Figure 2. Full-length FVIII can be expressed at levels as high as BDD FVIII. (A, B) Total mRNAs were extracted from 293T cells (A) or human iPS cells (B), and the level of expression was assessed by quantitative RT-PCR. Expression values were normalized to the level of *GAPDH* mRNA. (C, D) Secretion of functional FVIII measured by aPTT assay. Culture supernatants of transfected cells were harvested 3 days after medium change and subjected to aPTT assay to measure the coagulation activity of secreted FVIII protein in *293T cells* (C) or human iPS cells (D). Recombinant FVIII product was used to generate a standard curve. Normal FVIII activity (100%) represents 1 U/ml (= 1000 mU/ml). *: $P<0.05$ by two-sided Student's *t* test (n = 3).
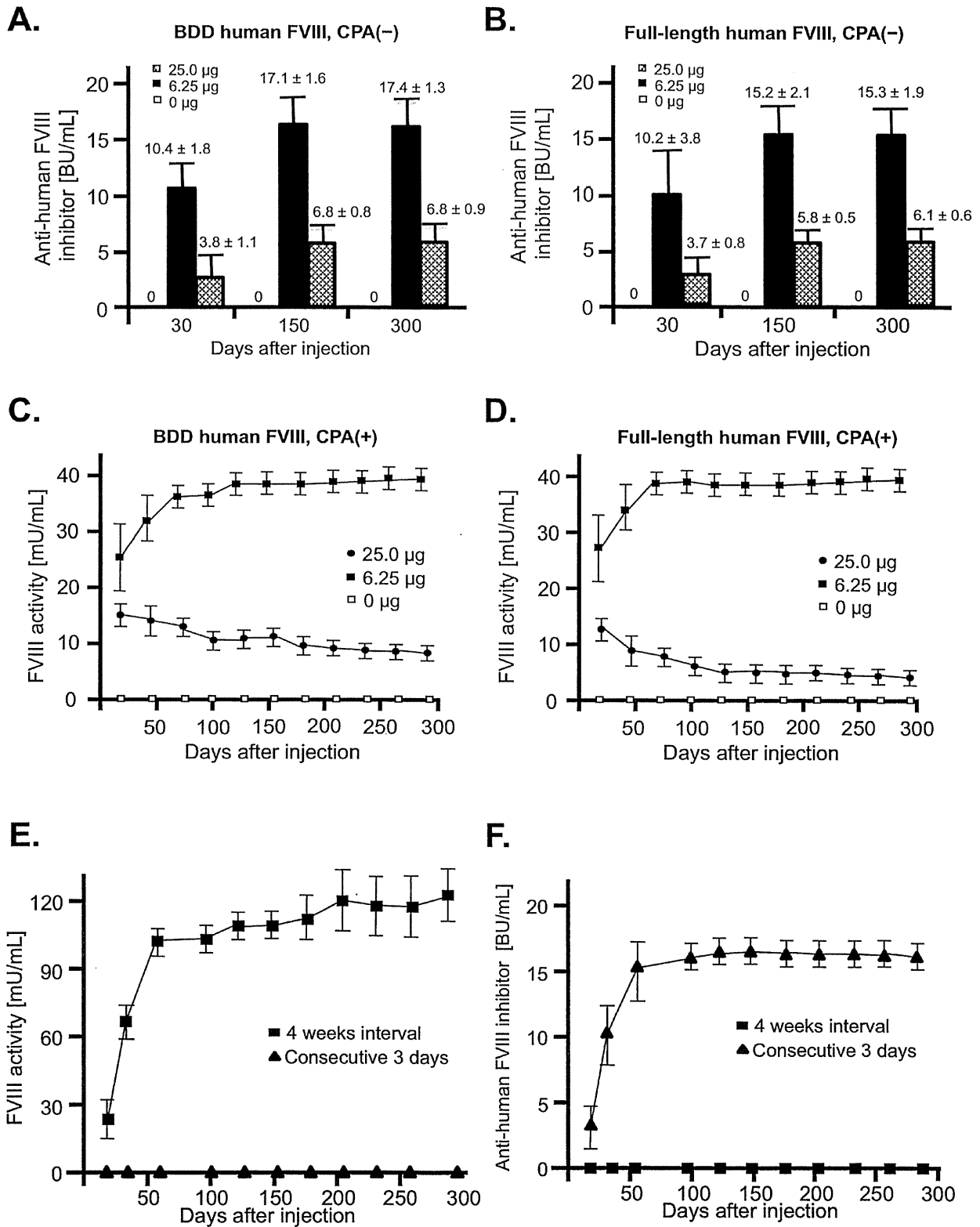doi:10.1371/journal.pone.0104957.g002

—478—

A.

BDD human FVIII, CPA(−)

B.

Full-length human FVIII, CPA(−)

C.

BDD human FVIII, CPA(+)

D.

Full-length human FVIII, CPA(+)

E.

F.

Figure 3. Long-term and stable expression of FVIII in hemophilia A mice. (A, B) *piggyBac* vector (25 µg of DNA) expressing either B-domain–deleted (A) or full-length FVIII (B) was introduced into hemophilia A mice (n = 5 each) by hydrodynamic injection without cyclophosphamide treatments. Amount of PBaseII *piggyBac* transposase (0 µg, 6.25 µg, or 25 µg of DNA) is indicated by symbols. Levels of anti-human FVIII inhibitors in

mouse plasma were measured by Bethesda assay. (C, D) *piggyBac* vector (25 μg of DNA) expressing either B-domain-deleted (C) or full-length FVIII (D) was introduced into hemophilia A mice (n=7 each) by hydrodynamic injection with cyclophosphamide treatments. Amount of PBaseII *piggyBac* transposase (0 μg, 6.25 μg or 25 μg of DNA) is indicated by symbols. Levels of FVIII activity in mouse plasma were measured by chromogenic assay. (E, F) Multiple injections boosted the level of FVIII in hemophilia A mice. PB-EF1α-hFVIII(Full)-iP (25 μg) and PBaseII (6.25 μg) vectors were hydrodynamically injected into hemophilia A mice (n=7 each) three times at intervals of 24 hours or 4 weeks. (E) Level of FVIII activity in mouse plasma was measured by chromogenic assay. (F) Level of anti-human FVIII inhibitor was measured by Bethesda assay. doi:10.1371/journal.pone.0104957.g003

produced in the liver, we targeted the liver by hydrodynamic injection via the tail vein. Human FVIII is inherently immunogenic in hemophilia A mice; consequently, all recipient mice that did not receive immunosuppresive treatment developed inhibitory anti–human FVIII antibodies, and there was no difference in the titer levels of FVIII antibodies between BDD and full-length FVIII vectors (Figure 3A, B). Therefore, under transient immunosuppression with cyclophosphamide, we injected either BDD or full-length FVIII *piggyBac* vectors (25 μg) with various amounts of transposase (0 μg, 6.25 μg, or 25 μg). As expected from the *in vitro* results (Supplemental Figure 2), we observed higher FVIII level in plasma of animals that received lower amounts of transposase. The levels of FVIII in plasma were 25–40 mU/ml (2.5–4.0% of normal level) in all hemophilia A mice injected with 6.25 μg of transposase (Figure 3C, D). Furthermore, we observed that the activity levels of full-length FVIII (Figure 3D) were comparable to those of BDD FVIII (Figure 3C), despite the size difference between the two proteins. In addition, the levels of FVIII antigen determined by human-specific FVIII ELISA corresponded with FVIII activity, as revealed by chromogenic assay in the same plasma samples (data not shown). The plasma levels of FVIII were stably maintained for more than 300 days.

In an attempt to boost the production of FVIII, we tested the effect of multiple injections of *piggyBac* vector. Surprisingly, when we injected the vector at 24-hour intervals, no FVIII production was observed (Figure 3E). Rather, we observed the production of FVIII inhibitors in response to the injections (Figure 3F). On the other hand, multiple vector injections at 4-week intervals boosted the levels of FVIII expression in hemophilia A mice without causing development of FVIII antibody (Figure 3E, F).

Next, to determine whether the *piggyBac* vector injection corrected the bleeding diathesis phenotype, we subjected the mice to tail-clip challenge. The tails of treated and untreated hemophilia A mice from Figure 3D were transected at a position corresponding to a specific diameter, and then bleeding times were monitored. Whereas untreated hemophilia A mice (n = 6) tended to lack stable clot formations, all hemophilia A mice treated with *piggyBac* vector (n = 5) formed a stable clot after tail clipping (Mean bleeding time; Untreated hemophilia A mice: 18 min 24 sec, Vector-treated hemophilia A mice: 6 min 13 sec, respectively) as shown in Figure 4A.

Finally, the mice were sacrificed, and multiple recovered organs were examined for FVIII expression by immunohistochemical staining. Among the organs we examined, only liver expressed detectable level of human FVIII protein (Figure 4B). We also confirmed the expression of human FVIII transcripts in homogenized liver by qRT-PCR (Figure 4C). Thus, even though FVIII expression was controlled by the ubiquitously active EF1α promoter, the expression pattern achieved by the hydrodynamic-injection method is largely restricted in liver.

Overall, these data indicate that *piggyBac* vectors can mediate efficient and sustained expression of the full-length FVIII gene, and thereby rescue the bleeding phenotype of hemophilia A mice.

