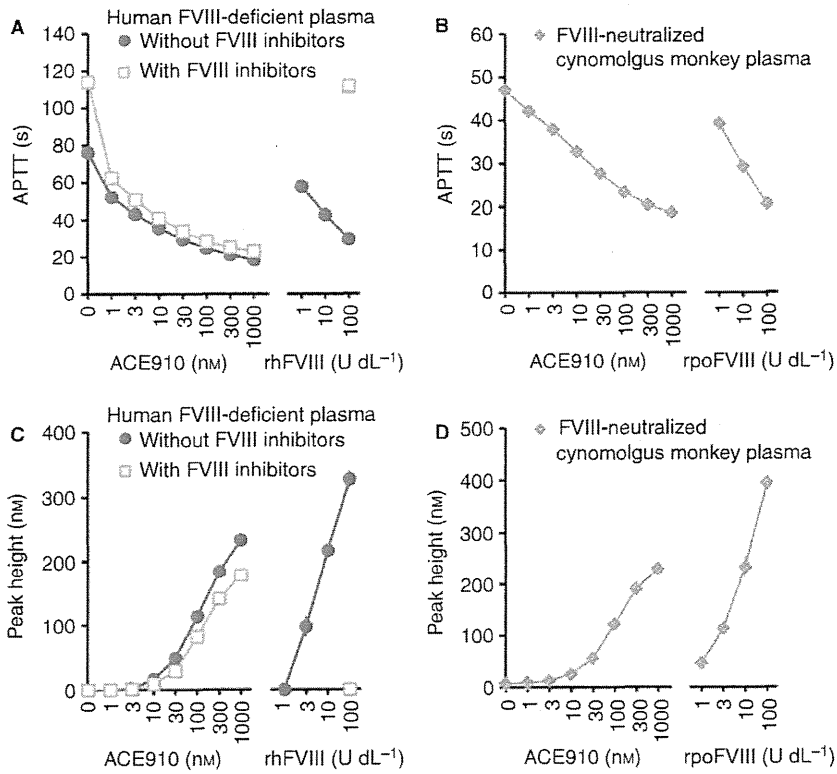

Fig. 1. FVIII-mimetic cofactor activity of ACE910 in human FVIII-deficient plasma without and with FVIII inhibitors and in FVIII-neutralized cynomolgus monkey plasma. Effects of ACE910, recombinant human FVIII (rhFVIII) or recombinant porcine FVIII (rpoFVIII) on activated partial thromboplastin time (APTT) (A, B) and on peak height of thrombin generation triggering the intrinsic pathway (C, D), in human FVIII-deficient plasma without and with FVIII inhibitors (A, C) and in FVIII-neutralized cynomolgus monkey plasma (B, D). Data are expressed as mean ± standard deviation (n = 3).

The experimental protocol is illustrated in Fig. 2A. An acquired hemophilia A status was first established by injecting an anti-primate FVIII antibody, VIII-2236, which neutralizes endogenous FVIII, but neither exogenous rpoFVIII nor ACE910 (Fig. S3). Then, bleeding was artificially induced by inserting a needle in the limb muscles and by subcutaneous exfoliation on the abdomen. The animals in the control group showed a progressive decrease in hemoglobin level (anemia associated with hemorrhage) and expansion of bruised areas (Fig. 2B,C). A single intravenous administration of ACE910 at 6–8 h after bleeding induction, when visible bleeding symptoms had emerged, tended to ameliorate the decrease in hemoglobin level (P = 0.0643 at 3 mg kg^{-1} vs. control). The expansion of bruised areas was significantly reduced at doses of 1 and 3 mg kg^{-1} ACE910 (P < 0.05 vs. control). These hemostatic effects of ACE910 at 1 and 3 mg kg^{-1} were comparable to the hemostatic effect of dosing twice daily with 10 U kg^{-1} rpoFVIII. In such a regimen, the plasma concentration of rpoFVIII would reach 25 U dL^{-1} just after the first injection, and would range between 7.4 and 46 U dL^{-1}, according to a simulation of multiple dosing of rpoFVIII based on the pharmacokinetic parameters obtained from the single-dose injection study of rpoFVIII in cynomolgus monkeys (Fig. S4). The mean plasma concentration of ACE910 (0.3, 1 or 3 mg kg^{-1}) was, respectively, 6.6, 26 or 61 μg mL^{-1} (45, 180 or 420 nM) just after administration, and 3.0, 8.4 or 34 μg mL^{-1} (21, 58 or 230 nM) on day 3 (Fig. 2D). In the clinical setting, 20 U dL^{-1} is often employed as the target initial FVIII level for treatment of ongoing bleeds [12]. Therefore, intravenous administration of 1–3 mg kg^{-1} ACE910, or a plasma concentration of 26–61 μg mL^{-1} (180–420 nM), is also expected to exert hemostatic activity against ongoing bleeds in the clinical setting.

Pharmacokinetic study and multiple-dosing simulation

In order to investigate the potency of ACE910 for routine supplementation, we performed a single-dose pharmacokinetic study of ACE910, to determine the pharmacokinetic parameters for simulating the plasma ACE910 concentration after multiple dosing.

The plasma half-life of ACE910 was 19.4 days after a single intravenous administration at 6 mg kg^{-1}, and in the range of 23.6–26.5 days after a single subcutaneous administration at 0.06, 0.6 or 6 mg kg^{-1} (Table S1). With

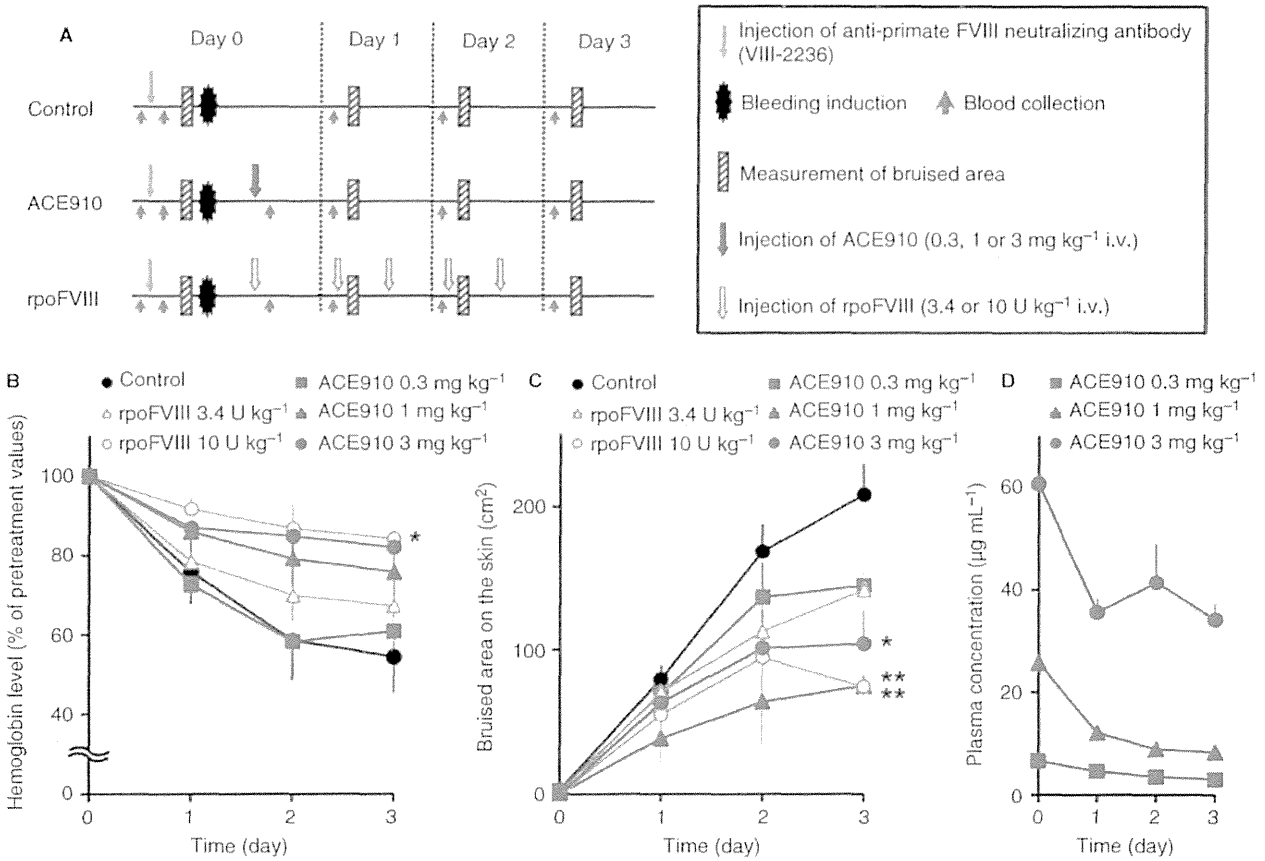

Fig. 2. *In vivo* hemostatic activity of ACE910 against ongoing bleeds in an acquired hemophilia A model. (A) The experimental protocol used. (B, C) Time course changes of (B) hemoglobin level and (C) bruised areas in the control group (no test item; $n = 6$), the ACE910 group (0.3, 1 or 3 mg kg^{-1}; $n = 4$ for each group), and the recombinant porcine FVIII (rpoFVIII) group (3.4 or 10 U kg^{-1}; $n = 4$ for each group). Asterisks show statistical significance of the data on day 3 (*$P < 0.05$ and **$P < 0.01$ vs. control). (D) Time course of plasma ACE910 concentration in the ACE910 groups. Data are expressed as mean ± standard error. i.v., intravenous.

subcutaneous administration, the maximum plasma concentration of ACE910 increased in approximate proportion to the dose increment. The subcutaneous bioavailability was 102.3% at the 6 mg kg^{-1} dose. These results were consistent with those of our previous study [9]. For these analyses, we excluded two animals in which anti-ACE910 alloantibodies were detected, respectively, from 28 days after the intravenous administration of 6 mg kg^{-1} and from 56 days after the subcutaneous administration of 0.06 mg kg^{-1}. Their plasma ACE910 concentrations decreased in association with the detection of anti-ACE910 alloantibodies.

