Studies on Factor VIII-mimetic Function of Anti-factor IXa/Factor X Bispecific Antibody

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Atsushi MUTO

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***In vivo* preventive effects of ACE910 prophylactic treatment on spontaneous bleeding including joint bleeds in a long-term non-human primate model of hemophilia A**

Abstract

Introduction and Aim

Hemophilia A is a bleeding disorder caused by an inherited deficiency of blood coagulation factor VIII (FVIII). FVIII functions as a cofactor to promote blood coagulation when activated by either thrombin or activated factor X (FXa). The resultant activated factor VIII (FVIIIa) binds to both factor IX (FIX) and factor X (FX) which have serine protease activity when activated. FX is a substrate for activated factor IX (FIXa) protease. The binding properties of FVIII to FIXa and FX contribute to FVIII's cofactor activity, modifying the interaction between FIXa and FX, then markedly enhancing the catalytic rate constant of FIXa against FX.

Patients with severe hemophilia A (<1% of normal FVIII level) typically suffer from recurrent bleeding episodes several times a month, primarily in the musculoskeletal system. Approximately 85% of the bleeding episodes are into joints, and repeated joint bleeding from early childhood results in a chronic degenerative arthritis and markedly reduces the patients' quality of life. Patients are primarily treated with FVIII agents (recombinant or plasma-derived). However, since FVIII agents are exogenous for severely affected patients, approximately 30% of them develop alloantibodies against FVIII (FVIII inhibitors). FVIII inhibitors largely restrict treatment with FVIII agents, and consequently make it difficult to control bleeding. Furthermore, in severe hemophilia A patients without FVIII inhibitors, routine prophylaxis with exogenous FVIII to maintain FVIII levels above 1% is beneficial to prevent bleeding; however, the need for frequent intravenous (IV) injections with FVIII agents also negatively affects patients' quality of life.

In order to overcome these shortcomings, we created a humanized anti-factor IXa/factor X (anti-FIXa/FX) bispecific IgG antibody, termed ACE910, which is expected to mimic the FVIII cofactor function as a kind of scaffold by binding and placing FIXa and FX into spatially appropriate positions [38]. In this study, I evaluated (i) *in vitro* FVIII-mimetic activities of ACE910 in plasma coagulation assays, (ii) *in vivo* hemostatic potency of ACE910 episodic treatment in a newly-established short-term non-human primate model of hemophilia A, (iii) pharmacokinetic (PK) properties of ACE910 in non-human primates for estimating the possibility of routine prophylactic use, and (iv) *in vivo* preventive effects of ACE910 prophylactic treatment on spontaneous bleeding including joint bleeds in a newly-established long-term non-human primate model of hemophilia A. By examining the *in vitro* FVIII-mimetic activities and *in vivo* hemostatic effects on bleeding in the hemophilia A models, I aimed at

elucidating the biological functions of ACE910 as a FVIII-cofactor mimetic which has a completely different protein structure from native FVIII.

Methods and Results

Chapter 1: *In vitro* FVIII-mimetic activity of ACE910 in plasma coagulation assays

Using an enzymatic assay with purified FIXa and FX, FVIII-mimetic activities had been evaluated in the previous study [40]. ACE910 greatly enhanced FX activation by FIXa, whereas could not promote FX activation without FIXa, indicating ACE910's binding to both FIXa and FX is necessary for its activity. These results also indicated ACE910 itself does not show a direct enzymatic action and therefore functions as a cofactor like FVIII. I next evaluated the activity of ACE910 in human FVIII-deficient plasma and cynomolgus monkey FVIII-inhibited plasma by using the unique anti-primate FVIII neutralizing antibody (termed VIII-2236). With respect to thrombin generation (TG) potency, ACE910 dose-dependently improved TG parameters in these plasma both in the presence and absence of FVIII inhibitors, whereas the effects of recombinant human FVIII (rhFVIII) on TG were blocked by the presence of FVIII inhibitors. These results indicate that ACE910 could enhance plasma coagulation even in patients with FVIII inhibitors who are nonresponsive to FVIII treatment.

Chapter 2: *In vivo* hemostatic potency of ACE910 episodic treatment in a short-term non-human primate model of hemophilia A

A short-term cynomolgus monkey model of acquired hemophilia A was established by injecting anti-primate FVIII neutralizing antibody (VIII-2236). When bleeds emerged following artificial bleed-inducing procedures in muscles and subcutis, 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 single bolus of 1 or 3 mg/kg of ACE910 showed hemostatic potency comparable to 10 U/kg (twice daily) of rpoFVIII against on-going bleeds.

Chapter 3: Pharmacokinetic properties of ACE910 in non-human primates for estimating the possibility of routine prophylactic use

A PK study and multiple dosing simulations of ACE910 were performed in cynomolgus monkeys. The determined ACE910 PK parameters included a long half-life (3 weeks) and high subcutaneous (SC) bioavailability (nearly 100%). The simulation results based on PK parameters indicated that the hemostatic level could be maintained with once-weekly SC administration of ACE910, suggesting the possibility of more effective prophylaxis.

Chapter 4: *In vivo* preventive effects of ACE910 prophylactic treatment on spontaneous bleeding including joint bleeds in a long-term non-human primate model of hemophilia A

In the Chapter 2 and 3 studies, I demonstrated in cynomolgus monkeys that a single IV dose of ACE910 exerted hemostatic activity against hemophilic bleeds artificially induced in muscles and subcutis, and that a SC dose of ACE910 showed a 3-week half-life and nearly 100% bioavailability, offering support for effective prophylaxis for hemophilia A by user-friendly SC dosing. However, there was no direct evidence that such SC dosing of ACE910 would prevent spontaneous bleeds occurring in daily life. In the Chapter 4 study, I newly established a long-term cynomolgus monkey model of hemophilia A by multiple IV injections of an anti-primate FVIII neutralizing antibody (termed cyVIII-2236) engineered in mouse–monkey chimeric form to reduce its antigenicity. The monkeys in the control group exhibited various spontaneous bleeding symptoms as well as continuous the activated partial thromboplastin time (APTT) prolongation; notably, all exhibited joint bleeds, which are a hallmark of hemophilia. Weekly SC doses of ACE910 (initial 3.97 mg/kg followed by 1 mg/kg) significantly prevented these bleeding symptoms; notably, no joint bleeding symptoms were observed. ACE910 is expected to prevent spontaneous bleeds and joint damage in hemophilia A patients even with weekly SC dosing, although appropriate clinical investigation is required.

Conclusions

We had generated a novel humanized anti-FIXa/FX bispecific IgG antibody, ACE910. By examining the *in vitro* FVIII-mimetic activities and *in vivo* hemostatic effects on bleeding in the hemophilia A models, I elucidated the biological functions of ACE910 as a FVIII-cofactor mimetic which has a completely different protein structure from native FVIII. In the general conclusions, (i) ACE910 improved coagulation in FVIII-deficient plasma both with and without FVIII inhibitors, (ii) a short-term hemophilia A model expressing stable artificial bleeds with proper severity was newly established in cynomolgus monkeys, and single episodic IV dose of ACE910 significantly inhibited these bleeding symptoms, (iii) ACE910 showed the 3-week plasma half-life and high SC bioavailability in cynomolgus monkeys, and possibility of effective prophylaxis by the once-weekly SC doses was simulated based on the PK parameters, and (iv) a long-term acquired hemophilia A model expressing reproducible spontaneous joint bleeds and other bleeds was newly established in cynomolgus monkeys, and weekly SC doses of ACE910 significantly prevented these bleeding symptoms. These results elucidated

biological functions of ACE910 as a FVIII-cofactor mimetic and suggested that ACE910 has the potential not only to ameliorate on-going bleeds, but also to offer a user-friendly and aggressive routine prophylaxis for hemophilia A patients both with and without FVIII inhibitors. Clinical investigation of ACE910 is currently on-going, based on the results in these studies.

Abbreviations

Abbreviation	Name
Abs	absorbance
APTT	activated partial thromboplastin time
BiAb	bispecific antibody
CHO	Chinese hamster ovary
ELISA	enzyme-linked immunosorbent assay
FIX	factor IX
FIXa	activated factor IX
FVIII	factor VIII
FX	factor X
FXa	activated factor X
FXIa	activated factor XI
HEK293	human embryonic kidney 293
Hgb	hemoglobin
hFIX	human factor IX
hFIXa	human activated factor IX
hFX	human factor X
hFXa	human activated factor X
IgG	immunoglobulin G
IV	intravenous
PK	pharmacokinetic
PL	phospholipid
rhFVIII	recombinant human factor VIII
rpoFVIII	recombinant porcine factor VIII
SD	standard deviation
SE	standard error
SC	subcutaneous
TF	tissue factor
TG	thrombin generation

General Introduction

Hemophilia A is a bleeding disorder caused by an inherited deficiency of blood coagulation factor VIII (FVIII). FVIII functions as a cofactor to promote blood coagulation when activated by either thrombin or activated factor X (FXa). The resultant activated factor VIII (FVIIIa) binds to both factor IX (FIX) and factor X (FX) which have serine protease activity when activated. FX is a substrate for activated factor IX (FIXa) protease. The binding properties of FVIII to FIXa and FX contribute to FVIII's cofactor activity, modifying the interaction between FIXa and FX, then markedly enhancing the catalytic rate constant of FIXa against FX [22].

The severity of hemophilia A is known to correlate with the plasma FVIII level: severe, moderate, and mild phenotypes are defined by a plasma FVIII level of < 1, 1 to 5, and > 5 to < 40%, respectively. Patients with severe hemophilia A (<1% of normal FVIII level) typically suffer from recurrent bleeding episodes several times a month, primarily in the musculoskeletal system, whereas moderate cases typically experience far fewer bleeding episodes and mild cases rarely bleed spontaneously [3, 8]. Approximately 85% of the bleeding episodes are into joints, and repeated joint bleeding from early childhood results in a chronic degenerative arthritis and markedly reduces the patients' quality of life.

Patients are primarily treated with FVIII agents (recombinant or plasma-derived). However, since FVIII agents are exogenous for severely affected patients, approximately 30% of them develop alloantibodies against FVIII (FVIII inhibitors) [1, 3]. 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 (e.g. half a day in FVIII agent, 2–3 hours in a recombinant activated factor VII agent) and are not always effective [1, 19]. Attempts to eradicate FVIII inhibitors with high doses of FVIII are tried, but these are very expensive and do not always work [14]. In severe hemophilia A patients without FVIII inhibitors, while traditional episodic treatment by a FVIII agent cannot prevent hemophilic arthropathy, routine prophylaxis with exogenous FVIII to maintain FVIII levels above 1% is beneficial to prevent bleeding [25, 30]; however, the need for frequent IV injections with FVIII agents also negatively affects patients' quality of life and their adherence to the routine prophylactic regimen, typically IV injections 3 times per week, which is particularly problematic when treating pediatric patients at home [35].

Therefore, a novel drug is needed: one that is long-lasting, subcutaneously injectable, effective regardless of FVIII inhibitors, and does not induce FVIII inhibitors [21, 23, 33, 43]. In

order to overcome these shortcomings, we created a humanized anti-factor IXa/factor X (anti-FIXa/FX) bispecific IgG antibody, termed ACE910, which is expected to mimic the FVIII cofactor function as a kind of scaffold by binding and placing FIXa and FX into spatially appropriate positions [22, 38]. One mechanism of the cofactor action of FVIII is to maintain FIXa and FX in the appropriate positional relationship in the enzyme reaction on activated phospholipid membranes (e.g. activated platelet membrane at hemostatic site in blood) in which FIXa activates FX. Based on this scientific assumption, the concept of "an antibody that is a functional mimetic for FVIII cofactor" was devised, by which the cofactor function of FVIII is substituted via "a bispecific antibody that binds to FIXa with one arm and binds to FX with the other arm" (Figure 1).

Given its characteristics as an IgG molecule, the bispecific antibody, finally identified ACE910, is expected to have the potential to help overcome a number of challenges in the current standard therapy for hemophilia A, including: (a) It has a long half-life in blood and, therefore, a potentially low dosing frequency; (b) It can be administered by SC injection because general antibody drugs are absorbed by SC administration; (c) It has the potential to be effective in patients with FVIII inhibitors because an antibody has a completely different protein sequences and structure from those of FVIII, therefore its immunogenicity is totally different; and (d) It is not expected to induce FVIII inhibitors. A series of investigations to seek such a bispecific antibody, we finally identified ACE910 with the capacity to be a functional mimetic for FVIII and with the potential to be effective in routine supplementation for hemophilia A patients.

ACE910 is a clinical investigation candidate which was highly optimized using a multidimensional approach to improve the FVIII-mimetic cofactor activity, PK properties, immunogenicity, physicochemical stability, and industrial manufacturability for clinical application [38]. ACE910 is a humanized monoclonal modified IgG4 antibody. By incorporation of a modified IgG4 constant region, the molecule has also been designed to reduce constant region-mediated effector activity. With respect to industrial manufacturability, difficulties in manufacturing the bispecific antibody were overcome by identifying a common light chain for the anti-FIXa and anti-FX heavy chains through framework/complementarity determining region shuffling, by pI engineering of the two heavy chains to facilitate ion exchange chromatographic purification of the bispecific antibody from the mixture of byproducts, and by enhancing preferential hetero-dimerization of the two heavy chains through charge attractive interaction in the CH3 region of each heavy chain (Figure 2). These

antibody-engineering technologies incorporated into ACE910 molecular design greatly contributed to realize large-scale manufacturing of ACE910 [17, 38].

Using an enzymatic assay with purified FIXa and FX, FVIII-mimetic activities of ACE910 had been evaluated in the previous study [40]. ACE910 greatly enhanced FX activation by FIXa, whereas could not promote FX activation without FIXa, indicating ACE910's binding to both FIXa and FX is necessary for its activity. These results also indicated ACE910 itself does not show a direct enzymatic action and therefore functions as a cofactor like FVIII (Figure 3).

Moreover, it was necessary to conduct *in vitro* assays to fully characterize the biological functions of ACE910 as a FVIII-cofactor mimetic which has a completely different protein structure from native FVIII. In the Chapter 1 study, I evaluated *in vitro* FVIII-mimetic activities of ACE910 in plasma coagulation assays.

It also remained unproven whether this bispecific antibody approach possessed the potency to ameliorate on-going bleeds, which would require higher levels of FVIII, or how much hemostatic potency it had in comparison with FVIII. In the Chapter 2 study, I elucidated the *in vivo* hemostatic potency of ACE910, including that against on-going bleeds compared to rpoFVIII, using a non-human primate model of acquired hemophilia A. Because ACE910 does not cross-react with mouse or rat, and cross-reacts only with primate, the cynomolgus monkey was selected as the animal species for evaluations in *in vivo* non-clinical studies. In primates, animal models of hemophilia had been unknown until then, I newly established a short-term (4 days) cynomolgus monkey model of acquired hemophilia A by injecting a unique FVIII-neutralizing antibody, VIII-2236, and evaluated *in vivo* hemostatic potency of ACE910 episodic treatment in this newly-established model.

Furthermore, in order to evaluate the potency of ACE910 in routine supplementation, I performed a PK study of ACE910 in cynomolgus monkeys to determine its PK parameters and conducted multiple dosing simulations using these parameters in the Chapter 3 study. The routine supplement of exogenous FVIII is aimed at keeping the FVIII level at 1% or above to convert a severe disease to a moderate one [3, 25]. This strategy successfully reduces bleeding episodes and the risk of developing hemophilic arthropathy [25]. However, the effect is not necessarily perfect: a recent report suggests that the risk of joint damage remains until the baseline factor level is 10 to 15% or higher [9]. In the Chapter 3 study, I also discuss the possibility of a once-weekly SC 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.

In the Chapter 2 study, by using a short-term cynomolgus monkey model of hemophilia A, ACE910 at a single IV dose of 1 or 3 mg/kg exerted hemostatic activity against artificial on-going bleeds in muscles and subcutis to the same extent as rpoFVIII at twice-daily IV doses of 10 U/kg. Further, in the Chapter 3 study, a multiple-dosing simulation calculated from the PK parameters of ACE910 in cynomolgus monkeys suggested that the plasma ACE910 concentration capable of stopping even on-going bleeds would be maintained by once-weekly SC administration of 0.64 to 1.5 mg/kg ACE910.