Discussion

The continuous improvement of gene-transfer vectors has broad implications for genetic studies and gene-therapy applications. In the gene-therapy field, host immunoresponse to viruses pose a major challenge for virus-mediated gene transfer, especially in the case of adenoviral or AAV vectors [2,3,6,14,35,36]. In addition, some hemophilia patients still suffer from AIDS due to the contamination of HIV in clotting factor concentrates that were used for the treatment. To avoid the risk of recombination between viral vectors and wild-type viruses, the use of relevant lentiviral vectors [8-11] should be avoided in patients who have a history of infection with HIV. Another limitation of viral vectors relates to the costly and cumbersome manufacturing process, which poses significant regulatory hurdles. Therefore, there is a need to develop safe and efficient non-viral gene delivery approaches that diminish the immunoresponse against viral components, overcome some of the manufacturing and regulatory hurdles, and allow for stable expression of the therapeutic gene(s).

Currently available non-viral plasmids integrate inefficiently, and gene expression from these plasmids typically declines within days after transfection. To overcome these issues, we have attempted to establish non-viral gene delivery approaches that take advantage of the latest transposon technology [19,20]. Because *piggyBac* transposons integrate into the target-cell genome, they have the potential for long-term expression of therapeutic genes.

Here, we performed hydrodynamic injection of *piggyBac* vectors into hemophilia A mice, resulting in stable and long-term expression of BDD and full-length FVIII in plasma. Furthermore, the bleeding phenotype of hemophilia A mice was almost completely abolished after transduction with *piggyBac* vectors. Although hydrodynamic transfection is not readily applicable to a clinical setting, our study demonstrates proof-of-principle that *piggyBac* vectors could be used for hemophilia gene therapy, because these vectors enjoy several advantages relative to previously developed viral vector–mediated gene transfer approaches.

First, we took advantage of the large cargo size of *piggyBac* vectors to deliver full-length FVIII cDNA. Despite the difference in the sizes of the two vectors (8.1 kb and 10.6 kb), we observed comparable transduction efficiencies between BDD and full-length FVIII vectors. The B-domain of FVIII is not essential for coagulation activity; however, previous reports have suggested that it plays a role in protein secretion and quality control [15]. Such a function of the B-domain may influence the overall FVIII activity under some circumstances. Our *piggyBac* vector should provide a powerful platform for investigating the effect and biochemical properties of the B-domain and its variants.

Second, *piggyBac* vector–mediated gene transfer offers a simple and cost-effective gene delivery system, because gene transfer can be achieved simply by co-transfection of vector DNA and transposase. This is in contrast to viral vector preparation, which requires a packaging cell line, several helper plasmids, concentration, and purification. The simplicity of the production procedure and lower cost should facilitate the manufacture of *piggyBac* vectors for clinical applications.
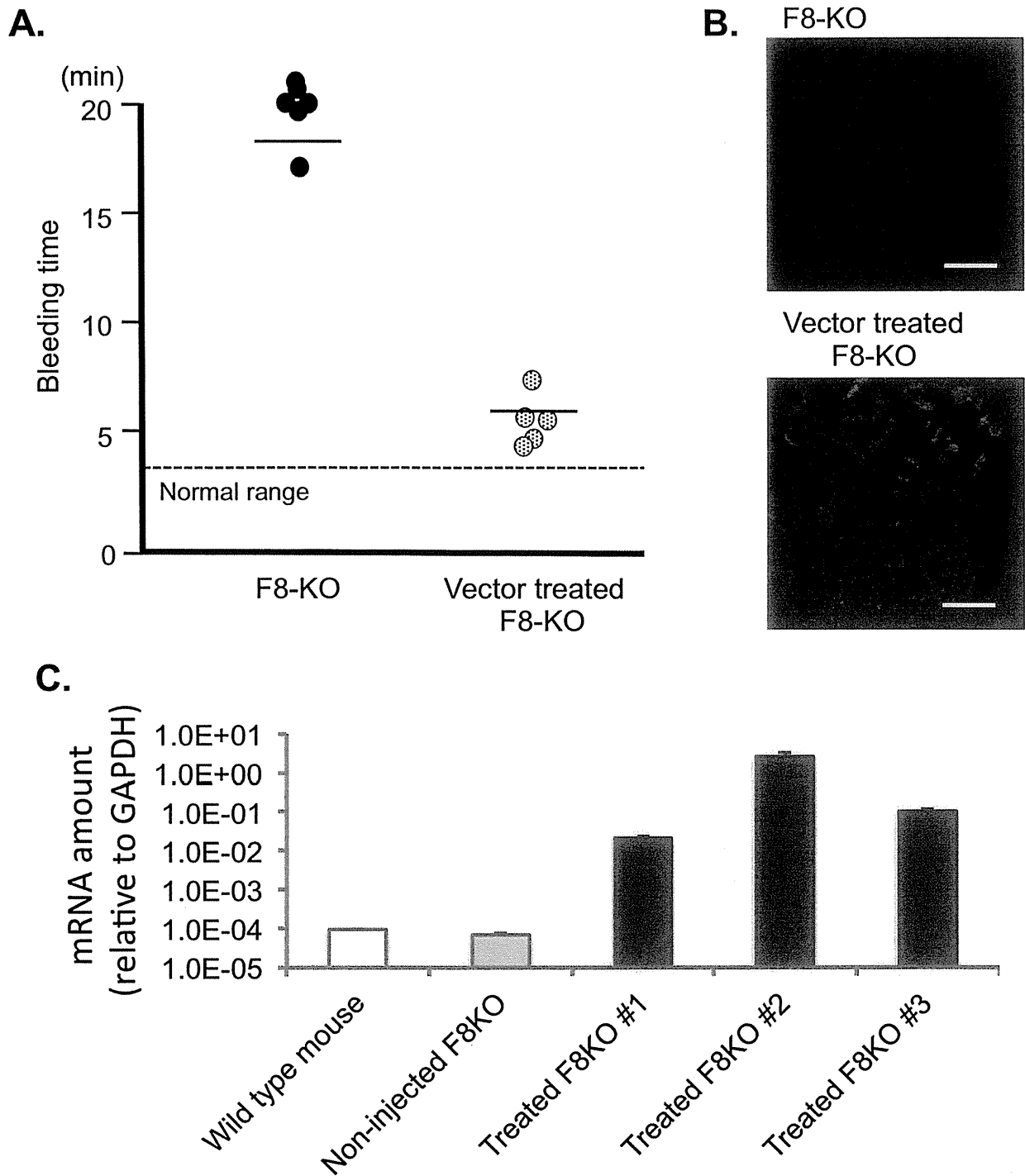
A.

B. F8-KO

Vector treated
F8-KO

C.

Figure 4. Phenotypic correction of hemophilia A mice by injection of *piggyBac* vectors. (A) Bleeding time of both hemophilia A mice (n = 6) and hemophilia A mice treated with *piggyBac* vector expressing full-length Factor VIII (n = 5) assessed by tail-clip assay. (B) Immunohistochemical analysis of liver tissue from non-treated hemophilia A mice and hemophilia A mice treated with *piggyBac* vector expressing full-length FVIII. Scale bar represents 50 μm (x400: original magnification). (C) Total RNA were extracted from the mouse liver treated with *piggyBac* vectors with 4 weeks interval, and quantified the level of transgene mRNA by qRT-PCR using human FVIII light-chain primers. Expression values were normalized to the level of *GAPDH* mRNA.
doi:10.1371/journal.pone.0104957.g004

−481−

On the other hand, several problems must be solved before transposon-based vectors can be used in therapeutic applications. Similar to gammaretroviruses or lentiviruses, *piggyBac* vector preferentially integrate their DNA sequences into host chromosomes near transcriptional start sites [21]; therefore, insertional mutagenesis and genotoxicity remain significant concerns. Indeed, insertion of non–self-inactivating retroviral vectors has resulted in the activation of oncogenes in human patients [37,38]. More recently designed self-inactivating retroviral vectors are less likely to activate surrounding genes via their LTR promoters; however, the possibility of *trans*-activation by internal enhancers/promoters must still be taken into account [39]. The degree of genotoxicity mediated by our *piggyBac* vector remains to be determined.

Hemophilia A patients require a continuous supply of active FVIII protein; therefore, for gene-therapy applications, it is preferable to achieve stable and sustained expression of FVIII in order to achieve consistent therapeutic outcomes and reduce the risk of developing inhibitory antibodies. Our approach to *in vivo* delivery by hydrodynamic injection cannot control the integration sites or targeted cells. However, the *ex vivo* gene therapy approach using tissue stem cells, BOECs [11,12], or patient-derived iPS cells enables us to pre-determine the integration sites of the vectors. The *ex vivo* approach allows us to select for cell clones that effectively secrete active FVIII and the vector has integrated into a "safe harbor" in the human genome, however, suitable cell type(s) for *ex vivo* targeting need to be determined.

Another limitation of the *piggyBac* vector is its delivery method. Hydrodynamic injection is a well-established and efficient delivery system in small animals [31,40], but not readily applicable to a human clinical setting for hemophilia patients as the method carries a risk of liver damage and bleeding. This warrants the development of alternative *in vivo* DNA delivery approaches. More controlled (smaller buffer volume, but with similar hydrodynamic pressure for purposes of gene transfer) and local hydrodynamic injection methods using catheter are preferred [41,42]. Transposon and transposase constructs can be delivered to target cells by chemical transfection with carrier molecules that facilitate their entry. Physical transfection methods such as electroporation have been used for *ex vivo* gene delivery of transposon/transposase constructs, resulting in long-term and efficient transgene expression. Furthermore, recent advances in tissue-specific DNA delivery methods based on nanotechnology [43] or chemical modifications may accelerate the delivery of *piggyBac* vectors *in vivo*.

