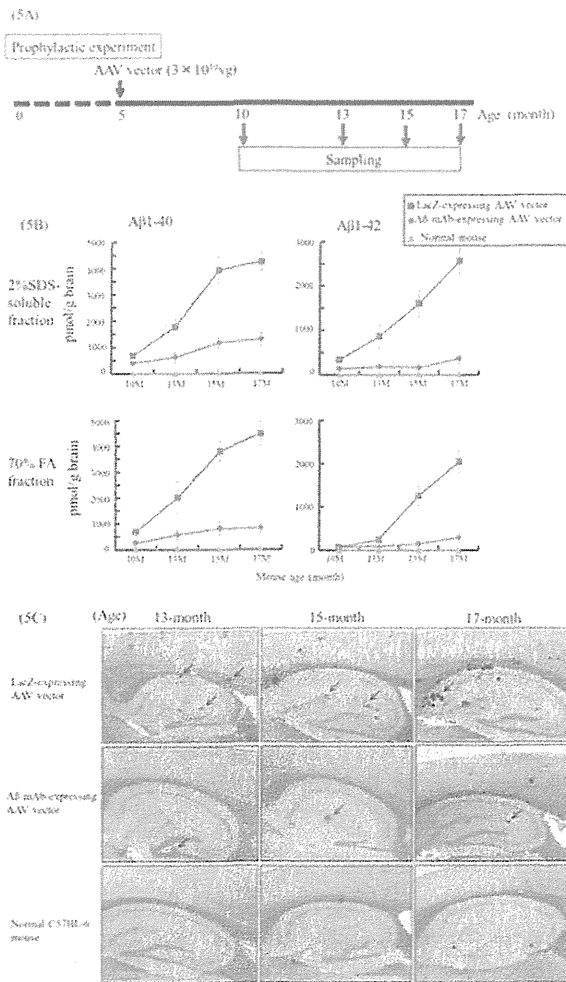

Figure 5. Prophylaxis of AD using Aß mAb-expressing AAV vector. Five-month old Tg2576 mice were injected i.m. with 3×10^{10} Aß mAb-expressing AAV. (A) Scheme of experiment. (B) The amount of Aß protein in brain extracts was determined using anti-Aß Ab-coated ELISA plates at age 10-, 13-, 15- and 17-month old (4–5 mice per time point). C57BL/6 mice were used as controls. Data are presented as mean ± SE. (C) Brain sections from Tg2576 or C57BL/6 mice were examined for Aß deposits by immunohistostaining using rabbit anti-human beta amyloid 1–42 polyclonal antibody.
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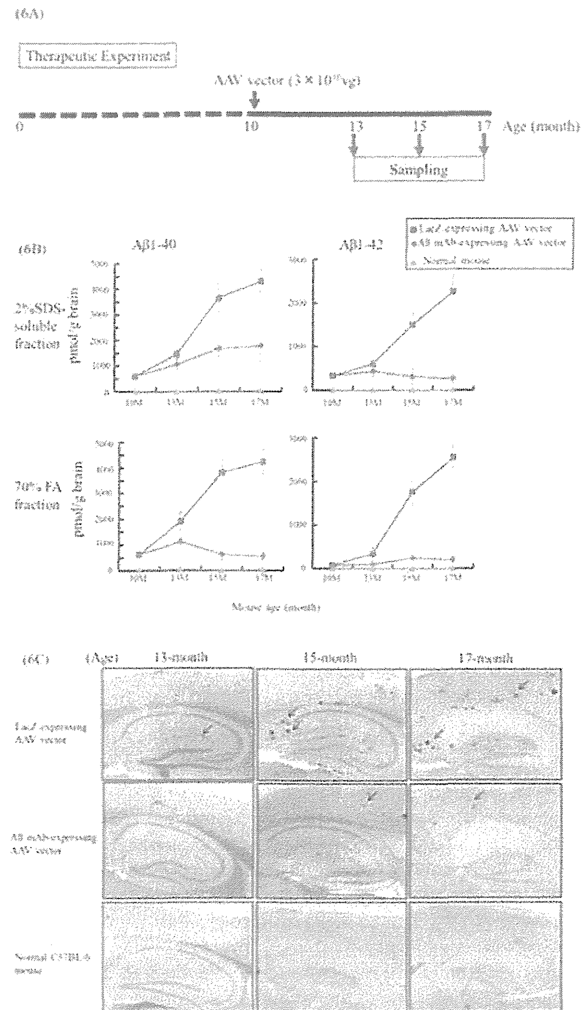

Figure 6. Therapy of AD using Aß mAb-expressing AAV vector. Tem-month old Tg2576 mice were injected i.m. with 3.0×10^{10} vg of the Aß mAb - expressing AAV vector. (A) Scheme of experiment. (B) The amount of Aß protein in brain extracts was determined using anti-Aß Ab-coated ELISA plates at age 10-, 13-, 15- and 17-month old (4–5 mice per time point). C57BL/6 mice were used as controls. Data are presented as mean ± SE. (C) Brain sections from Tg2576 or C57BL/6 mice were examined for Aß deposits by immunohistostaining using rabbit anti-human beta amyloid 1–42 polyclonal antibody.
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Recent reports suggest that mAb therapy is effective only in AD patients possessing e4/e4 proteins [12,41]. In this context, ApoE4 (+) individuals develop AD more often than ApoE4 (−) individuals [42–43]. Such observations help inform the design of trials with AAV vectors intended for human use, as it facilitates the identification of individuals at high risk for developing AD. In addition, previous studies documented that Aß oligomers are toxic to neuronal cells [44–45]. That finding is consistent with current results showing that aggregated Aß proteins are toxic to primary culture neuronal cells and that anti-Aß mAbs prevent this toxicity (Figure 3).