In the Chapter 4 study, I actually evaluated the preventive effect of once-weekly SC dosing of 1 mg/kg ACE910 in a long-term cynomolgus monkey model of hemophilia A. Prevention of joint bleeding is of major importance in the care of hemophilia A patients [36]. However, it remained unproven whether repeated SC dosing of ACE910 could actually prevent spontaneous bleeding episodes, including the joint bleeds that are a pathologic hallmark of hemophilia A. To address this question non-clinically, I required a newly-established primate model, because ACE910 is highly species-specific in its FVIII-mimetic cofactor activity and a primate model of hemophilia A which developed joint bleeding is unknown. Therefore, in the Chapter 4 study, I aimed firstly to establish a long-term hemophilia A model expressing spontaneous bleeding episodes, including joint bleeds, in cynomolgus monkeys, and secondly to actually evaluate the preventive effect of once-weekly SC dosing of ACE910 in this model for investigating the potential of a prophylactic treatment in hemophilia A patients.

By examining the *in vitro* FVIII-mimetic activities and *in vivo* hemostatic effects on bleeding in the hemophilia A models in the Chapters 1, 2, 3, and 4 studies, I aimed at elucidating the biological functions of ACE910 as a FVIII-cofactor mimetic which has a completely different protein structure from native FVIII.

Chapter 1

In vitro FVIII-mimetic activity of ACE910 in plasma coagulation assays

1-1. Materials and Methods

Materials

ACE910 was expressed in human embryonic kidney (HEK) 293 or Chinese hamster ovary (CHO) cells which 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 chromatographies from the culture supernatant [38]. B domain-deleted rpoFVIII [22] was prepared as described in the following section of "Preparation and analysis of recombinant porcine FVIII, rpoFVIII". Briefly, I expressed it in CHO cells by stable transfection. Then, rpoFVIII was purified from the supernatant by using ion-exchange and gel permeation chromatographies. I confirmed its purity by SDS-PAGE under reducing conditions and determined its activity (% = U/dL) by an APTT-based one-stage coagulation assay. The preparations of ACE910 and rpoFVIII were supported mainly by T. Wakabayashi and the protein purification team in Chugai Pharmaceutical. Recombinant human FVIII (rhFVIII) was purchased from Bayer HealthCare (Leverkusen, Germany). Anti-primate FVIII neutralizing antibody (VIII-2236) was prepared as previously presented [22]. ACE910, rpoFVIII, and VIII-2236 used in the later studies (the Chapters 2, 3, and 4) were prepared in the similar ways as described in the Chapter 1. The other purchased reagents are described in the following each section.

Preparation and analysis of recombinant porcine FVIII, rpoFVIII

I expressed a B domain-deleted form of rpoFVIII in CHO cells by stable transfection as previously described [22]. In detail, the vector employed for the transfection was designed on the basis of a previously reported sequence of the F8 (Sus scrofa) gene (GenBank: NM_214167) and the sequence of B domain-deleted human F8 gene [15, 44]. rpoFVIII was purified from the supernatant of rpoFVIII-containing culture medium by using Q Sepharose Fast Flow column (GE Healthcare Biosciences, Wauwatosa, WI, USA) with a linear 0 to 1 M NaCl gradient and then gel permeation chromatography on HiLoad 26/60 Superdex200pg column (GE Healthcare Biosciences) equilibrated in 150 mM NaCl, 10 mM histidine-HCl, 5 mM CaCl2, 0.1% (wt/vol) sucrose and 0.02% (wt/vol) polysorbate 80 (Junsei Chemical, Tokyo, Japan) (pH 7.0). I confirmed that the purified rpoFVIII showed double bands under reducing conditions in SDS-PAGE on which the actual molecular weight of the heavy chain and light chain of the rpoFVIII was consistent with the theoretical one calculated from the amino acid sequences,

respectively (Figure 6). I determined the FVIII activity (U/dL) of the purified rpoFVIII in an APTT-based one-stage coagulation assay. Fifty µL of diluted rpoFVIII or serial dilutions of standard reference (normal human plasma, Siemens Healthcare, Erlangen, Germany) were added to 50 µL of human FVIII-deficient plasma (Siemens Healthcare), followed by addition of 50 µL of an APTT reagent (Thrombocheck APTT-SLA, Sysmex, Kobe, Japan). Activity units (U) of rpoFVIII were calculated from the APTT-based coagulation times of the diluted rpoFVIII and the standard normal human plasma, and 1 U/dL of rpoFVIII was defined as the equivalent 1 U/dL of human FVIII in normal human plasma. The specific activity (U/mg) was calculated by dividing the activity of rpoFVIII with the protein amount determined by the PACE method using the absorbance at 280 nm [31]. The specific activity of rpoFVIII was 3610 U/mg which was comparable to previous reported values of rpoFVIII [10, 32] and recombinant human FVIII (rhFVIII) [5].

Activated partial thromboplastin time (APTT) and thrombin generation (TG) assays

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

I employed commercially available human FVIII-deficient plasma (< 1 U/dL) without or with FVIII inhibitors (53 Bethesda units) (George King Bio-Medical, Overland Park, KS, USA) in both the following APTT and TG assays. I also used the pooled citrated plasma of 3 male cynomolgus monkeys, which contained 300 µg/mL anti-primate FVIII neutralizing antibody (VIII-2236).

APTT was measured with a standard method using Thrombocheck APTT-SLA reagent by a semi-automated coagulation analyzer, KC4 delta (Stago, Paris, France). Data were collected in triplicate.

TG was measured by calibrated automated thrombography using a 96-well plate fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). In the TG assay, I employed two kinds of TG triggering solutions. One is a solution containing 0.16 nM human factor XIa (Enzyme Research Laboratories, Swansea, UK) and 20 µM synthetic phospholipids (10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine; Avanti Polar Lipids, Alabaster, AL, USA) which was prepared as previously described [29]. Another is the

purchased TF-containing reagent, PPP-Reagent LOW (Thrombinoscope BV, Maastricht, the Netherlands). Briefly, each well in a 96-well plate was dispensed with 80 μL of the plasma solution containing ACE910, rhFVIII or rpoFVIII to which was then added 20 μL of a triggering solution. For calibration, 20 μL of Thrombin Calibrator (Thrombinoscope BV) was added instead of the triggering solution. To initiate the reaction, 20 μL of FluCa reagent prepared from FluCa kit (Thrombinoscope BV) was dispensed by the instrument as programmed. I analyzed the thrombograms and peak height by the instrument's software. Data were collected in triplicate.

Confirmation of non-reactivity of an anti-primate FVIII neutralizing antibody, VIII-2236, to rpoFVIII and ACE910

As previously described, a neutralizing antibody against human FVIII, termed VIII-2236, was cross-reactive to cynomolgus monkey FVIII but not to porcine FVIII [22]. To confirm thoroughly that VIII-2236 has no neutralizing activity against rpoFVIII or ACE910, I examined the influence of VIII-2236 against the APTT-shortening activity of rpoFVIII, ACE910 or rhFVIII (control) in human FVIII-deficient plasma. Briefly, various concentrations of rhFVIII, rpoFVIII, or ACE910 was added to human FVIII-deficient plasma without or with 300 μg/mL of VIII-2236, which was incubated at 37°C for 2 hours. Then, APTT of the prepared plasma samples was measured.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The mean and SD were calculated by using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

1-2. Results

***In vitro* FVIII-mimetic activity of ACE910 in plasma coagulation assays**

First, I examined the effects of ACE910 on APTT in a plasma coagulation assay. ACE910 shortened APTT in human FVIII-deficient plasma with a similar concentration-dependency to that in FVIII-neutralized cynomolgus monkey plasma (Figure 4A, 4B). ACE910 also shortened APTT in human FVIII-deficient plasma in the presence of FVIII inhibitors, whereas the effects of rhFVIII on APTT were blocked by the presence of FVIII inhibitors (Figure 4A).

To further examine the FVIII-mimetic activity of ACE910, I next examined the effects on thrombin burst using one of the global assays, TG assay [39]. ACE910 improved the peak height of FXIa-triggered TG assay in human FVIII-deficient plasma in a similar concentration-dependent manner to that in FVIII-neutralized cynomolgus monkey plasma (Figure 4C, 4D). In comparison with rhFVIII or rpoFVIII, ACE910 had a roughly comparable cofactor activity of improving the peak height in each species. ACE910 also improved the peak height in human FVIII-deficient plasma in the presence of FVIII inhibitors, whereas the effects of rhFVIII on TG were not observed in the presence of FVIII inhibitors (Figure 4C).

I employed FXIa as a trigger in the above TG assay, because I had not clearly detected rpoFVIII activity in the standard low TF triggering condition in FVIII-neutralized cynomolgus monkey plasma. Beforehand, I had confirmed in human FVIII-deficient plasma that the two triggering conditions showed a similar concentration-dependency in the results of peak height for the purpose of comparing ACE910 with rhFVIII (Figure 4C, 5). I also beforehand confirmed rpoFVIII that I in-house prepared had been analyzed for qualification (Figure 6).

I had also preliminarily evaluated 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, the addition of rpoFVIII, ACE910, and rhFVIII concentration-dependently shortened the APTT of human FVIII-deficient plasma. The presence of VIII-2236 (300 μg/mL) canceled the APTT-shortening activity of rhFVIII, but not that of rpoFVIII or ACE910 at all. These results indicate that the anti-primate FVIII neutralizing antibody, VIII-2236, has no neutralizing activity against rpoFVIII and ACE910 (Figure 7).

Chapter 2

In vivo hemostatic potency of ACE910 episodic treatment in a short-term non-human primate model of hemophilia A

2-1. Materials and Methods

Animals and ethics

I used 26 male cynomolgus monkeys for the *in vivo* hemostatic study (2.6–4.0 kg, 3 years; Hamri, Ibaraki, Japan). 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 (AAALAC) International.

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

Since ACE910 is highly species-specific in its FVIII-mimetic cofactor activity, cynomolgus monkeys were used. On Day 0, the animals received an IV injection of VIII-2236 (10 mg/kg). Two hours thereafter, the animals were anesthetized by isoflurane inhalation and bruises on the body surface that might possibly have emerged by 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 (2 sites in each upper arm, 3 sites in each forearm, 4 sites on the inside of each thigh, and 2 sites on the outside of each thigh), and SC exfoliation by inserting the tip of forceps beneath the abdominal skin for 3 cm at 2 sites. After administering buprenorphine, an analgesic drug, the animals were allowed to recover from the anesthesia. They received this analgesic treatment twice daily (morning and evening) from Days 0 to 2; a total of 6 doses, and their conditions were daily observed with caution. After the bleeding recognition, about 6 to 8 h after injuring, the animals received IV administration of ACE910 (0.3, 1, or 3 mg/kg; n = 4 for each group), rpoFVIII (3.4 or 10 U/kg; 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 5 administrations), because the half-life of rpoFVIII had been beforehand confirmed to be approximately half a day in the pharmacodynamic study of rpoFVIII in cynomolgus monkeys, which is described in the later part of this section. In the morning on Days 1, 2 and 3, the animals were anesthetized to measure the bruised areas. After the evaluation on Day 3, the animals were euthanized humanely. Citrated blood was collected before and 2 h after the VIII-2236 injection, about 10 min after the test item administration on Day 0, and before measuring the bruised area on Days 1, 2, and 3. The change in blood hemoglobin (Hgb) level was expressed as a percentage of that

on Day 0 (2 h after the VIII-2236 injection).

Measurement of plasma ACE910 concentration

In the *in vivo* hemostatic study, the plasma ACE910 concentration was determined with a sandwich enzyme-linked immunosorbent assay (ELISA) to quantify human IgG. I used an anti-human IgG gamma-chain F(ab')2 fragment antibody produced in goat (Sigma Aldrich, St. Louis, MO, USA) as the solid phase antibody for capturing ACE910 and an anti-human IgG4 monoclonal antibody-alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL, USA) as the detection antibody.

Pharmacodynamic study of rpoFVIII in cynomolgus monkeys and multiple dosing simulation

Three male cynomolgus monkeys (2.7–2.8 kg, 3 years; Hamri) received a single IV administration of rpoFVIII (25 U/kg). Citrated blood sampling was performed at 0.25, 0.5, 1, 2, 4, 7, 24, 48, 72 h and 1 month after rpoFVIII administration. The plasma samples were stored at –80°C until measurement of rpoFVIII activity. rpoFVIII activity in each plasma sample was measured by APTT assay in which endogenous cynomolgus monkey FVIII was neutralized by the *in vitro* addition of VIII-2236 (300 μg/mL). The plasma samples at 1 month after rpoFVIII administration, to which were added VIII-2236 (300 μg/mL) and various concentrations of rpoFVIII *in vitro*, were used for preparing the standard curve for the respective animals. Based on the determined rpoFVIII activity, the PK parameters were analyzed. To simulate the plasma rpoFVIII concentrations after multiple dosing, the data of plasma rpoFVIII activity (regarded as rpoFVIII concentration) were analyzed using two-compartment model with first-order absorption, and the resulting PK parameters were used in simulation performed with SAAM II version 1.2 software (SAAM Institute). The PK analysis and simulation were particularly supported by K. Haraya in Chugai Pharmaceutical.

Statistical analysis

In the *in vivo* hemostatic study, data are presented as mean ± standard error (SE). The parametric Dunnett's multiple comparison test (2-tailed) (SAS Preclinical Package version 5.00; SAS Institute Japan, Tokyo, Japan) was used to determine P value. $P < 0.05$ was considered statistically significant.

2-2. Results

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

To examine the *in vivo* hemostatic potency of ACE910, including that against on-going bleeds, I newly established the cynomolgus monkey model of acquired hemophilia A by modifying that we previously reported [22]. Briefly, more intense injuring 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 of rpoFVIII could be properly evaluated.

The experimental protocol is illustrated in Figure 8A. An acquired hemophilia A status was first established by injecting anti-primate FVIII antibody, VIII-2236, which neutralizes endogenous FVIII but neither exogenous rpoFVIII nor ACE910 at all (Figure 7). Then, bleeding was artificially induced by inserting a needle in the limb muscles and by SC exfoliation on the abdomen. The animals in the control group showed a progressive decrease in Hgb level (anemia associated with hemorrhage) and expansion of bruised areas (Figure 8B, 8C). A single IV administration of ACE910 at 6 to 8 h after bleeding induction, when visible bleeding symptoms had emerged, tended to ameliorate the decrease in Hgb level ($P = 0.0643$ at 3 mg/kg vs. control). The expansion of bruised area was significantly reduced at doses of 1 and 3 mg/kg of ACE910 ($P < 0.05$ vs. control). These hemostatic effects of ACE910 at 1 and 3 mg/kg were comparable to the hemostatic effect of dosing twice daily with 10 U/kg of rpoFVIII. In such a regimen, plasma concentration of rpoFVIII would reach 25 U/dL just after the first injection and would range between 7.4 and 46 U/dL, according to a simulation of multiple dosing of rpoFVIII based on the PK parameters obtained from the single-dose injection study of rpoFVIII in cynomolgus monkeys (Figure 9). The mean plasma concentration of ACE910 (0.3, 1, or 3 mg/kg) was, respectively, 6.6, 26, or 61 μg/mL (45, 180, or 420 nM) just after administration, and 3.0, 8.4, or 34 μg/mL (21, 58, or 230 nM) on Day 3 (Figure 8D). In the clinical setting, 20 U/dL is often employed as the target initial FVIII level for treatment against on-going bleeds [41]. Therefore, IV administration of 1 to 3 mg/kg of ACE910, or a plasma concentration of 26 to 61 μg/mL (180 to 420 nM), is expected to exert hemostatic activity against on-going bleeds also in the clinical setting.

Chapter 3

Pharmacokinetic properties of ACE910 in non-human primates for estimating the possibility of routine prophylactic use

3-1. Materials and Methods

Animals and ethics

I used 12 male cynomolgus monkeys for the PK study (2.9–5.0 kg, 4–5 years; Japan Laboratory Animals, Tokyo, Japan). I used 24 female mice (5 weeks; Charles River, Yokohama, Japan and SLC, Hamamatsu, Japan) and 24 female rats (4 weeks; Charles River) for the immunization to generate anti-idiotype antibodies to the variable region of ACE910. The details of anti-idiotype antibodies are described in the following section of "Generation and preparation of anti-idiotype antibodies to each variable region of ACE910" in this Chapter 3 study.

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 (AAALAC) International.

Pharmacokinetic study

Animals received IV administration of ACE910 (6 mg/kg; n = 3) or SC administration of ACE910 (0.06, 0.6, or 6 mg/kg; 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, as well as at 7, 14, 28, 42, 56, 70, and 84 days post-dose. For animals dosed subcutaneously, blood was collected in the same way without sampling at 0.25 h. The plasma concentration of ACE910 and anti-ACE910 alloantibodies was measured by the methods described in the following sections. This PK study was particularly supported by Y. Sakamoto and the PK analysis team in Chugai Pharmaceutical.