In conclusion, we successfully achieved long-term therapeutic expression of full-length FVIII gene *in vitro* and *in vivo* by non-viral *piggyBac* vectors. The present study is the first report demonstrating that *piggyBac* vectors can mediate sustained FVIII expression *in vivo*, and provides evidence that *piggyBac* is a promising tool for various *in vivo* gene-transfer applications. We believe that the present study will encourage the development of *piggyBac*-based vectors, and contribute to future *in vivo* gene and cell therapy for genetic diseases including hemophilia.

Supporting Information

Figure S1 Optimization of internal promoters. *piggyBac* vectors expressing an EGFP-IRES-puro cassette under the control of various promoters (no internal promoter, PGK, CAG, or EF1α) were transfected into human iPS cells (A). The GFP fluorescence intensities from three samples were assessed by flow cytometry, and error bars represent the standard deviation (B).
(EPS)

Figure S2 DNA ratio of *piggyBac* vector and transposase. Either wild-type *piggyBac* transposase (A) or hyperactive *piggyBac* transposase (B) was co-transfected with various amount of *piggyBac* vector (PB-EF1α-EiP) into 293T cells. Transduction efficiencies were measured by flow cytometry 14 days after transfection. Transduction efficiencies for each condition are indicated by percentage (%) and grayscale (low transduction efficiency: light gray, high transduction efficiency: dark gray). N.E.: Not examined.
(TIFF)

Figure S3 Size of *piggyBac* vectors and transduction efficiency. Twenty-eight *piggyBac* vectors of various sizes (PB-EF1a-cDNA-IRES-mCherry) were transfected into 293T cells. Percentages of mCherry-positive cells were measured by flow cytometry, and each vector is plotted on the graph as a gray diamond. Transduction efficiency (E [%]) of the *piggyBac* vectors and their vector sizes (S [bp]) were correlated with the approximate power function (dotted line, $E = 3,380,333S^{-1.401}$); the coefficient of determination (R^2) was 0.717.
(EPS)

Table S1 List of primers used in this study.
(TIFF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: HM M. Shima M. Sugimoto SY AH. Performed the experiments: HM NF NS AH. Analyzed the data: HM AH. Contributed reagents/materials/analysis tools: YO. Contributed to the writing of the manuscript: HM AH.

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—483—

REVIEW ARTICLE

Novel products for haemostasis

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Summary. The primary major issue in haemophilia treatment remains the development of inhibitors. Recently two novel bypassing products have been developed. First, a humanized bispecific antibody against FIXa and FX, termed hBS23, was produced utilizing these two molecules placed into a spatially appropriate position to mimic FVIIIa, and recently this mimetic activity and the pharmacokinetics of the original antibody were improved by engineering the charge properties of the variable region within the immunoglobulin. Using the new antibody, termed ACE910, a phase 1 study in 64 Japanese and Caucasian healthy adults was performed and data from this trial suggested that the product had medically acceptable safety and tolerability profiles. The other new bypassing agent is named MC710, and consists of a mixture of plasma-derived FVIIa and FX. Preclinical studies using *in vitro* and *in vivo* haemophilia B inhibitor monkey models indicated that the haemostatic effects of FVIIa and FX were enhanced by simultaneous administration. Results from phase I and II clinical studies suggested that MC710 had equal or greater pharmacokinetic (PK), pharmacodynamic (PD), efficacy and safety profiles than conventional bypassing agents in the treatment of joint bleeding in haemophilia patients with inhibitors. Another significant current issue in this context is the increased medical cost of conventional treatment due to the higher consumption of concentrates. Biosimilar products may offer advantages in these circumstances and may offer a less expensive alternative. Regulatory issues, however, together with acceptability of biosimilar materials and reimbursement policies as well as supply and demand incentives remain to be considered. Rare bleeding disorders (RBDs) have attracted less attention from the pharmaceutical industry than haemophilia or von Willebrand disease due to the limited number of patients involved. Many cases of this type have been treated, therefore, using fresh frozen plasma (FFP) or prothrombin complex concentrates (PCCs) which carry serious risks of infections, allergic reactions and fluid overload. Several specific plasma-derived or recombinant products including fibrinogen, FVIIa, FXI and FXIII have now become available, however, and a phase III clinical study of recombinant FXIIIa has recently been completed demonstrating safety and efficacy of substances of this nature.

Keywords: biosimilars, bispecific antibody, bypassing therapy, haemophilia, rare bleeding disorders (RBDs)

Introduction

The introduction of highly purified and recombinant products has facilitated the use of regular prophylaxis as the principal type of haemostasis therapy especially in paediatric and young adult patients. The number of spontaneous and life-threatening bleeds has been remarkably reduced in these individuals compared to those treated on-demand. Furthermore, randomized prospective studies have revealed that primary prophylaxis may protect from the development and progress of haemo-arthropathy. However, several issues still remain unsolved in the treatment of haemophilia. For example, the need for frequent venous access for FVIII or FIX infusions can result in a significant physical and mental burden. Central venous catheters may be helpful, but these involve a risk of infection and thrombosis. In addition, the development of inhibitors presents the major clinical challenge. Once an inhibitor develops, haemostatic control becomes difficult and complicated. Immune tolerance treatment (ITI) is effective in over half of the patients with inhibitor, but clinical management in the unsuccessful patients is extremely difficult. In such cases, bypassing therapies

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29

with activated prothrombin complex concentrates (APCC) or recombinant factor VII (rFVIIa) are usually used. The haemostatic effects of these materials are limited, however, when compared to replacement therapy with FVIII or FIX concentrates in patients without inhibitor. Economic considerations may also be important due to the increased utilization of FVIII or FIX concentrates. This can cause substantial stress to haemophilia treaters, governments and insurance companies even in developed countries. Ideally, therefore, the criteria for therapeutic products in patients with haemophilia include longer acting coagulation activity, enhanced efficacy in patients with inhibitor, ease of venous access and economical competitiveness.

The past few years have reflected a second landmark in the development of therapeutic agents, and many new products are now being introduced into the market for patients with or without inhibitor. This article discusses progress with the development of a range of modern haemostasis products, and includes descriptions of new bypassing agents, biosimilar substances and materials for the treatment of rare bleeding disorders.

New bypassing agents

Essential considerations for the current treatment of haemophilia patients include the requirement for frequent intravenous injections and the development of inhibitors. Although longer acting FVIII or FIX products offer a very promising improvement for regular prophylactic treatment, physical and mental burdens remain especially in paediatric and old patient groups. Furthermore, the risk of inhibitor development remains a serious problem. Existing bypassing agents such as rFVIIa and APCC do not always provide adequate haemostasis, and clinical management is more challenging in patients with high-responding inhibitors who fail ITI. In this context, therefore, more potent and longer acting bypassing agents are being investigated. Recently, two novel bypassing agents have been developed in Japan; an intrinsic bypassing agent, hBS23, which is a humanized bispecific antibody to FIXa and FX mimicking FVIIIa, and a plasma-derived extrinsic bypassing agent (MC710) comprising a mixture of FVIIa and FX. Clinical trials using these agents are ongoing or recently completed.

The principle of the bispecific antibody is based on the hypothesis that FVIII co-factor function is enhanced by interactions between FIXa and FX. The humanized IgG antibody, designated hBS23, targets both proteins, and effectively acts as FVIIIa in the blood clotting cascade by spatially arranging the two target molecules, in correct contact with each other, to facilitate FXIa-catalyzed conversion of FX to its activated form FXa. [1]. Kinetic studies of FXa generation by FIXa in the presence of phospholipid

indicated that hBS23 increased the k_{cat}/K_m by 2×10^4 -fold equivalent to 7.3% FVIIIa.

Conventionally, native FVIII is activated by thrombin generated in the initial coagulation process triggered by extrinsic TF/FVIIa. In contrast, the bispecific antibody mimics FVIIIa and is not dependent on thrombin activation. In consequence, the haemostatic effectiveness of the antibody is rapid and does not need stabilization by VWF. Furthermore, the reaction is not affected by APC/PS or by the presence of FVIII inhibitors. Initial studies demonstrated that the antibody shortened the APTT of haemophilia A plasma with inhibitor to within normal range and an intravenous dose of 0.3 mg kg^{-1} exerted haemostatic activity preventing the progression of bleeding symptoms in a non-human primate model of acquired haemophilia A to the same extent as recombinant porcine FVIII maintained at a plasma level of ≥ 1 U dL^{-1}.

Recently a more potent bispecific antibody, ACE910, has been produced by optimizing its multidimensional profile for clinical applications [2], and a single intravenous dose of 1 or 3 mg kg^{-1} of ACE910 was shown to substantially reduce the bleeding symptoms in a non-human primate model of acquired haemophilia A. The haemostatic effect was comparable to that of 10 U kg^{-1} rpoFVIII given twice daily. Pharmacokinetic parameters indicated that the half-life of AC910 was approximately 3 weeks for both single intravenous and subcutaneous administrations. The subcutaneous bioavailability was almost 100%. These data suggested that effective haemostatic levels might be maintained by once-weekly subcutaneous administration of ACE910, offering the possibility of more effective and easier prophylactic treatment from early childhood [3]. Furthermore, the APTT was shortened and thrombin generation was increased in artificial FVIII deficient plasma samples spiked with two anti-FVIII neutralizing antibodies, suggesting that similar prophylactic properties could be expected in patients with inhibitors.

