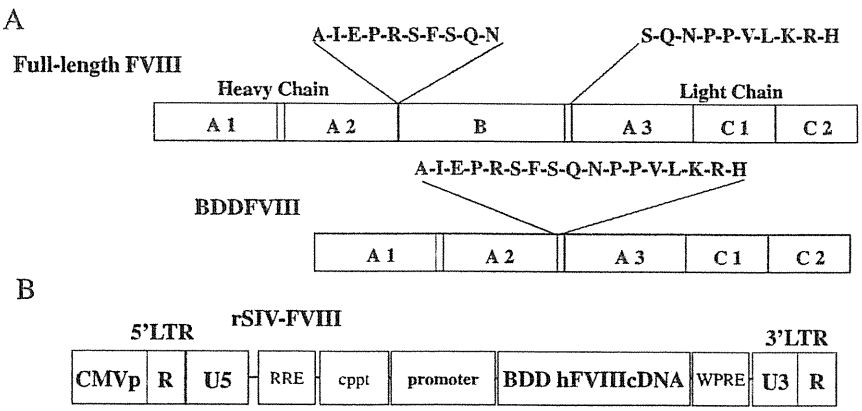


図3 第VIII因子遺伝子搭載SIVベクター
A: 完全長FVIIIとBドメイン欠如型FVIII (B domain deleted FVIII, BDDFVIII)の構造を模式的に示す。Bドメインは細胞で産生される時に部分的に切除され，活性化反応で完全に除去され，FVIII活性発現には直接は関係しない。FVIII cDNAには遺伝子発現を抑制する配列がBドメインをコードする塩基配列にあるため，BDDFVIIIを発現させるBDDFVIII cDNAが血友病A遺伝子治療研究で主として用いられている
B: 第VIII因子遺伝子を搭載する第三世代化された自己不活型SIVベクターを模式的に示す。LTRは修飾され自己不活型となり，遺伝子発現に重要なcentral poly purine tract (cppt)と遺伝子発現増強のためのWPRE配列を搭載している。BDDFVIII遺伝子を搭載するように示してあるが，Bドメインを部分的に欠如する第VIII因子遺伝子も搭載可能である。

マウスの出血傾向を改善させることができ[11,12]，自己骨髄移植を応用した血友病A遺伝子治療の可能性を示し得た。また，同じベクターを用いて脂肪細胞への第VIII因子遺伝子導入と第VIII因子の発現にも成功している[13]。SIVベクターは，まもなく眼科領域での臨床治験に用いられることになっており，標的細胞によっては良いベクターといえる。

(3) アデノウイルスベクター

アデノウイルスベクターは，免疫原性が高いこと，肝臓障害や造血障害が発生することが問題とされてきた。アデノウイルス由来の遺伝子の大部分を除去し，ベクター投与に伴う免疫反応や細胞障害性を低減した第2世代ベクター（Gutless adenovirus vector）を用い，血友病Aモデルマウスと血友病Aイヌに対してイヌ第VIII因子遺伝子を導入し有効性が示されているがヒトへ傷害性のない投与量までは減量できていない[14]。ポリエチレングリコール付加などの化学修飾で生体反応を軽減されたアデノウイルスベクターの開発が試みられているとされていたがその後の報告はない。

2) 体外法における細胞とベクターの選択

免疫学的排除を考えると，自己細胞移植が選択される。細胞治療においても細胞に遺伝子操作を加える必要がある。ウイルスベクターも非ウイルスベクターも用いることができるが，細胞治療においては染色体へ治療遺伝子を組み込む必要がある。遺伝子導入に伴う変異の影響を最小限にすることと，導入遺伝子のサイレンシングは解決されなくてはならない問題である。移植後の細胞寿命も考慮すると幹細胞の移植が，また移植後の増殖を懸念すれば幹細胞で遺伝子導入し終末分化させて移植する方法が考えられる。ES細胞やiPS細胞も研究されているが，腫瘍形成性の問題が解決される必要がある。ただし，遺伝子導入した自己iPS細胞由来の血小板や赤血球などは無核で腫瘍形成性の問題がなく，将来的には細胞治療の標的となりえる可能性を秘めている。