Generation and preparation of anti-idiotype antibodies to each variable region of ACE910

I established each hybridoma producing a mouse anti-idiotype monoclonal antibody against FIXa-binding arm Fab (termed anti-FIXa Fab) of ACE910 or a rat anti-idiotype monoclonal antibody against FX-binding arm Fab (termed anti-FX Fab) of ACE910. Briefly, 12 mice and 12 rats were immunized with the F(ab')2 fragment consisting of anti-FIXa Fab, and 12 mice and 12 rats were immunized with the F(ab')2 fragment of anti-FX Fab. The lymph node cells and/or spleen cells were isolated from the immunized animals, and fused with murine myeloma P3U1 or SP2/0 cells to establish hybridoma clones. The supernatants of the

hybridoma clones that should contain an antibody were applied to two kinds of ELISA to detect binding to anti-FIXa Fab or anti-FX Fab. Then, specific binders, a mouse antibody specific to anti-FIXa Fab (AQ8) and a rat antibody specific to anti-FX Fab (AJ540), were selected. The establishment of hybridoma clones was particularly supported by T. Suzuki and the hybridoma preparation team in Chugai Pharmaceutical.

Next, the variable regions of AQ8 and AJ540 were cloned. Then, I assembled the genes of recombinant chimeric AQ8 (rAQ8-mIgG2b) consisting of the variable regions of AQ8 and the constant region of mouse IgG2b (heavy chain: EMBL accession No. J00461, light chain: EMBL accession No. V00807) and recombinant chimeric AJ540 (rAJ540-rbtIgG) consisting of the variable regions of AJ540 and the constant region of rabbit IgG (heavy chain: EMBL accession No. L29172, light chain: EMBL accession No. X00231), and constructed the expression vectors for them. The vectors were transfected into HEK293 cells to express rAQ8-IgG2b and rAJ540-rbtIgG. After culturing the transfectants, rAQ8-IgG2b and rAJ540-rbtIgG were purified with recombinant Protein A and gel permeation chromatography. Preparation of the anti-idiotype antibodies was particularly supported by T. Kuramochi and the antibody engineering team in Chugai Pharmaceutical.

Measurement of plasma ACE910 concentration

In the *in vivo* hemostatic study of the Chapter 2, the plasma ACE910 concentration was determined with a sandwich ELISA to quantify human IgG as described in the Chapter 2 methods. I used an anti-human IgG gamma-chain F(ab')2 fragment antibody produced in goat (Sigma Aldrich, St. Louis, MO, USA) as the solid phase antibody for capturing ACE910 and an anti-human IgG4 monoclonal antibody-alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL, USA) as the detection antibody.

In the PK study of the Chapter 3, the plasma ACE910 concentration was measured with the bridging ELISA employing rAJ540-rbtIgG as solid phase antibody for capturing ACE910 and rAQ8-mIgG2b as detection antibody. Alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used as secondary detection antibody.

I confirmed that both ELISA methods indicated a consistent plasma concentration of ACE910. The bridging ELISA using the anti-idiotype monoclonal antibodies can be used to measure the concentration of ACE910 specifically in human plasma.

Detection of anti-ACE910 alloantibodies using electrochemiluminescent bridging immunoassay

Anti-ACE910 alloantibodies in cynomolgus monkey plasma were detected by an electrochemiluminescent bridging immunoassay. Anti-ACE910 alloantibodies in plasma samples or serially-diluted plasma samples were incubated with biotin-labeled ACE910 and ruthenium-labeled ACE910, and then the immune complexes, which form in proportion to the concentration of anti-ACE910 alloantibodies, were captured on a streptavidin-immobilized plate and detected with an electrochemiluminescence detector (Meso Scale Discovery, Rockville, MD, USA). A confirmation test was performed to check whether each screened anti-ACE910 alloantibodies-positive sample had a specific response to ACE910 by ascertaining whether the addition of a fixed amount of ACE910 to the above test system inhibited the binding of anti-ACE910 alloantibodies to the two types of ACE910-labelled molecules. Detection of anti-ACE910 alloantibodies was particularly supported by Y. Sakamoto and the PK analysis team in Chugai Pharmaceutical.

Statistical analysis, pharmacokinetic analysis and multiple-dosing simulation

In the PK study, the plasma ACE910 concentration data were analyzed by non-compartmental analysis with Phoenix WinNonlin software version 6.2 (Pharsight, St. Louis, MO, USA). Multiple dosing simulations were performed with SAAM II version 1.2 software (SAAM Institute, Seattle, WA, USA). Data are presented as mean ± SD. The PK analysis and simulation were particularly supported by K. Haraya in Chugai Pharmaceutical.

3-2. Results

Pharmacokinetic study and multiple dosing simulation

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

The plasma half-life of ACE910 was 19.4 days after a single IV administration at 6 mg/kg, and in the range of 23.6 to 26.5 days after a single SC administration at 0.06, 0.6, or 6 mg/kg (Table 1). As for the SC administration, the maximum plasma concentration (Cmax) of ACE910 increased in approximate proportion to the dose increment. The SC bioavailability was 102.3% at the 6 mg/kg dose. These results were consistent with those of our previous study [38]. For these analyses, I excluded two animals in which anti-ACE910 alloantibodies were detected respectively from 28 days after the IV administration of 6 mg/kg and from 56 days after the SC administration of 0.06 mg/kg. Their plasma ACE910 concentration decreased in association with detection of anti-ACE910 alloantibodies.

In the *in vivo* hemostatic study, the mean initial plasma concentrations of ACE910 were 26 and 61 μg/mL (180 and 420 nM) in the 1 mg/kg and 3 mg/kg groups, respectively. The hemostatic effect in these groups was comparable to that in the rpoFVIII 10 U/kg group, in which the FVIII level would lie within the range of a mild phenotype (Figure 9). I considered that if, by routine supplementation, a plasma ACE910 level of 26 μg/mL or above were maintained at all times in patients, a severe phenotype would be possibly converted to a mild phenotype beyond a moderate phenotype. To examine this possibility, multiple dosing simulations of ACE910 were performed using the parameters obtained from the PK study. The result of the simulations indicated that, if the target trough plasma level of ACE910 were set to 26 μg/mL or 61 μg/mL, it could be maintained by once-weekly SC administrations of 0.64 mg/kg or 1.5 mg/kg at a steady state, respectively (Figure 10).

Chapter 4

In vivo preventive effects of ACE910 prophylactic treatment on spontaneous bleeding including joint bleeds in a long-term non-human primate model of hemophilia A

4-1. Materials and Methods

Anti-primate FVIII neutralizing antibodies

A mouse monoclonal anti-primate FVIII neutralizing antibody, termed VIII-2236, was prepared from hybridoma culture supernatants as previously presented [22, 27]. A chimeric mouse–monkey anti-primate FVIII neutralizing antibody, termed cyVIII-2236, was constructed comprising the mouse variable region from VIII-2236 and the monkey constant region of IgG, which I originally cloned from cynomolgus monkey thymus. The cyVIII-2236 antibody was produced in HEK293 cells and isolated by protein A and gel permeation chromatography from the culture supernatants. Preparation of cyVIII-2236 was particularly supported by T. Kuramochi and A. Sakamoto in Chugai Pharmaceutical.

Comparison of cyVIII-2236 with VIII-2236 in APTT assay

Firstly, to compare the FVIII-neutralizing activity between cyVIII-2236 and VIII-2236, each was added to citrated plasma pooled from 3 normal male cynomolgus monkeys. Then, APTT was measured with a standard method using Thrombocheck APTT-SLA (Sysmex) and coagulation analyzer KC4 Delta (Stago). Secondly, to compare the effect of cyVIII-2236 and VIII-2236 on the APTT-shortening activity of ACE910, each was added to FVIII-deficient human plasma (George King) at the final plasma concentration of 300 μg/mL together with various concentrations of ACE910, then APTT was measured.

Animals and ethics

Ten male cynomolgus monkeys (2.35–4.25 kg, aged 3–4 years) were purchased from Hamri. 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 (AAALAC) International.

Long-term primate model of acquired hemophilia A

In the first part of this Chapter 4 study, 2 cynomolgus monkeys received weekly IV injections (3 or 10 mg/kg each) of VIII-2236. Citrated blood was collected over time. Blood Hgb concentration was measured by hematology analyzer SF-3000 (Sysmex) to monitor

hemorrhagic anemia. APTT was assessed with a standard method using Data-Fi APTT (Siemens) and coagulation analyzer AMAX CS-190 (Trinity). In addition, bleeding symptoms (bruises [dark-red areas on the body surface], hematuria, limping, and general condition including joint swelling) were monitored on 21 working days from Day 0 until Day 28. To observe bruises easily, the body hair was sheared on Day 0, and the area of bruises and the number of days when bruises had been detected were assessed.

In the second part of this Chapter 4 study, another 8 cynomolgus monkeys received weekly IV injections (10 mg/kg) of cyVIII-2236. Two groups (n = 4 each) were established: a control group (the vehicle group), treated with vehicle, and a test group (the ACE910 group), treated with ACE910. ACE910 was administered as an initial 3.97 mg/kg SC dose 2 h after cyVIII-2236 injection on Day 0, and thereafter as weekly 1 mg/kg SC doses. The same dosing regimen of vehicle (histidine buffer containing a surfactant) was applied to the vehicle group. Citrated blood was collected over time. Blood Hgb concentration was measured; the change in blood Hgb level was expressed as a percentage of that on Day 0 (2 h after cyVIII-2236 injection). APTT was measured with Thrombocheck APTT-SLA and KC4 Delta. Bleeding symptoms were monitored on 41 working days until Day 56. Necropsy was performed on Day 56; organs and tissues from the whole body were macroscopically examined, and hemorrhagic findings in joints (shoulder, elbow, wrist, hip, knee, and ankle on both sides) were scored. Then, they were histopathologically examined. The histopathological evaluation was particularly supported by K. Adachi in Chugai Pharmaceutical.

I assessed cyVIII-2236 concentration, FVIII-neutralizing titer of cyVIII-2236, ACE910 concentration, and development of anti-ACE910 alloantibodies. Briefly, cyVIII-2236 concentration was determined with a sandwich ELISA using recombinant human FVIII and an anti-human IgG antibody. FVIII-neutralizing titers of cyVIII-2236 on Days 0 (just after cyVIII-2236 injection) and 56 were assessed with a modified Bethesda assay [28]. ACE910 concentration was determined with a sandwich ELISA to quantify human IgG [27]. Anti-ACE910 alloantibodies present at necropsy were examined with an electrochemiluminescent bridging immunoassay using labeled ACE910 [27]. Detection of anti-ACE910 alloantibodies was particularly supported by Y. Sakamoto and the PK analysis team in Chugai Pharmaceutical.

In both parts, the monkeys were carefully monitored under the supervision of the attending veterinarian to determine the necessity of pain relief or other treatment. Consequently, no monkeys received an analgesic drug.

Plasma cyVIII-2236 concentration

In the *in vivo* efficacy study, the plasma cyVIII-2236 concentration was determined with a sandwich ELISA using recombinant human FVIII (rhFVIII) (Bayer HealthCare) as the solid phase and a goat anti-human IgG1 (gamma-chain specific) antibody biotin conjugate (Southern Biotechnology Associates) and Streptavidin-PolyHRP80 (Stereospecific Detection Technologies) as the detection system.

Determination of the *in vitro* correlation between plasma cyVIII-2236 concentration and one-stage clotting assay-based FVIII:C

In an *in vitro* APTT-based one-stage clotting assay, FVIII activity (FVIII:C) in the presence of cyVIII-2236 was assessed. Samples of cyVIII-2236 at various concentrations were mixed with citrated plasma pooled from 3 normal male cynomolgus monkeys. After the mixture was incubated at 37°C for 2 h, 50 µL of the cyVIII-2236-containg plasma diluted tenfold with imidazole dilution buffer (or the original pooled plasma serially diluted as standard) was added to 50 µL of human FVIII-deficient plasma (George King), followed by addition of 50 µL of an APTT reagent (Thrombocheck APTT-SLA, Sysmex). The FVIII:C was determined on the basis of the standard curve.

FVIII-neutralizing titer of cyVIII-2236

In the *in vivo* efficacy study, the FVIII-neutralizing titers of cyVIII-2236 on Day 0 (just after cyVIII-2236 injection) and Day 56 were assessed with a modified Bethesda assay. The diluted plasma sample or imidazole dilution buffer (as a control sample) was mixed with an equal amount of normal human plasma (Siemens) to which an anti-ACE910 FIXa-arm Fab antibody (termed AQ8) and an anti-ACE910 FX-arm Fab antibody (termed AJ540) were added (each at 300 µg/mL concentration) to completely inhibit any ACE910 activity in the sample. After the mixture was incubated at 37°C for 2 h, 50 µL of the plasma samples diluted appropriately (or serial dilutions of normal human plasma as standard) was added to 50 µL of human FVIII-deficient plasma (Sysmex), followed by addition of 50 µL of an APTT reagent (Thrombocheck APTT-SLA, Sysmex). The FVIII:C of each sample was determined, and Bethesda unit (BU) was calculated [28].

Plasma ACE910 concentration

In the *in vivo* efficacy study, the plasma ACE910 concentration was determined with a sandwich ELISA to quantify human IgG using a goat anti-human IgG gamma-chain F(ab')2 fragment antibody (Sigma Aldrich) and an anti-human IgG4 monoclonal antibody‐alkaline phosphatase conjugate (Southern Biotechnology Associates) [27].

Anti-ACE910 alloantibodies in plasma

In the *in vivo* efficacy study, anti-ACE910 alloantibodies in plasma collected at necropsy were examined by an electrochemiluminescent bridging immunoassay [27]. Plasma was incubated with biotin-labeled ACE910 and ruthenium-labeled ACE910, then the immune complexes, which form in proportion to the concentration of anti-ACE910 alloantibodies, were captured on a streptavidin-immobilized plate and detected with an electrochemiluminescence detector (Meso Scale Discovery). Detection of anti-ACE910 alloantibodies was particularly supported by Y. Sakamoto and the PK analysis team in Chugai Pharmaceutical.

Simulation of plasma rpoFVIII concentration

Based on the PK parameters of rpoFVIII obtained from the previous cynomolgus monkey PK study [27], the plasma rpoFVIII concentration in the *in vivo* efficacy study with a twice-weekly 20 U/kg IV dosing regimen was simulated using SAAM II version 1.2 software (SAAM Institute). It has been confirmed that the half-life of rpoFVIII in cynomolgus monkeys is about 14 h, which is almost the same as the half-life of rhFVIII in humans, and that rpoFVIII activity in cynomolgus monkeys and rhFVIII activity in humans are nearly identical at the same dosage [27]. The PK analysis and simulation were particularly supported by K. Haraya in Chugai Pharmaceutical.

Effects of rpoFVIII on spontaneous bleeds in a long-term primate model of acquired hemophilia A

In an attempt to further validate the long-term hemophilia A model by looking at reactivity to a FVIII agent, the effects of rpoFVIII were examined.

The B-domain–deleted form of rpoFVIII was expressed in CHO cells by stable transfection [22, 27]. The transfection vector was designed on the basis of a reported sequence of the porcine F8 (Sus scrofa) gene (GenBank: NM_214167) and the B-domain–deleted human F8 gene [15, 44]. rpoFVIII was purified by ion-exchange and gel permeation chromatography from the

culture supernatants. Activity units (U) of purified rpoFVIII were determined in an APTT-based one-stage clotting assay, and 1 U/dL rpoFVIII was defined as the equivalent of 1 U/dL human FVIII in normal human plasma (Siemens). The specific activity of rpoFVIII was 3610 U/g which was comparable to previous reported values of rpoFVIII and rhFVIII [5, 10, 32]. It was confirmed that the anti-primate FVIII neutralizing antibody cyVIII-2236 has neutralizing activity against rhFVIII (Figure 12B), but not against rpoFVIII.

Four male cynomolgus monkeys (2.50–3.15 kg, aged 3–4 years) were purchased from Hamri. As a supplement to the second part of the study, these 4 animals received a weekly IV injection (10 mg/kg) of cyVIII-2236 on Days 0, 7, 14, 21, 28, 35, 42, and 49. rpoFVIII was administered at a 20 U/kg IV dose 2 h after cyVIII-2236 injection on Day 0 and subsequently twice-weekly on Days 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, 42, 46, 49, and 53. This dosing regimen was selected to maintain ≥1% rpoFVIII:C for 6 days a week. For an 8-week observation period, citrated blood was collected on Days 0, 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, 42, 46, 49, 53, and 56 (before and 2 h after cyVIII-2236 injection on Day 0; immediately after rpoFVIII injection on Days 4, 11, 18, 25, 32, 39, 46, and 53; just before cyVIII-2236 and rpoFVIII injections on Days 7, 14, 21, 28, 35, 42, and 49). Blood Hgb concentration was measured; the change in blood Hgb level was expressed as a percentage of that on Day 0 (2 h after cyVIII-2236 injection). APTT was measured in plasma from the blood collected. In addition, limping was monitored on 41 working days until Day 56. Necropsy was performed on Day 56; hemorrhagic findings in joints (shoulder, elbow, wrist, hip, knee, and ankle on both sides) were scored.