In the *in vivo* hemostatic study, the mean initial plasma concentrations of ACE910 were 26 and 61 μg mL^{-1} (180 and 420 nM) in the 1 and 3 mg kg^{-1} groups, respectively. The hemostatic effect in these groups was comparable to that in the rpoFVIII 10 U kg^{-1} group, in which the FVIII level was within the range of a mild phenotype (Fig. S4B). We considered that if, by routine supplementation, a plasma ACE910 level of 26 μg mL^{-1} or above were maintained at all times in patients, a severe phenotype would possibly be converted to a mild phenotype beyond a moderate phenotype. To examine this possibility, multiple-dosing simulations of ACE910 were performed with the parameters obtained from the pharmacokinetic study. The results of the simulations indicated that, if the target trough plasma level of ACE910 were set to 26 or 61 μg mL^{-1}, it could be maintained by once-weekly subcutaneous administrations of 0.64 or 1.5 mg kg^{-1} at a steady state, respectively (Fig. 3).

Discussion

We previously reported the creation of an anti-FIXa/FX bispecific antibody, named hBS23, which restored FVIII cofactor function [8]. Although hBS23 had meaningful hemostatic activity, its molecular structure would have required further optimization in terms of manufacturing efficiency, immunogenicity, pharmacokinetic profile, physicochemical properties, and FVIII-mimetic cofactor activity. To address these remaining issues, we continued to

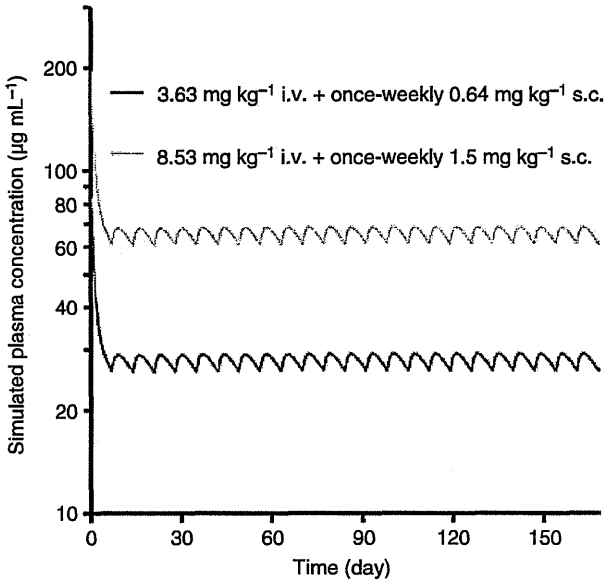

Fig. 3. Examples of simulations; plasma ACE910 concentration after multiple dosing in cynomolgus monkeys. The time course of plasma ACE910 concentration was simulated by use of the pharmacokinetic study data in cynomolgus monkeys for the case of once-weekly subcutaneous (s.c.) administration at 0.64 or 1.5 mg kg^{-1}, starting 7 days after the initial bolus intravenous administration of 3.63 or 8.53 mg kg^{-1}, respectively. i.v., intravenous.

optimize the bispecific antibody multidimensionally, and finally identified an improved one, ACE910, for clinical investigation [9]. ACE910 had twice the effect on increasing catalytic efficiency, 1.5 times the *in vivo* half-life and higher subcutaneous bioavailability than hBS23. Furthermore, ACE910 was able to be purified on a large manufacturing scale and formulated into a subcutaneously injectable liquid formulation. However, the degree of *in vivo* hemostatic potency of ACE910 remained unproven. We hypothesized that approximately 300 nM (44 μg mL^{-1}) of plasma ACE910 would exert an *in vivo* hemostatic activity equivalent to 10 U dL^{-1} FVIII, as 300 nM ACE910 showed *in vitro* cofactor activity similar to that of 10 U dL^{-1} FVIII, in terms of the peak height in the TG assay in human FVIII-deficient plasma (Fig. 1C) [9]. When making this hypothesis, we did not use the APTT data. ACE910 strongly shortened APTT, even beyond the level achieved with 100 U dL^{-1} FVIII at more than 300 nM (Fig. 1A), but we considered that this phenomenon could be attributed to the fact that FVIII requires additional time to be activated by thrombin or FXa, whereas ACE910 does not.

In order to prove the hypothesis, we had to detect appropriately the *in vivo* hemostatic activity of a plasma level of approximately 10 U dL^{-1} rpoFVIII. For this purpose, we employed more intensive injury procedures, and changed the timing of administration of the test items to after bleeding symptoms had emerged. In the clinical setting, the treatment of ongoing bleeds minimally requires a plasma FVIII level of 10–20 U dL^{-1}, which is much higher than the level required for prophylactic bleeding prevention (1 U dL^{-1}) [12]. As a result, intravenous administration of 10 U kg^{-1} (twice daily) of rpoFVIII showed a significant hemostatic effect, whereas a hemostatic effect of 3.4 U kg^{-1} (twice daily) of rpoFVIII was not clearly detected in this model. The multiple-dosing simulations of rpoFVIII in cynomolgus monkeys indicated that, with twice-daily doses of 3.4 or 10 U kg^{-1}, the plasma rpoFVIII level would be, respectively, 8.5 or 25 U dL^{-1} at the outset, would remain at more than 2.5 or 7.4 U dL^{-1}, and would reach a maximum of 16 or 46 U dL^{-1} by the end of the observation period (Fig. S4B). Therefore, we judged that this re-established model was well validated in terms of the reactivity to FVIII. Using this validated model, we elucidated the *in vivo* hemostatic potency of ACE910. A single intravenous administration of ACE910 at 1 or 3 mg kg^{-1} ameliorated bleeding symptoms to an extent equivalent to that achieved with twice-daily doses of 10 U kg^{-1} rpoFVIII. Among the results, it seems contradictory that the mean bruised area of the ACE910 1 mg kg^{-1} group was smaller than that of the 3 mg kg^{-1} group. From the viewpoint of ethics for primates, we employed the minimum number of animals possible to detect a hemostatic effect. Therefore, we think that this variation in dose dependency occurred incidentally, because the deviation in the bruised area was rather large.

The pharmacokinetic profiles of ACE910 and rpoFVIII were different, and therefore it is quite difficult to compare their *in vivo* hemostatic activities in terms of plasma level. However, to say the least, the hemostatic activity at the maximum plasma level of ACE910, 26 or 61 μg mL^{-1}, would have reached that at the minimum plasma level of rpoFVIII, 7.4 U dL^{-1}. If the two agents were compared according to their initial plasma levels, 26 or 61 μg mL^{-1} plasma ACE910 would have shown hemostatic activity equivalent to that of 25 U dL^{-1} rpoFVIII. Given that ACE910 should work equivalently in humans and cynomolgus monkeys (Fig. 1C,D), and that ACE910 fully exerted its activity even in the presence of FVIII inhibitors (Fig. 1A,C), ACE910 could be possibly an effective and long-acting treatment option to ameliorate ongoing bleeds in patients with FVIII inhibitors.

We also consider that ACE910 will be highly valuable for routine prophylaxis against bleeding. Current routine prophylaxis with exogenous FVIII is aimed at converting a severe disease (< 1 U dL^{-1} FVIII) to a moderate one (1–5 U dL^{-1}), but it requires frequent venous access, typically three times weekly. This negatively affects both the implementation of and adherence to the supplementation routine, particularly for pediatric patients treated at home [7]. In addition, the development of FVIII inhibitors deprives them of this treatment option. As ACE910 is expected to be a long-acting, subcutaneously injectable agent that is unaffected by the presence of FVIII inhibi-

tors, it will be able to resolve the drawbacks inherent to exogenous FVIII and its prophylactic use [13,14]. Furthermore, although routine prophylaxis with exogenous FVIII effectively reduces joint bleeds and prevents joint damage, its prophylactic effect is not necessarily perfect [5,10]. In line with this, the clinical outcomes of patients with moderate hemophilia A vary, and the proportion of them who suffer from joint impairment is not negligible [1]. Therefore, keeping FVIII levels within the range of a mild phenotype (> 5 U dL^{-1}) may provide patients with substantial benefits in terms of preserving joint status and enabling patients to participate in physical activities [15]. As mentioned above, even by a conservative estimate, 61 μg mL^{-1} plasma ACE910 would be expected to show hemostatic activity within the range of a mild phenotype.

Generally, pharmacokinetic data of therapeutic antibodies from cynomolgus monkeys can be scaled to project human pharmacokinetic profiles [16], and the simulated plasma concentration–time profiles from the pharmacokinetic parameters are known to be comparable to the actual observed profiles for therapeutic antibodies [17]. Therefore, we conducted multiple-dosing simulations with the pharmacokinetic study data in cynomolgus monkeys, and found that 61 μg mL^{-1} plasma ACE910 would be maintained at a steady state by once-weekly subcutaneous administration of 1.5 mg kg^{-1} (Fig. 3). The simulation is, of course, not the same as actual data, but we have since confirmed that the simulation of the time profile of plasma ACE910 concentration with the above pharmacokinetic parameters gave a good prediction of the actual data in another cynomolgus monkey study employing multiple dosing with ACE910 (Y. Sakamoto, unpublished data). Therefore, we think that the simulation would work well.

In the pharmacokinetic study, two of 12 animals developed anti-ACE910 alloantibodies. In cynomolgus monkeys, the development of anti-humanized antibody alloantibodies is theoretically inevitable, and their reported incidence rates vary (0–100%) [18]. Unfortunately, it has been found that the immunogenicity in cynomolgus monkeys cannot predict that in humans [18].

In terms of subcutaneous injection, the upper limit of the dosing amount is generally considered to be 1 mL or less than 2 mg kg^{-1} of therapeutic antibodies [19], and ACE910 has a sufficiently high solubility for such a subcutaneous dosage to be obtained with a small injection volume [9]. Thus, we expect that once-weekly subcutaneous administration of ACE910 will provide more aggressive routine prophylaxis aimed at achieving a mild phenotype in hemophilia A patients both without and with FVIII inhibitors.