We hypothesize that the ongoing accumulation of Aß aggregates is responsible for widespread neuronal cell degeneration and the subsequent dementia characteristic of AD. We believe that the failure of clinical trials involving anti-Aß mAb may reflect the late initiation of such treatment as it is important to eliminate Aß oligomers during the early stages of AD. Evidence that ApoE4 and other factors can predict individuals at high risk [34] lead us to recommend clinical trials of Aß mAb - expressing AAV vector commence at an early age for prophylaxis of AD. Current results show that the production of anti-Aß mAbs persists for an extended period (Figure 4). Thus, a single early treatment may yield long term clinical benefit. Further study that includes behavioral and memory testing combined with even longer follow should help clarify the value of AAV vector mediated in the prophylaxis and/or therapy of AD treatment.

Taken together, we constructed an anti-Aβ Ab-expressing AAV vector. A single intramuscular injection of the vector generated high serum anti-Aß Ab level for up to 64 weeks, and significantly

decreased Aß levels in the brain of AD model mice treated at 5 months (prophylactic) or 10 months (therapeutic) of age. Our present results clearly demonstrated that the Aß mAb-expressing AAV vector may be of prophylactic and/or therapeutic value for AD treatment.

Materials and Methods

Ethics Statement

All animal work has been conducted according to relevant Japan and international guidelines. All experimental procedures were carried out in accordance with the Administrative Panel on Laboratory Animal Care (APLAC) protocol and the institutional guidelines set by Yokohama City University and Chyoju Medical Institute. The protocols used in this study were proved by Institutional Animal Care and Use Committee (IACUC)/Ethics committee of Yokohama City University (No. 0741, 0875 and 0974) and Chyoju Medical Institute (No. B-22).

All animal work has been conducted according to relevant U.S. and international guidelines. Specifically, all experimental procedures were carried out in accordance with the Administrative Panel on Laboratory Animal Care (APLAC) protocol and the institutional guidelines set by the Veterinary Service Center at Stanford University (Animal Welfare Assurance A3213-01 and USDA License 93-4R-00). Stanford APLAC and institutional guidelines are in compliance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals. The Stanford APLAC approved the animal protocol associated with the work described in this publication.

Genes of mAbs against Aß

A hybridoma producing anti-Aß1–13 mAb (IIA2) [14] was cultured in KBM 450 medium (Kohjin Bio Co., Ltd., Saitama, Japan) in the absence of fetal bovine serum (FBS). The antibody was concentrated and partially purified from culture supernatant by ammonium sulfate precipitation and was used for the experiments as a positive control.

Aß peptide synthesis and aggregated Aß or oligomer formation

The Aß1-42 peptide used in these studies was produced by chemical synthesis (American Peptide, Sunnyvale, CA, USA). Reverse - phase high performance liquid chromatography showed that the synthesized peptide has >95% purity, and mass spectrometry analysis verified the molecular mass. Oligomer formation was done using the method described previously (Stine et al., 2003). Briefly, Aß oligomers were prepared by diluting 5 mM Aß1–42 in Me2SO to 100 µM in ice-cold cell culture supernatant (phenol red - free Ham's F-12; BioSource, CA, USA), immediately vortexing for 30 s, and incubating at 4°C, room temperature or 37°C for 24 h. The aggregated Aß or oligomer solution was used for the Western blotting analysis, as well as cytotoxic tests of neural cells.

Construction of an expression vector for the anti-Aß mAb gene

Total RNA was extracted from a hybridoma producing anti-Aß1–13 mAb (IIA2) [14] using TRIzol Reagent (Gibco BRL, Grand Island, NY, USA). Full-length heavy (H) chain and light (L) chain cDNA was transcribed with 5′-RACE primer and 3′-RACE primer using BD SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. H chain cDNA was amplified with sense primer (5′ CGG GGT ACC ATG GGC AGG CTT ACT TCT TC 3′) and antisense primer (5′ CCC AAG CTT TTT ACC AGG AGA GTG GGA GA 3′). L chain cDNA was amplified with sense primer (5′ CCG GAA TTC ATG GAG ACA GAC ACA CTC CT 3′) and antisense primer (5′ ATA AGA ATG CGG CCG CA G TCG ACG CTA ACA CTC ATT CCT GTT GA 3′). The Furin 2A fragment [46–47] from the foot and mouth disease virus was synthesized with complementary oligo (5′ CCC AAG CTT CGC GCC AAG CGC GCC CCC GT 3′ and 5′ CCG GAA TTC GGG GCC GGG GTT GGA CTC CA 3′). The H chain–Furin 2A–L chain fusion fragment was subcloned into proviral plasmid pW1 controlled by the CMV promoter. The AAV vectors were prepared by the previously described three - plasmid transfection adenovirus-free protocol [16,48]. Briefly, 60% confluent human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's modified Eagle's medium – nutrient mixture F-12 (1:1) (DMEM/F-12; GIBCOBRL, New York, NY) supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in an atmosphere of 5% CO2 in air. Subconfluent HEK293 cells were co-transfected by the calcium phosphate co-precipitation method with the AAV shuttle plasmid pW1 (containing LacZ or antibody heavy chain-F2A-light chain), the AAV-1 chimeric helper plasmid p1RepCap (provided by Dr. James M. Wilson, University of Pennsylvania, Philadelphia, PA, USA), and the adenoviral helper plasmid pAdeno (provided by Avigen, Inc., Alamada, CA, USA). After 48 h, the cells were harvested and lysed in Tris buffer (10 mM Tris-HCl, 150 mM NaCl, pH 8.0) by three cycles of freezing and thawing. One round of sucrose precipitation and two rounds of CsCl density-gradient ultracentrifugation were sufficient to isolate the AAV vector from the lysates. The vector titer was determined by quantitative PCR and presented as vg.