Statistical analysis and multiple-dosing simulation

In the *in vivo* efficacy study, some were analyzed by 2-tailed Student's *t* test (SAS Preclinical Package, version 5.00) between the vehicle and the ACE910 groups. A *P* value of 0.05 or less was considered statistically significant. Multiple doses of ACE910 were simulated with SAAM II version 1.2 software (SAAM Institute) on the basis of the PK parameters determined in the Chapter 3 study. The data are presented as individual values or as mean ± SD. The PK analysis and simulation were particularly supported by K. Haraya in Chugai Pharmaceutical.

4-2. Results

Failure of long-term neutralization of endogenous FVIII by VIII-2236 in cynomolgus monkeys

First, I attempted to establish a long-term acquired hemophilia A model by weekly IV injection of VIII-2236 in cynomolgus monkeys. APTT prolongation was observed for the first 2 weeks (Figure 11), but gradually shortened after the third injection, and finally disappeared by the end of the fourth week, suggesting the development of alloantibodies to VIII-2236. VIII-2236 is a mouse IgG, and therefore entirely foreign to primates. In addition, no sign of joint bleeds was observed, although some bruises and a transient decrease in blood Hgb were detected. Consequently, I sought an alternative way of establishing a longer-term primate model of acquired hemophilia A.

Chimerization of VIII-2236 to cyVIII-2236

As an attempt to avoid anti-VIII-2236 alloantibody development, I chimerized VIII-2236 (mouse IgG) by replacing its constant region with that of cynomolgus monkey IgG. The chimerized VIII-2236 (cyVIII-2236) successfully prolonged APTT in cynomolgus monkey plasma *in vitro* with a similar concentration dependency to that of original VIII-2236 (Figure 12A). Additionally, cyVIII-2236 did not interfere with the APTT-shortening activity of ACE910, the same results were also found with VIII-2236 (Figure 12B). These results indicate that VIII-2236 was successfully chimerized to cyVIII-2236 while retaining the original characteristics.

Establishment of a long-term acquired hemophilia A model expressing spontaneous joint bleeds in cynomolgus monkeys

Using cyVIII-2236, I retried to establish long-term neutralization of endogenous FVIII, expecting reproducible development of spontaneous joint bleeds. The experimental protocol is illustrated in Figure 13A. In all the monkeys given vehicle (n = 4), APTT was prolonged to approximately twice the normal baseline for 8 weeks (Figure 13B). Plasma cyVIII-2236 concentration fluctuated, but remained over 58.6 μg/mL (nearly 400 nM) (Figure 14). As over 400 nM cyVIII-2236 prolonged APTT by 2-fold *in vitro* (Figure 12A), these observation seemed consistent. Thus, an acquired hemophilia A state was successfully maintained by using cyVIII-2236.

In this long-lasting acquired hemophilia A state, all the control monkeys given vehicle developed abnormal leg motion known as limping: a behavior to avoid using the affected leg (Figure 13C). The limping continued until the experiments ended, with days when limping was detected reaching 25.0 ± 8.3 days (61% ± 20%) of the 41 observation days. Two control monkeys (#3 and #4) developed joint swelling at the ankle in the limping limb from Days 28 to 42 and from Days 18 to 42, respectively. Macroscopic observation at necropsy on Day 56 revealed intra-articular dark red areas (consistent with hemorrhagic findings in the histopathological examination) in joints (elbow, hip, or ankle) of all the control monkeys; the number of bleeding joints was 2.0 ± 0.8 per head (Figure 13D and Table 2). Bleeding was detected in the hip or ankle joint of the side that limped. Representative macroscopic findings of the hip joints are shown in Figures 13E (i) and (ii). Hemorrhagic findings or reactive findings associated with hemorrhage were histopathologically confirmed in the joints that limped: hemorrhage/hemosiderin deposition, mononuclear cell infiltration, and vascular proliferation in the synovial membranes; synovial hyperplasia; granulation tissue; and destruction of articular cartilage or underlying bone (Figure 15A, B, and C). Thus, a long-term acquired hemophilia A model expressing spontaneous joint bleeds was successfully established in cynomolgus monkeys.

Other spontaneous bleeding symptoms, i.e. bruises and hematuria, were detected by daily observation in the vehicle group. By macroscopic observation at necropsy, hemorrhagic findings were also observed in joint, skeletal muscle (lower abdomen and femoral region), urinary bladder, seminal vesicle, rectum, and subcutis (back) in 1, 2, or 4 of the 4 vehicle-control monkeys (Table 2). Additionally, blood Hgb temporarily decreased during the observation period with the lowest values reaching 64% to 87% (Figure 16), suggesting substantial hemorrhagic blood loss.

Bleeding preventive effect of ACE910 in the long-term acquired hemophilia A model

The preventive effect of a weekly SC dose of ACE910 on spontaneous joint bleeds was evaluated in the model established above. The dosing regimen was simulated to maintain the plasma concentration trough at >30 μg/mL from the first week after administration using the PK parameters of ACE910 in cynomolgus monkeys previously obtained, because around 26 μg/mL of plasma ACE910 would show hemostatic potency comparable to that of 10 U/kg (twice-daily) rpoFVIII in a short-term primate model of acquired hemophilia A [27].

In all the monkeys treated with ACE910 (n = 4), APTT was initially prolonged to approximately twice the normal baseline after the first IV injection of cyVIII-2236, as in the group treated with vehicle. Then, repeated ACE910 administration shortened the prolonged APTT to the baseline level over the entire dosing period (Figure 13B), although careful observation indicates that the APTT-shortening effect was slightly reversed in one monkey (ACE910 #4) on Days 25 and 28. It should be noted that the APTT of FVIII-neutralized cynomolgus monkey plasma was normalized even at around 30 μg/mL (nearly 200 nM) of ACE910, which also indicated rpoFVIII relative activity of <10% on the basis of peak height in the TG assay (Figure 4B). This is assumedly because ACE910 does not require activation process to exert its cofactor activity, whereas FVIII needs additional time to be activated by thrombin or FXa in the APTT assay. Therefore, the effect of ACE910 on APTT should be carefully interpreted in relation to the hemostatic efficacy. Plasma cyVIII-2236 in the ACE910 group remained over 96.1 μg/mL, which exceeded the minimal cyVIII-2236 level (58.6 μg/mL) in the vehicle group (Figure 14). Further, in all monkeys other than the ACE910 #4 monkey, the FVIII-neutralizing titer measured by the modified Bethesda assay was maintained on Day 56. These results suggest that endogenous FVIII was also successfully neutralized in the ACE910 group.

The ACE910 #4 monkey showed a rapid and significant decrease in plasma ACE910 concentration from Day 21 to Day 28, on which it was only 2.8 μg/mL (Figure 17), and anti-ACE910 alloantibodies were detected in the plasma. Furthermore, this monkey accidentally experienced a fracture of the mandibular cuspid on Day 28, although no other monkeys experienced such a rare accident. Owing to the consequent massive bleeding in the oral cavity, the blood Hgb concentration decreased to 2.2 g/dL (19% of Day 0 values, Figure 16). Therefore, in consideration of animal ethics and the impossibility of maintaining an effective ACE910 level, this monkey was euthanized humanely on Day 28 and macroscopic observation of its organs and tissues was carried out at necropsy.

In the other 3 monkeys, plasma ACE910 level remained around the target concentration (30 μg/mL) from Day 4 to Day 56 (Figure 17), demonstrating that the multiple-dosing simulation worked very well. Anti-ACE910 alloantibodies were examined in plasma collected at necropsy (Day 28 for the #4 monkey; Day 56 for the other 3 monkeys). In addition to the #4 monkey, one other monkey (ACE910 #3) had anti-ACE910 alloantibodies. In this monkey, however, the plasma ACE910 level slightly decreased only after Day 49, and remained around 26 μg/mL, a level which is expected to show hemostatic activity [27].

Regarding the joint bleeding symptoms, no limping and no macroscopic bleeding joints at necropsy were observed in any of the ACE910-treated monkeys, including monkey #4. In the ACE910 group (n = 3; excluding #4 statistically), the number of limping days (Figure 13C) and the number of bleeding joints at necropsy (Figure 13D and Table 2) significantly decreased ($P < 0.01$, 2-tailed Student's *t* test), compared to those of the vehicle group (n = 4). In the ACE910 group, synovial hyperplasia and vascular proliferation in the synovial membranes of joints were histopathologically noted (Figure 15D, E, and F). However, these findings were less severe and less frequent in the ACE910 group than those in the joints with neither macroscopic bleeding nor limping in the vehicle group.

Regarding the other spontaneous bleeding symptoms, bruises (the maximum value of areas and the number of days detected during the observation period) were ameliorated, and no hematuria and no organ bleeds at necropsy were found except for the oral cavity bleeds of the ACE910 #4 monkey (Table 2). Further, monkeys #1–3 in the ACE910 group exhibited blood Hgb of 90% or above for 8 weeks (Figure 16). The minimum value of blood Hgb during the observation period was 94.9% ± 4.9% in the ACE910 group (n = 3, excluding monkey #4), which was significantly higher than 75.2% ± 11.3% in the vehicle group (n = 4) ($P < 0.05$, 2-tailed Student's *t* test).

General Discussion

Chapter 1: *In vitro* FVIII-mimetic activity of ACE910 in plasma coagulation assays

To address the remaining issues (FVIII inhibitor, frequent IV injection with FVIII and bypassing agents etc) in current treatment of hemophilia A, we created a humanized anti-FIXa/FX bispecific IgG antibody, ACE910, which is expected to mimic the FVIII cofactor function as a kind of scaffold by binding and placing FIXa and FX into spatially appropriate positions [22]. ACE910 is a clinical investigation candidate which was highly optimized using a multidimensional approach to improve the FVIII-mimetic cofactor activity, PK properties, immunogenicity, physicochemical stability, and industrial manufacturability for clinical application [38].

However, it was necessary to clarify whether the biological functions of ACE910 can actually achieve the target profiles which are expected by its basic characteristics as a FVIII-function mimetic IgG molecule. Therefore, I conducted various *in vitro* and *in vivo* assays to fully characterize the biological functions of ACE910 which has a completely different protein structure from native FVIII. In the enzymatic assay with purified FIXa and FX, ACE910 greatly enhanced FX activation by FIXa, whereas could not promote FX activation without FIXa, indicating ACE910's binding to both FIXa and FX is essential for its function and ACE910 is a cofactor like FVIII which has no direct enzymatic activity (Figure 3).

Furthermore, in the Chapter 1 study, I demonstrated ACE910 promotes plasma coagulation, e.g. shortening APTT and enhancing TG, in human FVIII-deficient plasma with a concentration-dependency similar to that in FVIII-neutralized cynomolgus monkey plasma (Figure 4). ACE910 also could promote plasma coagulation in human FVIII-deficient plasma in the presence of FVIII inhibitors, whereas the effects of rhFVIII on TG were not observed in the presence of FVIII inhibitors (Figure 4A, 4C). These results suggest that ACE910 has the potential to be effective in patients with FVIII inhibitors because an antibody has a completely different protein sequences and structure from those of FVIII, therefore its immunogenicity is totally different.

In comparison with TG potential between ACE910 and rhFVIII/rpoFVIII, I hypothesized that around 300 nM (44 μg/mL) of plasma ACE910 would exert an *in vivo* hemostatic potency equivalent to 10 U/dL (10%) of FVIII, since 300 nM of ACE910 exhibited *in vitro* cofactor activity similar to that of 10 U/dL of FVIII in terms of the peak height in TG assays (Figure 4C, 4D). When making this hypothesis, I did not use the APTT data. ACE910 strongly shortened

APTT even beyond the level achieved by 100 U/dL of FVIII at more than 300 nM (Figure 4A, 4B), but I considered that this phenomenon should 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, I next examined the *in vivo* hemostatic activity of ACE910 on injury-induced on-going bleeds in a short-term non-human primate model of hemophilia A in the Chapter 2 study.

Chapter 2: *In vivo* hemostatic potency of ACE910 episodic treatment in a short-term non-human primate model of hemophilia A

In the Chapter 2 study, I elucidated the *in vivo* hemostatic potency of ACE910, including that against on-going bleeds compared to rpoFVIII, using a short-term non-human primate model of hemophilia A. An acquired hemophilia A status was established by injecting anti-primate FVIII antibody, VIII-2236, which neutralizes endogenous primate's FVIII but neither exogenous rpoFVIII nor ACE910 at all (Figure 7). Therefore, rpoFVIII can work as a positive reference in this model.

It had remained unproven whether the bispecific antibody approach possessed the potency to ameliorate on-going bleeds, which would require higher levels of FVIII, or how much hemostatic potency it had in comparison with FVIII. In the clinical setting, the treatment against on-going bleeds minimally requires 10 to 20 U/dL of the plasma FVIII level, which is much higher than the level required for prophylactic bleeding prevention (1 U/dL). However, the degree of *in vivo* hemostatic potency of ACE910 remained unproven.

As a result, IV administration of 10 U/kg (twice daily) of rpoFVIII showed a significant hemostatic effect, whereas the hemostatic effect of 3.4 U/kg (twice daily) of rpoFVIII was not clearly detected in this model. The multiple dosing simulations of rpoFVIII in cynomolgus monkeys indicated that, by twice-daily doses of 3.4 or 10 U/kg, the plasma rpoFVIII level would be, respectively, 8.5 or 25 U/dL at the outset, would remain at more than 2.5 or 7.4 U/dL, and would reach a maximum of 16 or 46 U/dL by the end of the observation period (Figure 9). Therefore, I judged that this established model was well validated in terms of the reactivity to FVIII. Using this validated model, I elucidated the *in vivo* hemostatic potency of ACE910. A single IV administration of ACE910 at 1 or 3 mg/kg ameliorated bleeding symptoms to an extent equivalent to that achieved by twice-daily doses of 10 U/kg of rpoFVIII. Among the results, it seems contradictory that the mean bruised area of the ACE910 1 mg/kg group was smaller than the 3 mg/kg group. From the viewpoint of ethics for primates, I employed the

minimum number of animals possible to detect a hemostatic effect. Therefore, I think that this variance of dose dependency occurred incidentally, because the deviation in bruised area was rather large.

The PK profiles of ACE910 and rpoFVIII were different, and therefore it is quite difficult to compare their *in vivo* hemostatic activity 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, would have reached that at the minimum plasma level of rpoFVIII, 7.4 U/dL. If the two agents were compared by their initial plasma levels, 26 or 61 μg/mL of plasma ACE910 would have exhibited a hemostatic activity equivalent to that of 25 U/dL of rpoFVIII. Considering that ACE910 should work equivalently in human and cynomolgus monkey (Figure 4C, 4D) together with the fact that ACE910 fully exerted its activity even in the presence of FVIII inhibitors (Figure 4A, 4C), ACE910 will be possibly an effective and long-acting treatment option to ameliorate on-going bleeds in patients both with and without FVIII inhibitors.

Chapter 3: Pharmacokinetic properties of ACE910 in non-human primates for estimating the possibility of routine prophylactic use

I 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 of FVIII) to a moderate one (1–5 U/dL), but it requires frequent venous access, typically 3 times a week. This negatively affects both the implementation of and adherence to the supplementation routine, particularly for pediatric patients treated at home [1]. In addition, 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 inhibitors, it will be able to resolve the drawbacks inherent to exogenous FVIII and its prophylactic use [21, 23, 33]. Furthermore, although routine prophylaxis with exogenous FVIII effectively reduces joint bleeds and prevents joint damage, its prophylactic effect is not always perfect [25, 30]. In line with it, the clinical outcomes of patients with moderate hemophilia A vary, and the proportion of them that suffers from joint impairment is not negligible [8]. Therefore, keeping FVIII levels within the range of a mild phenotype (> 5 U/dL) may provide patients with substantial benefits in terms of preserving joint status and enabling patients to participate in physical activities [6]. As mentioned in the Chapter 2 study, even if conservatively estimated, 61 μg/mL of plasma ACE910 would be expected to exhibit a hemostatic activity within the range of a mild phenotype.