In conclusion, this study suggests that ACE910 has the potential not only to ameliorate ongoing bleeds, even in patients with FVIII inhibitors, but also to offer a user-friendly and aggressive routine prophylaxis for patients both without and with FVIII inhibitors. ACE910 may provide great benefits to all patients with severe hemophilia A, including pediatric patients and patients with FVIII inhibitors.

Addendum

A. Muto, K. Yoshihashi, M. Takeda, T. Kitazawa, T. Soeda and Y. Kawabe designed and performed the pharmacologic studies. T. Igawa, Y. Sakamoto and K. Haraya designed and performed the pharmacokinetic studies. M. Shima and A. Yoshioka provided advice from the viewpoints of their medical expertise in hemophilia. K. Hattori provided direction and organized the program. A. Muto and T. Kitazawa wrote the manuscript.

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Disclosure of Conflict of Interests

A. Muto, K. Yoshihashi, T. Kitazawa, T. Soeda, T. Igawa, K. Hattori, M. Takeda, Y. Sakamoto, K. Haraya and Y. Kawabe are employees of Chugai Pharmaceutical, and the first six of these authors are inventors of the patents relating to anti-FIXa/FX bispecific antibodies, all rights for which have been assigned to the company. M. Shima receives consulting honoraria and research support from Chugai Pharmaceutical. A. Yoshioka previously received research support from Chugai Pharmaceutical.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Pharmacokinetic parameters of ACE910 in cynomolgus monkeys.
Fig. S1. Effects of ACE910 or rhFVIII on peak height of thrombin generation triggered by low TF in human FVIII-deficient plasma without FVIII inhibitors.
Fig. S2. SDS-PAGE analysis of rpoFVIII. Purified rpoFVIII (0.95 μg) was analyzed by SDS-PAGE with 4–20% gradient gel under reducing conditions, followed by staining with Coomassie brilliant blue.

Fig. S3. Influence of VIII-2236 on the APTT-shortening activity of rpoFVIII, ACE910 or rhFVIII in human FVIII-deficient plasma. In the absence of VIII-2236, rpoFVIII, ACE910 and rhFVIII concentration-dependently shortened the APTT of human FVIII-deficient plasma.

Fig. S4. Pharmacodynamic study and multiple dosing simulations of rpoFVIII in cynomolgus monkeys.

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A Novel Cell-Sheet Technology That Achieves Durable Factor VIII Delivery in a Mouse Model of Hemophilia A

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Abstract

Gene- or cell-based therapies aimed at creating delivery systems for coagulation factor VIII (FVIII) protein have emerged as promising options for hemophilia A treatment. However, several issues remain to be addressed regarding the efficacies and adverse events of these new classes of therapies. To improve an existing cell-based therapy involving the subcutaneous transplantation of FVIII-transduced blood outgrowth endothelial cells (BOECs), we employed a novel cell-sheet technology that allows individual dispersed cells to form a thin and contiguous monolayer without traditional bioabsorbable scaffold matrices. Compared to the traditional methodology, our cell-sheet approach resulted in longer-term and 3–5-fold higher expression of FVIII (up to 11% of normal) in recipient hemophilia A mice that lacked a FVIII humoral immune response due to transient immunosuppression with cyclophosphamide. Histological studies revealed that the transplanted BOEC sheets were structured as flat clusters, supporting the long-term expression of therapeutic FVIII in plasma from an ectopic subcutaneous space. Our novel tissue-engineering approach using genetically modified BOEC sheets could aid in development of cell-based therapy that will allow safe and effective *in vivo* delivery of functional FVIII protein in patients with hemophilia A.

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Introduction

Hemophilia A is an inherited bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII). Currently, patients with hemophilia A are treated with plasma-derived or recombinant FVIII concentrates [1]. This form of protein-replacement therapy has improved management of bleeding in hemophilia A patients. However, this method is also problematic because of the requirement for frequent venous access as well as the limited availability and high costs of FVIII concentrates. To address such problems, gene- or cell-based therapies are attractive alternative strategies, and such methods are now expansively being in the progress for the disease. Indeed, continuous expression of FVIII levels as low as 1–5% of normal substantially ameliorates the bleeding phenotype and improves quality of life in preclinical [2–5] and clinical settings [6–8].

We previously reported that therapeutic levels of plasma FVIII can be successfully achieved in hemophilia A mice by subcutaneous implantation of lentivirally engineered blood outgrowth endothelial cells (BOECs) mixed with Matrigel [9]. However, in that system we observed gradual loss of plasma FVIII, probably due to breakdown of the scaffold material or cell death.

To overcome these issues, we employed cell-sheet technology, an innovative tissue-engineering approach that allows individual dispersed cells to form a thin and contiguous monolayer; this method has recently shown great promise in regenerative medicine [10–11]. In fact, our previous studies [12–13] indicated that cell sheets engineered from a number of sources have considerable benefits, and can strengthen the viability and functionality of cells implanted in the subcutaneous space for therapeutic purposes. Here, we report a unique and effective tissue-engineering approach using BOEC sheets as a new class of potential cell-based treatment for hemophilia A.

Materials and Methods

Animals

Immunocompetent C57Bl/6 hemophilia A mice with targeted destruction of exon 16 of the *FVIII* gene [14] were a kind gift from Prof. Yoichi Sakata (Jichi Medical University, Shimotsuke, Japan). Wild-type C57Bl/6 mice syngenic to the hemophilia A mice were

used as donors of normal mouse plasma. All animal procedures were reviewed and approved by the Animal Care Committee at Nara Medical University.

Isolation and lentiviral vector transduction of BOECs *in vitro*

Isolation of BOECs from hemophilia A mice and *in vitro* FVIII transduction of hemophilia A mouse BOECs, using a lentiviral vector that encodes the canine B-domain deleted FVIII (BDD-FVIII) under the control of the EF1-alpha (EF1α) promoter, were described previously [9,15]. In brief, cultured murine BOECs (1×10^5) were transduced following single exposure of the Lenti-EF1α-cFVIII viral vectors at increasing multiplicities of infection (MOI). After transduction, cells were expanded, and assessment of FVIII expression from BOECs was carried out using a functional chromogenic assay described below.

Fabrication of genetically modified BOEC sheets

The lentivirally modified hemophilia A mouse BOECs expressing canine FVIII were seeded on temperature-responsive culture dishes (UpCell, CellSeed, Tokyo, Japan) [10–11]. The dishes were created by covalently grafting Poly (N-isopropylacrylamide) (PIPAAm) by electron-beam irradiation. Normal- and large-sized cell sheets were generated using 35-mm and 100-mm dishes, respectively. When cultured BOECs reached confluency, they were detached from PIPPAm dishes as uniformly connected tissue sheets by lowering the culture temperature to 20°C for 30 min.

Transplantation of BOEC sheets to hemophilia A mice

Cell counting revealed that normal-sized and large-sized BOEC sheets consisted of $2.8\pm0.4\times10^5$ and $2.0\pm0.2\times10^6$ cells, respectively. BOEC sheets were recovered with support membranes for transplantation into subcutaneous sites in hemophilia A mice. To avoid excessive surgical procedure–related bleeding, all recipient hemophilia A mice received an intraperitoneal injection of 0.5 mL pooled normal mouse plasma 30 min prior to surgical procedures. All surgeries were conducted under general anesthesia using isoflurane. Because canine FVIII is inherently immunogenic in hemophilia A mice, some recipient mice also received intraperitoneal injection of cyclophosphamide (20 mg/kg per injection) administered on the day of transplantation and then biweekly for 4 weeks. All recipient hemophilia A mice that did not receive this treatment developed an anti–canine FVIII humoral immune response.

FVIII activity, FVIII antigen and FVIII antibody assays

Functional FVIII was quantified by a chromogenic assay as previously described [9]. FVIII antigen was calculated by canine FVIII ELISA kit (Affinity Biologicals, Ancaster, ON, Canada). Development of anti–canine FVIII humoral response was detected and quantitated by the Bethesda assay [16]. The standard curve was generated with pooled normal canine plasma. Same mouse plasma samples were used in these assays.

Tail-clip bleeding tests

Successful long-term phenotypic correction was tested in both untreated wild-type mice and hemophilia A mice that received transplants of BOEC sheets. At the termination of the experiments, phenotypes were analyzed by anesthetizing the mice with isoflurane and clipping the tails at the position where the tail diameter was 0.5 mm. The mice were then observed for 1 hour to determine the bleeding time.

Subcutaneous implant removal and immunohistochemical analysis

Eight weeks after transplantation, some mice were sacrificed. The implants of sacrificed mice were recovered, fixed with 4% formalin, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E) staining. To assess FVIII expression in BOECs, specimens were characterized with double immunostaining for FVIII and vWF as previously described [9]. Specimens were viewed with a confocal laser scanning microscope (CLSM, FV300; Olympus Co., Tokyo, Japan). H&E and immunostaining were performed on sequential sections.

Results and Discussion

Recent preclinical and clinical studies using adeno-associated viral (AAV) vectors for hemophilia B demonstrated that the safety profile is partly determined by vector dose, and that immune responses to AAV-capsid proteins with subsequent hepatocyte toxicity require transient immunosuppression in order to achieve sustained transgene expression [17–20]. However, some concerns still remain regarding the safety of systemic injection of viral vectors. Potential side effects include adverse immunological reactions, vector-mediated cytotoxicity, germ-line transmission, and insertional oncogenesis [21–23]. Moreover, especially in hemophilia A, an alternative transgene delivery approach may be necessary due to the large size of the FVIII cDNA. Therefore, considering the aforementioned issues, we elected to investigate an *ex vivo* gene-transfer strategy that avoids systemic administration of a viral vector.

In the Transkaryotic Therapy study, the first *ex vivo* gene-transfer strategy for hemophilia A patients in the clinic, the limited viability of the implanted autologous fibroblasts failed to provide sustained therapeutic levels of FVIII [8]. In this regard, tissue-engineering approaches using cell-sheet technology have already been applied in different clinical settings as therapeutic modalities for several diseases, including corneal disease [24], wounds of the esophageal mucosa [25], heart failure [26], and periodontitis [27]. In addition, we recently demonstrated that cell-sheet transplantation using pancreatic islet cells can successfully improve disease in a mouse model of diabetes mellitus [13]. Thus, cell-sheet technology represents a new class of drug-delivery system, allowing engineering of tissues that can secrete therapeutical proteins such as insulin.