A phase I study in 64 Japanese and Caucasian healthy adults indicated that ACE910 at doses up to 1 mg kg^{-1} had medically acceptable safety and tolerability profiles, and recently a new phase I study has been initiated to assess prophylactic efficacy as well as safety and PK in patients with/without inhibitors.

MC710 was developed for the purpose of providing more potent and longer acting haemostatic effects of FVIIa by mixing it with FX. Preclinical studies *in vitro* and animal studies *in vivo* using a haemophilia B inhibitor monkey model confirmed that administration of FVIIa and FX enhanced haemostatic potential to a greater extent than rFVII. [4,5]. These effects of MC710 were also confirmed in a study using haemophilia inhibitor-like plasma. In a multicentre, open-labelled, non-randomized, active-controlled crossover phase I trial, MC710 was intravenously administered

at single escalating doses of FVIIa (five doses from 20 to 120 μg kg^{-1}) to non-bleeding patients to evaluate product safety, pharmacokinetic (PK) and pharmacodynamic (PD) parameters. NovoSeven (120 μg kg^{-1}) and/or FEIBA (50 or 75 U kg^{-1}) were used as comparative controls. Ten minutes after the administration of MC710, APTT measurements were dose-dependently improved and the PT tests were shortened to approximately 6 s. The effects were maintained for 12 h after administration at all doses. No serious or severe adverse events were observed [6]. A further analysis in this study demonstrated that Clot Waveform (CWA) parameters including clotting time, maximum clot velocity and maximum clot acceleration were significantly improved after administration of 80 μg kg^{-1} MC710 compared with FEIBA and Novo-Seven. Furthermore, MC710 demonstrated a significantly greater effect than the control products on thrombin generation tests (TGT) [7]. These phase I results again suggested that MC710 has equal or greater haemostatic potential than the current bypassing agents. In a subsequent phase II study, the haemostatic efficacy, safety and kinetics of two doses of MC710 (60 and 120 μg kg^{-1}) were investigated during the treatment of joint bleeding in haemophilia patients with inhibitors [8]. The results demonstrated that in nine bleeding episodes seven treatments were clinically rated as 'excellent' or 'effective' 8 h after administration without any serious adverse reactions or laboratory evidence of disseminated intravascular coagulation (DIC). More recently a phase III study has been completed utilizing one to two injections of MC710 for joint, muscle and subcutaneous bleeding in haemophilia patients with inhibitor. The results demonstrated that 19 out of a total of 21 treatments were rated 'excellent' or 'effective'.

The results obtained of these clinical trials indicated that MC710 could have considerable potential as a bypassing agent in haemophilia A and B patients with inhibitor.

Further studies are warranted to firmly establish optimum therapeutic protocols and reliable monitoring tests for these new bypassing agents.

Assessment and impact of biosimiliars products

Throughout the last few decades, the development and validation of several commercial brands of Factor VIII (FVIII) concentrates either extracted from human plasma or engineered from mammalian cell cultures by means of recombinant DNA technology has greatly improved the safety and availability of therapy for patients with haemophilia [9,10]. At least in high-income countries, patients with haemophilia enjoy the benefits of a long-term substitutive treatment that allows them to reach the same life expectancy of their normal male peers. However, rationing of healthcare costs and the current global economic crisis is triggering containment which could impinge on an expensive treatment, such as that of haemophilia. In many middle- or low-income countries, availability of clotting factor concentrates is limited because of the high cost of haemophilia therapy and priorities given in the frame of healthcare budgets to other more frequent communicable and non-communicable diseases [11].

In analogy with the introduction of generics for chemically derived medicines, the expiration of patents of several biological medicines opens hopes for affordable copies and increased competition. Replicate versions of biological medicines 'so-called biosimilars' are available on the European market for growth hormone, erythropoiesis-stimulating agents and granulocyte-colony stimulating factors. In June 2013, the Committee for Medicinal Products for Human Use (CHMP) recommended granting marketing authorizations for the first two monoclonal antibody biosimilars (infliximab) [12]. In this context, one could wonder whether the availability of biosimilars of clotting concentrates would represent an opportunity or a threat for patients with haemophilia.

A brief reminder of the differences between generics and biosimilars is required before considering the specific implications for the haemophilia community. By contrast with small molecule drugs (aspirin, statins, antibiotics...) that can typically be described by a single chemical formula and duplicated relatively easily by chemical synthesis (also referred to as non-biological medicine), the development and manufacturing process of biologics are considerably more complex [13–15]. Biologics are either derived or extracted from a living organism such as plasma-derived coagulation factors and heparins or produced through recombinant DNA methodology, which typically involves cloning and expression of the protein molecule into a carefully chosen host cell line (i.e. yeast, mammalian, bacterial). This is followed by a specifically designed expansion, production, recovery, purification and packaging process; all of these conditions must be controlled if the efficacy and safety of the final product are to be retained. Also integral to the function and safety of biologic drugs are different types of posttranslational modifications (e.g. glycosylation) [16]. Biologicals are used for the treatment of chronic and life-threatening diseases such as cancer, multiple sclerosis and rheumatoid arthritis. Treatment with biologicals is usually expensive and represents ever increasing pharmaceutical expenditures for the third-party payer.

Recombinant full-length FVIII was first approved to be marketed in 1992–1993 with the international non-proprietary name 'octocog alfa' [10]. Since then other drugs based on recombinant FVIII have been developed and are currently available. They, however, differ with respect to the producing cell line (BHK,

CHO), the genes expressed (full-length FVIII, B-domain deleted FVIII, VWF), the presence of proteins in the culture medium (human plasma proteins, bovine serum albumin, aprotinin, none), the purification method (affinity chromatography using monoclonal antibodies or synthetic ligands), the stabilizing agent in the final formulation (human serum albumin, sucrose, mannitol), the viral inactivation steps (treatment with solvent-detergent, pasteurization, nanofiltration. Because of these many differences in the manufacturing of blood clotting factors, all currently available products are not the same and should be considered as specific and unique entities. These differences will be greater in the future considering the multiple strategies of extending half-life that are currently being applied to FVIII (pegylation, Fc-fusion, single-chain molecule) [17].

The term 'biosimilar' (also referred to as 'follow-on biologic' (FOB), 'subsequent entry biologics' or 'generic biologic') refers to a biological product developed to be highly similar as opposed to identical to an existing licensed biological product. Biosimilars (rather than 'bioidenticals') are generic or similar versions of an original biological drug (also referred to as the 'reference', 'innovator', 'brand-name' biological product) but are not an exact replication although there should not be clinically meaningful differences between the biological product and the reference product in terms of safety, purity and potency of the product. Due to the inherent complexity of biologics, while 'generic' versions cannot be produced, a similar product (biosimilar) is produced. One of the most significant challenges in developing a biosimilar product is designing the manufacturing process to achieve comparability to the reference product. All development activities starting as early as the generation of the production cell line through definition of the final purification and process conditions must focus on mimicking the host cell line and process conditions of the reference product to drive the process towards producing a similar product. It is rare for innovators to provide details about their manufacturing processes publicly, so the challenge for biosimilar companies is to figure out what the process conditions are likely to be and then mimic them.

For the time being, no biosimilar of FVIII is currently available. However, several principles appear to be crucial to ensuring that biosimilars are as safe and effective as the innovative products on which the haemophilia community presently relies:

Clinical trials. Robust human clinical trials are essential to the approval process to ensure that biosimilars are safe, effective and meet an appropriate standard of immunogenicity. The consequences of non-bioequivalence could be severe for clotting factor therapy. There is the potential for adverse reactions whenever an individual uses a new factor product for the first time or is switched to a new treatment. The inclusion of additional post marketing surveillance and pharmacovigilance activities is essential to detect any potential safety issues associated with a biosimilar product. These processes will depend on a globally standardized system for naming biosimilars that will enable the rapid identification of a specific biosimilar relative to its reference biological(or another biosimilar), so that any unique adverse events can be correctly identified and associated with the correct product.

Assessment of immunogenicity. Patients using biologics face increased risk of an inhibitor, an immune response to a biological that can have critical adverse health impacts and limit the effectiveness of the product. Research must prove that patients will not suffer from adverse effects of immunogenicity for biosimilars products. Given the high immunogenicity of exogenous FVIII given to patients with haemophilia, demonstration that biosimilars of FVIII are not more immunogenic than the currently available treatments is critical.

Interchangeability. Whether insurance companies, pharmacies or other providers can switch a patient from one therapy to another at their discretion is another critical issue. Currently, there is little consensus within the scientific community as to the resulting immunogenicity risk when randomly switching patients between products or product classes. People with bleeding disorders respond differently to innovator products, so it is crucial that this treatment decision is left to a physician and patient. Substitution should not be permitted except with the input and consent of the patient and the treating haematologist.