Generally, PK data of therapeutic antibodies from cynomolgus monkeys can be scaled to project human PK profiles [7], and the simulated plasma concentration-time profiles from the PK parameters are known to be comparable to actual observed profiles for therapeutic antibodies [11]. Therefore, I conducted multiple dosing simulations using the PK study data in cynomolgus monkeys, and found that 61 μg/mL of plasma ACE910 would be maintained at a steady state by once-weekly SC administration of 1.5 mg/kg (Figure 10). The simulation is, of course, not actual data, but I have since confirmed that the simulation of time profile of plasma ACE910 concentration using the above PK parameters well predicted the actual data in another cynomolgus monkey study employing multiple dosing of ACE910 (unpublished data). Therefore, I think that the simulation would work well.

In the PK study, 2 out of 12 animals developed anti-ACE910 alloantibodies. In cynomolgus monkeys, development of anti-humanized antibody alloantibodies is theoretically inevitable, and reports of their incidence rate varies (0% to 100%) [34]. Unfortunately, it has been found that the immunogenicity in cynomolgus monkeys cannot predict that in human, because a humanized antibody is a foreign protein in cynomolgus monkeys [34].

In terms of SC injection, the upper limit of the dosing amount is generally considered to be 1 mL or less than 2 mg/kg of therapeutic antibodies [18], and ACE910 has solubility sufficiently high to realize such a SC dosage with a small injection volume [38]. Thus, I expect that once-weekly SC administration of ACE910 will realize a more aggressive routine prophylaxis aimed at achieving a mild phenotype in hemophilia A patients both with and without FVIII inhibitors.

In conclusion, the Chapter 1, 2, and 3 studies suggested that ACE910 has the potential not only to ameliorate on-going bleeds even in patients with FVIII inhibitors, but also to offer a user-friendly and aggressive routine prophylaxis for patients both with and without FVIII inhibitors [27].

Chapter 4: *In vivo* preventive effects of ACE910 prophylactic treatment on spontaneous bleeding including joint bleeds in a long-term non-human primate model of hemophilia A

Joint damage is one of the most problematic bleeding-related complications in hemophilia A patients. Manco-Johnson et al. demonstrated in a prospective clinical study that the progression of joint damage could be decreased by routine alternate-day doses of FVIII from infancy [25]. Although this dosing regimen could render a severe disease (<1% FVIII:C) into a moderate one (1% – 5%), its preventive effect on joint bleeds was not perfect [6, 25] and an

analysis by den Uijl et al. determined that the threshold FVIII:C level required to be free from joint bleeds would be 12% [9]. The Chapter 2 study in a primate model of acquired hemophilia A predicted that ≥26 μg/mL ACE910 would exhibit hemostatic activity within the range of the mild phenotype (>5%) (Figure 8) and that such a hemostatic level would be maintained by weekly SC doses of 0.64 mg/kg ACE910 (Figure 10). Therefore, in the Chapter 4 study, I investigated weekly SC doses of 1 mg/kg ACE910 and actually demonstrated that such a regimen significantly prevented spontaneous joint bleeding, which also confirmed that the simulation of plasma ACE910 concentrations worked well. Because PK data of therapeutic antibodies from cynomolgus monkeys can be scaled to project human PK profiles [7, 34], a similar PK profile and efficacy are expected for ACE910 in a clinical setting.

In the Chapter 4 study, a long-term acquired hemophilia A model was newly established. The FVIII:C in this model was speculated to keep <4% and <3% in the vehicle and ACE910 groups, respectively, according to plasma cyVIII-2236 level and the cyVIII-2236 concentration vs FVIII:C correlation in the pooled cynomolgus monkey plasma (Figures 14, 18).

In the case of congenital hemophilia A, a small % of FVIII:C is considered to render the severity of bleeding; however, in acquired hemophilia A, measured FVIII:C does not necessarily correlate with the severity [12]. Although cyVIII-2236 would not have necessarily decreased FVIII:C in the one-stage clotting assay to <1% in the monkeys, the control monkeys actually presented severe bleeding symptoms. Thus, I considered that this model was a severe acquired hemophilia A one. In an attempt to further validate this model by looking at reactivity to injected FVIII, I examined the effects of rpoFVIII, which is not neutralized by cyVIII-2236. Aiming to evaluate the marginal level of rpoFVIII efficacy, I administered rpoFVIII for 8 weeks with a twice-weekly 20 U/kg IV dosing regimen, which was set to keep ≥1% rpoFVIII:C for 6 days a week (Figures 19 and 20A) [41].

The prolonged APTT was shortened immediately after the initial rpoFVIII injection (Figure 20B); however, in 3 of 4 monkeys, the APTT-shortening effect gradually disappeared in the middle of the dosing period, suggesting that anti-rpoFVIII alloantibodies had developed. In terms of bleeding symptoms, the number of days with limping was lower in the rpoFVIII group than in the vehicle group (Figure 20C); further, the course of blood Hgb tended to worsen when anti-rpoFVIII alloantibodies presumably emerged (Figure 20D). In the monkey in which rpoFVIII maintained the APTT-shortening effect, limping and decrease in blood Hgb were not observed; nevertheless, 2 bleeding joints were noted by macroscopic observation at necropsy. Although it was difficult to fully evaluate the effect of rpoFVIII because anti-rpoFVIII

alloantibodies developed, these results suggest that increasing FVIII activity could ameliorate the bleeding tendency in this model; however, the twice-weekly 20 U/kg IV dosing regimen was not sufficient to fully prevent joint bleeding.

ACE910 and rpoFVIII are proteins foreign to cynomolgus monkeys; therefore, the development of alloantibodies to these is theoretically inevitable [11]. Incidence rates of anti-humanized antibody alloantibodies in cynomolgus monkeys are reported to vary (0% to 100%), and antigenicity in cynomolgus monkeys cannot predict that in humans [34]. Although the antigenicity risk score of ACE910 in an in silico T-cell epitope prediction system was comparable to that of trastuzumab and palivizumab, which are non-immunogenic in a clinical setting [38], the rate of anti-ACE910 alloantibody development must be evaluated in the actual clinical setting.

In this long-term acquired hemophilia A model, joint damage involving abnormal motion and histopathological features associated with intra-articular hemorrhage were similar to those of hemophilia A patients [36, 37]. While a number of animal models have been reported for hemophilia A, it has been difficult to develop reproducible spontaneous joint bleeds [26, 42]. The FVIII-deficient mouse model of hemophilia A needs an artificial injuring procedure to induce joint bleeding [16, 26]. Although congenital hemophilia A models in dogs, sheep, rats, and pigs, have been reported to develop joint bleeds, it seems difficult to express reproducible joint bleeds in individuals and to efficiently evaluate drug efficacy in a practicable experimental period [4, 16, 20, 24]. Our model stably developed leg-joint damage within 8 weeks, which was possibly produced by the severe acquired hemophilic state and by bodyweight loading due to the bipedal motion of the monkeys. Thus, this model may be particularly useful in testing the efficacy of therapeutic agents from the orthopedic aspect of hemophilia A. In addition, this primate model should be useful for the many therapeutic antibodies that have poor interspecies cross-reactivity. Moreover, along with our model of hemophilia A, the strategy of using a mouse–host animal chimeric antibody may be beneficial for establishing long-term acquired animal models of other protein-deficiency diseases in the hematology research field.

Although limping and macroscopic intra-articular hemorrhage at necropsy were observed in all 4 control monkeys, joint swelling was noted in only 2 monkeys. Therefore, it was not so reproducible and may be inappropriate for quantitative assessment. The joint swelling was linked to limping in the both monkeys. However, in one of the monkeys (vehicle #4), the ankle joint that had swollen did not present macroscopic intra-articular hemorrhage. I assume that the intra-articular hemorrhage may have been absorbed during 2 weeks after the remission of joint

swelling.

In the Chapter 4 study, although ACE910 completely prevented the macroscopic joint impairment, subclinical histopathological changes in the joint synovium were found, despite the presence of ACE910. I assume that the subclinical or small joint bleeds would occur in this model, and ACE910 prevented them from becoming larger and from leading to clinical symptoms, but did not provide a histopathological complete recovery at around 30 μg/mL of plasma ACE910 concentration. An appropriate clinical investigation will be required to elucidate whether such an action of ACE910 can completely prevent macroscopic and clinical joint damage in the long run.

In conclusion of the Chapter 4 study, a long-term acquired hemophilia A model expressing reproducible spontaneous joint bleeds and other bleeds was newly established in cynomolgus monkeys, and weekly SC doses of ACE910 significantly prevented these bleeding symptoms [28]. The difficulty in venous access negatively affects the adoption of and adherence to the routine prophylaxis regimen in home settings [2]. ACE910 is expected to provide hemophilia A patients, regardless of FVIII inhibitors, with a more effective and user-friendly way of bleeding prophylaxis.

General conclusion

We had generated a novel humanized anti-FIXa/FX bispecific IgG antibody, ACE910 [38]. By examining the *in vitro* FVIII-mimetic activities and *in vivo* hemostatic effects on bleeding in the hemophilia A models, I elucidated the biological functions of ACE910 as a FVIII-cofactor mimetic which has a completely different protein structure from native FVIII. In the general conclusions (Figure 21), (i) ACE910 improved coagulation in FVIII-deficient plasma both with and without FVIII inhibitors, (ii) a short-term acquired hemophilia A model expressing stable artificial bleeds with proper severity was newly established in cynomolgus monkeys, and single episodic IV dose of ACE910 significantly inhibited these bleeding symptoms, (iii) ACE910 showed the 3-week plasma half-life and high SC bioavailability in cynomolgus monkeys, and possibility of effective prophylaxis by the once-weekly SC doses was simulated based on the PK parameters, (iv) a long-term acquired hemophilia A model expressing reproducible spontaneous joint bleeds and other bleeds was newly established in cynomolgus monkeys, and weekly SC doses of ACE910 significantly prevented these bleeding symptoms. These results elucidated biological functions of ACE910 as a FVIII-cofactor mimetic and suggested that ACE910 has the potential not only to ameliorate on-going bleeds, but also to offer a

user-friendly and aggressive routine prophylaxis for hemophilia A patients both without and with FVIII inhibitors. Clinical investigation of ACE910 is currently on-going, based on the results in these studies.

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Tables

Table 1 (page 23). Pharmacokinetic parameters of ACE910 in cynomolgus monkeys

(A) Intravenous administration

Dose (mg kg^{-1})		$t_{1/2}$ (day)	$AUC_{0\text{-inf}}$ (μg·day mL^{-1})
6.0	Mean[a)]	19.4	1630
		–	–

(B) Subcutaneous administration

Dose (mg kg^{-1})		$t_{1/2}$ (day)	$AUC_{0\text{-inf}}$ (μg·day mL^{-1})	t_{max} (day)	C_{max} (μg mL^{-1})	Bioavailability (%)
0.060	Mean[a)]	26.5	24.6	3.00	0.602	–
		–	–	–	–	–
0.60	Mean[b)]	24.7	189	5.00	5.10	–
	SD	8.1	45	1.73	0.21	–
6.0	Mean[b)]	23.6	1670	5.33	45.7	102.3
	SD	3.2	140	2.89	5.2	–

a) $n = 2$, b) $n = 3$

The plasma half-life of ACE910 was 19.4 days after a single IV administration at 6 mg/kg, and in the range of 23.6 to 26.5 days after a single SC administration at 0.06, 0.6, or 6 mg/kg in cynomolgus monkeys. As for the SC administration, the maximum plasma concentration (Cmax) of ACE910 increased in approximate proportion to the dose increment. The SC bioavailability was 102.3% at the 6 mg/kg dose.

Table 2 (page 31). Macroscopic hemorrhagic findings at necropsy on Day 56 in a long-term primate model of acquired hemophilia A

Organ/tissue	Site		Number of animals with findings	
			Vehicle (*n* = 4)	ACE910 (*n* = 3)
Joint	Elbow	left	1	0
		right	1	0
	Hip	left	4	0
		right	1	0
	Ankle	left	1	0
Skeletal muscle	Lower abdomen		2	0
	Femur		1	0
Urinary bladder	Wall		2	0
Seminal vesicle	Serosa		1	0
Rectum	Serosa		1	0
Skin	Back	subcutis	1	0

By macroscopic observation at necropsy on Day 56 in the long-term primate model of acquired hemophilia A, hemorrhagic findings were observed in joint, skeletal muscle (lower abdomen and femoral region), urinary bladder, seminal vesicle, rectum, and subcutis (back) in 1, 2 or 4 of the 4 vehicle-control monkeys. No macroscopic organ bleeds at necropsy were found in any of the ACE910-treated monkeys (n = 3; excluding #4 monkey).

Figures

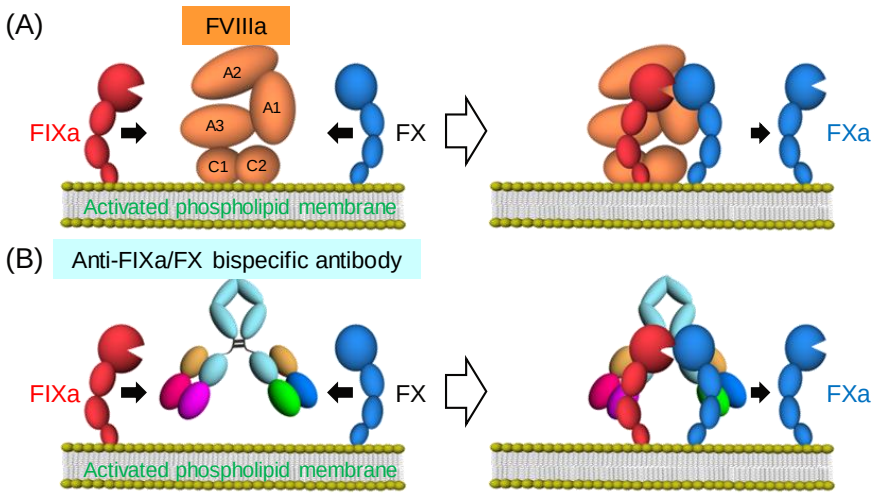

Figure 1 (page 7). Schematic illustration of the FVIII-mimetic cofactor action of an anti-FIXa/FX bispecific antibody. (A) Activated FVIII (FVIIIa) supports the interaction between activated factor IX (FIXa) and factor FX (FX) through its binding to both factors on the activated phospholipid membrane at a coagulation site. (B) An anti-FIXa/FX bispecific antibody binds to FIXa and FX, promoting the interaction between FIXa and FX, enhancing the generation of activated FX (FXa), and exerting FVIII-mimetic cofactor activity.

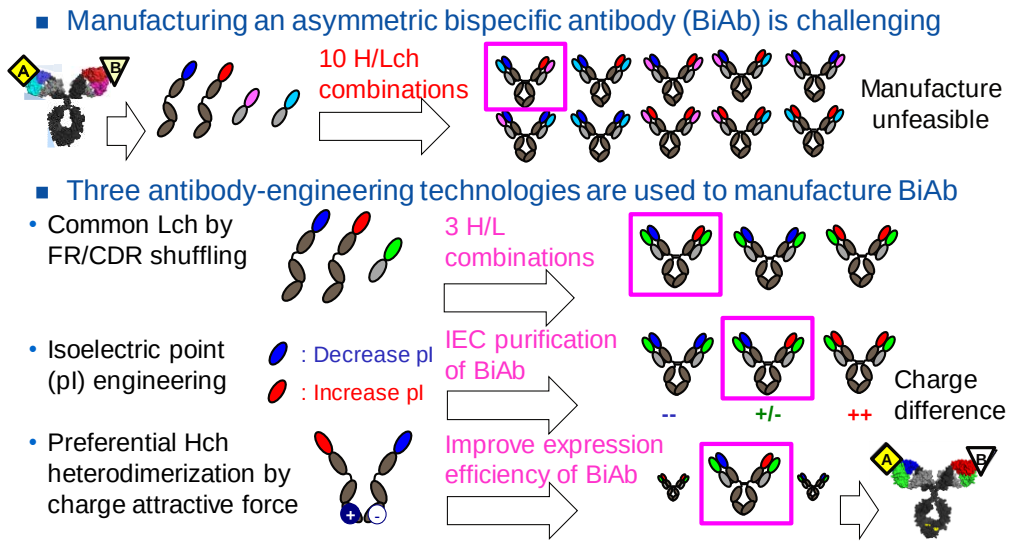

Figure 2 (page 7). Antibody-engineering technologies incorporated into ACE910 molecular design [17]. The difficulties in manufacturing the asymmetric bispecific antibody were overcome by identifying a common light chain for the anti-FIXa and anti-FX heavy chains through framework/complementarity determining region shuffling, by pI engineering of the two heavy chains to facilitate ion exchange chromatographic purification of the bispecific antibody from the mixture of byproducts, and by enhancing preferential hetero-dimerization of the two heavy chains through charge attractive interaction in the CH3 region of each heavy chain.

