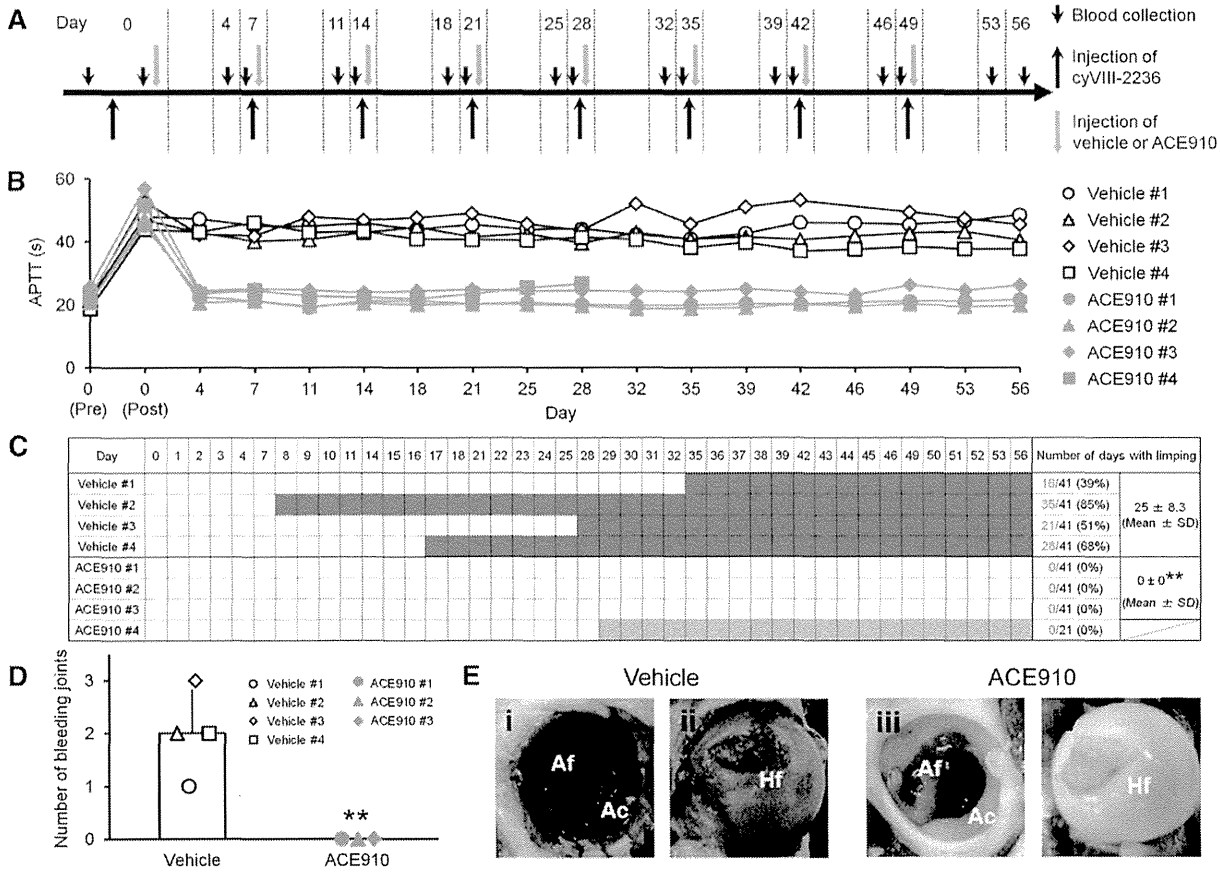

Figure 3. Preventive effects of ACE910 on spontaneous joint bleeds in a long-term primate model of acquired hemophilia A. (A) Experimental protocol used for evaluating preventive effects of ACE910 in a long-term hemophilia A model induced by the weekly IV doses of 10 mg/kg cyVIII-2236. The 8 cynomolgus 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 hours 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 hours 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 gray boxes indicate that no data are available because the ACE910 #4 monkey was killed for humane reasons on day 28 (See "Results"). The number of days with limping (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). $^{**}P < .01$ indicates significant differences from the vehicle group (2-tailed Student *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). Ac, acetabulum; Af, acetabular fossa; Hf, head of femur.

comparable to that of 10 U/kg (twice-daily) rpoFVIII in a short-term primate model of acquired hemophilia A.[16]

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 3B), although careful observation indicates that the APTT-shortening effect was slightly reversed in 1 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 thrombin generation assay.[16] This is assumedly because ACE910 does not require an 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 (supplemental Figure 2). Furthermore, in all monkeys other than the ACE910 #4 monkey, the FVIII-neutralizing titer measured by the modified Bethesda assay was maintained on day 56 (data not shown). 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 6), and anti-ACE910 alloantibodies were detected in the plasma (data not shown). 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 5). Therefore, in consideration of animal ethics and the impossibility of maintaining an effective ACE910 level, this monkey was killed humanely on day 28 and macroscopic observation of its organs and tissues was carried out at necropsy.