4. 血友病A遺伝子治療

1) 治療遺伝子における最近の進歩

(1) 改変第VIII因子遺伝子

第VIII因子（FVIII）遺伝子（cDNA）は翻訳領域でも7.1 kbあり，ウイルスベクターへの搭載も困難であること，またB domainをコードする領域を除くとFVIIIの発現も増加することから，B domainをコードする領域をほぼ完全に除いたB domain deleted FVIII cDNA（BDDFVIII cDNA）が一般的に用いられてきた。B domainの様々な領域を残したFVIIIの発現を検討した結果，B domainを部分的に残すと，B domainを完全

に除いたBDDFVIII cDNAよりもFVIIIの発現が上昇するとの報告があるが[15]，これに対しては異論もある[16,17]。発現されるFVIII分子のBドメインの配列によりインヒビター発生も差異があるとされるが，ヒトでインヒビターが出来やすいのはBドメインではないので疑問もある。いずれにしても，遺伝子導入に際し，完全長のFVIII cDNAを用いるよりもB domainをコードする塩基配列を部分的ないし完全に除去するとFVIII発現が上昇するのは間違いない。また，BDDFVIIIは組換第VIII因子製剤として既に臨床で用いられており，治療効果あるいはインヒビター発生率ともnative FVIIIと差がないことが報告されていることから治療遺伝子としてBDDFVIII cDNAを用いることには問題はないと考えられる。さらにコドンの最適化や発現抑制に働く可能性のある配列を変更することで遺伝子発現が数倍に上昇することが報告され[17]，今後は最適化された塩基配列のFVIII遺伝子が用いられる可能性がある。

(2) 改変第VII因子遺伝子

活性化第VII因子は，高濃度では第IX因子（FIX），FVIIIに依存せずに第X因子を活性化することができるため，インヒビターのある血友病患者の出血時の治療に使用されている。この理論に基づき，改変第VII因子遺伝子を用いて活性化型第VII因子を発現させる試みが血友病Bモデルの第IX因子欠損マウスで行われた。同じく，AAVベクターでイヌ活性化第VII因子を発現させ，血友病Aイヌおよび血友病Bイヌでも試みられ，止血効果がえられている[18]。

2) ベクターの進歩

非ウイルスベクターとしてhyaluronanとasialoorsomucoidでコートされたnano-capsuleにFVIII遺伝子を内包したベクターを血友病Aマウスに投与しtransposonを用いてFVIII発現が得られた報告があり，今後の展開が期待されるがtransposonを用いることによる染色体へのランダムな遺伝子組込の懸念は依然として残る[19]。

3) 遺伝子導入の標的臓器の最新の知見

第VIII因子の細胞内輸送にはERGIC-53（シャペロン分子）などが関わっており，第VIII因子の細胞からの分泌が全ての細胞でも効率よく行われるかは明らかでない。BDD FVIIIの細胞内輸送が完全長FVIIIと同じか否かも明らかではない[13]。第VIII因子遺伝子導入が高効率に行われても必ずしもFVIIIが血流へスムースに移行するとは限らず，FVIII遺伝子導入の標的細胞・臓器は慎重に選択する必要がある。肝臓，血液細胞，脂肪細胞，骨格筋，血管内皮細胞などが標的細胞・臓器として考えられている。

4) 血友病A遺伝子治療の臨床治験の最新の知見

現在までに3つの臨床治験（TKTトライアル，Chironトライアル，アデノウイルスベクターをもちいたトライアル）が行われている[14]。一時的な効果が認められたが，いずれも1年以上に及ぶ長期間の第VIII因子の上昇は得られていない。これ以後血友病A遺伝子治療臨床治験は行われていないのが現状である。遺伝子導入方法，標的組織・臓器，ベクター，プロモーターの選択と改良が必要と考えられ，今後の展開は，AAVベクターを用いる血友病B遺伝子治療臨床治験の成果次第と思われるが，血友病B遺伝子治療と同じストラテジーが用いることが出来るかは確実でない。

5. 血友病B遺伝子治療

血友病A遺伝子治療に比較し，血友病B遺伝子治療研究は進んでおり，臨床治験も進行中である。FIX遺伝子搭載AAV2ベクターを用いた血友病B遺伝子治療臨床治験（Avigen trial）は，血友病遺伝子治療で最も期待されていた。最初は骨格筋へFIX遺伝子導入が行われたが十分な成果が得られなかった。続いて同じベクターを用いて肝臓へのFIX遺伝子導入の臨床治験が行われたが，肝臓へのFIX遺伝子導入にともないT細胞性免疫反応によるAAV2ベクター感染肝細胞の排除という傷害事象が発生したため，臨床治験は中断されていた[14]。