In this context, we employed endothelial cells formed into a contiguous monolayer sheet, which can be readily transplanted into the subcutaneous space for the production of FVIII (Figure 1A–1E). Under transient immunosuppression with cyclophosphamide, plasma FVIII levels up to 11% of normal were detected 3 weeks after transplantation in immunocompetent hemophilia A mice receiving transplantation of BOEC sheets. These levels were sustained for at least 300 days of observation without the development of anti-FVIII antibodies (Figure 1F–G). In addition, the levels of canine FVIII antigen by canine FVIII-specific ELISA are corresponded with canine FVIII activity by chromogenic assay in same plasma samples (data not shown). The levels and duration of FVIII expression achieved using this method were much higher than those observed in our previous BOEC studies [9], in which cell-sheet technology was not used. In the earlier study, elevated FVIII in plasma (maximum activity: 2% of normal) fell to zero 180 days after transplantation of BOECs. Consistent with increased FVIII activity, tail-clipping tests revealed that bleeding was significantly shortened in hemophilia A mice that received BOEC sheet transplants (Figure 1H). Together, these results clearly demonstrate that long-term

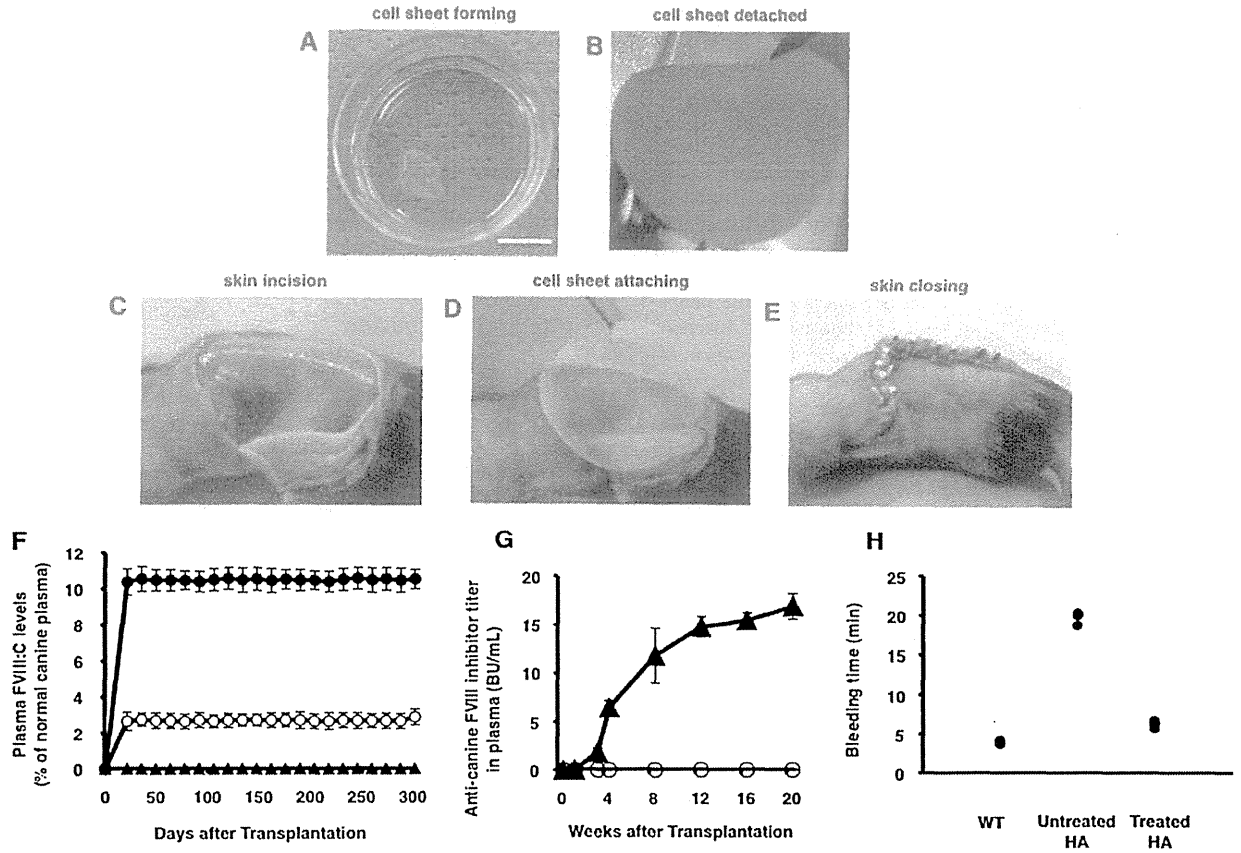

Figure 1. Subcutaneous transplantation of canine FVIII (cFVIII)-transduced blood outgrowth endothelial cell (BOEC) sheets in hemophilia A mice. (A–E) Schematic procedure for BOEC sheet transplantation. BOECs from hemophilia A mice were transduced using a lentiviral vector expressing the canine FVIII gene. The cells were cultured on temperature-responsive culture dishes. (A) Cell sheets were detached from the culture dishes by lowering the culture temperature, and (B) harvested as monolayer sheets using a support membrane. The scale bar represents 10 mm. (C) L-shaped skin incisions were made on the left dorsal regions of hemophilia A mice. (D) BOEC sheets were transplanted into the sites. After 5 min of attachment, the support membrane was carefully removed. (E) Thereafter, the skin flap was returned to its original position, and the skin wound was closed. (F) Plasma FVIII activity (FVIII: C) levels after cFVIII-transduced BOEC sheet transplantation in hemophilia A mice. Original-size sheets (open circles, n = 7) and large-size sheets (filled circles, n = 5) were fabricated on 35-mm and 100-mm-sized culture dishes, respectively. BOEC sheets not subjected to gene transduction were also transplanted (filled triangles, n = 2). (G) Anti-cFVIII inhibitor titers after transplantation of cFVIII-transduced (filled triangles, n = 4) or non-transduced (open circles, n = 3) BOEC sheets in hemophilia A mice that did not receive cyclophosphamide. (H) Bleeding time after tail clipping in wild-type mice (n = 4), hemophilia A mice (n = 5), and hemophilia A mice that were treated with large-sized cFVIII-expressing BOEC sheets and cyclophosphamide administration (n = 5).
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phenotypic correction of hemophilia A in this mouse model was successfully achieved using endothelial cells in conjunction with a novel cell-sheet technology.

Histological observations confirmed the superior outcome of our novel cell-sheet approach. In particular, histological studies revealed clear tube formation by FVIII-positive BOECs in the sub-adipose tissue layer, suggesting that the transplanted BOECs could integrate efficiently into the subcutaneous space and differentiate into mature endothelial cells, leading to formation of new blood vessels without any cellular response (Figure 2A–2F). Furthermore, these histological observations verified that cell viability was much improved in the novel cell-sheet approach, resulting in longer-term and 3–5-fold higher expression of plasma FVIII per numbers of transplanted BOECs, relative to our previous Matrigel transplantation approach [9].

The use of a temperature-responsive poly (N-isopropylacrylamide) (PIPAAm)-grafted dish may also explain the superior outcome of our novel BOEC sheet approach. Such dishes allow simple detachment of cultured cells without the use of proteolytic enzymes such as trypsin and the efficient harvest of a cell sheet as a contiguous monolayer that retains its native intercellular communications and intracellular microstructure. These properties of PIPAAm-grafted dishes could contribute to the preservation of normal cellular functions. In addition, BOECs in monolayer sheet configuration may facilitate oxygen delivery within the tissue microenvironment. In our previous study, in which BOECs were transplanted with Matrigel [9], the generation of BOEC clusters might not have provided adequate perfusion of the cells with nutrients, because the subcutaneous space was not as actively vascularized. By contrast, our novel cell-sheet approach allows unlimited diffusion of gases required for cell survival, thereby contributing to improved cell viability. Several previous studies have been designed around the development of vascular platforms within the subcutaneous space in hopes of enhancing cell survival