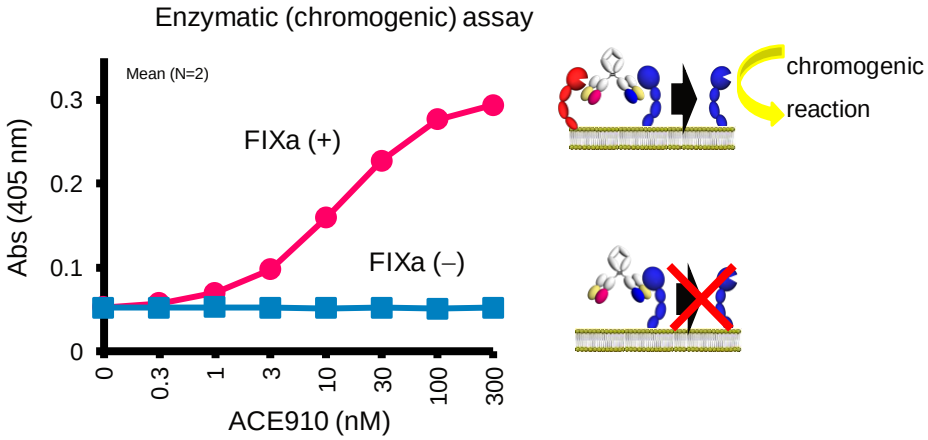

Figure 3 (page 8). ACE910 enhances FX activation by FIXa in the enzymatic assay, indicating FVIII-mimetic cofactor function [40]. ACE910's action depends on the presence of FIXa, suggesting ACE910 is a cofactor but not an active enzyme.

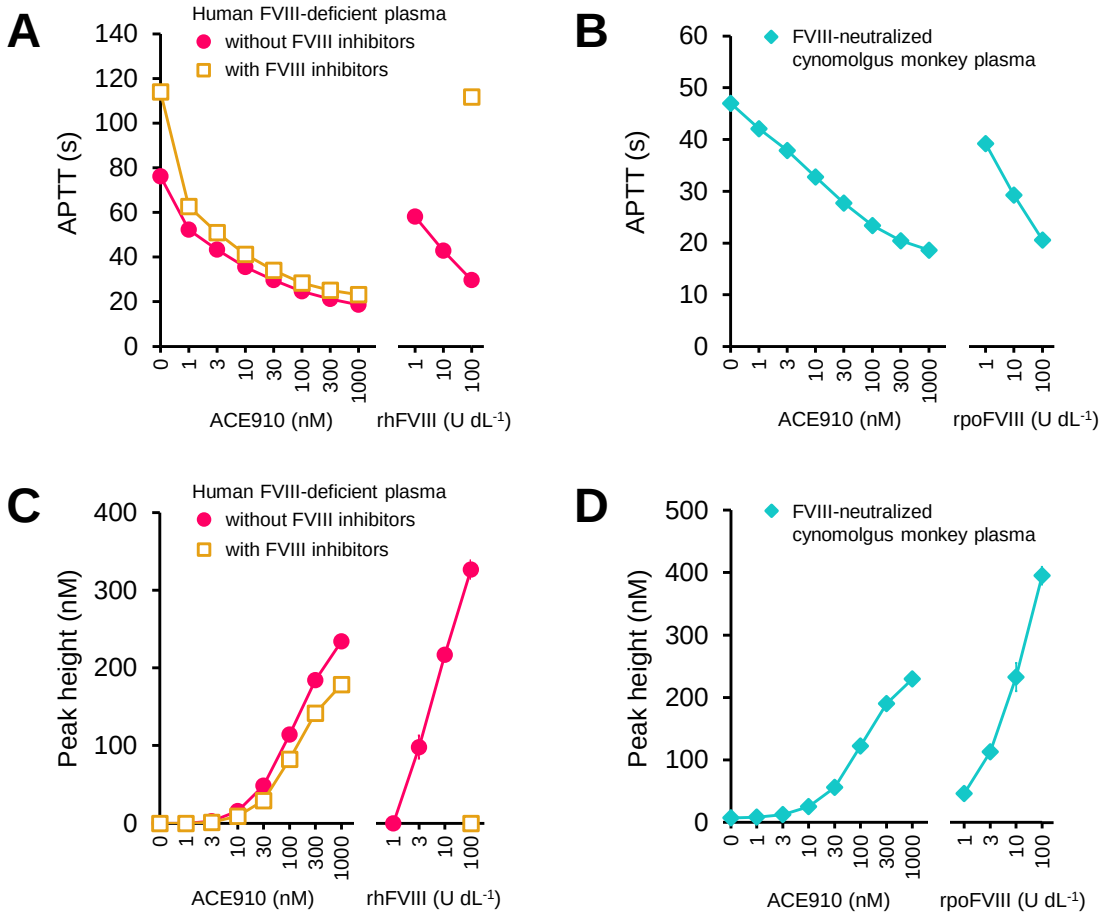

Figure 4 (page 14). FVIII-mimetic cofactor activity of ACE910 in human FVIII-deficient plasma without and with FVIII inhibitors and in FVIII-neutralized cynomolgus monkey plasma. Effects of ACE910, rhFVIII, or rpoFVIII on APTT (A, B) and on peak height of thrombin generation triggering the intrinsic 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 ± SD (n = 3). In the some data plotted on the Figure 4, the bars depicting SD are shorter than the height of the symbols.

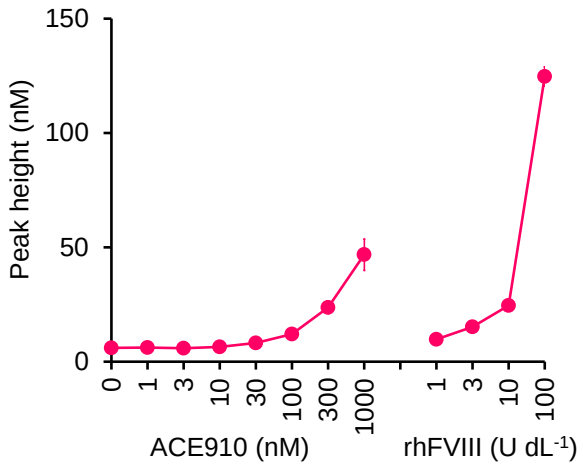

Figure 5 (page 14). Effects of ACE910 or rhFVIII on peak height of thrombin generation triggered by low TF in human FVIII-deficient plasma without FVIII inhibitors. Data are expressed as mean ± SD (n = 3). In the some data plotted on the Figure 5, the bars depicting SD are shorter than the height of the symbols.

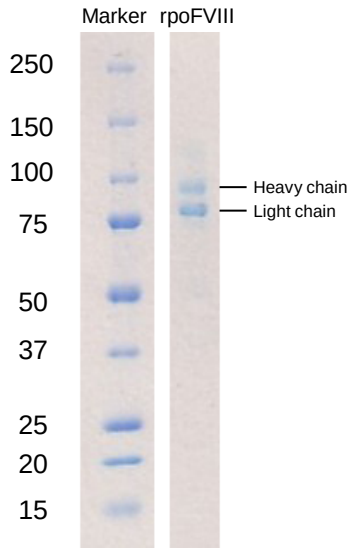

Figure 6 (page 14). SDS-PAGE analysis of rpoFVIII. Purified rpoFVIII (0.95 µg) was analyzed by SDS-PAGE using 4–20% gradient gel under reducing conditions, followed by staining with coomassie brilliant blue. The positions of molecular mass markers in kDa are indicated to the left. SDS-PAGE analysis represented two bands of apparent mass approximately 90 and 80 kDa, which were consistent with the theoretical molecular weight of the heavy chain and light chain of rpoFVIII respectively.

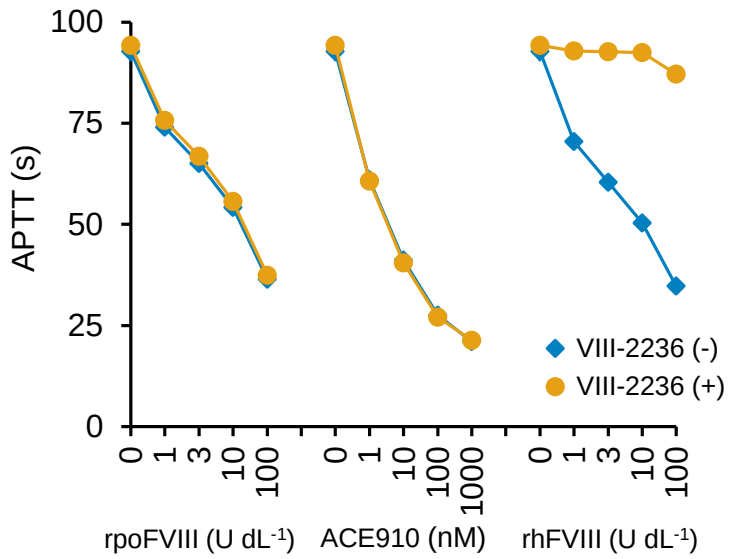

Figure 7 (page 14). Influence of VIII-2236 on the APTT-shortening activity of rpoFVIII, ACE910 or rhFVIII in human FVIII-deficient plasma. In the absence of VIII-2236, the addition of rpoFVIII, ACE910 and rhFVIII concentration-dependently shortened the APTT of human FVIII-deficient plasma. The presence of VIII-2236 (300 μg/mL) canceled the APTT-shortening activity of rhFVIII, but not that of rpoFVIII or ACE910 at all. These results indicate that the anti-primate FVIII neutralizing antibody, VIII-2236, has no neutralizing activity against rpoFVIII and ACE910. Data are expressed as mean ± SD (n = 3). The bars depicting SD are shorter than the height of the symbols.

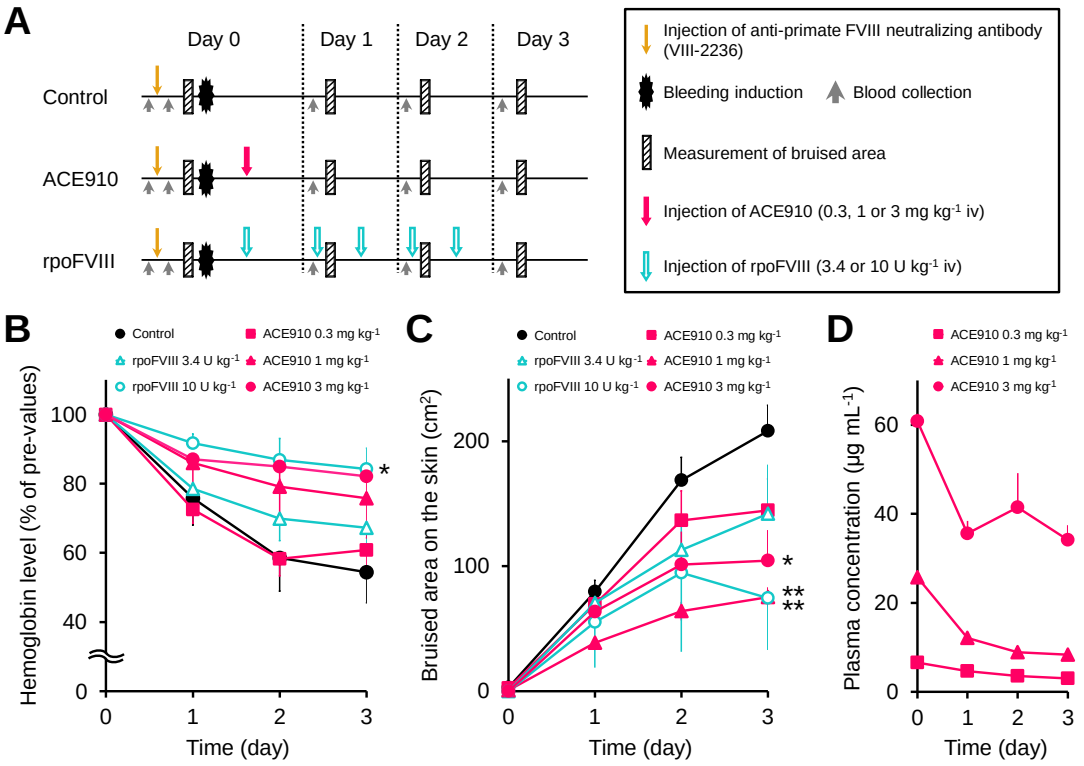

Figure 8 (page 18). ***In vivo*** **hemostatic activity of ACE910 against on-going bleeds in an acquired hemophilia A model.** (A) The experimental protocol used. Time course changes of (B) hemoglobin level and (C) bruised areas in the control group (no test item; n = 6), the ACE910 group (0.3, 1, or 3 mg/kg; n = 4 for each group), and the rpoFVIII group (3.4 or 10 U/kg; n = 4 for each group). Asterisks show statistical significance of the data on Day 3 (**P* < 0.05, ***P* < 0.01 vs. control). (D) Time course of plasma ACE910 concentration in the ACE910 groups. Data are expressed as mean ± SE.

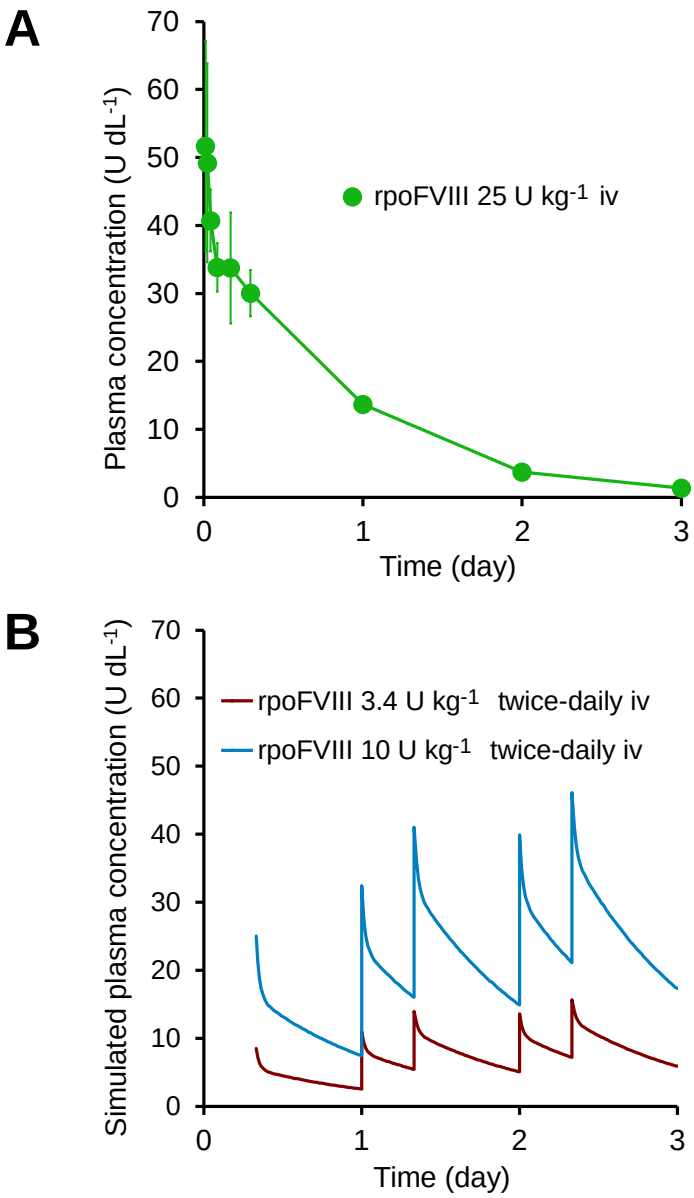

Figure 9 (page 18). Pharmacodynamic study and multiple dosing simulations of rpoFVIII in cynomolgus monkeys. (A) Time course of rpoFVIII activity in plasma after a single intravenous administration of 25 U/kg of rpoFVIII. Data are expressed as mean ± SD (n = 3). (B) Simulated plasma levels of rpoFVIII are shown for the dosing regimens (twice-daily intravenous doses of 3.4 and 10 U/kg) adopted in the *in vivo* hemostatic study. The intervals between the doses were set as follows: 16 h for the 1^{st}–2^{nd} doses and the 3^{rd}–4^{th} doses, and 8 h for the 2^{nd}–3^{rd} doses and the 4^{th}–5^{th} doses.