Western blot analysis

To confirm the expression of the Ab proteins, HEK293 cells were transduced with the AAV vector encoding the mAb genes (Aß mAb-expressing vector) in a 6-well plate. After transduction of AAV vector for 2 h, the cells were washed twice with PBS and cultured with Ex-CELL CD CHO serum-free medium (Life Technologies Japan Ltd, Tokyo, Japan) for another 2 days. Then, antibody protein was detected in culture supernatant and cell lysates with Western blot. The cells were washed with PBS and lysed with 0.1 M Tris-HCl (pH 7.8) and 0.125% Nonidet P-40 2 days after transduction. The cell lysates were mixed with an equal volume of 2× SDS buffer (125 mM Tris-HCl [pH 6.8], 4% SDS) with 100 mM of DTT (reducing condition) or without DTT (non-reducing condition) and boiled for 10 min. The cell lysates were loaded on an 8% polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane. After rinsing with PBS, the membrane was probed with HRP-labeled goat anti-mouse IgG1 or IgκAbs (Ig; ICN Pharmaceuticals Inc., Solon, OH, USA). The protein was detected using the ECL Plus Western Blotting Detection System (Amersham Pharmacia Biotech, Uppsala, Sweden).

Animals and administration

Heterozygous Tg2576 mice were obtained from Taconic Farms Inc. (Germantown, NY, USA) [14]. Normal C57BL/6 female mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mice were housed in the animal centers located at Yokohama City University and Chyoju Medical Institute, and maintained on a 12-h day–night cycle. Five-month and 10-month old mice were used for prophylactic and therapeutic experiments, respectively. The mice were received a single injection in quadriceps muscles

with a dose of 3.0×10^{10} vg of the Aß mAb-expressing vector. We used 20–25 mice in each group. The mice for prophylactic experiments were sacrificed 5, 8, 10 and 12 months after Aß mAb - expressing vector administration and the mice for therapeutic experiments were sacrificed 0, 3, 5 and 7 months after Aß mAb-expressing vector administration. To explore the magnitude and duration of antibody expression, we injected 3.0×10^{9}, 3.0×10^{10} and 3.0×10^{11} vg of Aß mAb-expressing vector or 3.0×10^{11} vg of AAV vector expressing LacZ gene (LacZ-expressing vector) to mouse quadriceps muscles, and blood was collected at indicated time points for Aβ-specific antibody detection. The animal experiments were approved by the Animal Ethical Committees of Yokohama City University School of Medicine and and Chyoju Medical Institute.

Enzyme-linked immunosorbent assay (ELISA) for Ab titers

ELISA was performed as described previously [14]. Briefly, 96-well microtiter plates were coated with 40 µg/ml of Aß1-42 peptide in 0.15 M phosphate-buffered saline (PBS). The wells were rinsed with 0.15 M PBS and then blocked with 3% FBS in 0.15 M PBS for 1 h. Appropriately diluted mAb as well as the serum from AAV vector-administered mouse were incubated on antigen-coated plates for 6 h at 4°C. Then, wells were rinsed with 0.15 M PBS and the bound Abs were detected using HRP-coupled goat anti-mouse IgG1 (Pierce Chemical Co., Rockford, IL, USA). We detected IgG1 titer rather than total IgG titer, because transgene of the AAV vector was isolated from the mouse IgG1-secreting hybridoma (IIA2). The anti-Aß1–13 mAb (IIA2) purified from the hybridoma was used as a standard control.

Brain sample preparation for histochemical studies

Brains were removed and divided sagittally along the interhemispheric fissure. The right hemisphere was dissected from the cerebella. Brain samples were snap frozen in large test tubes containing n-hexane, immersed in a dry ice/acetone mixture, and stored at −80°C until processing. The left hemisphere was fixed with formalin and then embedded in paraffin for histochemical studies. To test whether Aß mAb-expressing vector-produced antibody can be used for immunostaining against human Aß, Culture supernatant of Aß mAb - expressing vector-transduced HEK293 cells was used as a first antibody and HRP-Goat ant-mouse IgG1 antibody used as a second antibody. Rabbit anti-human beta amyloid (1–42) antibody (Genetex, Inc., Irvine, CA) and mAb purified from hybridoma IIA2 were used as controls. Paraffin samples from AAV vector administrated mice were stained with the rabbit anti-human beta amyloid (1–42) antibody.

ELISA to measure Aß protein levels in the brain

The frozen left cerebra were obtained from each mouse and homogenized with a homogenizer in Tris-buffered saline buffer (TBS, 50 mM Tris, 150 mM NaCl, pH 7.6) containing protease inhibitor cocktail (Nacalai, San Diego, CA, USA) with 20 µg/ml pepstatin A, then centrifuged at 100,000 *g* for 1 h at 4°C using an Optima TLX ultracentrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The pellets were homogenized in TBS buffer containing 2% SDS and protease inhibitor cocktail (Nacalai, San Diego, CA, USA) following incubation at 37°C for 15 min. Then the solution was centrifuged again at 100,000 *g* for 1 h at 25°C. The supernatant and pellet correspond to the soluble (2% SDS soluble fraction) and insoluble fraction, respectively.

The insoluble fraction was washed, then extracted again with 70% formic acid and centrifuged at 100,000 *g* for 1 h. The supernatants of 70% formic acid extracts were neutralized with 1 M Tris-HCl, pH 8.0 at a dilution of 1:20 (70% FA fraction). The dissolved samples of Aß1–42 or Aß1–40 protein were quantified using Human Amyloid ß (N3pE-42) Kit and Human Amyloid ß (N3pE-40) assay kit, respectively (IBL Co., Ltd., Gunma, Japan). The values obtained were corrected with the wet weight of each brain hemisphere sample and expressed as pmol/g brain.