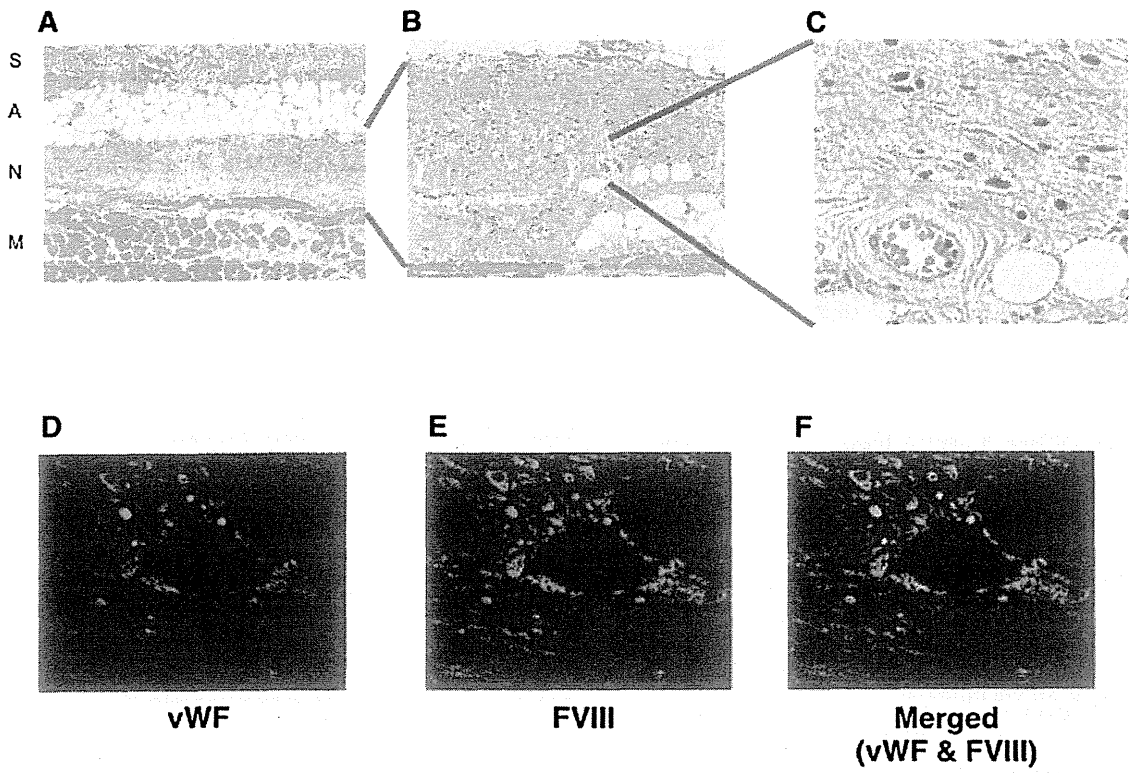

Figure 2. Histological analyses of subcutaneously transplanted genetically modified blood outgrowth endothelial cell (BOEC) sheets in hemophilia A mice. Eight weeks after transplantation of canine FVIII-transduced BOEC sheets, several recipient hemophilia A mice were sacrificed, and the implant tissue sections were subjected to (A–C) hematoxylin and eosin staining and immunostaining for (D) von Willebrand Factor (vWF) or (E) FVIII. (F) Merged image of vWF and FVIII staining. Magnification: (A) ×10, (B) ×20, (C) ×40, (D–F) ×60. S, skin; A, adipose tissue; N, newly generated tissues including BOEC sheet transplants and connective tissues; M, muscle. Each scale bar represents 30 μm. Engrafted BOEC implants were structured as flat sheets without any cell infiltration. Moreover, FVIII and vWF double-positive vessels were observed in newly generated tissues derived from the implanted BOECs. Abbreviations: H&E, hematoxylin and eosin; vWF, von Willebrand factor; FVIII, factor VIII.
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[28]. In this regard, it is noteworthy that our novel approach does not require the preparation of a vascular platform before cell transplantation.

Compared to recently developed gene therapies that employ systemic administration of viral vectors, our novel BOEC sheet approach has considerable benefits. Indeed, this cell-sheet transplantation approach can be repeated several times in a single recipient, if necessary, in order to increase the therapeutic efficacy.

In order to advance our mouse study into the clinic, there remain several issues to be addressed. Perhaps most importantly, the size of cell sheets used for transplantation must be significantly enlarged for use in human hemophiliacs. Development of multilayer cell-sheet transplantation within a confined space may provide a solution to this problem, and research on this topic is now underway in our laboratory.

Conclusion

We have succeeded in long-term phenotypic correction of hemophilia A in a mouse model by *ex vivo* engineering of genetically modified endothelial cells in an ectopic subcutaneous space. Our novel approach using cell sheet technology could represent an initial basis for curative treatment of hemophiliacs in the near future.

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Author Contributions

Conceived and designed the experiments: KT M. Sugimoto M. Shima KO TO HM. Performed the experiments: KT HM. Analyzed the data: KT HM. Contributed reagents/materials/analysis tools: KT DL HM. Wrote the paper: KT HM. Provided the constructs for lentiviral vectors used in this study: DL. Provided insights on experimental design: M. Sugimoto M. Shima KO TO. Edited the manuscript: DL M. Sugimoto M. Shima KO TO. Directed and performed the experiments: HM. Conducted data analysis and interpretation: HM.

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特 集
血液内科医が知っておくべき小児血液学

血友病ワールド

嶋　緑倫

Key words : Hemophilia, Regular prophylaxis, Comprehensive care

はじめに

血友病は第VIII因子あるいはIX因子の低下～欠乏に基づく先天性の凝固障害症で出血傾向は最も重篤である。代表的な出血症状は皮下，関節内および筋肉内出血である。関節内出血を重篤反復すると滑膜の炎症起点が進み滑膜炎を発症するために出血頻度がさらに増加すると標的関節を形成する。進行すると軟骨や骨の変性も伴う血友病性関節症を発症し，不可逆的な関節の可動制限をきたす。治療は第VIII因子製剤あるいは第IX因子製剤による止血と出血の予防が基本である。現在，わが国の小児科領域における血友病患者の過半数は定期的に製剤を投与する定期補充療法を実施している。血友病の治療方針は各年齢により変化する。すなわち，幼少期は定期補充療法の指導・実践とインヒビターのチェック，学童期は自己注射の指導，思春期以降は定期補充の遵守の指導と関節症のフォローが中心になる。小児から成人への移行期は特に治療の継続と治療遵守に対する包括的な指導が重要になる。この移行期の治療が以後の患者の日常生活における活動性を左右するといっても過言ではない。さらに，年齢が進むにつれて血友病診療は医療面のみならず，社会的心理的な対応も必要になる。そのためには，包括的な医療を実施する院内や施設間の連携体制が重要である。わが国は血友病治療・ケア施設と地域診療施設の国家的連携体制の構築の点では海外から遅れている。また，国家的登録システムもなく，諸外国の事情と比較するエビデンスにも乏しい。本稿では，小児期から成人への移行期に焦点をあて治療や診療連携体制の課題などについて考察を試みる。

奈良県立医科大学　小児科

I　小児から成人移行期の血友病診療

1. 移行期の特徴

小児期から成人に移行する時期は，以後の関節症の予後を決定する極めて重要な時期である。しかしながら，この時期は自立心が極度に高まるために対応が困難になりやすい。これまでに定期補充療法を受けていた場合，出血症状がほとんどみられないために治療の必要性がわからず，治療の継続を望まなくなるためにコンプライアンスが低下する。また出血の経験が少ないために症状の判断が難しく，重篤な出血の場合でも対応が遅れる危険性がある。この時期は運動活動や健康的な体重を維持しなければならない。さらに，さまざまな心理社会的問題が出てくる時期でもある。例えば，学校や職場で血友病であることを“暴露する”か“しないか”あるいは“いつ暴露するか”などの判断が難しい問題も出てくる。また，この時期は治療の責任が親から自分自身に代わって行く時期でもある。これらの様々な問題に対応するためには，医師のみではなく看護師やカウンセラーなどと協力して指導に当たることが必要である[1]。

2. 定期補充療法の継続と成人の定期補充療法

小児期におけるインヒビターのない血友病患者の治療は，オンデマンド投与療法から定期補充療法へ移行している。定期補充療法は大きく1次定期補充療法と2次定期補充療法に大別される（表1）。我が国でも小児の定期補充療法の実施率は，2歳から20歳では6～7割と過半数を超えている[2]。また2歳までに実施する1次定期補充療法も増加傾向にある。しかしながら，実施率は20歳を超えると激減しているのが現状である。いつまで定期補充療法を継続すべきかについては未だに議論の多いところである。従来，オンデマンドで治療を受けていた患者が定期補充を成人期になってから開始する是非についても検討課題である。

表 1　定期補充療法の定義と目標[11]

	1次定期補充療法	2次定期補充療法	3次定期補充療法
定義	<2歳または関節内出血>1回で開始	≧2歳，関節内出血≧2回で開始	≧18歳から開始
目的	関節症の発症と重篤な出血を防ぐ	出血回数，重篤な出血の回数を減らす	関節症の症状を阻止し他の合併症を防ぐ
目標	重篤な出血を防ぐ 本来の関節を維持する 出血頻度を減らす 高いQOLを維持する 社会的活動の参加，勉学や就労を支持する 運動活動を可能にする	重篤な出血を防ぐ 関節症のリスクを減らす 出血頻度を減らす 高いQOLを維持する 社会的活動の参加，勉学や就労を支持する 運動活動を可能にする 標的関節を防ぐ	重篤な出血症状を防ぐ 関節症の悪化を軽減する 出血回数を減らす QOLを改善する 社会的活動の参加，勉学や就労を改善する 活動と自立性を高める 標的関節の出血を減らす 疼痛のコントロールを改善する 運動療法・訓練が可能になる 合併疾患に起因する出血を減らす

成人の定期補充療法の有効性については報告例が増加している[3~6]。成人の定期補充療法を実施する際，いくつかのハードルがある。まず，第一は医療経済的制約とその重圧である。オンデマンド群と比較すると定期補充療法実施群では医療費は圧倒的に高くなる。第二はコンプライアンスが低下しやすいことが挙げられる。特に，自己注射の場合，介助者がいない場合，勤務中，出張時等に自己注射を実施する場合など時間的・空間的な制約がコンプライアンスの維持を困難にする。第三は頻回の静脈注射に伴う侵襲的苦痛，第四は投与回数や投与量の決定の指標が確立されていないことがあげられる。20歳までの定期補充療法では国際的な投与量25～40単位/kg，週3回（血友病A），50単位/kg週2回（血友病B）が標準的であるが，成人の場合はより個別化が必要である。その際，回収率や半減期などの薬物動態，出血頻度，関節症の重症度，活動性など様々な要因を考慮して決定する必要がある。

欧州の21センターによる思春期から成人期にかけての218症例を対象としたコホート調査では，半数が思春期に達した時点で予防投与を中止または投与回数が減らされたが，中止した28%が予防投与を再開し，投与回数を減らした症例の20%が出血症状の増悪のために元の投与に戻っていた[4]。したがって，本調査研究は思春期まで定期補充を継続していた患者が成人期に向けて定期補充を中止や投与回数を減ずると，一部の症例では出血症状が増悪することを示している。米国10センターで実施された調査では，1次定期補充療法を実施した症例の1/4が中止したが，その半数で出血症状が増悪していた[5]。Nooneらは18～35歳の重症血友病患者計124例を対象に比較検討を行った。長期間の定期補充療法群では，標的関節が少ない（$p<0.001$），重篤な出血が少ない（$p<0.05$），反復出血が少ない（$p<0.01$），外科手術が必要ではない（$p<0.05$）という結果であった。さらに，移動性，日常の活動性，疼痛や不安感などを評価する健康に関する有用性においてはオンデマンド群が明らかに低かった（$p<0.01$）。したがって，成人患者においても定期補充療法は，出血回数を減少させるのみならず，日常生活のQOLも向上することが示唆される[6]。