At this stage, there is a lot of uncertainty about the savings that could be achieved following the introduction of biosimilars for patients with haemophilia. As for generics, the biggest advantage of biosimilars is that they may offer a less expensive alternative to an existing medicine and, therefore, reduce pharmaceutical expenditure for the third-party payer. However, regulatory issues, biosimilar acceptability among physicians, price and reimbursement policies as well as supply- and demand-side incentives will ultimately determine the level of biosimilar-related savings [17]. Theoretical models predicted that biosimilar competition will lead to less price erosion than that obtained through generic competition. In line with this theoretical prediction, although price erosion arising from generic competition of up to 90% has been reported in countries like the UK and Germany, reported price erosion from biosimilar competition has not exceeded 15–30%. This reduction should be compared to that obtained through competitive tendering and national procurement schemes such as that in place in the UK.

This system, following EU procurement rules, evaluated products technically and by price. Considerable cost reductions were achieved while retaining all suppliers and maintaining a degree of prescribing freedom [18].

Expiry of market exclusivity of major biological blockbusters is the main driver surrounding the interest in the development of the biosimilar industry. Many leading 'traditional' originator companies are already developing biosimilars. Companies' experience in the production of complex biologicals may lead to optimized production of biosimilars at low cost and even drive originators to reconsider their production method. Originator companies will probably produce biosimilars in new product classes (for instance monoclonal antibodies) and may have different marketing strategies towards health professionals than current biosimilar manufacturers.

One should also consider that there must be an appropriate balance between incentives for companies to innovate and improve products and the benefits individuals with bleeding disorders could see from lower cost products. Haemophilia and the related bleeding disorders are very rare. Given the small total number of patients living with a bleeding disorder worldwide, a global approach to product development is required. The exclusivity period afforded in different countries should be harmonized and probably given longer to products that treat rare diseases. It is important that incentives are adequate to make the development of a therapy for a rare condition such as haemophilia sufficiently appealing, given the risks of developing products for small patient populations.

In conclusion, the aim of biosimilars is to provide safe, effective and less expensive biological drugs and to increase patient access to these medications. However, the extent to which this goal can be accomplished for patients with haemophilia remains to be seen. Although the approval process for biosimilars is expected to be less than that for a new biologic, it is still considerably more extensive than that of a generic drug, and therefore the extent of savings over the reference product is yet to be determined. A range of other factors are also expected to affect the economic success of biosimilars, including clinician and patient attitudes about switching to an unbranded product and safety issues that may emerge with biosimilars (mainly immunogenicity) as they enter the market. Other issues to consider include formulary and insurance coverage for biosimilars and possible price reductions by the reference product manufacturer that may be implemented to dissuade switching to biosimilar versions.

Factor concentrates for rare bleeding disorders

Due to the limited number of patients, rare bleeding disorders (RBDs) have drawn less attention from the industry than haemophilia or von Willebrand disease.

In all RBDs (fibrinogen, FII, FV, FV+VIII, FX, combined vitamin K-dependent factors, FXI and FXIII deficiencies), fresh frozen plasma (FFP) is a possibility when no concentrates are available but FFP bring unnecessary factors and proteins, carry the risk of infections, allergic reactions and fluid overload (in the event of volume overload diuretics are sometimes used). Cryoprecipitates are used for fibrinogen disorders and sometimes for FXIII deficiencies. A low cost minipooled solvent-detergent filtered cryoprecipitate FVIII has been developed that is also used for fibrinogen and FXIII deficiencies in countries with limited resources [19]. However, if there is no cost limitation, the best solution for a specific deficiency is to bring the missing factor, so we will focus primarily on available concentrates. A list of products is regularly updated by the WFH [20]. A common problem for the RBDs is the difficulty to register new products when authorities require inclusion of many patients to show their efficacy and safety, particularly when paediatric data are also required. Studies can be performed in countries where these disorders are more prevalent (especially in countries where consanguineous marriages are frequent) but often the same countries do not have the appropriate logistics. Because most RBDs are recessive disorders special attention has to be paid to affected women who suffer particularly (menorrhagia, ovarian haemorrhage, failures of pregnancy, post-partum haemorrhage). We will briefly consider all these deficiencies separately because each one has its particular feature and treatment.

Fibrinogen disorders include quantitative (afibrinogenemia and hypofibrinogenemia) and qualitative disorders. Several plasma concentrates are now available [21]. Fibrinogen substitution should be performed carefully due to the risk of thrombotic complications. The industry is keen to develop new products (many new fibrinogen concentrates are under evaluation) because there is an increasing demand for acquired fibrinogen disorders, particularly in the setting of surgery and trauma. Patients with congenital fibrinogen deficiencies take advantage of this situation. Some recombinant fibrinogen preparations exist [22] but no data have been published so far for patients with congenital deficiencies.

There is no specific factor II concentrate available for FII deficiencies so patients are often treated either with FFP or with various prothrombin complex concentrates (PCCs). Most PCCs contain several factors which could potentially induce thrombotic complications although the link between these events and PCC infusion has often been brought into question [23].

Fresh frozen plasma and PCCs are also given for FX deficiency as well as for patients with vitamin K combined-dependent factors deficiencies who respond poorly to vitamin K1 administration. There is a factor IX concentrate which contains high amount of factor

X. Recently a specific FX concentrate has been developed and several data will be presented at the WFH 2014 World Congress.

Specific plasma-derived FVII and recombinant FVIIa are currently available for FVII deficiencies. As for fibrinogen deficient patients, patients with FVII deficiencies take advantage of the interest of the industry to develop FVIIa concentrates as bypassing agents for persons with haemophilia with inhibitors as well as 'universal' agents in case of refractory bleeding (with the associated risk of thrombotic complications). Due to the short half-life of FVII some long-lasting products (pegylated, site-specific pegylated, N-linked glycan, Fc or albumin fusion FVIIa) as well as other modified FVII are currently under development [24].

Until recently no specific FV concentrate was available, so patients with FV deficiencies are treated with FFP (and sometimes with platelets which contain FV). The same is true for combined FV and FVIII deficiencies (DDAVP or FVIII concentrates are also given for these patients). However, the situation may change since a factor V concentrate is under evaluation. Patients with mild to moderate deficiencies benefit from tranexamic acid and, as for all RBDs, menorrhagia can usually be managed using oral contraceptives.

FXI deficient patients are mainly treated with tranexamic acid but sometimes FXI concentrates are required. Two concentrates are available. Factor XI concentrates should be used sparingly due to the risk of FXI inhibitors and at low dose due to the risk of severe thrombotic complications, particularly in elderly patients, in those with cardiovascular diseases or surgery with thrombotic potential and in case of venous thrombotic risk factors [25,26]. Indeed the potential thrombotic risks must be weighed up carefully against the potential haemostatic benefits of concentrate. FXI deficient patients with inhibitors may benefit from FVIIa used at low dose $(15–30 \ \mu g \ kg^{-1})$ which has been shown *in vitro* to normalize thrombin generation and *in vivo* to be efficacious [26].

FXIII deficient patients benefit from a plasma concentrate with a long-lasting efficacy and safety record. A phase III clinical trial has recently been completed with a recombinant FXIII which has been proven to be safe and effective in preventing bleeding episodes in patients with congenital FXIII-A subunit deficiency [27].

In future, patients with RBD could take advantage of the many bioengineering as well as alternative strategies (aptamers, RNAi, inhibition of TFPI, etc.), which are under development for persons with haemophilia [28].

Disclosures

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Addendum

A.S. Clere collected, assembled the data, analysed and interpreted the results, wrote the manuscript and approved the final version; I. Diaz analysed and interpreted the results and approved the final version, A. Lebreton analysed and interpreted the results and approved the final version. G. Lavigne-Lissalde provided critical revision of the article and approved the final version; J.F. Schved provided critical revision of the article and approved the final version; C. Biron-Andreani conceived and designed the research, analysed and interpreted the results, wrote the manuscript and approved the final version.

Disclosures

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

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The first case of int1h-related inversion in Japanese haemophilia A patients

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Haemophilia A (HA) is a coagulation disorder caused by defect or impairment of factor (F) VIII as a result of mutation(s) broken into FVIII gene (*F8*) located at Xq28. The inversions of intron 1 and 22 are the major causative *F8* mutations of severe HA, and the