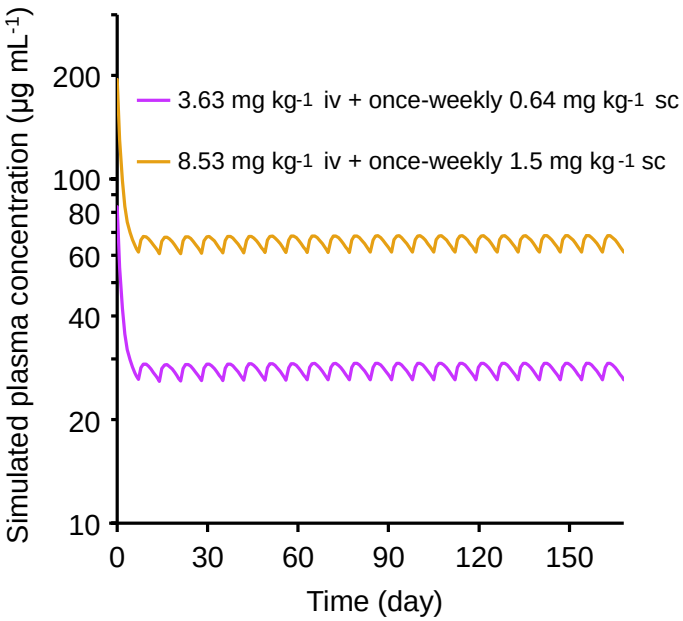

Figure 10 (page 23). Examples of simulations; plasma ACE910 concentration after multiple dosing in cynomolgus monkeys. The time course of plasma ACE910 concentration was simulated using the pharmacokinetic study data in cynomolgus monkeys for the case of once-weekly subcutaneous administration at 0.64 or 1.5 mg/kg starting 7 days after the initial bolus intravenous administration of 3.63 or 8.53 mg/kg, respectively.

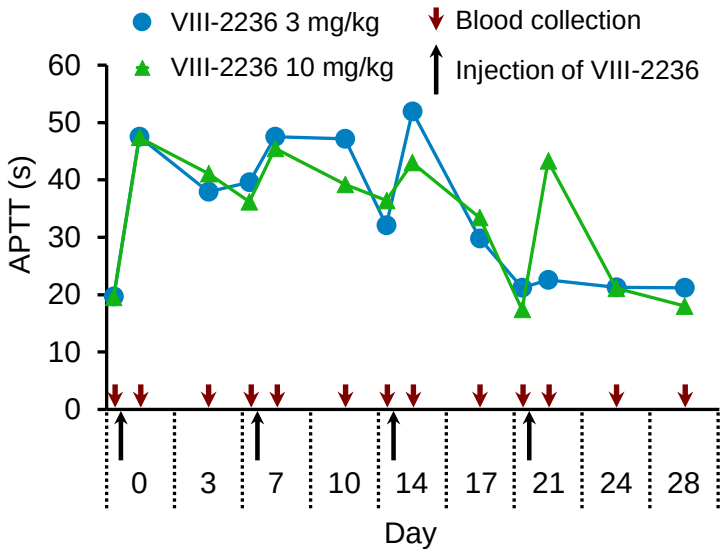

Figure 11 (page 30). Change in APTT after weekly IV injection of the mouse anti-primate FVIII neutralizing antibody VIII-2236 in cynomolgus monkeys. VIII-2236 was injected at 3 or 10 mg/kg IV doses to cynomolgus monkeys on Days 0, 7, 14, and 21. Citrated blood was collected on Days 0, 3, 7, 10, 14, 17, 21, 24, and 28 (before and 2 h after VIII-2236 injection on Days 0, 7, 14, and 21). The time course of APTT is shown for each monkey.

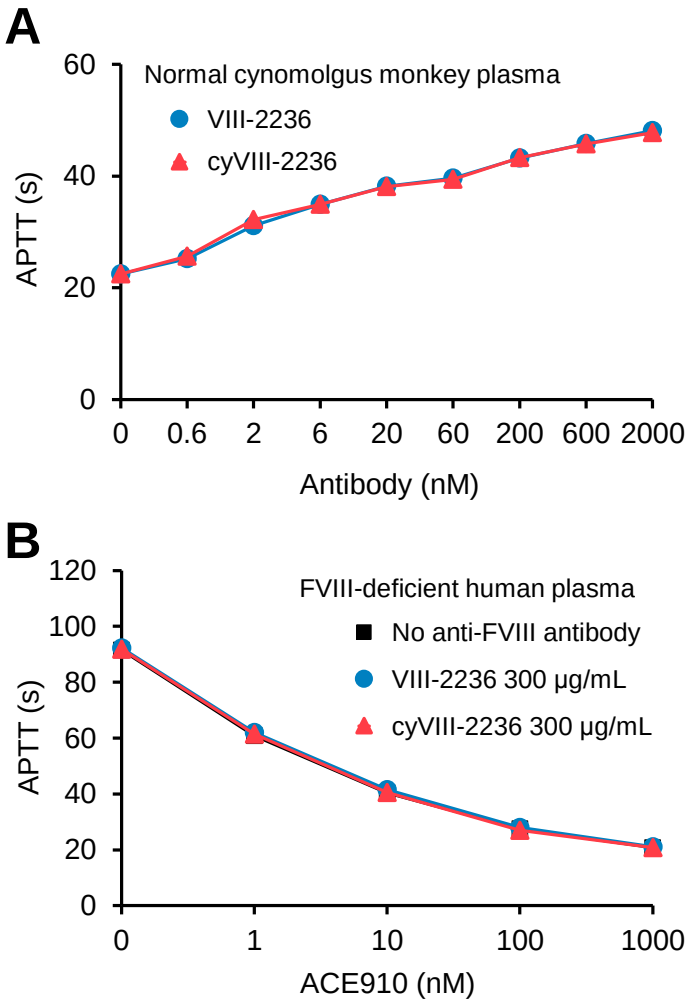

Figure 12 (page 30). Comparison of the mouse–monkey chimeric anti-primate FVIII neutralizing antibody cyVIII-2236 with the original mouse antibody VIII-2236 in an APTT assay. (A) Effects of cyVIII-2236 and VIII-2236 on APTT in normal cynomolgus monkey plasma. (B) Influence of 300 µg/mL cyVIII-2236 and VIII-2236 on APTT-shortening activity of ACE910 in FVIII-deficient human plasma. Data are expressed as means ± SD (n = 3). The bars depicting SD are shorter than the height of the symbols. The symbols for the group without anti-FVIII antibody are hidden behind the symbols for the other groups in Figure 12B.

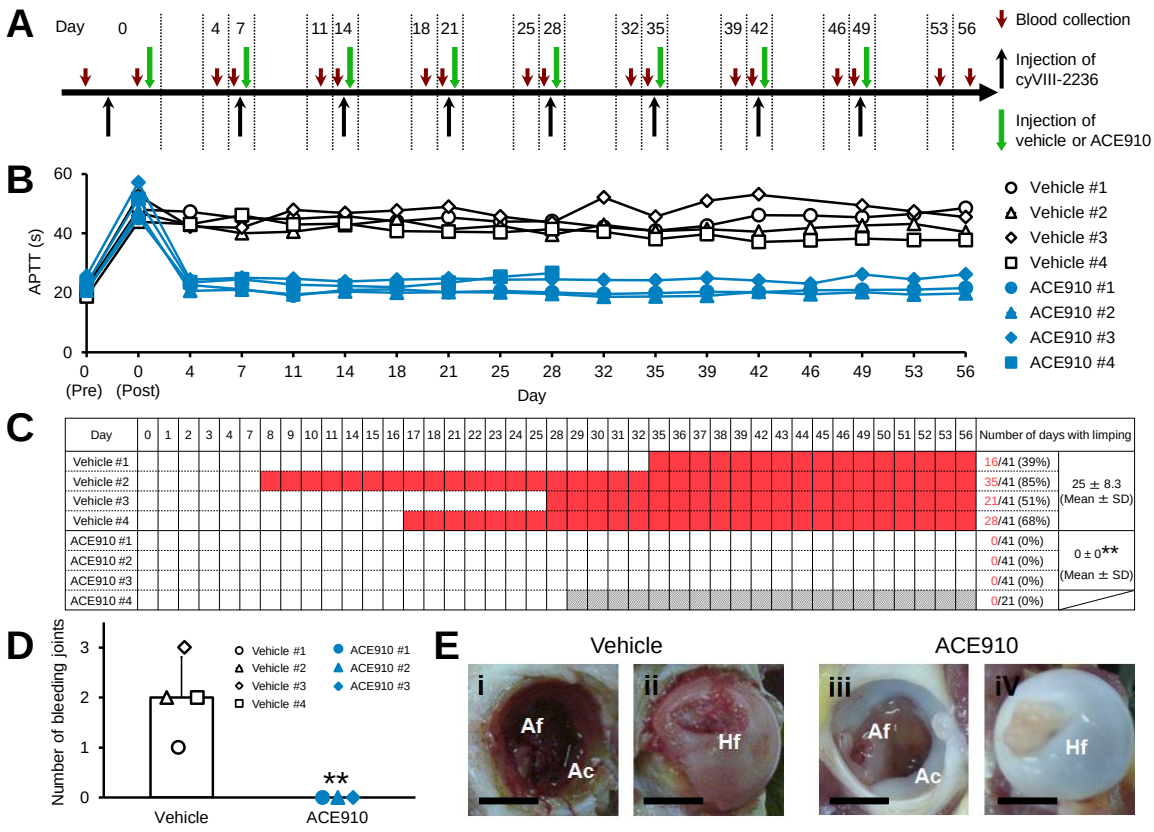

Figure 13 (page 30). Preventive effects of ACE910 on spontaneous joint bleeds in a long-term primate model of acquired hemophilia A. (A) Experimental protocol used for evaluating preventive effects of ACE910 in a long-term hemophilia A model induced by the weekly IV doses of 10 mg/kg cyVIII-2236. The 8 cynomolgus monkeys received weekly IV injections of cyVIII-2236 on Days 0, 7, 14, 21, 28, 35, 42, and 49. ACE910 was administered as an initial 3.97 mg/kg SC dose 2 h after cyVIII-2236 injection on Day 0, and thereafter as a weekly 1 mg/kg SC dose on Days 7, 14, 21, 28, 35, 42, and 49. Citrated blood was collected on Days 0, 4, 7, 11, 14, 18, 21, 25, 28, 32, 35, 39, 42, 46, 49, 53, and 56 (before and 2 h after cyVIII-2236 injection on Day 0; just before cyVIII-2236, vehicle, and ACE910 injections on Days 7, 14, 21, 28, 35, 42, and 49). (B) The time courses of APTT are shown as individual values for respective cynomolgus monkeys (#1–4) of the vehicle and ACE910 groups. The APTT of the vehicle #3 monkey on Day 46 was not determined due to a handling failure. (C) The days in which limping was observed are shown in red for the individual cynomolgus monkeys. The grey boxes indicate that no data is available because the ACE910 #4 monkey was euthanized for humane reasons on Day 28 (See Results). The number of days with limping (panel C) and the number of bleeding joints at necropsy (D) are shown as individual values (#1–4) and as means ± SD in the vehicle group (n = 4) and the ACE910 group (n = 3). $^{**}P < 0.01$ indicates significant differences from the vehicle group

(2-tailed Student's *t* test). (E) Representative macroscopic findings of the joints at necropsy. Left hip joint with limping in the vehicle #1 monkey; dark-red area in the Af (i) and Hf (ii) is detected. Left hip joint without limping in the ACE910 #3 monkey; no abnormalities are noted in the Af (iii) and Hf (iv). The scale bar in each panel indicates 10 mm length. Abbreviations: Ac, acetabulum; Af, acetabular fossa; Hf, head of femur.

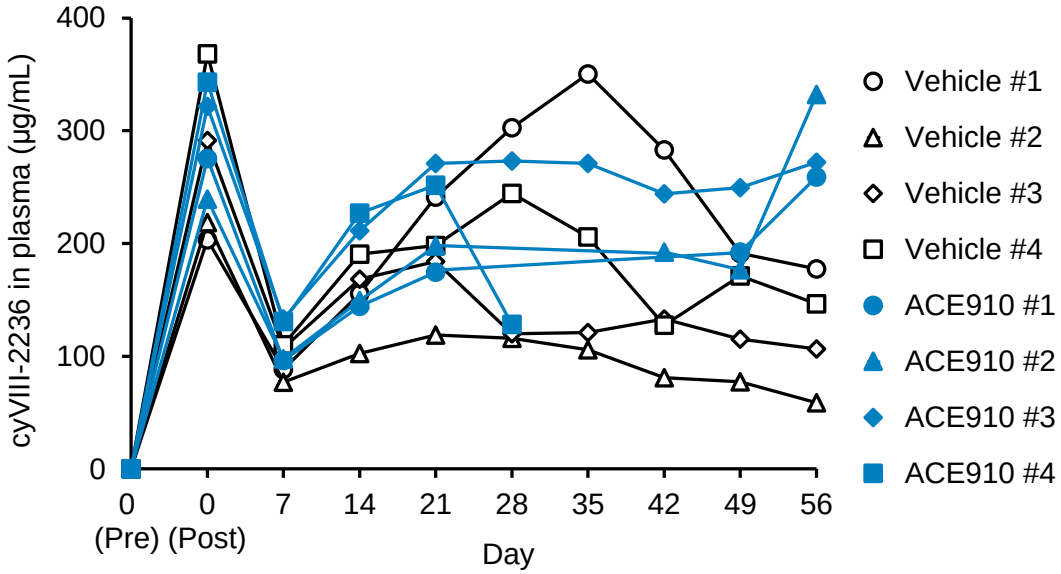

Figure 14 (page 30). Plasma cyVIII-2236 concentrations in a long-term primate model of acquired hemophilia A. The time courses of plasma cyVIII-2236 concentration in the *in vivo* efficacy study are shown. Data are expressed as individual values in cynomolgus monkeys (#1–4) of the vehicle and ACE910 groups. The ACE910 #4 monkey was euthanized humanely on Day 28 after anti-ACE910 alloantibodies developed, plasma ACE910 concentration decreased, and accidental bleeding occurred. Thus, the measurement values for the ACE910 #4 monkey are not present after Day 28. The other missing values at various time points are due to handling failures of measurement or an insufficient volume of sample plasma.

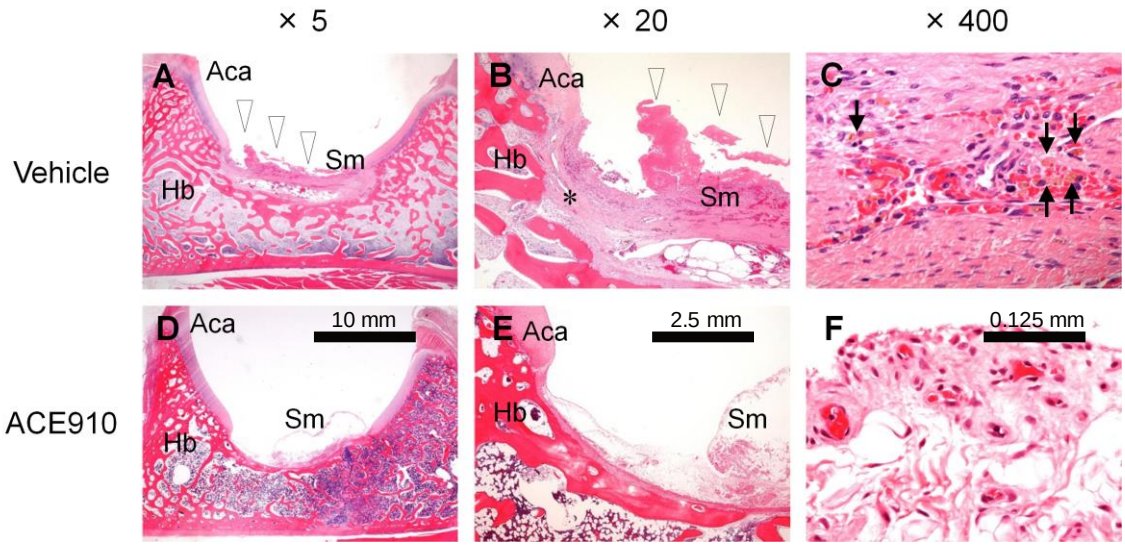

Figure 15 (page 31). Histopathological findings in a representative joint in a long-term primate model of acquired hemophilia A. Left hip joint with limping in the vehicle #1 monkey (A, B, and C) and left hip joint without limping in the ACE910 #3 monkey (D, E, and F) are shown at original magnification ×5 (A and D), ×20 (B and E), and ×400 (C and F). Hemorrhagic changes (arrowheads) including hemosiderin deposition (arrows) and destruction of articular cartilage/underlying bone (*) are detected in the joint with limping (A, B, and C). No hemorrhagic changes are noted in the joint without limping (D, E, and F). Abbreviations: Aca, articular cartilage of acetabulum; Hb, hip bone; Sm, synovial membrane. Hematoxylin and eosin stain.