Cytotoxicity inhibition test by Abs from AAV-transduced cells using primary culture hippocampal cell

Hippocampal cells were collected from 15-day-old fetal mouse brains and gently minced. The samples were incubated at 37°C for 5 min in 9 ml of 0.15 M PBS and 1 ml of 2.5% trypsin, followed by addition of 1 ml 0.5% trypsin inhibitor on ice. The cells were washed twice with 0.15 M PBS and resuspended in media stock (MS) supplemented with 20% FBS, 10 ng/ml epidermal growth factor, 50 IU/ml penicillin, and 50 µg/mL streptomycin. MS is composed of modified Eagle's medium supplemented with 2 mM glutamine and 20 mM glucose [49].

We reacted 5.0×10^{4} primary culture hippocampal cells in 100 µl MS per well with each concentration of aggregated Aß solution with or without Aß mAb - expressing vector-transduced culture supernatant in a humidified atmosphere (37°C, 5% CO2). After 6 and 24 h incubation, 10 µL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reagent (final concentration 0.5 mg/ml) was added to each well. The microplate was incubated for 4 h in a humidified atmosphere [50]. Then, 100 µl of the solubilization solution was added into each well to solubilize the purple formazan crystals, and the absorbance of the samples was measured using an ELISA microplate reader [51].

Data analysis

All values were expressed as the mean ± standard error (SE). Statistical analysis (Student's t-test) of the experimental data and controls was conducted using two-way factorial analysis of variance. Significance was defined as $P<0.05$ for statistical analysis using all time points in each group.

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Author Contributions

Conceived and designed the experiments: K. Ozawa K. Okuda. Performed the experiments: MS SA TT. Analyzed the data: KS DMK. Contributed reagents/materials/analysis tools: MO HM. Wrote the paper: MS K. Okuda.

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ORIGINAL ARTICLE

Anti-factor IXa/X bispecific antibody (ACE910): hemostatic potency against ongoing bleeds in a hemophilia A model and the possibility of routine supplementation

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Summary. *Background:* We previously reported that a humanized anti-factor IXa/X bispecific antibody, hBS23, mimics the function of FVIII even in the presence of FVIII inhibitors, and has preventive hemostatic activity against bleeding in an animal model of acquired hemophilia A. After further molecular engineering of hBS23, we recently identified an improved humanized bispecific antibody, ACE910, for clinical investigation. *Objectives:* To elucidate the *in vivo* hemostatic potency of ACE910 by examining its effect against ongoing bleeds, and to determine its pharmacokinetic parameters for discussion of its potency for prophylactic use. *Methods:* A non-human primate model of acquired hemophilia A was established by injecting anti-primate FVIII neutralizing antibody. When bleeds emerged following an artificial bleed-inducing procedure, either ACE910 or recombinant porcine FVIII (rpoFVIII) was intravenously administered. rpoFVIII was additionally administered twice daily on the following 2 days. Bleeding symptoms were monitored for 3 days. A pharmacokinetic study and multiple-dosing simulations of ACE910 were also performed. *Results:* A single bolus of 1 or 3 mg kg^{-1} ACE910 showed hemostatic activity comparable to that of 10 U kg^{-1} (twice daily) rpoFVIII against ongoing bleeds. The determined ACE910 pharmacokinetic parameters included a long half-life (3 weeks) and high subcutaneous bioavailability (nearly 100%). The simulation results based on pharmacokinetic parameters indicated that the above hemostatic level could be maintained with once-weekly subcutaneous administration of ACE910, suggesting the possibility of more effective prophylaxis. *Conclusions:* ACE910 may offer an alternative on-demand treatment option for patients with hemophilia A, as well as user-friendly and aggressive routine supplementation.

Keywords: antibodies, catalytic; factor VIII; hemophilia A; hemostasis; therapeutics.

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Introduction

Hemophilia A is a bleeding disorder caused by an inherited deficiency of factor VIII. The severity is known to correlate with the plasma FVIII level: severe, moderate and mild phenotypes are defined by plasma FVIII levels of < 1, 1–5 and > 5 to < 40 U dL^{-1}, respectively. Severe cases have a high propensity to suffer bleeds, including joint bleeds, whereas moderate cases typically experience far fewer bleeding episodes, and mild cases rarely bleed spontaneously [1,2].

Patients are primarily treated with FVIII agents. However, as FVIII agents are exogenous for severely affected patients, ~ 30% of them develop alloantibodies against FVIII (FVIII inhibitors) [2]. FVIII inhibitors largely restrict treatment with FVIII agents, and consequently make it difficult to control bleeding, because alternative bypassing agents have shorter *in vivo* half-lives and are not always effective [3]. The eradication of FVIII inhibitors with high doses of FVIII is very expensive, and does not always work [4]. In patients with severe hemophilia A without FVIII inhibitors, routine prophylaxis with exoge-

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nous FVIII to maintain FVIII levels above 1 U dL^{-1} is beneficial to prevent bleeding [5,6]; however, the need for frequent intravenous injections negatively affects patients' quality of life [7].

In order to overcome these shortcomings, we previously created a humanized anti-FIXa/FX bispecific IgG antibody, termed hBS23, which replicated FVIII cofactor function by binding and placing FIXa and FX into spatially appropriate positions [8]. hBS23 had cofactor activity *in vitro*, even in the presence of FVIII inhibitors, and, in a non-human primate model of acquired hemophilia A, hBS23 at an intravenous dose of 0.3 mg kg^{-1} exerted hemostatic activity to prevent the progression of bleeding symptoms to the same extent as recombinant porcine FVIII (rpoFVIII) maintained at a plasma level of $\geq$ 1 U dL^{-1}. However, it remained unproven whether this bispecific antibody approach possessed the potency to ameliorate ongoing bleeds, which would require higher levels of FVIII, or how much hemostatic potency it had in comparison with FVIII. We recently modified hBS23 with a multidimensional optimization approach to improve the FVIII-mimetic cofactor activity, pharmacokinetic properties, immunogenicity, physicochemical stability, and ease of industrial manufacture for clinical application; we consequently identified an improved humanized anti-FIXa/FX bispecific IgG antibody, termed ACE910 [9]. In this study, we elucidate the *in vivo* hemostatic potency of ACE910, including that against ongoing bleeds as compared with rpoFVIII, by using a non-human primate model of acquired hemophilia A.