1次定期補充療法は関節症の発症を防ぎ，頭蓋内出血などの生命の危険を伴う重篤な出血を防ぐために実施する。その目標は，出血を最小限度に減少させて関節症の発症を抑制するのみならず通常の社会的，学校生活と就労を可能とすることにある。一方，2次定期補充療法については1次定期補充療法の目標と同様であるが，それ以上の出血症状や関節症の進展を防ぐことにある（表1）[7]。成人期に実施する3次定期補充療法とはすでに関節症を有する18歳以上の成人から開始する定期補充療法であるが，治療目標は1・2次定期補充療法とは異なり，重症の臨床的フェノタイプをより軽症に変えることにより重度の出血症状や関節症状を防ぐとともに，よりQOLを高めることにある[7]。しかしながら，すでに発症した関節症についてはその進展を防ぐことはできない。成人期に開始する定期補充療法に関するエビデンスはまだ少ないが，出血回数が減少しQOLを高める効果はあるが，関節症に対する影響については一定した見解が得られていない。さらに，製剤の消費量は明らかに

増加するのは事実である。最近，Valentinoらは従来オンデマンド止血療法を実施していた第VIII因子活性<2%の計66例の血友病A患者（7～59才）を対象に6か月間のオンデマンド治療後，2つの定期補充療法群（標準的投与群：20～40単位/kg　隔日投与，薬物動態力学により決定された群：20～80単位/kg 3日毎投与）に振り分けられる前向き調査をした。年間の出血回数では両定期補充療法群で差は見られなかったが，オンデマンド時より出血回数は有意に減少した[8]。すでに米国のガイドライン（the Medical and Scientific Advisory Council of the US National Haemophilia Foundation; MASAC）は全年齢の重症血友病患者定期補充療法の実施を薦めている。（http://www.hemophilia.org/NHFWeb/MainPgs/MainNHF.aspx?menuid=57&contentid=1007）。全例に定期補充療法を実施することは現時点では困難であるが，今後，活動性や出血症状，関節症の程度，薬物動態などを個別に評価してその適応を決定する必要があると思われる。

3. 中等症/軽症の問題

最近，軽症や中等症の患者も関節症が進行しているケースが少なからず存在することが明らかになってきた[9]。我が国の血友病患者のQOL調査[10]によると，定期補充なしの1年間の出血回数は，重症，中等症，軽症でそれぞれ30.0±17.2回，20.3±16.9回，6.6±7.5回であった。1年間の関節内出血の回数でみると重症，中等症，軽症でそれぞれ17.5回，18.5回，5.0回と軽症でも関節出血の頻度は少ないものの認められること，また，中等症は重症と同様の出血回数がみられることが分かる。定期補充療法の影響を除くために定期補充療法なし患者で比較しても，重症，中等症，軽症の出血回数はそれぞれ24.0±17.2回，18.5±15.8回，4.0±3.7回で，中等症では相当数の関節内出血を発生していることが明らかになった。これは，特に関節内出血歴のある中等症患者においてもオンデマンド治療では関節症が重症より遅いものの確実に進行することが示されている。したがって，全例ではないが，少なくとも関節内出血を反復する症例は中等症や軽症でも定期補充療法を考慮することも必要であると考えられる。

また，インヒビターの発生率は重症と比較して少ないものの，年齢とともに増加していることも明らかにされている。報告によると軽症血友病A患者（第VIII因子活性5～40%）297名中231名（78%）が補充療法を受けており，14例（6.1%）でインヒビターが発現している。投与日数の中央値は502日で，発生年齢の中央値は66歳であった。したがって，中等症/軽症であっても小児期のみならず成人期でもフォローアップは必要であることを示している。また，整形外科手術などのピーク治療やインヒビター発生因子として知られる第VIII因子Arg593Cys変異は特に要注意である。

以上より，思春期から成人の移行期には中等症や軽症例の出血エピソードや関節評価，さらにはインヒビターのチェックも必要である。

II 小児期から成人移行期における血友病診療の連携と標準化

1. 国際的位置づけ

わが国の血友病診療は特に小児では定期補充療法の実施率も向上し，成人の実施率も増加しつつある。また，20歳未満は小児慢性特定疾患研究事業，20歳以上の成人期は先天性凝固因子障害等治療研究事業の対象疾患になっており，公費のサポートも完備している。したがって，我が国の血友病診療も欧米先進国レベルに近づきつつある。世界血友病連盟（World Federation of Hemophilia: WFH）は，国別の患者の発生率と国民一人あたりの製剤使用量を指標に各国の血友病診療レベルを評価している。前者は，それぞれの国における血友病の診断率と患者をどの程度把握しているかを考える目安になる（表2）。我が国では，23,470人に一人で，英国では9,527人に一人で発生率は半分以下である。また，中国では132,010人に一人で，それぞれの国における患者の把握度を反映しているものと考えられている。我が国では血友病の全国調査が毎年実施され年々患者数が増加しているが，わが国の全患者を反映していない（図1）。今後我が国でも国家ベースの調査あるいはデータベースの構築が必要であろう。後者は，血友病治療の程度をある程度は間接的に評価できる（図2）。我が国の国民一人あたりの製剤使用量は欧米の約半分である。この原因として，平均体重の差，実際の製剤使用料が少ないこと，成人の定期補充療法の実施率が少ないことなどの原因が考えられる。特に，小児期では多くの症例で定期補充療法が国際的基準で実施されている事実を考慮すると，成人における製剤の投与量が少ないと想像される。

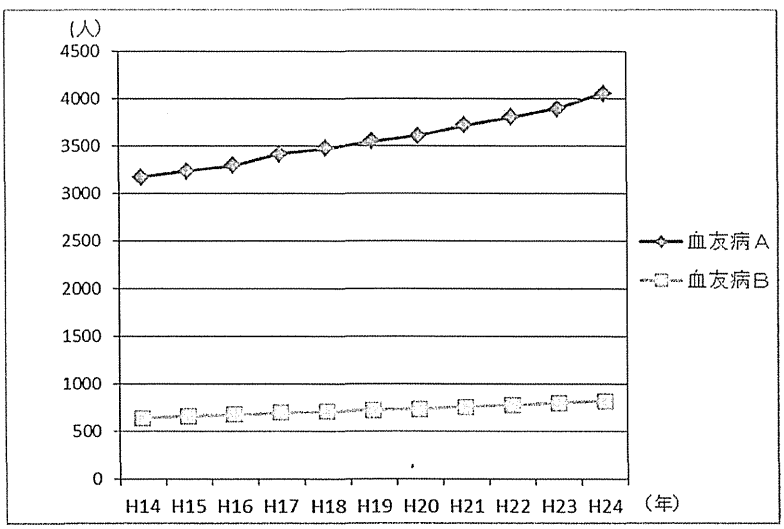
2. 血友病の専門診療・ケア施設

小児期の適切な診療は成人期の予後に大きく作用する[11,12]。さらに，血友病患者の平均余命は専門的ケアと関連するとの報告もある[13]。血友病患者のQOLを高めるために適正な治療を実施していくためには包括的ケアシステムの構築が望ましい。欧州14か国の血友病治療標準化ボード（The European Haemophilia Therapy Standardization Board; EHTSB）では血友病診療・ケアに必要な原則を表のようにあげている（表3）[14]。参加国で実際血友病ケアに関する中央的組織が存在する国は

表 2　各国の血友病患者数と人口に対する比率

国	人口	患者数	人口に対する比率 (小数点以下切り上げ)
United Kingdom	62,641,000	6,575	9,527
Canada	34,482,779	3,380	10,202
Switzerland	7,907,000	701	11,280
France	65,436,552	5,735	11,410
Denmark	5,574,000	477	11,686
Germany	81,726,000	4,654	17,560
United States	311,591,917	17,485	17,821
Brazil	196,655,014	10,558	18,626
Argentina	40,764,561	2,133	19,111
Japan	127,817,277	5,446	23,470
Korea, Republic of	49,779,000	1,908	26,090
Russia	141,930,000	5,421	26,182
China	1,344,130,000	10,182	132,010

WFH 2011



瀧 正志. 厚生労働省委託事業 血液凝固異常症全国調査

図 1　わが国の血友病患者登録数

79%，中央的患者登録システムが完備されているのは57%であった。すべての患者が包括的ケアセンター（Comprehensive care centres: CCCs）あるいは血友病治療センター（Hemophilia treatment centre; HTC）でみられている国は64%であった。我が国では，血友病専門医のボードは，たとえば日本血栓止血学会標準化委員会血友病部会や小児血液がん学会止血血栓委員会が相当するが，患者の登録システムは確立されていない。英国ではHTCが40，CCCが29施設あり，また血友病専門医は United Kingdom Haemophilia Centre Doctors'

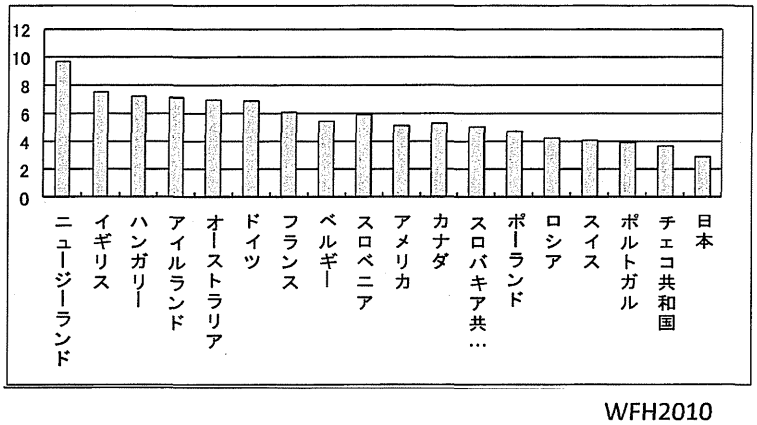


図2　国別一人当たりの製剤使用量

表3　包括的治療センターと血友病治療センターの定義[14]