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prevalence of them in severe HA patients are reported as 2–5% and 40–50%, respectively [1]. These inversions are also known to be relatively high-risk factors for the anti-FVIII inhibitor development [2]. The development of the inhibitor is still one of the serious events to complicate the treatment in HA patients. The detection of the inversions is significant for the anticipation of the prognosis and for the carrier diagnosis. In Japan, the study on the intron 22 inversions in severe haemophilia A patients has been already reported by Fukuda et al. [3], and the incidence of the intron 22 inversion was 42%, consistent with the prevalence reported in the world. However, no case

with intron 1 inversion was reported in Japan. We found the first intron 1 inversion positive HA case in Japan. The case was a boy having a 2 years elder healthy brother and there was no family history of the haemorrhagic disorders. He was diagnosed as severe HA with repetitive subcutaneous haemorrhage at ten months old. He started to take infusions of rFVIII concentrates on demand after diagnosis. The anti-FVIII inhibitor [max 12 Bethesda unit (BU) mL^{-1}] was detected at ~10th exposure day. After the development of the inhibitor, the bypassing agents, the activated prothrombin complex concentrates were utilized for haemostatic treatment on bleeding events and the inhibitor titre was gradually declined to <5 BU mL^{-1}. For immune tolerance induction (ITI) therapy, he visited our hospital at seven years old. The clinical data on admission in our clinic were summarized in Table 1. The inhibitor titre was 0.9 BU mL^{-1} and the IgG subclass of the inhibitor antibody was identified as IgG1 by an ELISA. The body weight of the case was 26.4 kg and rFVIII (100 units kg^{-1} day^{-1}) concentrates were administrated for 20 days followed by administration of the same amount of rFVIII every other day for a week. After the ITI therapy, the inhibitor was diminished to the undetectable level and the 81.4% of recovery in FVIII:C was observed post infusion of rFVIII concentrates. To investigate the molecular features of the case, we performed genotyping in this case. The institutional review board of the Nara Medical University approved this study and appropriate informed consent was obtained from the analysed individual. Firstly, we investigated PCR tests for the FVIII intron 1 inversion according to the method suggested by Bagnall et al. [1], using the genomic DNA extracted from the citrated whole blood collected from the patient. The reaction for int1h-1 (NCBI Reference sequence: NT_167198.1, nt 5.152320-5.153360) using the primers 9F, 9cR and int1h-2F (Table 2), and int1h-2 (NCBI Reference sequence: NT_167198.1, nt 5.293994-5.295034) using the

primers int1h-2F, int1h-2R and 9F with normal DNA yielded a 1908 bp product and a 1191 bp product respectively, whilst the reaction with DNA from the case yielded a 1776 bp product from int1h-1/2 and a 1323bp product from int1h-2/1 consistent with the non-aberrant pattern for intron 1 inversion status (Fig. 1). In addition, the reaction with DNA from the mother of the case yielded both products, indicating a carrier status (Fig. 1). Furthermore, we studied the breaking point of the inversion according to the method as described [1]. The intron 1 was sectioned

Table 1. The clinical data of the case.

Vital signs	
Body temperature: 36.5°C	CRP 0.0 mg dL^{-1}
Blood pressure: 100/60 mmHg	TP 6.9 g dL^{-1}
Heart rate: 76 min^{-1}	Alb 4.4 g dL^{-1}
Physical findings	AST 27 IU L^{-1}
Cardiac sounds: clear, no murmur	ALT 14 IU L^{-1}
Respiratory sounds: clear	LDH 240 IU L^{-1}
Abdomen: tense moderate	BUN 13 mg dL^{-1}
Liver, Spleen: impalpable	Cr 0.3 mg dL^{-1}
Left ankle joint: dorsiflexion limitation(+)	Na 139 mEq L^{-1}
Laboratory data	K 4.0 mEq L^{-1}
Blood type: O (Rh+)	Cl 103 mEq L^{-1}
WBC 6600 μL^{-1}	PT 12.2 s
RBC 45.5 × 10^3 μL^{-1}	APTT 90.2 s
Hb 12.8 g dL^{-1}	fibrinogen 296 mg dL^{-1}
Ht 37.8%	D-dimer 0.1 μg mL^{-1}
Plt 264 × 10^3 μL^{-1}	FVIII:C 0.4 IU dL^{-1}
	anti-FVIII inhibitor 0.9 BU mL^{-1}

Table 2. The sequence of the primers.

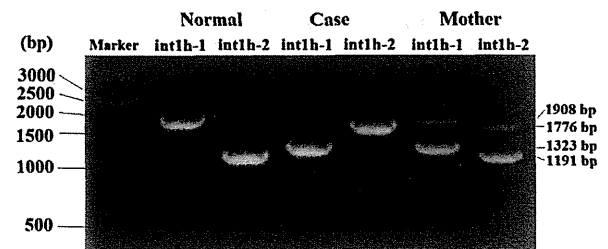| Primer	Primer sequence (5' to 3')
FVIII intron primers	
z1F	GGGAGCTAAAGATATTTTAGAGAA
1R	AAATAAATCCAATAGCCAACAAG
2F	TTGTCATATGGCATGAGAGAGGGAT
2R	CAATTCATTTGATAAAGCCAAC
3F	GCAGCTGGTATCATCTGAATTG
3R	ATGCACCATTGCTGGTAGAGG
4F	AGGGCTGCACTCCTTAGTGA
4R	GAACTTGAGGTGGGCAAAGATTTC
5F	GAAATCTTTGCCCACCTCAA
5R	GGTTTTCCCATGCTGTTCAC
6F	AATCATGGTGGAAGGCAAAG
6R	GGAACACTCATACTTTGGTAGTG
7F	TTGGATTGTTTCCCCTTTTG
7R	CCAACATGAAGAAACCCTGTCTAC
8F	GCTCTTGATCCTGTTCCCTCTCTG
8R	GATCTGAGGCGGACAGTTTC
9F	GTTGTTGGGAATGGTTACGG
9R	CACCACATCCAAACTCATGC
10F	GAGGCAAGTTGTCTGGACCTATATAG
10R	CCATCCTGGCTAACATGGTGAAACCC
11F	TTTGTTTGTTCGTTTGCATCT
11R	GCATTTGTCTTTTCATGACTGG
12F	CATGCTACGACATAGATGAACC
12R	TGGCAGCTGCACTTTTTAACTGC
FVIII intron1 segment 9 primers	
9aF	TTCCTAACAGGCCACAGACC
9aR	CACCCGAGAAAGCACGTAGT
9bF	GGAGTGAAATGCAGGGCTAC
9bR	TCAGGTTGCAGACAAAGCAG
9cF	GCGTTTTCCCTCAGTTACCA
9cR	CTAGCTTGAGCTCCCTGTGG
9dF	GAGGCAAGTTGTCTGGACCT
9dR	ATGGCTTACAAAGGGCATGA
int1h-2 primers	
int1h-2F	GGCAGGGATCTTGTTGGTAAA
int1h-2R	TGGGTGATATAAGCTGCTGAGCTA

Fig. 1. Detection of the FVIII int1h-related inversion by PCR. Amplification of int1h-1 using 9cR, 9F and int1h-2F primers and int1h-2 using int1h-2F, int1h-2R and 9F primers for Normal control, this case and the mother are displayed.
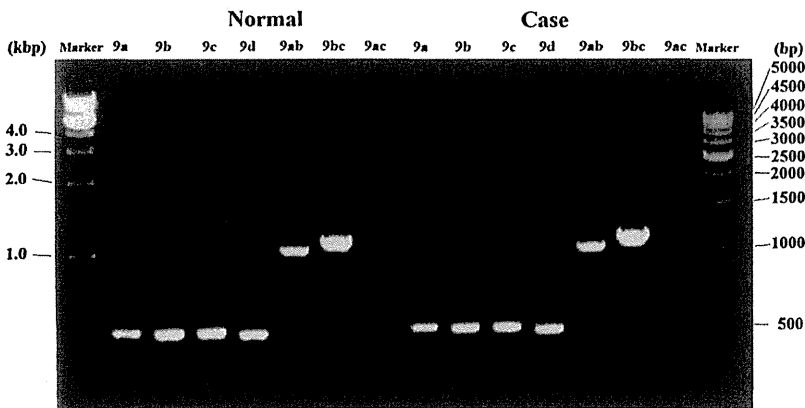
Fig. 2. PCR for the sections of segment 9 within intron 1. The sections of segment 9 within intron1 in control (left) and the case (right) were amplified by PCR. The 1.5 kb comprising sections a to c (9ac) could not be amplified from the DNA of the case. However, the 1-kb overlapping segments comprising sections a+b and b+c (9ab and 9bc) were amplified as readily as the individual sections 9a to d.

into overlapping 12 segments and the segment 9 was further divided into the sequential 4 sections (9a–d). Each segment was amplified with PCR using the primers shown in Table 2 according to the method reported previously [1]. The PCR reactions but for 9ac segment in this case with intron 1 inversion were successful while the PCR reactions for all segments in the normal DNA were successful (Fig. 2). The patterns of the PCR products indicated that the homologous recombination occurred at 9b, the same breaking point as described [1]. Neither intron 22 inversion nor any other mutation(s) in all exons and their flanking regions in *F8* was detected in this case. Furthermore, we analysed a group of Japanese 50 HA patients including this case consisting of 36 severe, 8 moderate and 6 mild forms from 45 unrelated families with the fully informed consent from January 2007 to March 2012 in our centre. The rest 49 of 50 patients were found to be not related to intron 1 inversion and the genotypes of them could be identified, including 11 of intron 22 inversions, 15 of missense mutation, 7 of nonsense mutation, 4 of small deletion, 5 of insertion and 7 of splicing variants, as a result of a direct sequencing of 26 exons and their flanking regions in *F8* after the screening of inversions in intron 22 and intron 1 performed by long distance PCR and multiplex PCR method respectively as described [1,4]. Thus, we found the first Japanese HA case with the int1h-related inversion. Taking it into account that the mother of this case was confirmed to be a carrier, it suggested that there should be more patients with intron 1 inversion in our country. The risk for inhibi-

tor development associated with int1h-related inversion is known to be as high as that with intron 22 inversion [2], and the inhibitor was actually developed in our case. As the ITI therapy is not always complete and the haemostatic management in HA patients with inhibitors is still complicated and expensive, the anticipation of inhibitor development by genotyping would be significant and tailor-made therapy for each genotype would be expected. In addition, the fact that the patient with intron 1 inversion exists also in our country may renovate the variety of *F8* mutations in Japanese haemophilia patients. A nation-wide prospective cohort study on the genotypes of Japanese haemophilia patients is being in progress, led by our centre, and it is expected that the results of the study will reveal the precise population of *F8* mutations in Japan.