Furthermore, in order to elucidate the potency of ACE910 in routine supplementation, we performed a pharmacokinetic study of ACE910 in non-human primates to determine its pharmacokinetic parameters, and conducted multiple dosing simulations with those parameters. Routine supplementation with exogenous FVIII is aimed at keeping the FVIII level at 1 U dL^{-1} or above to convert a severe disease to a moderate one [2,5]. This strategy successfully reduces bleeding episodes and the risk of developing hemophilic arthropathy [5]. However, the effect is not necessarily perfect: a recent report suggested that the risk of joint damage remains until the baseline factor level is 10–15 U dL^{-1} or higher [10]. In this study, we also discuss the possibility of once-weekly subcutaneous administration of ACE910 for routine supplementation that is more aggressive than the current one with exogenous FVIII; in other words, a regimen that can convert a severe disease to a mild disease.

Materials and methods

Materials

ACE910 was expressed in human embryonic kidney 293 or Chinese hamster ovary (CHO) cells, which were co-transfected with the mixture of plasmids encoding the humanized anti-FIXa heavy chain, anti-FX heavy chain, and common light chain. ACE910 was purified by protein A and ion exchange chromatography from the culture supernatant [9]. B domain-deleted rpoFVIII [8] was prepared as described in the supporting information (Preparation and analysis of rpoFVIII). Briefly, we expressed it in CHO cells by stable transfection. Then, rpoFVIII was purified from the supernatant by using ion exchange and gel permeation chromatography. We confirmed its purity with SDS-PAGE under reduced conditions, and determined its activity (U dL^{-1}) with an activated partial thromboplastin time (APTT)-based one-stage coagulation assay. Recombinant human FVIII (rhFVIII) was purchased from Bayer HealthCare (Leverkusen, Germany). Anti-primate FVIII neutralizing antibody (VIII-2236) [8] and the other purchased reagents are described in the supporting information (Confirmation of non-reactivity of VIII-2236 to rpoFVIII and ACE910, and the other supporting methods).

Animals and ethics

We used 26 and 12 male cynomolgus monkeys for the *in vivo* hemostatic study (2.6–4.0 kg, aged 3 years; Hamri, Ibaraki, Japan) and for the pharmacokinetic study (2.9–5.0 kg, aged 4–5 years; Japan Laboratory Animals, Tokyo, Japan), respectively. We used 24 female mice (aged 5 weeks; Charles River, Yokohama, Japan, and SLC, Hamamatsu, Japan) and 24 female rats (aged 4 weeks; Charles River) for the immunization to generate anti-idiotype antibodies against the variable region of ACE910. The details of anti-idiotype antibodies are described in the supporting information (Generation and preparation of anti-idiotype antibodies to each variable region of ACE910).

All animal studies were approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical, and were conducted in accordance with the approved protocols and the Guidelines for the Care and Use of Laboratory Animals at the company. Chugai Pharmaceutical is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

APTT and thrombin generation (TG) assays

APTT and TG assays were performed with standard equipment. In the TG assay, we employed two kinds of triggering solutions that contained, respectively, FXIa and tissue factor (TF). The solution containing human FXIa (Enzyme Research Laboratories, Swansea, UK) was prepared in-house, and the solution containing TF, PPP-Reagent LOW (Thrombinoscope BV, Maastricht, the Netherlands) was purchased. The details are described in the supporting information (APTT and TG assays).

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In vivo *hemostatic study in an acquired hemophilia A model*

As ACE910 is highly species-specific in its FVIII-mimetic cofactor activity, non-human primates were used. On day 0, the animals received an intravenous injection of VIII-2236 (10 mg kg^{-1}). Two hours later, the animals were anesthetized with isoflurane inhalation, and bruises on the body surface that might possibly have emerged because of FVIII neutralization were measured. Then, the following two surgical procedures were performed: a 18G-needle was inserted 1 cm deep into the muscle at 22 sites (two sites in each upper arm, three sites in each forearm, four sites on the inside of each thigh, and two sites on the outside of each thigh); and subcutaneous exfoliation was performed by inserting the tip of forceps beneath the abdominal skin to 3 cm at two sites. After administration of buprenorphine, an analgesic drug, the animals were allowed to recover from the anesthesia. (They received this analgesic treatment twice daily [morning and evening] from day 0 to day 2; six doses were given, and the condition of the animals was observed daily.) After recognition of bleeding, approximately 6–8 h after injury, the animals received intravenous ACE910 (0.3, 1 or 3 mg kg^{-1}; $n = 4$ for each group), rpoFVIII (3.4 or 10 U kg^{-1}; $n = 4$ for each group), or no test item (control; $n = 6$). In the rpoFVIII group, rpoFVIII was also intravenously administered in the morning and evening on days 1 and 2 (a total of five administrations). In the morning on days 1, 2, and 3, the animals were anesthetized for measurement of the bruised areas. After the evaluation on day 3, the animals were killed humanely. Citrated blood was collected before and 2 h after the VIII-2236 injection, ~ 10 min after the test item administration on day 0, and before measurement of the bruised area on days 1, 2, and 3. The change in blood hemoglobin level was expressed as a percentage of that on day 0 (2 h after the VIII-2236 injection). The plasma ACE910 concentration was determined with the method described in the supporting information (Measurement of plasma ACE910 concentration).