Table 1. Macroscopic hemorrhagic findings at necropsy on day 56 in a long-term primate model of acquired hemophilia A

			No. of animals with findings	
Organ/tissue	Site		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

In the other 3 monkeys, plasma ACE910 level remained around the target concentration (30 μg/mL) from day 4 to day 56 (Figure 6), 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, 1 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.[16]

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 3C) and the number of bleeding joints at necropsy (Figure 3D, Table 1) significantly decreased ($P < .01$, 2-tailed Student *t* test), compared with 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 4D-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 1). Furthermore, monkeys #1 to 3 in the ACE910 group exhibited blood Hgb of 90% or above for 8 weeks (Figure 5). 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) ($P < .05$, 2-tailed Student *t* test).

Discussion

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.[4] 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,[4,17] 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%.[18] Our previous 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%) and that such a hemostatic level would be maintained by weekly SC doses of 0.64 mg/kg ACE910.[16] Therefore, in the present study, we investigated weekly SC doses of 1 mg/kg ACE910 and 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,[19,20] a similar PK profile and efficacy are expected for ACE910 in a clinical setting.

In this 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 (supplemental Figures 2 and 3). In the case of congenital hemophilia A, a small percentage 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.[21] Although cyVIII-2236 would not have necessarily decreased FVIII:C in the 1-stage clotting assay to <1% in the monkeys, the control monkeys actually presented severe bleeding symptoms. Thus, we considered that this model was a severe

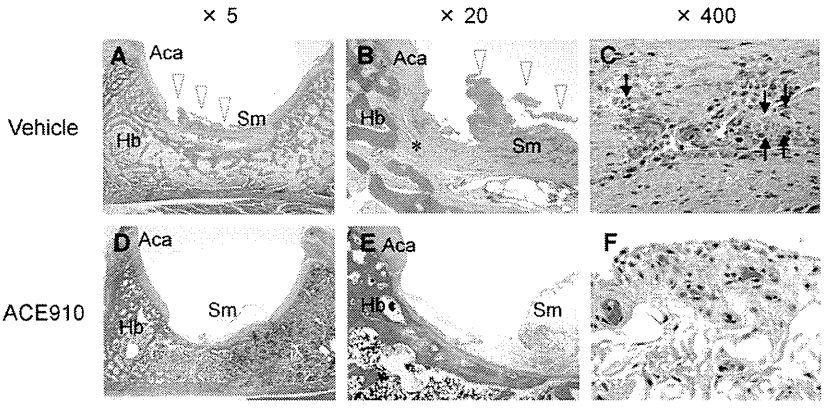

Figure 4. Histopathological findings in a representative joint in a long-term primate model of acquired hemophilia A. Left hip joint with limping in the vehicle #1 monkey (A-C) and left hip joint without limping in the ACE910 #3 monkey (D-F) are shown at original magnification ×5 (A, D), ×20 (B, E), and ×400 (C, F). Hemorrhagic changes (arrowheads) including hemosiderin deposition (arrows) and destruction of articular cartilage/underlying bone (*) are detected in the joint with limping (A-C). No hemorrhagic changes are noted in the joint without limping (D-F). Hematoxylin and eosin stain. Aca, articular cartilage of acetabulum; Hb, hip bone; Sm, synovial membrane.

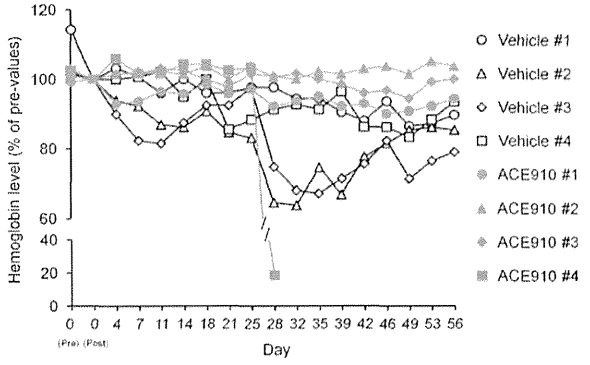

Figure 5. Relative blood Hgb concentrations in a long-term primate model of acquired hemophilia A. The time courses of blood Hgb concentration relative to concentration on day 0 (2 hours after cyVIII-2236 injection) are shown as individual values in respective cynomolgus monkeys (#1-4) of the vehicle and ACE910 groups.