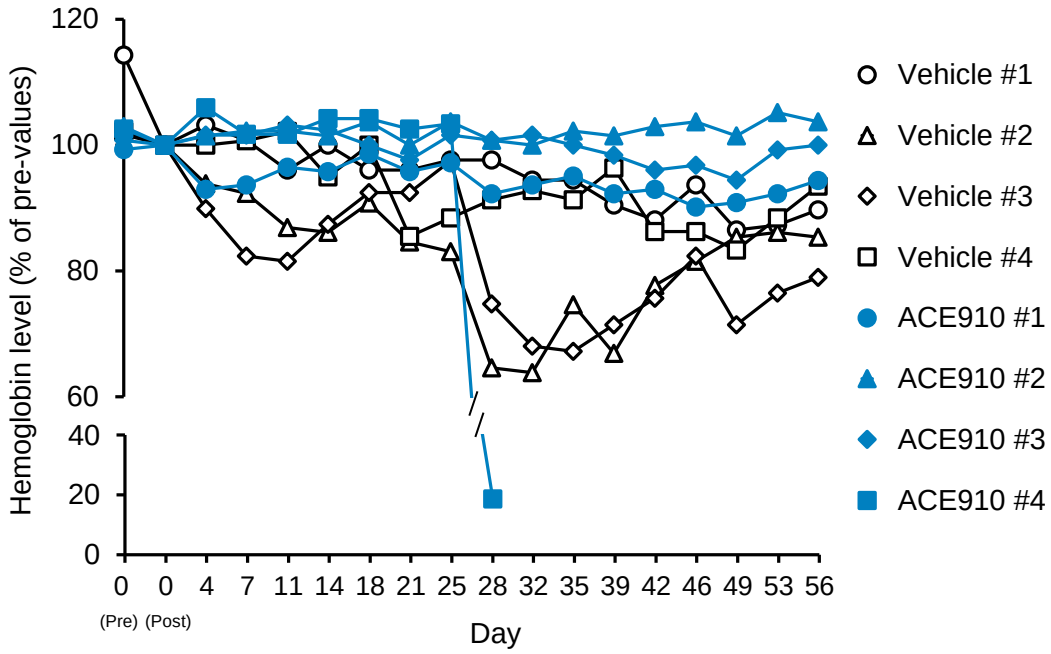

Figure 16 (page 31). Relative blood hemoglobin concentrations in a long-term primate model of acquired hemophilia A. The time courses of blood hemoglobin concentration relative to concentration on Day 0 (2 h after cyVIII-2236 injection) are shown as individual values in respective cynomolgus monkeys (#1–4) of the vehicle and ACE910 groups.

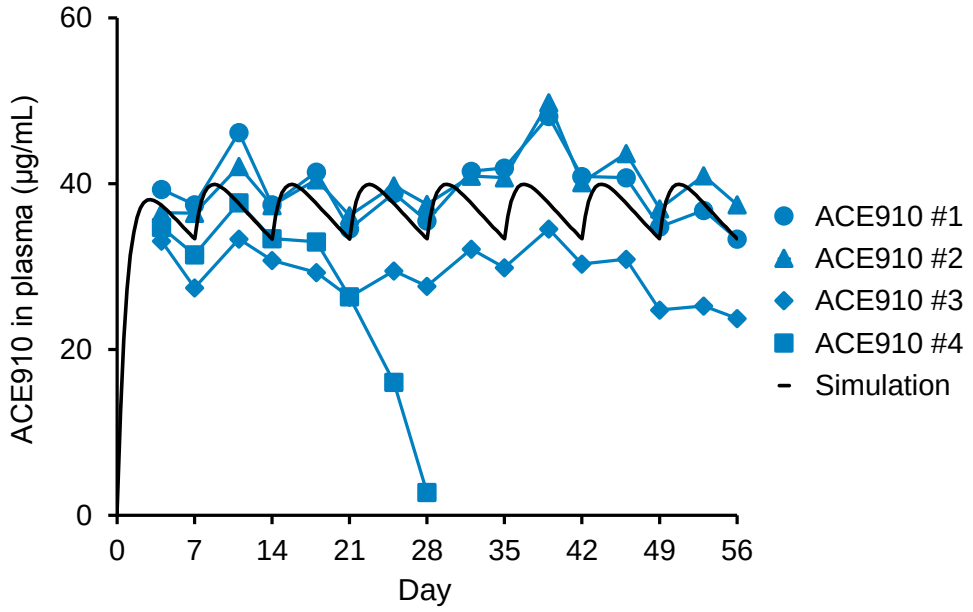

Figure 17 (page 32). Plasma ACE910 concentrations in a long-term primate model of acquired hemophilia A. ACE910 was administered at an initial SC dose of 3.97 mg/kg on Day 0 followed by weekly SC doses of 1 mg/kg on Days 7, 14, 21, 28, 35, 42, and 49. The time courses of actual measured and simulated plasma concentrations of ACE910 are shown. The actual measured concentrations are presented as individual values for cynomolgus monkeys (#1–4) of the ACE910 group.

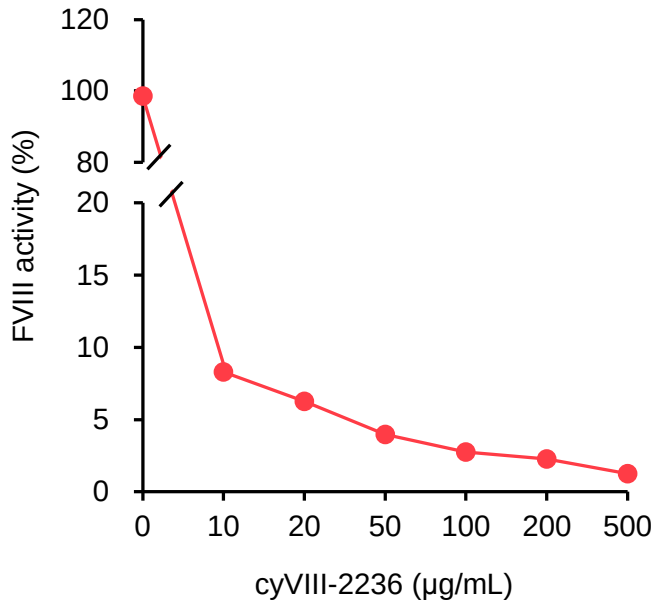

Figure 18 (page 38). FVIII:C measured in an APTT-based one-stage clotting assay in the presence of cyVIII-2236. Data are expressed as means ± SD (n = 3). The bars depicting SD are shorter than the height of the symbols.

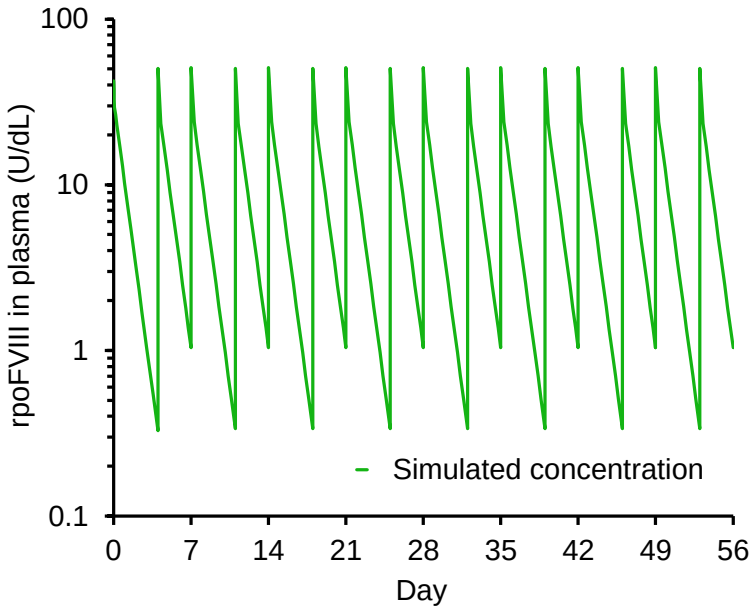

Figure 19 (page 38). Simulated plasma rpoFVIII concentration in a long-term primate model of acquired hemophilia A. The simulated course of plasma rpoFVIII concentration is shown for a twice-weekly 20 U/kg IV dosing regimen.

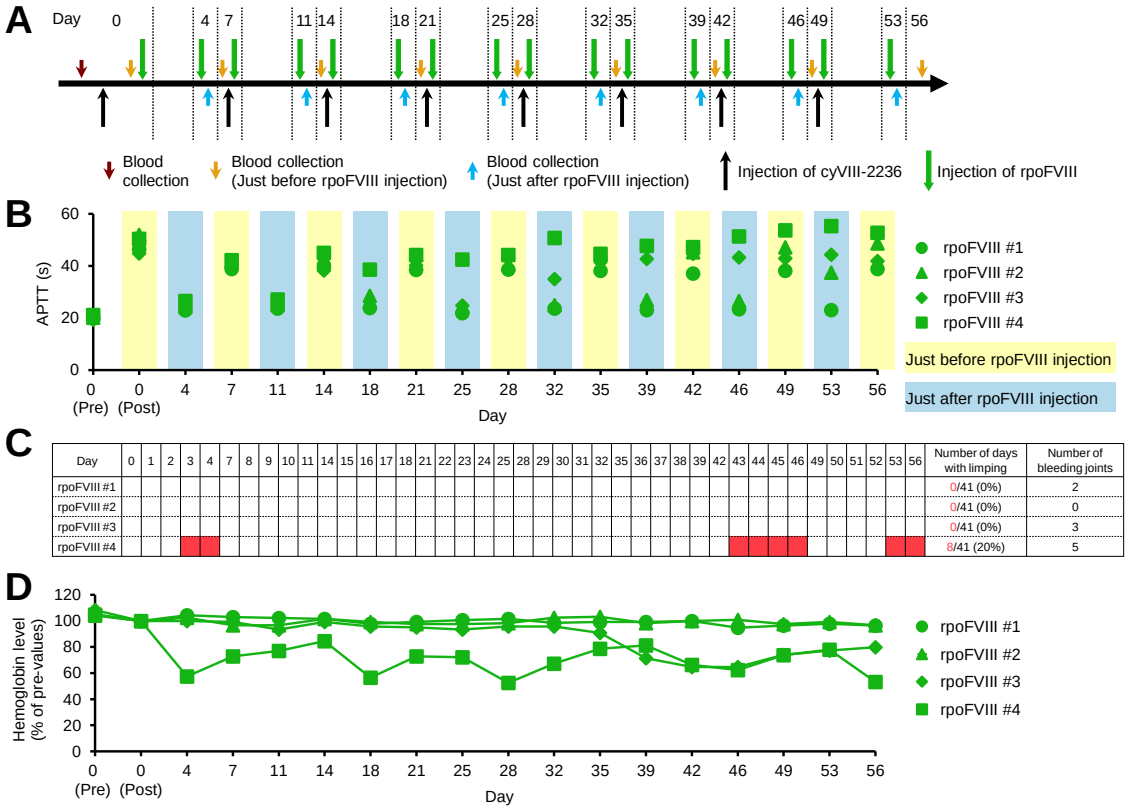

Figure 20 (page 38). Effects of rpoFVIII on spontaneous bleeds in a long-term primate model of acquired hemophilia A. (A) Experimental protocol used for evaluating the preventive effects of rpoFVIII in a long-term hemophilia A model induced by the weekly IV dose of 10 mg/kg cyVIII-2236. rpoFVIII was administered for 8 weeks on a twice-weekly 20 U/kg IV dosing regimen. (B) The time courses of APTT are shown as individual values (#1–4) of the rpoFVIII group. (C) The days in which limping was observed are shown in red for the individual cynomolgus monkeys. The number of limping days and the number of bleeding joints at necropsy are shown as individual values (#1–4) of the rpoFVIII group. (D) The time courses of blood hemoglobin concentration relative to concentration on Day 0 (2 h after cyVIII-2236 injection) are shown as individual values (#1–4) of the rpoFVIII group.

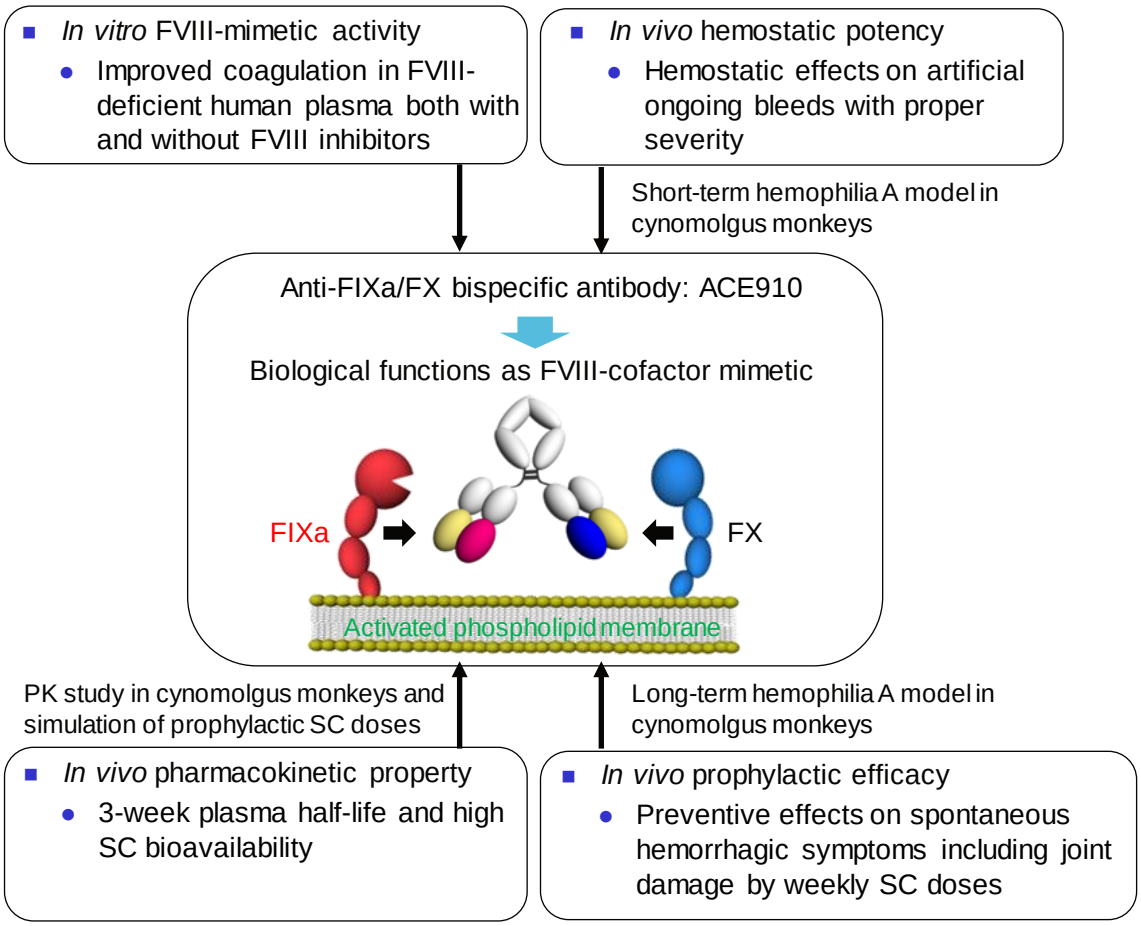

Figure 21 (page 40). Biological functions of anti-FIXa/FX bispecific antibody, ACE910, as a FVIII-cofactor mimetic. In the general conclusions, (i) ACE910 improved coagulation in FVIII-deficient plasma both with and without FVIII inhibitors, (ii) a short-term acquired hemophilia A model expressing stable artificial bleeds with proper severity was newly established in cynomolgus monkeys, and single episodic IV dose of ACE910 significantly inhibited these bleeding symptoms, (iii) ACE910 showed the 3-week plasma half-life and high SC bioavailability in cynomolgus monkeys, and possibility of effective prophylaxis by the once-weekly SC doses was simulated based on the PK parameters, (iv) a long-term acquired hemophilia A model expressing reproducible spontaneous joint bleeds and other bleeds was newly established in cynomolgus monkeys, and weekly SC doses of ACE910 significantly prevented these bleeding symptoms. These results elucidated biological functions of ACE910 as a FVIII-cofactor mimetic and suggested that ACE910 has the potential not only to ameliorate on-going bleeds, but also to offer a user-friendly and aggressive routine prophylaxis for hemophilia A patients both with and without FVIII inhibitors.