Pharmacokinetic study

Animals received intravenous ACE910 (6 mg kg^{-1}; $n = 3$) or subcutaneous ACE910 (0.06, 0.6 or 6 mg kg^{-1}; $n = 3$ for each group) in a single dose. For animals dosed intravenously, blood was sampled with a heparinized syringe at 0.25, 2, 8, 24, 48, 72 and 96 h postdose, as well as at 7, 14, 28, 42, 56, 70 and 84 days postdose. For animals dosed subcutaneously, blood was collected in the same way without sampling at 0.25 h. The plasma concentrations of ACE910 and anti-ACE910 alloantibodies were measured with the methods described in the supporting information (Measurement of plasma ACE910 concentration and detection of anti-ACE910 alloantibodies).

Statistical analysis, pharmacokinetic analysis, and multiple-dosing simulation

For the *in vivo* hemostatic study, data are presented as mean ± standard error. Other data are presented as mean ± standard deviation. The parametric Dunnett multiple comparison test (two-tailed) (SAS preclinical package version 5.00; SAS Institute Japan, Tokyo, Japan) was used to determine *P*-values. $P < 0.05$ was considered to be statistically significant.

In the pharmacokinetic study, the plasma ACE910 concentration data were analyzed by non-compartmental analysis with PHOENIX WINNONLIN version 6.2 (Pharsight, St Louis, MO, USA). Multiple-dosing simulations were performed with SAAM II version 1.2 (SAAM Institute, Seattle, WA, USA).

Results

In vitro *cross-reactivity of ACE910 with cynomolgus monkeys*

First, we examined the cross-reactivity of ACE910 with cynomolgus monkeys by use of an APTT assay. ACE910 shortened APTT in FVIII-neutralized cynomolgus monkey plasma with a concentration dependency similar to that in human FVIII-deficient plasma (Fig. 1A,B).

We next examined the cross-reactivity of ACE910 with cynomolgus monkeys by use of one of the global assays, the TG assay [11]. Because we had not clearly detected rpoFVIII activity in the standard low-TF triggering condition in FVIII-neutralized cynomolgus monkey plasma, we instead employed FXIa as a trigger. Beforehand, we had confirmed, in human FVIII-deficient plasma, that the two triggering conditions produced similar peak heights for the purpose of comparing ACE910 with rhFVIII (Figs 1C and S1). ACE910 improved the peak height of the FXIa-triggered TG assay in FVIII-neutralized cynomolgus monkey plasma in a similar concentration-dependent manner to that in human FVIII-deficient plasma (Fig. 1C,D). ACE910 had a similar cofactor activity as rhFVIII or rpoFVIII in improving the peak height in each species. Beforehand, the rpoFVIII that we prepared had been analyzed for qualification (Fig. S2).

In vivo *hemostatic study in an acquired hemophilia A model*

To examine the *in vivo* hemostatic potency of ACE910, including that against ongoing bleeds, we modified the non-human primate model of acquired hemophilia A that we previously reported [8]. Briefly, more intense injury procedures were employed, and the dose timing of the test item was set to after the emergence of bleeding symptoms, so that the hemostatic action of 10 U kg^{-1} rpoF-VIII could be properly evaluated.

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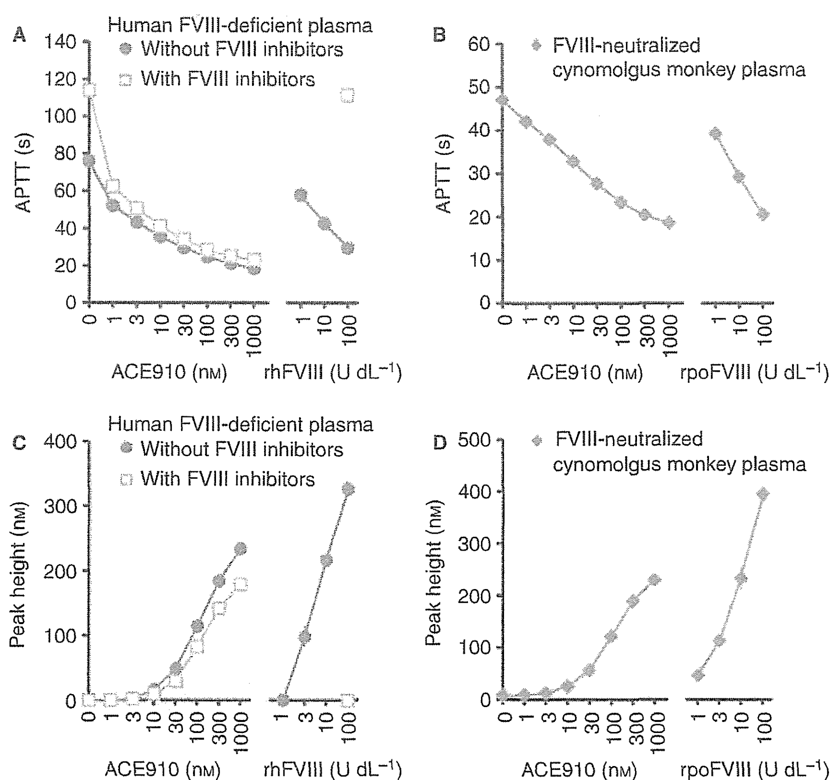

Fig. 1. FVIII-mimetic cofactor activity of ACE910 in human FVIII-deficient plasma without and with FVIII inhibitors and in FVIII-neutralized cynomolgus monkey plasma. Effects of ACE910, recombinant human FVIII (rhFVIII) or recombinant porcine FVIII (rpoFVIII) on activated partial thromboplastin time (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