acquired hemophilia A one. In an attempt to further validate this model by looking at reactivity to injected FVIII, we examined the effects of rpoFVIII, which is not neutralized by cyVIII-2236 (data not shown). Aiming to evaluate the marginal level of rpoFVIII efficacy, we 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 (supplemental Figures 4 and 5A).[22] The prolonged APTT was shortened immediately after the initial rpoFVIII injection (supplemental Figure 5B); 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 (supplemental Figure 5C); furthermore, the course of blood Hgb tended to worsen when anti-rpoFVIII alloantibodies presumably emerged (supplemental Figure 5D). 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.[23] Incidence rates of anti-humanized antibody alloantibodies in cynomolgus monkeys are reported to vary (0%-100%), and antigenicity in cynomolgus monkeys cannot predict that in humans.[19] 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 nonimmunogenic in a clinical setting,[15] 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 intraarticular hemorrhage were similar to those of hemophilia A patients.[3,24,25] Although a number of animal models have been reported for hemophilia A, it has been difficult to develop reproducible spontaneous joint bleeds.[26-28] The FVIII-deficient mouse model of hemophilia A needs an artificial injuring procedure to induce joint bleeding.[27,29] 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.[28-34] 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 intraarticular 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 both monkeys. However, in one of the monkeys (vehicle #4), the ankle joint that had swollen did not present macroscopic intraarticular hemorrhage. We assume that the intraarticular hemorrhage may have been absorbed during 2 weeks after the remission of joint swelling.

In this study, although ACE910 completely prevented the macroscopic joint impairment, subclinical histopathological changes in the joint synovium were found, despite the presence of ACE910. We 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, 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. The difficulty in venous access negatively affects the adoption of and adherence to the routine prophylaxis regimen in home settings.[35]

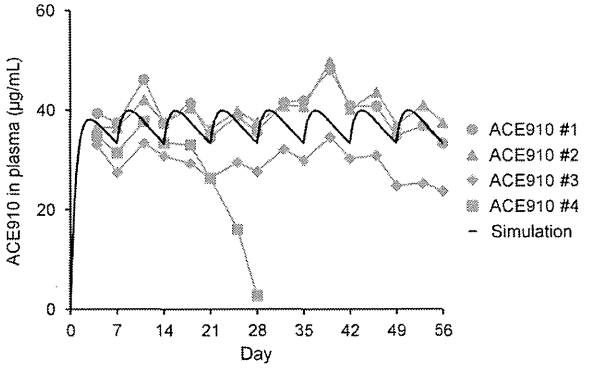

Figure 6. Plasma ACE910 concentrations in a long-term primate model of acquired hemophilia A. ACE910 was administered at an initial SC dose of 3.97 mg/kg on day 0 followed by weekly SC doses of 1 mg/kg on days 7, 14, 21, 28, 35, 42, and 49. The time courses of actual measured and simulated plasma concentrations of ACE910 are shown. The actual measured concentrations are presented as individual values for cynomolgus monkeys (#1-4) of the ACE910 group.

ACE910 is expected to provide hemophilia A patients, regardless of FVIII inhibitors, with a more effective and user-friendly way of bleeding prophylaxis. Clinical investigation of ACE910 is currently ongoing.

Acknowledgments

The authors thank their colleagues at Chugai Pharmaceutical Co, Ltd: T. Matsuura, M. Hiranuma, R. Takemoto, T. Koike, H. Kitamura, and T. Houjo for the in vivo pharmacologic experiments; T. Kojima, T. Wakabayashi, E. Tanaka, K. Esaki, Y. Kikuchi, M. Wada, M. Goto, H. Tsunoda, T. Suzuki, Y. Okuyama-Nishida, A. Harada, M. Funaki, S. Suzuki, T. Toyoda, and M. Ijiri for the preparation of test items and the in vitro experiments; Y. Sakamoto, T. Tachibana, and M. Nanami for the PK studies; and H. Saito for providing advice for the various experiments. The work was carried out at the Research Division, Chugai Pharmaceutical Co, Ltd.

This work was supported by research funding from Chugai Pharmaceutical Co, Ltd.

Authorship

Contribution: A.M., K.Y., M.T., T. Kitazawa, T.S., and Y.K. designed, performed, and analyzed the pharmacologic studies; T.I., Z.S., T. Kuramochi, and A.S. prepared the test items; K. Haraya conducted the PK studies; K.A. conducted the histopathological studies; K.N. and M.S. interpreted data and provided advice from the viewpoints of their medical expertise in hemophilia; K. Hattori provided direction and organized the program; and A.M. and T. Kitazawa wrote this manuscript.