1) ベクターの進歩

AAVベクターの血清型とプロモーターなどがみなおされた結果，肝臓へのFIX遺伝子導入にはAAV8ベクターが優れ，免疫反応を考慮するとAAV5ベクターも選択肢に入ると思われ，実際にAAV8ベクターが血友病B遺伝子治療臨床治験に用いられ始めた。

新規血清型AAV8ベクターに加えAAVベクター構築の進歩もある。AAVそのものは一本鎖DNAウイルスであり，AAVベクター感染細胞内でベクター搭載遺伝子を発現するためには二本鎖となる必要があるとされる（二本鎖DNAになる機序は議論がある）。近年開発された自己相補型AAVベクターself complementary AAV (scAAV) vector（二本鎖AAVベクターdouble strand AAV vector，ds AAV vectorとも呼ばれる）は，AAVベクターに搭載される遺伝子の後半部分の塩基配列が前半部分の相補的配列になっているため，ベクター内ですでに二本鎖DNA構造をとり，AAVベクターが感染した細胞内で相補鎖の合成などのステップを取る必要がなく効率よく遺伝子発現を行えると考えられている[20]。

2) 非ヒト霊長類を用いた血友病B遺伝子治療研究

これまで血友病遺伝子治療研究は血友病マウスや血友病イヌを用いて前臨床研究を進めてきた。しかし，これらの実験動物とヒトとの種差は大きく，これらを用いて得られた研究成果をヒトへ外挿できないことも多い。そ

の意味で，非ヒト霊長類を用いた血友病遺伝子治療研究を行うことは意義が大きい。我々も一部をヒト型にしたカニクイザル FIX 遺伝子を搭載した AAV8 ベクターをカニクイザルへ投与し前臨床研究を進めてきた[21]。詳細な成果は示せないが，肝臓へ効率の良い遺伝子導入を行いうる AAV8 ベクターも，カニクイザル肝臓への遺伝子導入はマウス肝臓への遺伝子導入効率著しく異なることが分かった。また，血中に存在する抗 AAV 抗体の影響も大きいことも明らかとなった[22]。このようにヒトでの血友病遺伝子治療の前臨床研究を非ヒト霊長類で行うことは重要であると思われる。

3) AAV ベクターによる遺伝子導入に対する既感染の影響

ヒトはウイルスから自己を守ため抗体を産生し，またウイルス感染細胞を排除する。野生型 AAV の既感染があると中和抗体が形成され，中和抗体が低力価でも存在すると AAV ベクターの遺伝子導入は著しく阻害されることがサルを用いた研究から明らかとなっている。特に，AAV8 ベクターのように血中に投与し肝臓へ遺伝子導入するタイプのベクターでは，短時間でも血中の中和抗体と反応すると遺伝子導入効率が著しく低下する。AAV のうち AAV2 に対するヒトの中和抗体陽性率は世界的にも高いものがある[23]。また，各血清型 AAV のキャプシドのアミノ酸配列は相同性が高く，ある血清型の AAV に対する抗体は，他の血清型 AAV に対しても阻害活性を有する可能性があり，一般人と血友病患者の抗 AAV 抗体価測定など，今後の検討が必要である。野生型 AAV 既感染はベクター感染細胞の免疫学的排除にも関わるとされる。scAAV8 ベクターを用いた FIX 遺伝子導入は血友病 B マウスやサルでの発現実験でも優れた FIX 発現能力を示したことを受け[24, 25]，臨床治験が開始され 2 例以上で臨床効果がえられている[26]。しかし，AAV8 中和抗体陽性の患者では有効性が得られなかったと報告されている。

6. 終わりに

血友病は遺伝子治療のよいモデルとされ，精力的に研究され臨床治験も行われてきた。現在進行している血友病 B 遺伝子治療では成果が得られつつあり，今後の展開が待たれる。血友病遺伝子治療における導入遺伝子由来の凝固因子に対するインヒビター産生の問題と，ベクター感染細胞に対する免疫反応など安全性の問題は今後さらに検討されるべきものと思われる。

著者の COI (conflicts of interest) 開示：本論文発表内容に関連して特に申告なし

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