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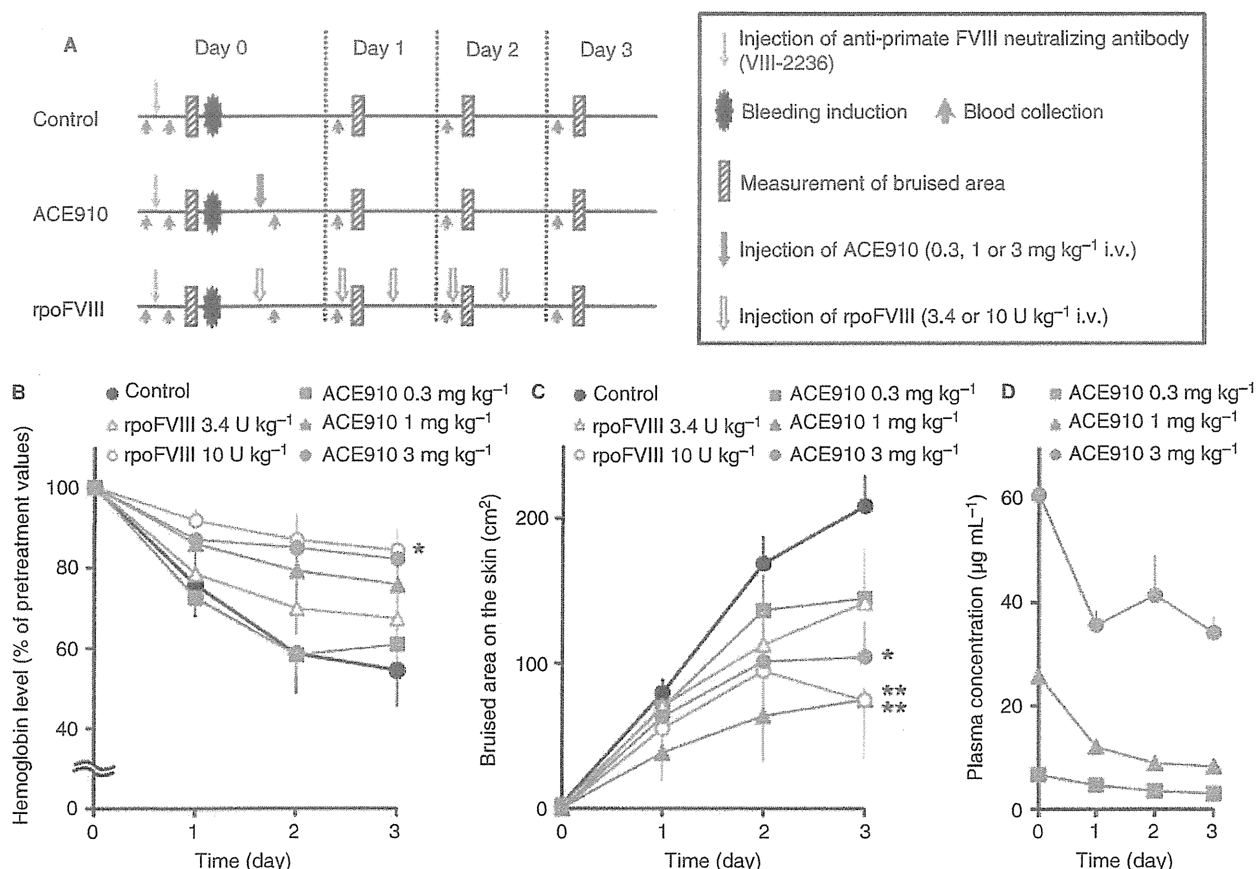

Fig. 2. *In vivo* hemostatic activity of ACE910 against ongoing bleeds in an acquired hemophilia A model. (A) The experimental protocol used. (B, C) Time course changes of (B) hemoglobin level and (C) bruised areas in the control group (no test item; n = 6), the ACE910 group (0.3, 1 or 3 mg kg^{-1}; 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

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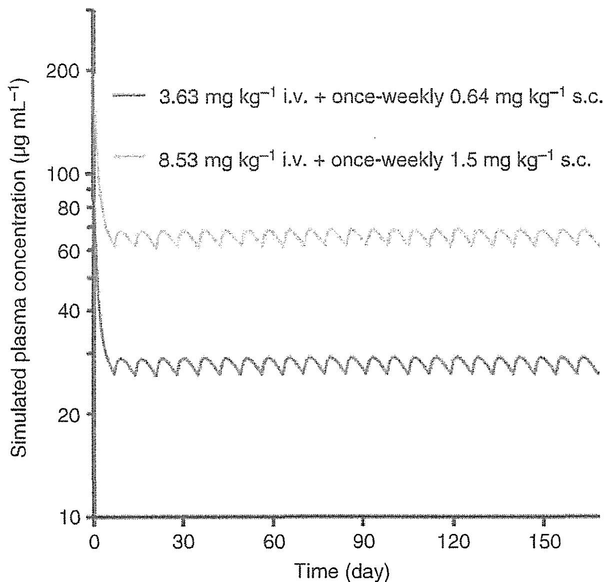

Fig. 3. Examples of simulations; plasma ACE910 concentration after multiple dosing in cynomolgus monkeys. The time course of plasma ACE910 concentration was simulated by use of the pharmacokinetic study data in cynomolgus monkeys for the case of once-weekly subcutaneous (s.c.) administration at 0.64 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-

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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.

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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