Conflict-of-interest disclosure: A.M., K.Y., T. Kitazawa., T.S., T.I., Z.S., K. Hattori, M.T., T. Kuramochi, A.S., K. Haraya, K.A., and Y.K. are employees of Chugai Pharmaceutical, and the former 7 authors are inventors of the patents relating to anti-FIXa/X bi-specific antibodies, of which all rights have been assigned to the company. K.N. and M.S. receive research support paid to their institution from Chugai Pharmaceutical, and have received consulting honoraria from Chugai Pharmaceutical and F. Hoffmann-La Roche.

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blood 2014 124: 3165-3171
doi:10.1182/blood-2014-07-585737 originally published online October 1, 2014

Anti-factor IXa/X bispecific antibody ACE910 prevents joint bleeds in a long-term primate model of acquired hemophilia A

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.

Biochimica et Biophysica Acta 1844 (2014) 1943–1950

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Review

pH-dependent antigen-binding antibodies as a novel therapeutic modality☆

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ARTICLE INFO

Article history:
Received 31 January 2014
Received in revised form 31 July 2014
Accepted 5 August 2014
Available online 12 August 2014

Keywords:
Monoclonal antibody
Antibody engineering
pH dependent

ABSTRACT

Monoclonal antibodies have become a general modality in therapeutic development. However, even with infinite binding affinity to an antigen, a conventional antibody is limited in that it can bind to the antigen only once, and this results in antigen-mediated antibody clearance when the a membrane-bound antigen is targeted, or in antibody-mediated antigen accumulation when a soluble antigen is targeted. Recently, a pH-dependent antigen-binding antibody that binds to an antigen in plasma at neutral pH and dissociates from the antigen in endosome at acidic pH has been reported to overcome this limitation and to reduce antigen-mediated antibody clearance and antibody-mediated antigen accumulation. A pH-dependent binding antibody against a soluble antigen can be further improved by Fc engineering to enhance the Fc receptor binding. Various approaches, including histidine-based engineering, direct cloning from immunized animals, and synthetic and combinatorial libraries, have been successfully applied to generate pH-dependent binding antibodies against various antigens. This review discusses the features, approaches, advantages, and challenges of developing a pH-dependent binding antibody as a novel therapeutic modality. This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

1. Introduction

With more than 30 monoclonal antibodies already approved for therapeutic use [1] and more than 300 under clinical development [2], monoclonal antibodies are clearly expected to play an important role in future therapeutics. Therefore, antibody engineering technologies that improve the therapeutic potency of monoclonal antibodies have been extensively studied in this decade. Such technologies include improving the binding affinity to a target, the specificity, the pharmacokinetics, and the effector function mediated by the Fc region of an antibody. Binding affinity can be improved by several methods, such as *in vitro* affinity maturation by library display [3] and computer-based *in silico* design [4]. For pharmacokinetics, lowering the isoelectric point of the variable region of an antibody is reported to prolong the antibody half-life by reducing the non-specific clearance of the antibody [5]. Fc engineering to enhance the binding affinity to FcRn at acidic pH also improves pharmacokinetics by increasing the recycling efficiency from the endosomes after non-specific uptake into cells [6]. Modulation of the antibody effector function by optimizing the interaction between the Fc region and an Fc gamma receptor is reported to improve the therapeutic efficacy of a monoclonal antibody [7]. Another noteworthy technology that has recently been reported provides bispecific antibodies with a unique function that conventional monoclonal antibodies cannot achieve; they can neutralize two different disease-related cytokines [8]. In addition, bispecific antibodies can also redirect cytotoxic T cells to cancer cells by binding to CD3 and a tumor antigen [9], and they can mimic the function of coagulation factor VIII by binding to coagulation factor IXa and X [10].

Although these technologies have bestowed improved potency on therapeutic antibodies, it is now becoming more important to generate monoclonal antibodies that have further improved properties and are differentiated from conventional high-affinity monoclonal antibodies. In terms of modulating the interaction between an antigen and an antibody, achieving a high affinity binding to the antigen by affinity maturation has been the only approach for improvement. However, even with an infinite binding affinity to the antigen, a conventional antibody can bind to the antigen only once during its lifetime in plasma, and it is fundamentally limited by its consequent inability to neutralize further antigens, if the *in vivo* produced molar amount of antigen is larger than the amount of antibody injected.