包括的ケアセンター（CCC）	血友病治療センター（HTC）
• 最低 40 人の重症血友病患者（FVIII/IX <1%）	• 最低患者数の指定なし
• 24 時間　専門的治療が可能	• 24 時間 専門家がカバー
• 24 時間　検査が可能	• 検査対応可能（遅れあり）
• 以下を含む，多くの専門分野に渡る包括的ケアチームによる治療を実施 フルタイムの血液学者，あるいは小児科医を 1 人は採用 熟練した看護士 経験豊富な理学療法士 ソーシャルワーカー データ管理	• 以下を含む，多くの専門分野に渡る包括的ケアチームによる治療を実施 フルタイムの血液学者，あるいは小児科医を 1 人は採用 熟練した看護士 経験豊富な理学療法士 ソーシャルワーカー 十分な記録の保持
• 在宅自己注射，予防処置，インヒビター治療，および ITI の実行	• CCC と協力して 在宅自己注射，予防処置，インヒビター治療，および ITI の実行
• 産婦人科，整形外科，歯科，遺伝子学との連携	• CCC と協力して 産婦人科，整形外科，歯科，遺伝子学との連携
• 診療監査の実行 （内部監査は必須，外部監査は実施するのが望ましい）	• 診療監査の実行
• コンセンサスガイドラインを順守し，医学教育も提供	• コンセンサスガイドラインを順守し，医学教育も提供
• 研究も実施する	

Organisation（UKHCDO）を中心に組織されている。米国では 141 の HTC があり，CDC（Centers for Disease Control and Prevention）や HRSA（Health Resources and Services Administration）よりサポートされている。我が国ではまだ CCCs の基準を満たす施設はない。人口 100 万人あたりの HTCs は欧州では中央値 0.84（0.62～1.11）である。欧州の基準から考えると我が国では 80～145 施設必要なことになる。

3. 診療連携

前述したように，我が国では諸外国のように血友病治療センターや血友病包括的ケアセンターが確立していない。したがって，血友病診療の質的差が生じることはいなめない。定期補充療法の普及により，患者の関節症の予後やQOLは改善しており特に1次的補充療法は血友病性関節症の発症や進行を抑制する。しかしながら，Manco Johnsonらが実施したオンデマンド治療法と1次定期補充療法群とのランダム化前向き調査では定期補充群でも多少関節スコアが悪化している[15]。さらに，思春期から若年成人年齢にかけてコンプライアンスが低下するために包括的な治療・ケア体制が必要である。したがって，血友病診療は小児科/血液内科/内科での止血治療のみならず，関節症の評価のための整形外科やリハビリテーション科，さらに口腔外科など他科との診療連携が必須である。また，日常の注射指導など看護部との連携や大手術の止血管理やインヒビター陽性例の治療あるいは救急対応などの専門施設との連携も重要である。

診療連携の第一歩は，院内連携システムの構築である。特に看護部，整形外科，内科/血液内科，医療相談課などとの連携を構築する。院内包括外来を実施するのが院内連携構築の第一歩になる（図3）。また，実際の生活，学校や職場などにおける様々な相談については患者会の紹介も重要である（全国ヘモフィリア友の会ネットワーク：http://hemophilia.web.fc2.com）。院内連携とともに，都道府県内あるいは地域の専門施設との連携体制も必要である。日本小児血液がん学会止血血栓委員会の小児血友病診療ネットワーク（小児血液がん学会止血血栓委員会，瀧　正志　委員長，事務局 m3asa@marianna-u.ac.jp）では全国の血友病診療施設321施設で計520名の医師が参加している（図4）。ブロック長及び都道府県代表が決められており，地域診療連携の基盤となる。また，インヒビターの手術などさらに高い専門性が必要な場合には，専門施設と患者在住の診療施設との連携で実施することも可能である。図5に奈良医大小児科と他施設との連携により整形外科手術の実施体制を示している。まずかかりつけの診療施設から血友病性関節症の手術に関するコンサルトを受け，あらかじめ凝血学的評価および整形外科的評価のために受診してもらい，小児科ではインヒビターの評価，バイパス製剤の効果判定を実施して整形外科では当該関節の評価と手術計画をたてる。手術時は小児科が止血管理を実施して，リハビリ科と連携して術後のリハビリを実施する。リハビリが安定したころに退院して，紹介先の診療施設でリハビリを継続する。専門施設と診療施設が連携することで高度の治療もよりスムーズに実施することができると思われる。

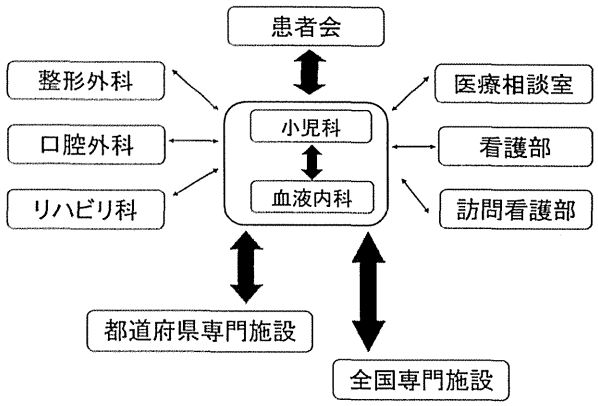


図3　院内外診療連携の構築

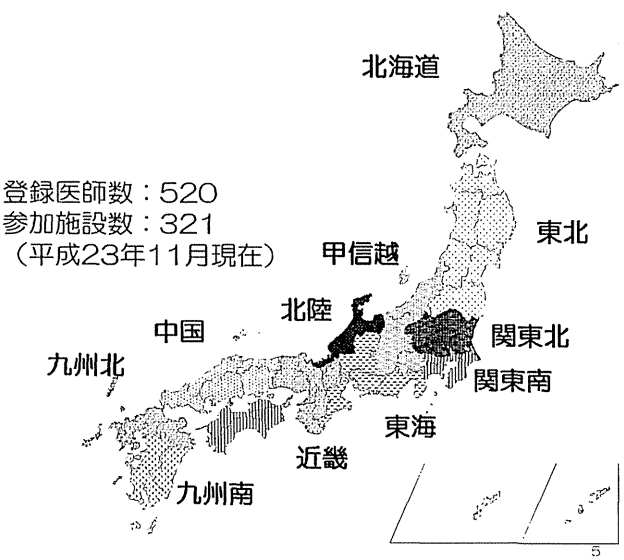


図4　小児血友病診療ネットワーク

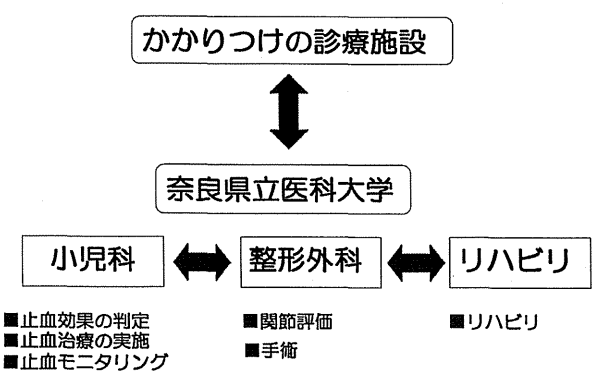


図5　血友病Aインヒビターの滑膜切除術の診療連携例

表4 UKHCDO Guideline リスト

Year	Reference	Title
2011	Haemophilia, 17, e877-883	UKHCDO guidelines on the management of HCV in patients with hereditary bleeding disorders 2011.
2011	Br J Haematol, 154, 208-215	Guideline on the management of haemophilia in the fetus and neonate
2010	CMGS Website	Practice Guidelines for the Molecular Diagnosis of Haemophilia A
2010	CMGS Website	Practice Guidelines for the Molecular Diagnosis of Haemophilia B
2010	Br J Haematol, 149, 498-507	A United Kingdom Haemophilia Centre Doctors' Organization guideline approved by the British Committee for Standards in Haematology: guideline on the use of prophylactic factor VIII concentrate in children and adults with severe haemophilia A
2009	UKHCDO Website	Emergency and out of hours care for patients with bleeding disorders–Standards of care for assessment and treatment
2008	Haemophilia, 14, 1099-1111	The molecular analysis of von Willebrand disease: a guideline from the UK Haemophilia Centre Doctors' Organisation Haemophilia Genetics Laboratory Network
2008	Haemophilia, 14, 671-684	Guideline on the selection and use of therapeutic products to treat haemophilia and other hereditary bleeding disorders. A United Kingdom Haemophilia Center Doctors' Organisation (UKHCDO) guideline approved by the British Committee for Standards in Haematology
2006	Haemophilia, 12, 301-336	The obstetric and gynaecological management of women with inherited bleeding disorders-review with guidelines produced by a taskforce of UK Haemophilia Centre Doctors' Organization
2006	Br J Haematol, 133, 591-605	The diagnosis and management of factor VIII and IX inhibitors: a guideline from the United Kingdom Haemophilia Centre Doctors Organisation
2006	Br J Haematol, 135, 603-633	A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO
2005	Haemophilia, 11, 145-163	A framework for genetic service provision for haemophilia and other inherited bleeding disorders
2004	Haemophilia, 10, 218-231	Management of von Willebrand's disease: a guideline from the UK Haemophilia Centre Doctors' Organisation
2004	Haemophilia, 10, 199-217	The diagnosis of von Willebrand's disease: a guideline from the UK Haemophilia Centre Doctors' Organisation
2004	Haemophilia, 10, 593-628	The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation

4. 血友病の診療ガイドライン

血友病診療の標準化において診療ガイドラインは重要である。国際的には最近 WFH から非常に詳細なガイドラインが発表されている[16]。英国では血友病専門医による United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO) から血友病診療に関する様々なガイドラインが発表されている（表 4)。わが国では，日本血栓止血学会標準化委員会血友病部会が中心となって，文献検索，実態調査および専門家の意見に基づきインヒビター非保有例[17] および保有例の診療ガイドラインが作成された[18]。現在，改訂版を製作中である。