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Authorship

K.Y. performed the research, analysed the data and wrote the paper; K.N. designed the research study and wrote the paper; T.K. and H.M. performed the research; M.S. interpreted data and edited the manuscript.

Disclosures

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

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ORIGINAL ARTICLE

Coagulation function and mechanisms in various clinical phenotypes of patients with acquired factor V inhibitors

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Summary. *Background:* The clinical phenotype of individuals with acquired factor V (A-FV) inhibitors varies from asymptomatic (non-B group) to life-threatening bleeding (B group), but the mechanism(s) underlying this variation in hemorrhagic phenotype are poorly understood. *Objective:* To investigate coagulation mechanistically in a range of patients with A-FV antibodies. *Methods and Results:* Ten cases of A-FV inhibitors in the non-B (*n* = 5) and B groups (*n* = 5) were studied. Thrombin generation assays in these plasmas revealed little thrombin generation, despite similar FV activity levels in both groups. However, prothrombin time-based clot waveform analysis revealed that the clot times were significantly prolonged and the maximum velocity and acceleration of coagulation were lower in the B group than in the non-B group, suggesting that this technique might be useful for predicting and monitoring hemorrhagic symptoms. A-FV inhibitors from the non-B group recognized predominantly the FV heavy chain, whereas those from the B group recognized the light chain. Purified anti-FV autoantibodies (autoAbs) from the B group inhibited FV binding to phospholipid by 60–90%, whereas there was little effect on this reaction in the non-B group. In addition, anti-FV autoAbs from the non-B group impaired the activated protein C (APC) cofactor activity of FV in FVIIIa inactivation mechanisms, and delayed APC-catalyzed cleavage of FVa at Arg306, but not at Arg506, indicating the presence of APC resistance in the non-B group. *Conclusions:* The results suggest that the different hemorrhagic phenotypes in A-FV inhibitors depend on the specific epitope of anti-FV autoAbs, and appear to be associated with an imbalance of procoagulant and anticoagulant function.

Keywords: APC resistance; blood coagulation factor inhibitors; clinical laboratory techniques; factor V; hemostatic techniques.

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Introduction

Factor V is a single-chain molecule consisting of 2196 amino acids arranged in six domains, A1–A2–B–A3–C1–C2 [1,2]. FV governs the balance of coagulation by regulating opposing functional mechanisms. The procoagulant action of FV is associated with cofactor activity for FXa in the prothrombinase complex, which catalyzes the conversion of prothrombin to thrombin on a phospholipid (PL) surface [3]. Thrombin proteolyses FV, generating the activated form (FVa), a heterodimer composed of a 105-kDa heavy chain (HCh), containing the A1 and A2 domains, and 71/74-kDa light chain (LCh), containing the A3, C1 and C2 domains. The development of a hypercoagulant state is controlled, however, by downregulation of cofactor activity by activated protein C (APC) with protein S (PS). FVa is rapidly inactivated by proteolytic cleavage at Arg506, and then at Arg306 and Arg679 [4]. Cleavage at Arg506 is essential for the exposure of other cleavage sites, but is unlikely to contribute significantly to the reduction in activity. Cleavage at Arg306 results in almost complete loss of FVa activity (FVa:C), but that at Arg679 has a more modest impact [5]. Irregularities in the mechanism of APC-mediated inactivation of FVa are therefore associated with thrombotic episodes in the presence of sustained prothrombin activation.

An alternative function of FV is an as anticoagulant cofactor of APC in the inactivation of FVIIIa [6]. FVIIIa is inactivated by cleavage at Arg336 by APC [7,8]. In the process of APC-mediated inactivation of FVIIIa, FV functions as an anticoagulant cofactor of APC, resulting in acceleration of FVIIIa inactivation [9]. This anticoagulant activity of FV is mediated by a product of proteoly-

sis by APC, prior to cleavage by thrombin. Cleavage at Arg506 of FV attached to the B domain is essential for the anticoagulant FV activity, whereas cleavage at Arg306 appears to be unlikely to contribute to this mechanism [9–12]. Any molecular defect of these cleavage reactions confers APC resistance (APCR). Clinically, individuals with the Arg506Gln mutation (FV Leiden) have a poor anticoagulant response to APC, which is associated with a significant increase in the risk of deep vein thrombosis (DVT) [13,14].

Acquired FV (A-FV) inhibitors occur rarely, but may develop spontaneously as autoantibodies (autoAbs) in previously normal individuals, after exposure to topical hemostatic agents containing bovine thrombin, antibiotic administration, cancer, and autoimmune disorders [15–18]. These anti-FV autoAbs are frequently associated with hemorrhagic symptoms, which are usually mild but are occasionally severe. Some patients remain asymptomatic, however, and hemorrhagic symptoms appear to be limited in ~ 20% of patients diagnosed with A-FV inhibitors [17,18]. It is of note that only four patients with A-FV inhibitors have presented with thrombotic manifestations [18]. Among these cases with DVT, Kalafatis *et al.* reported that the anti-FV autoAbs from one individual diminished both APC-mediated FVa inactivation and FV cofactor activity in APC-mediated FVIIIa inactivation, reflecting APCR [19]. Thrombotic mechanism(s) in other three cases appear not to have been explored, however. Moreover, the precise reasons for the variation in hemorrhagic phenotype in patients with A-FV inhibitors are poorly understood. We therefore investigated coagulation mechanisms in a range of patients with A-FV inhibitors, using a combination of established functional techniques.

Materials and methods

Reagents

Recombinant FVIII was a generous gift from Bayer (Osaka, Japan). Purified FV/FVa, FIXa, FX/FXa, prothrombin, α-thrombin, APC, PS, 5-dimethylamino-naphthalene-1-sulfonylarginine-N-(3-ethyl-1,5-pentanediyl)-amide (DAPA) and anti-FV HCh and LCh mAbs, AHV-5146 and AHV-5112, respectively (Hematologic Technologies, Essex Junction, VT, USA), hirudin (Calbiochem, San Diego, CA, USA) and chromogenic substrates S-2222 and S-2238 (Chromogenix, Milano, Italy) were commercially purchased. The activated partial thromboplastin time (APTT) and prothrombin time (PT) reagents, ellagic acid (Sysmex, Kobe, Japan), FV-deficient plasma (George King Biomedical, Overland Park, KS, USA), lipidated tissue factor (Innovin, Dade Behring, Marburg, Germany) and the thrombin substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) were purchased. PL vesicles (phosphatidylserine/phosphatidylcholine/phosphatidyleth-

anolamine; 10% : 60% : 30%) were prepared with N-octylglucoside [20]. HBS buffer (20 mM Hepes, pH 7.2, 0.1 M NaCl, 0.01% Tween-20) containing 2.5 mM $CaCl_2$ was used for dilution.

Blood samples

Whole blood was obtained by venepuncture from patients into tubes containing a 1 : 9 volume of 3.8% (w/v) trisodium citrate. Platelet-poor plasma was recovered after centrifugation of citrated whole blood for 10 min at 1500 × g. Normal pooled plasmas were prepared from 30 normal healthy individuals (25 : 5 male/female). All plasmas were stored at − 80 °C and thawed at 37 °C immediately prior to the assays. All samples were obtained after informed consent had been obtained, following local ethical guidelines.

FV activity (FV:C), FV antigen (FV:Ag) and FV inhibitor levels

FV:C and FV:Ag were measured with PT-based clotting assays with FV-deficient plasma and with ELISAs, respectively. A-FV inhibitor titers were determined with the Bethesda method described for FVIII antibodies [21].

Anti-FV inhibitor autoAbs

Anti-FV IgGs were purified from the plasma of patients with A-FV inhibitors. IgG preparations were fractionated by affinity chromatography on protein G–Sepharose. F(ab′)₂ fragments were prepared by the use of immobilized pepsin–Sepharose (Pierce, Rockford, IL, USA). Specific regions of FV/FVa recognized by these antibodies were determined with SDS-PAGE and western blotting. The binding of FV/FVa fragments to anti-FV autoAbs was detected by the addition of anti-human peroxidase-linked antibody. The effects of FV inhibitors were expressed as a function of IgG concentration in this study, although the percentage of anti-FV in IgG preparations varied between patients.

Clot waveform analysis (CWA)

PT and APTT measurements were performed with the MDA-II Hemostasis System (Tcoag Ireland, Bray, Ireland). The clot waveforms obtained were computer-processed with the commercial kinetic algorithm [22]. The minimum value of the first derivative (min1) was calculated as an indicator of the maximum velocity of coagulation achieved. The minimum value of the second derivative (min2) was calculated as an indicator of the maximum acceleration of the reaction achieved. As the minima of min1 and min2 are derived from negative changes, the data were expressed as min1 and min2. The

clot time was defined as the time until the start of coagulation.

Prothrombinase assay

The rate of conversion of prothrombin to thrombin was monitored in a purified system. FVa (2 nM) was incubated with various concentrations of anti-FV autoAbs at 37 °C for 30 min. The reactants were mixed with prothrombin (1.4 µM), PL (20 µM), and DAPA (30 µM), and this was followed by initiation of the addition of FXa (10 pM). Aliquots were removed at appropriate times to assess the initial rates of product formation, and were mixed with EDTA (final concentration of 50 mM) to quench the reactions. Rates of thrombin generation were determined at an absorbance of 405 nm after the addition of S-2238 (final concentration of 0.46 mM). Thrombin generation was quantified from a standard curve prepared with known amounts of thrombin.