If an antibody is targeting a membrane-bound antigen with a high rate of synthesis, such as IL-6 receptor (IL-6R), EGFR, CD4, or CD40 [11–14], the antibody is rapidly eliminated from plasma through antigen-mediated clearance. As a result, targeting these types of membrane antigen cannot be improved by affinity maturation and requires a high dose. Similarly, a high antibody dose is also required to neutralize soluble antigen at a high plasma concentration. The plasma

☆ This article is part of a Special Issue entitled: Recent advances in molecular engineering of antibody.

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http://dx.doi.org/10.1016/j.bbapap.2014.08.003
1570-9639/

concentration of soluble antigen after antibody administration is determined by two factors: the baseline concentration and the antibody-mediated antigen accumulation. Some soluble antigens, such as IgE and C5, have a plasma baseline concentration that is already very high [15,16]; other soluble antigens without a high baseline concentration in plasma accumulate more than 1000-fold because the recycling function of an antibody inhibits degradation of the antigen and results in a very high concentration [17]. This accumulation after antibody administration occurs because an antigen in complex with an antibody has a longer half-life than the antigen alone [18,19]. The resulting high plasma antigen concentration requires a high antibody dose to neutralize the antigen. For both membrane-bound and soluble antigens, the limitation of conventional antibodies in only binding to the antigen once, even when the binding affinity is infinite, requires a high antibody dose.

Recently, we and others have reported that a pH-dependent antigen-binding property could overcome this limitation of conventional antibodies [20–22]. An antibody with a pH-dependent antigen-binding property dissociates the bound antigen in acidic endosomes after internalization into cells. Consequently, the dissociated antigen is trafficked to the lysosome and degraded, whereas the dissociated antibody, free of antigen, is recycled back to plasma by FcRn. The recycled free antibody can bind to another target antigen. By repeating this cycle, a pH-dependent antigen-binding antibody can bind to the target molecule more than once. Moreover, we have shown that the therapeutic potency of a pH-dependent antigen-binding antibody can be further enhanced by increasing its binding affinity to FcRn at neutral pH [23]. These studies demonstrate that pH-dependent antigen-binding antibodies can overcome the limitation of conventional antibodies and allow novel antibody therapeutics with differentiating properties to be generated. In this review, the features, advantages, and challenges of pH-dependent antigen-binding antibodies, and how they can be generated and optimized are discussed.

2. Effect of a pH-dependent antigen-binding antibody

2.1. Against a membrane-bound antigen

Fig. 1A describes the fate of a conventional antibody bound to a membrane-bound antigen. A conventional antibody bound to the cell surface antigen is internalized into cells, after which the antibody–antigen complex passes through the sorting endosome and is transferred to a lysosome, where eventually both the antibody and antigen are degraded by proteolysis. This means that, even with infinite binding affinity to the antigen, a conventional antibody is limited to binding to an antigen only once. Conventional antibodies against membrane-bound antigens, such as IL-6R, EGFR, CD4, and CD40, exhibit non-linear clearance *in vivo* through antigen-mediated antibody clearance [11–14]. When a membrane-bound antigen is highly expressed in the body, the antibody is rapidly cleared from plasma, thereby requiring a high dose to neutralize this type of antigen over a long period.

Fig. 1B describes the fate of a pH-dependent antigen-binding antibody, which we also refer to as a recycling antibody, bound to a membrane-bound antigen. The recycling antibody bound to the cell surface antigen is internalized into the cells in the same way as a conventional antibody. However, in the sorting endosome, where the pH of the vesicle is acidic, the pH-dependent binding antibody is dissociated from the antigen. The antigen is transferred to the lysosome and degraded by proteolysis, while the dissociated antibody binds to FcRn in the endosome and is recycled back to the cell surface. Since FcRn does not bind to the antibody at neutral pH, the antibody dissociates from FcRn at the cell surface and returns back to plasma. A recycling antibody exhibits less non-linear antigen-mediated antibody clearance *in vivo* than conventional antibody and shows prolonged pharmacokinetics by avoiding antigen-mediated clearance. By repeating this cycle of binding at the cell surface and dissociating within the endosomes, a recycling antibody can bind to multiple antigens and overcomes the limitation of a conventional antibody.