5. 血友病の登録システム

わが国では国家規模の血友病患者の登録システムは存

在しない。欧州では仏，独，ギリシア，伊，オランダ，ノルウェー，スロバキア，スペイン，英国で各患者が登録されている。血友病患者の登録システムは我が国における血友病の疫学に重要であるが，血友病診療の国際比較や血友病診療の政策においても必須である。現在，厚労省の研究班で新規発生血友病患者に関する前向き調査が実施されている（厚生労働科学研究事業　血友病の治療とその合併症の克服に関する研究　分担研究「第VIII，第IX因子製剤のインヒビター発生要因に関する研究」：事務局　奈良県立医科大学小児科　pedlab@naramed-u.ac.jp）。

最後に

血友病診療の基礎と，小児期から成人への移行期のポイントと我が国の課題について解説した。移行期の治療は小児科と内科/血液内科との谷間にあるが，以後の患者のQOLを大きく作用する重要な時期である。止血治療のみならず様々な領域や職種と連携して診療を進める必要がある。今後，血友病の治療は長時間作用型製剤などの新たな製剤の開発のみならず遺伝子治療など次世代の治療も期待されている。国際的にみて我が国の診療レベルが遅れないためにも我が国における血友病診療の標準化と連携体制の構築が急務であると思われる。

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bjh research paper

Different factor VIII neutralizing effects on anti-factor VIII inhibitor antibodies associated with epitope specificity and von Willebrand factor*

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Summary

Inhibitor neutralization therapy based on factor (F)VIII replacement is used for haemostatic treatment in haemophilia A patients with inhibitors on low responder, but effects appear to depend on various properties of inhibitors. We investigated this nature by evaluating the global coagulation function in timed-reactions after mixing FVIII (1 U/ml) with anti-FVIII alloantibodies containing distinct epitopes (2·5 Bethesda units/ml). Thrombin generation assays showed that peak thrombin and mean velocity to peak thrombin were depressed by anti-C2 type 1 inhibitors to significantly greater extents than by anti-A2 type 1 and anti-C2 type 2 (2- to 6-fold and 10- to 20-fold, respectively). In the presence of FVIII-von Willebrand Factor (VWF) complex, the anti-C2 type 1-mediated decreased thrombin generation was reduced by 20–40%, reflecting the protective function of VWF. However, the activities of anti-A2 type 1 were little affected, and that of anti-C2 type 2 was rather enhanced by *c.* 2·5-fold, relative to FVIII. Clot waveform analysis also showed similar patterns. Anti-FVIII monoclonal antibodies with well-defined characteristics demonstrated similar reactions to those with polyclonal inhibitors. In conclusion, the neutralizing effects of FVIII(-VWF) depending on epitopes could have significant therapeutic implications, and it could be important to determine inhibitor properties in order to predict the effects of infused FVIII in neutralization therapy.

Keywords: factorVIII, inhibitor, neutralization therapy, haemophilia A, epitopes.

Factor (F)VIII, a plasma protein deficient in individuals with the severe congenital bleeding disorder, haemophilia A (HA), functions as a cofactor in the tenase complex, responsible for phospholipid (PL)-dependent FIXa-mediated activation of FX (Mann *et al*, 1990). The FVIII molecule is arranged into three domains (A1-A2-B-A3-C1-C2) based on amino acid homology, and is processed into a series of heterodimers, generating a heavy chain (HCh) consisting of A1 and A2 domains together with heterogeneous fragments of proteolysed B domain linked to a light chain (LCh) consisting of A3, C1, and C2 domains. The catalytic efficiency of FVIII in the tenase complex is markedly enhanced by conversion into FVIIIa, through limited proteolysis by thrombin and FXa (Eaton *et al*, 1986). Both enzymes proteolyse at Arg^{372} and Arg^{740} in the HCh, resulting in the generation of 50-kDa A1 and 40-kDa A2 subunits. The 80-kDa LCh is proteolysed at Arg^{1689} producing a 70-kDa subunit. Proteolysis at Arg^{372} and Arg^{1689} is essential for generating FVIIIa cofactor activity (Fay, 2004). Cleavage at the latter site liberates FVIII from its carrier protein, von Willebrand factor (VWF; Lollar *et al*, 1988). FVIIIa activity is down-regulated by activated protein C, following cleavage at Arg^{336} (Eaton *et al*, 1986).

FVIII inhibitors develop as alloantibodies (alloAbs) in 20–30% of multi-transfused HA patients (Oldenburg *et al*, 2000). The reduction or disappearance of FVIII coagulant activity (FVIII:C) in the presence of anti-FVIII antibodies is associated with impairment of FVIII(a) cofactor function mediated by binding to functionally essential regions on FVIII. Anti-FVIII inhibitor antibodies either inhibit FVIII:C completely or incompletely at saturating concentrations, corresponding to a classification of type 1 or type 2, respectively (Gawryl & Hoyer, 1982). Major inhibitory epitopes have been localized to one or both of the A2 and C2 domains (Prescott *et al*, 1997). Anti-C2 antibodies sub-classified as

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type 1 prevent the binding of FVIII to PL and VWF (Shima *et al*, 1993), whilst those classified as type 2 prevent the association of FVIII with thrombin and FXa (Meeks *et al*, 2007; Matsumoto *et al*, 2012). Anti-A2 antibodies prevent the association of FVIIIa with FIXa (Fay & Scandella, 1999).

Clinical treatment protocols for HA patients with inhibitor are based on replacement therapy using FVIII concentrates ('neutralization therapy') and so called 'bypassing therapy' utilizing recombinant FVIIa (rFVIIa) and plasma-derived activated prothrombin complex concentrates (APCC). The former type of therapy is usually regarded as first choice for inhibitor patients classed as low responders, whilst the latter protocols are used for those classed as high responders. Klintman *et al* (2010) reported that mixtures of FVIII and bypassing agents (rFVIIa and APCC) significantly potentiated coagulation effects *in vitro* compared to bypassing agents alone in plasmas from HA patients with inhibitor. In addition, we have recently demonstrated that FVIII was activated by limit proteolysis by rFVIIa or APCC (Soeda *et al*, 2010; Yada *et al*, 2013), and that this activation was not impaired by the presence of anti-FVIII inhibitors. Furthermore, anti-C2 type 1 inhibitors depressed the inactivation phases in both rFVIIa-mediated and APCC-mediated reactions, resulting in relatively persistent, elevated levels of FVIII:C (Yada *et al*, 2011, 2013). These findings suggested further studies were warranted to determine if the coagulation effects of neutralization therapy in HA patients with inhibitor might be governed by various characteristics of the antibodies (epitope specificity, kinetics, mechanisms of inhibition of FVIII function, etc.). In the present study, we have examined different FVIII inhibitors in *in vitro* models of neutralization therapy using mixtures of FVIII and well-defined anti-FVIII antibodies.

Materials and methods

Reagents

Recombinant FVIII preparations (Kogenate FS®) and plasma-derived FVIII-VWF concentrates (Confact F®) were provided by Bayer Corp. Japan (Osaka, Japan) and Chemo-Sero-Therapeutic Research Inc. (Kumamoto, Japan), respectively. VWF was purified from FVIII-VWF concentrates by gel filtration using Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) and immune-beads coated with immobilized anti-FVIII mAb (Shima *et al*, 1992). Enzyme-linked immunosorbent assays of FVIII demonstrated VWF purity of >95%. Recombinant lipidated tissue factor (TF; Innovin®; Dade Behring, Marburg, Germany), ellagic acid (Sysmex, Kobe, Japan), thrombin-specific fluorogenic substrate (Bachem, Bubendorf, Switzerland), thrombin calibrator (Thrombinoscope, Maastricht, Netherlands), and FVIII-deficient plasma (George King Biomedical, Overland Park, KS, USA) were purchased from the indicated vendors. Anti-C2 mAbs ESH4 (epitope 2303–2332) and ESH8 (epitope 2248–2285) were purchased from American Diagnostica Inc. (Greenwich, CT, USA). An anti-A2 mAb, JR8, was obtained from JR Scientific Inc. (Woodland, CA, USA). PL vesicles (phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine; 10/60/30%; Sigma, St Louis, MO, USA) were prepared using *N*-octylglucoside (Mimms *et al*, 1981).

Anti-FVIII inhibitor alloAbs

Six anti-FVIII inhibitor alloAbs were obtained from Japanese patients with congenital severe HA. IgG fractions were prepared using protein A-Sepharose (Amersham Biosciences). The inhibitor titres of antibody IgGs were determined using Bethesda assays. The two kinetic patterns of FVIII inactivation by anti-FVIII antibodies (type 1 and type 2 behaviors) were determined in one-stage clotting assays. Epitopes of these antibodies were determined by sodium dodecyl sulfate polyacrylamide gel elctrophoresis and Western blotting using isolated FVIII fragments. The properties of these anti-FVIII antibodies used in the present study are summarized in Table I. All experiments were performed using blood samples obtained from patients enrolled in the Nara Medical University Haemophilia Programme after informed consent following local ethical guidelines.

In vitro *model of neutralization therapy*

Purified inhibitor IgG at a final concentration (f.c.) of 2·5 Bethesda units (BU)/ml was added to FVIII-deficient plasma. The reconstituted HA inhibitor plasma samples were then incubated with FVIII or FVIII-VWF (f.c. 1 U/ml) at 37°C. FVIII-VWF was prepared by a 1 h-incubation of equivalent amount of FVIII and VWF at 37°C. Aliquots were obtained at the indicated times and coagulation function assessed using global coagulation assays.

Thrombin generation assays

Calibrated automated thrombin generation assays were performed as previously described (Matsumoto *et al*, 2009).

Table I. Properties of anti-FVIII inhibitor antibodies.

Case	Type of Ab	Epitope	Type of kinetics	Inhibitor titre (BU/ml)
1	alloAb	A2	1	114
2	alloAb	A2	1	56
3	alloAb	C2	1	128
4	alloAb	C2	1	580
5	alloAb	C2	1	23
6	alloAb	C2	2	4
JR8	mAb	A2	1	620
ESH4	mAb	C2	1	33
ESH8	mAb	C2	2	840