FV–PL binding assay

Binding of FV to immobilized PL was examined in a solid-phase based ELISA [23]. α-Phosphatidyl-L-serine (5 µg mL^{-1}) in methanol was added to microtiter wells and air-dried at 4 °C overnight. After washing of the wells, the wells were blocked by the addition of gelatin solution (5 mg mL^{-1}) at 37 °C for 2 h. FV (1 nM) and anti-FV autoAbs were incubated for 2 h. After washing, the mixture reactants were added to the PL-coated well, and incubated for 2 h. Bound FV was quantified by the addition of the anti-FV mAb AHV-5146 (2.5 µg mL^{-1}), followed by a goat anti-mouse peroxidase-linked antibody (3 µg mL^{-1}) and O-phenylenediamine dihydrochloride substrate. Reactions were quenched by the addition of 2 M H$_2$SO$_4$, and absorbance at 492 nm was measured. The amount of non-specific IgG binding without FV was < 3% of the total signal. Specific binding was estimated by subtracting the amount of non-specific binding.

APCR assays

Chromogenic assay FXa generation in plasma samples was performed with the COATEST SP FVIII assay (Chromogenix). The test specifically quantifies FVIIIa activity in 16-fold-diluted plasma, by measuring intrinsic FXa generation mediated by excess exogenous FIXa and FX with PL and CaCl$_2$. The simultaneous addition of APC (40 nM) with the cofactors PS and FV contained in plasma inhibits intrinsic FXa generation by inactivating FVIIIa. The APC sensitivity ratio (APCsr) was expressed as the absorbance in the absence of APC divided by that in its presence. A low APCsr value indicates a defect in the inactivation of FVIIIa, reflecting the APCR.

APC cofactor activity of FV The APC cofactor activity of FV was measured with an FVIIIa degradation assay, as previously reported, with minor modifications [9]. FVIII (10 nM) and PL (20 µM) were activated by thrombin (5 nM) for 30 s, and the reaction was terminated by the addition of hirudin (2.5 U mL^{-1}). The generated FVIIIa was then incubated for 7 min with APC (0.5 nM), PS (5 nM), and the mixtures of FV (1 nM) and the indicated amounts of anti-FV autoAbs or normal IgG. The mixture was 10-fold-diluted prior to incubation with FIXa (2 nM) and FX (200 nM) for 1 min. After the addition of EDTA, generated FXa was evaluated by the use of S-2222 at 405 nm. Control experiments were performed without either APC or FV. FXa generation was quantified from a standard curve prepared with known amounts of FXa.

APC-catalyzed cleavage of FVa FV (8 nM) and anti-FV autoAbs (30 µg mL^{-1}) were incubated for 2 h at 37 °C. The mixtures were incubated with thrombin (30 nM) for 5 min at 37 °C, and the thrombin reaction was rapidly terminated by the addition of hirudin (10 U mL^{-1}). Samples containing the generated FVa (2 nM) were incubated with PL (20 µM), PS (30 nM), and APC (0.7 nM). Aliquots were removed from the mixtures at the indicated times, and reactions were immediately terminated by adding SDS and boiling for 3 min.

Western blot analysis

SDS-PAGE was performed with 8% gels, and this was followed by western blotting [24]. Protein bands were probed with the indicated anti-FV mAb, and this was followed by the addition of goat anti-mouse peroxidase-linked antibody. Signals were detected with enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA). Densitometric scans were quantified with IMAGEJ 1.34 (NIH, Bethesda, MD, USA).

Results

Global coagulation function in plasmas from patients with A-FV inhibitors

The properties of five patients with A-FV inhibitors in the asymptomatic group (non-B group) and five patients with severe bleeding symptoms (B group) are summarized in Table 1. To define the bleeding symptoms, we used the Vicenza Bleeding Score [25] to evaluate hemorrhage. In the B group, the scores ranged from 3 to 7, whereas in the non-B group the scores were 0. In both groups, both the PT and the APTT were markedly prolonged, and FV: C ranged from undetectable to low levels (~ 10 IU dL^{-1}), showing that the routine clotting tests and FV:C levels did not well reflect the clinical phenotype. FV:Ag levels

Table 1 Properties of patients' plasmas with acquired factor V inhibitors

Case	Sex	PT (s)	APTT (s)	FV:C (IU dL^{-1})	FV:Ag (IU dL^{-1})	Inhibitor titer (BU mL^{-1})	Ig subtype	Bleeding score*	Underlying disease
Asymptomatic (non-B group)									
1	M	53.5	> 150	2.3	93.8	4.3	IgG	0	Chronic thyroiditis
2	M	47.4	133	11.5	86.3	5.4	IgG	0	Progressive supranuclear palsy
3	M	67.7	> 150	< 1.0	137	11.8	IgG	0	Intraductal papillary mucinous tumor of pancreas
4	M	83.6	> 150	< 1.0	180	1.7	IgG	0	Atrial fibrillation
5	F	34.1	66.8	1.7	55.6	8.7	IgG	0	None
Severe bleeding (B group)									
6	M	94.5	> 150	8.0	81.3	118	IgG	4	Aspiration pneumonia, asthma
7	F	56.0	> 150	< 1.0	75.0	16	IgG	4	Surgery for valve replacement (TR, AS, MR)
8	M	> 100	> 150	< 1.0	57.8	64	IgG	3	Chronic renal failure
9	M	> 100	> 150	< 1.0	2.1	9.9	IgG	5	None
10	M	81.1	92.3	< 1.0	36.2	8.2	IgG	7	None
Control	–	12.1	30.3	–	–	–	–	–	–

APTT, activated partial thromboplastin time; AS, aortic stenosis; F, female; FV:Ag, FV antigen; FV:C, FV activity; M, male; MR, mitral regurgitation; PT, prothrombin time; TR, tricuspid regurgitation. *Numbers represent the bleeding score calculated according to Rodeghiero et al. [25].

(except for case 9) were not significantly different between groups, supporting the contention that low FV:C in patients was attributable to functional inhibition by A-FV inhibitors. FV inhibitor titers were 6.4 ± 3.9 and 43 ± 48 BU mL^{-1} in the non-B group and B group, respectively. The immunoglobulin class was IgG in all patients, and none had been exposed to antibiotic therapy or bovine thrombin products. In all cases, platelet counts, other coagulation proteins, anticoagulant proteins (protein C [PC]/PS, antithrombin) and fibrinolytic proteins showed normal plasma levels. No autoAbs, except for those against FV, were detectable (data not shown). These findings typified the difficulties in identifying the different clinical phenotypes on the basis of basic coagulation tests.

We compared the different inhibitor groups by using the following global coagulation assays: the thrombin generation test (TGT) and CWA. There was little evident thrombin generation in patient plasmas, even after 60 min of reaction time (data not shown), suggesting that evaluation of the different A-FV inhibitors with this technique was not informative. Unlike the TGT, CWA reflects the process of fibrin formation. Representative curves of the PT-based CWA are shown in Fig. 1 (upper). Because of the small sample volume from case 7, this case failed to show the clot waveform. The clot time observed in the non-B group was markedly shorter that that in the B group (56.5 ± 19.6 vs. 104 ± 20 s, $P = 0.0044$) (Fig. 1A), and the parameters min1 and min2 were significantly greater in the non-B group than in the B group (min1, 3.09 ± 1.00 vs. 0.87 ± 0.32, $P = 0.0022$; min2, 0.90 ± 0.48 vs. 0.15 ± 0.06, $P = 0.009$) (Fig. 1B,C). These findings appeared to be in keeping with the contrasting hemorrhagic symptoms observed in the different patients. The data suggested that CWA might be a useful

method for predicting and monitoring the bleeding tendency in patients with A-FV inhibitors.

Binding epitope(s) of A-FV inhibitors

To clarify the precise mechanism(s) involved in the distinct phenotypes in patients with A-FV inhibitors, we initially attempted to determine the FV epitopes of the antibodies by immunoblotting. Figure 2 shows that, in the non-B group, three inhibitors reacted with the HCh alone. In contrast, in the B group, two inhibitors reacted with the LCh alone, and two inhibitors reacted with both chains. The results suggested the possible presence of distinct epitopes of anti-FV autoAbs between both groups. The reason for the presence of some bands in FV lanes (cases 1 and 10) was unclear, but partially proteolysed FV may be a contaminant of the single-chain FV preparation. Binding to FV(a) fragments was not observed in two cases from the non-B group and in one case from the B group. This may be attributable to weak binding reactivity of individual inhibitors and/or to low sensitivity of this assay.

Effects of purified anti-FV autoAbs on FV–PL binding

The specific properties of A-FV inhibitors were further examined with immune-purified IgGs from the patients' plasmas. With mixtures of normal plasma and purified anti-FV IgG, all CWA parameters were similar to those obtained with native patients' plasmas (data not shown), confirming that the defective coagulation function in both groups was attributable to the presence of antibody. The LCh of FV, and in particular the C2 domain, contains PL-binding site(s) [26]. The effects of purified anti-FV autoAbs on FV binding to immobilized PL were therefore examined with a solid-phase-based ELISA. All IgGs from