The effect of a recycling antibody on a membrane-bound antigen was first demonstrated by our group using a monoclonal antibody against IL-6R [20]. A conventional antibody against IL-6R, tocilizumab, was engineered by a histidine mutagenesis approach (details described in section 4-1) to generate a pH-dependent binding antibody against IL-6R, named recycling tocilizumab. Importantly, tocilizumab and recycling tocilizumab have similar binding affinity to IL-6R at pH 7.4, while only recycling tocilizumab rapidly dissociates IL-6R at pH 6.0, allowing us to evaluate only the effect of pH-dependent antigen dissociation in the acidic endosomes. In a pharmacokinetic study in human IL-6R transgenic mice, the pharmacokinetics of recycling tocilizumab was better than that of tocilizumab, while in normal mice (whose IL-6R neither of the antibodies can bind) the pharmacokinetics of the two antibodies were similar, demonstrating that engineering the pH dependency reduced IL-6R-mediated clearance but did not influence non-specific clearance. By using a FcRn null variant of each antibody, we confirmed that the reduced IL-6R-mediated clearance by recycling tocilizumab was dependent on FcRn binding, indicating that recycling tocilizumab dissociated within the endosomes and

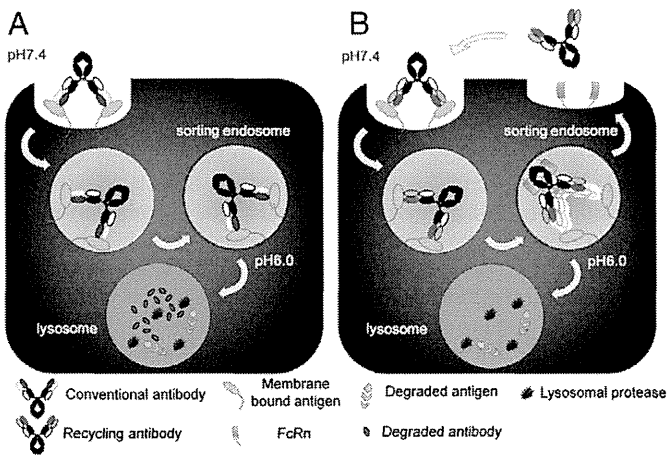

Fig. 1. Models showing the fate of (A) a conventional antibody and (B) a recycling antibody bound to a membrane-bound antigen. (A) A conventional antibody bound to a membrane-bound antigen is internalized into the cell, after which the antibody–antigen complex is trafficked to the sorting endosome and finally to lysosome, where eventually both the antibody and antigen are degraded by lysosomal protease. (B) A recycling antibody bound to a membrane-bound antigen is internalized into the cell in the same way as a conventional antibody. However, in the sorting endosome, where the pH of the vesicle is acidic, the recycling antibody is dissociated from the antigen by means of its pH-dependent antigen-binding property. The antigen is transferred to the lysosome and degraded by proteolysis, while the dissociated antibody is recycled back to the cell surface and plasma by utilizing FcRn.

was recycled back to plasma by FcRn (unpublished data). Chaparro-Riggers *et al.* also showed that FcRn is indispensable for a pH-dependent anti-PCSK9 antibody to be rescued from antigen-mediated clearance [21]. This was demonstrated by the fact that the half-life of the pH-dependent anti-PCSK9 antibody was the same as that of an antibody without pH-dependent binding (0.7 days) in FcRn knockout mice, while in wild-type mice, that of the pH-dependent antibody (14.4 days) was significantly longer than that of the antibody without pH-dependent binding (2.9 days).

A further engineered version of recycling tocilizumab, named SA237, which has a pH-dependent antigen-binding property and enhanced FcRn binding, demonstrated improved pharmacokinetics in cynomolgus monkeys compared to tocilizumab [20]. Whereas a subcutaneous injection of 2 mg/kg of tocilizumab inhibited C-reactive protein production for approximately 10 days, 2 mg/kg of SA237 was effective for more than 28 days. A Phase 1 clinical study of SA237 demonstrated significantly prolonged pharmacokinetics compared to tocilizumab, similar to that observed in cynomolgus monkeys (unpublished data). This study result clinically validated the concept of pH-dependent antigen-binding recycling antibodies.

2.2. Against a soluble antigen

Fig. 2A describes the fate of a conventional antibody bound to a soluble antigen. The conventional antibody bound to the soluble antigen in plasma is taken up into cells by non-specific endocytosis or pinocytosis. The antibody–antigen complex is transferred to a sorting endosome and binds to FcRn at acidic pH. FcRn recycles the antibody–antigen complex to the cell surface, where the complex is dissociated from FcRn back to plasma. Although the antibody is recycled, it is recycled in an antigen-bound form; therefore, the antibody cannot bind to the next antigen. This also limits a conventional antibody to binding only one soluble antigen, just as with a membrane-bound antigen. A soluble antigen in the absence of a conventional antibody is generally transferred to a lysosome and degraded, since most target antigens do not bind to FcRn (only IgG and albumin bind to FcRn). By binding to the soluble antigen, a conventional antibody inhibits the lysosomal degradation pathway of the soluble antigen. Thus, a conventional antibody against a soluble antigen *in vivo* generally results in an increase of total plasma antigen concentration or an accumulation of soluble antigen in plasma, an effect that has been reported for IL-6, MCP1, amyloid beta, hepcidin, and other soluble antigens [17,24–28]. When a soluble antigen is abundant in the body, antigen accumulation results in the antigen binding sites of the antibodies becoming rapidly saturated, and free antibodies that are capable of binding to the antigens is lost.