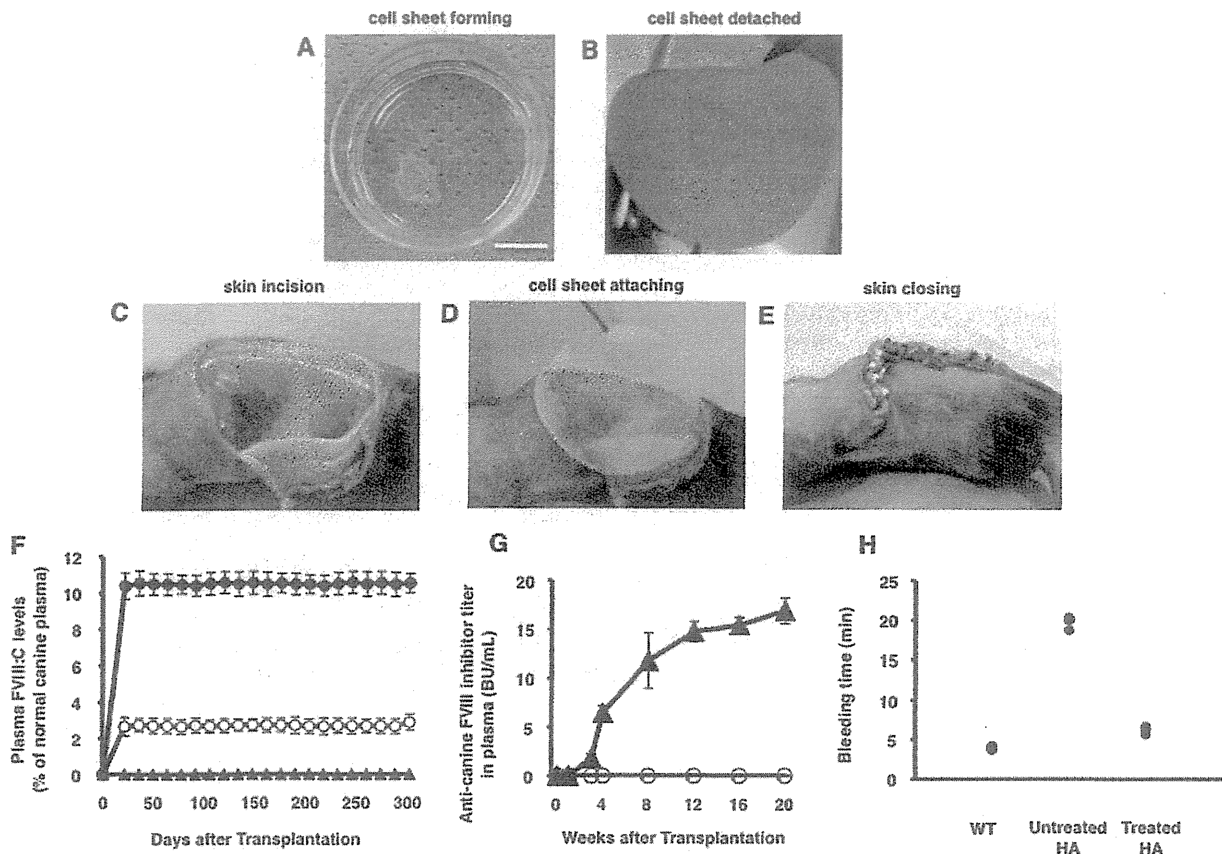

Figure 1. Subcutaneous transplantation of canine FVIII (cFVIII)-transduced blood outgrowth endothelial cell (BOEC) sheets in hemophilia A mice. (A–E) Schematic procedure for BOEC sheet transplantation. BOECs from hemophilia A mice were transduced using a lentiviral vector expressing the canine FVIII 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

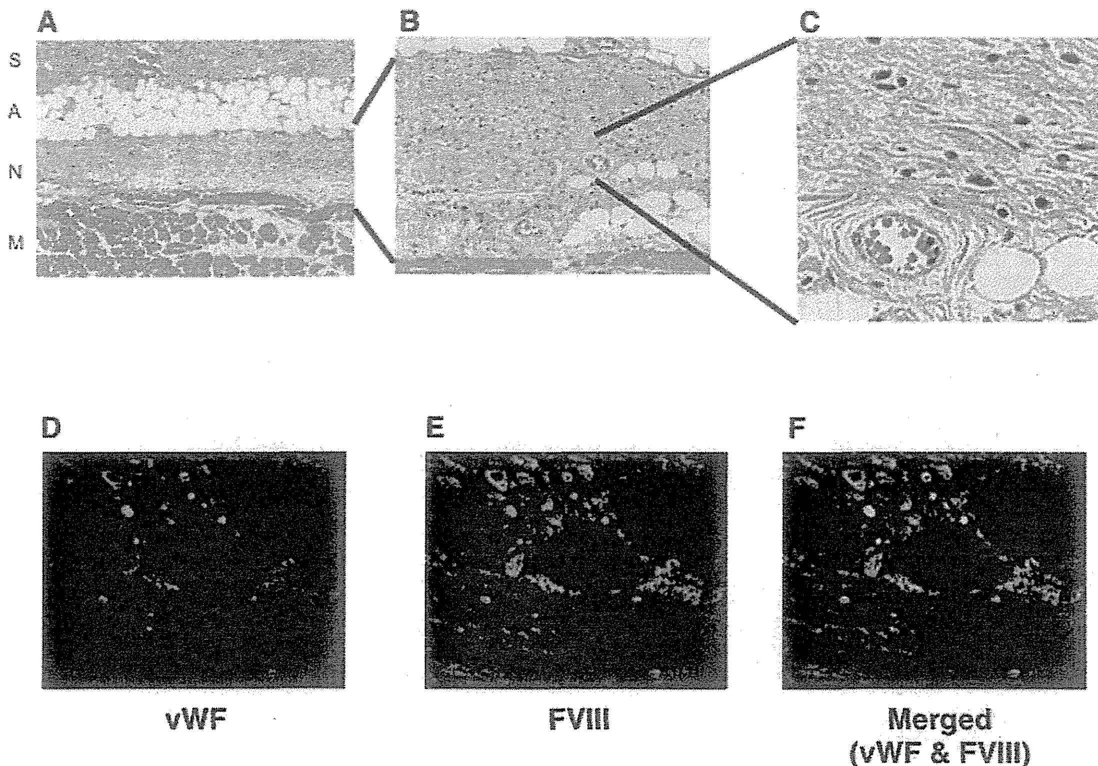

Figure 2. Histological analyses of subcutaneously transplanted genetically modified blood outgrowth endothelial cell (BOEC) sheets in hemophilia A mice. Eight weeks after transplantation of canine FVIII-transduced BOEC sheets, several recipient hemophilia A mice were sacrificed, and the implant tissue 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