Fig. 2B describes the fate of a recycling antibody bound to a soluble antigen. The antibody–antigen complex formed by the recycling antibody is also non-specifically taken up into cells in the same way as a conventional antibody. However, within the sorting endosome, the antibody pH-dependently dissociates from the soluble antigen, which is transferred to the lysosome and degraded by proteolysis. On the other hand, the free antibody is recycled back to plasma by FcRn. By repeating this cycle of binding in plasma and dissociating within the endosome, recycling antibodies against soluble antigens can also bind to multiple antigens and overcome the limitation of conventional antibodies. Since an antigen that forms a complex with a recycling antibody is degraded in the lysosome, recycling antibodies can degrade soluble antigens at the rate of non-specific uptake of the antibody–antigen complexes, and thus degrade the antigens more effectively than conventional antibodies. A recycling antibody exhibits reduced plasma antigen accumulation *in vivo* by enhancing soluble antigen clearance, and extends the half-life of the free antibodies.

The effect of a recycling antibody on a soluble antigen was also first demonstrated by the same monoclonal antibodies against IL-6R described above. Soluble IL-6R (sIL-6R) clearance was compared in normal mice that received sIL-6R alone, tocilizumab with sIL-6R, or recycling tocilizumab with sIL-6R. While sIL-6R alone was rapidly cleared from plasma, sIL-6R injected together with tocilizumab showed reduced clearance because it was bound to tocilizumab, which has a long half-life. In contrast, sIL-6R injected together with recycling tocilizumab showed accelerated sIL-6R clearance [20]. This antibody–antigen co-injection study can be generally applied to evaluate whether the clearance of a soluble antigen can be accelerated by pH-dependent antigen binding. However, this study does not mimic the clinical situation, where an antibody is injected to plasma at a steady-state soluble antigen concentration. To mimic the clinical situation, mice were implanted with an infusion pump filled with sIL-6R that continuously provided sIL-6R into plasma and maintained a steady-state sIL-6R concentration. In this model, an injection of tocilizumab resulted in approximately 20-fold increase of total plasma sIL-6R concentration, while recycling tocilizumab significantly reduced the accumulation and resulted in only a 2-fold increase of total plasma sIL-6R concentration [23].

Chaparro-Riggers *et al.* reported using a pH-dependent binding antibody against PCSK9 [21]. PCSK9 is a soluble antigen responsible for

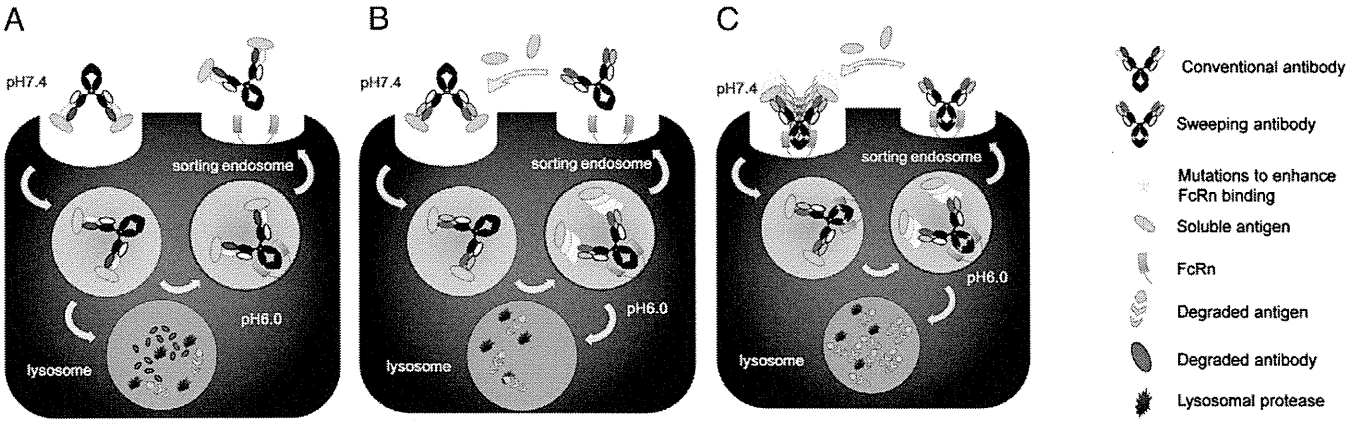

Fig. 2. Models showing the fate of (A) a conventional antibody, (B) a recycling antibody and (C) a sweeping antibody bound to a soluble antigen. (A) A conventional antibody bound to a soluble antigen in plasma is taken up into cells by non-specific endocytosis or pinocytosis. The antibody–antigen complex is transferred to a sorting endosome, where it binds to FcRn at acidic pH and is recycled back to the cell surface and plasma by utilizing FcRn. Although the antibody is recycled, it is recycled in an antigen-bound form; therefore, the antibody cannot bind to the next antigen. (B) A recycling antibody bound to a soluble antigen in plasma is taken up into cells in the same way as a conventional antibody. However, within the sorting endosome, the recycling antibody dissociates the soluble antigen by utilizing its pH-dependent antigen-binding property. The dissociated antigen is transferred to a lysosome and degraded by lysosomal protease. On the other hand, the free antibody is recycled back to plasma by FcRn. (C) A sweeping antibody with enhanced FcRn binding at neutral pH binds to a soluble antigen, and the antibody–antigen complex is rapidly taken up into the cell in a FcRn-mediated manner. By accelerating the uptake of antibody–antigen complexes into cells, a sweeping antibody can degrade a larger amount of soluble antigen than a recycling antibody.

enhancing the degradation of LDL receptors. Inhibition of PCSK9 inhibits LDL receptor degradation and thereby reduces the LDL cholesterol level in plasma. A conventional antibody against PCSK9, J10, showed non-linear clearance in cynomolgus monkeys, which was presumably caused by an accelerated clearance of PCSK9–antibody complexes via an unknown pathway. To overcome the relatively short pharmacokinetics of J10 at low dosages, J17, a histidine-engineered version of J10 with a pH-dependent PCSK9-binding property, was generated. J17 demonstrated significantly improved pharmacokinetics compared to J10 in cynomolgus monkeys, and the LDL cholesterol-lowering effect was longer than that of J10 at the same dose. In the microscopic assay using HepG2 cells, J10 was more efficiently trafficked to lysosomes than J17 when co-incubated with PCSK9, while the trafficking of PCSK9 to lysosomes was comparable in the presence of J10 and J17. The authors demonstrated that a PCSK9-bound antibody shows accelerated lysosomal degradation by an unknown pathway, and this degradation can be avoided by a pH-dependent binding antibody that dissociates from PCSK9 in the endosomes. This study also confirmed the favorable effect of pH-dependent binding against a soluble antigen. Recently, Devanaboyina *et al.* reported a pH-dependent binding antibody against IL-6. In a mouse co-injection study, a conventional antibody against IL-6, clone 0218, significantly reduced the clearance of IL-6, while the pH-dependent binding antibody derived from clone 0218, VH4, accelerated the clearance of IL-6 from plasma [22]. In a microscopic assay using GFP-fused human FcRn variant-transfected HMEC-1 cells, they demonstrated that the conventional antibody against IL-6, clone 0218, colocalized with IL-6 in early endosomes, while the pH-dependent binding antibody, clone VH4, dissociated IL-6 in the early endosomes, and the dissociated IL-6 was subsequently trafficked to lysosomes.

These studies clearly demonstrated that recycling antibody against soluble antigen generally clears soluble antigen faster than conventional antibody and reduces antigen accumulation in plasma. Because administering recycling antibody reduces the total soluble antigen concentration compared with conventional antibody, the antibody dose required to neutralize the soluble antigen can be reduced.

3. Improving a recycling antibody against soluble antigens into a sweeping antibody by Fc engineering

A recycling antibody, with the capability of dissociating soluble antigens in the endosomes, can accelerate the clearance of the soluble antigens from plasma. However, the rate of antigen clearance by a recycling antibody is limited by the slow rate of non-specific uptake of antibody–antigen complexes into cells, so further enhancing the rate of soluble antigen degradation would be very beneficial. To further enhance the clearance of soluble antigens from plasma, we have recently developed a sweeping antibody, which is an Fc-engineered pH-dependent binding antibody [23]. Since uptake of antibody–antigen complexes was the rate-limiting step for antigen degradation, we engineered the Fc region of a pH-dependent antigen-binding antibody to increase the binding affinity to FcRn at neutral pH (wild-type IgG1 just marginally binds to FcRn at pH 7.4). This sweeping antibody, a pH-dependent binding antibody with engineered IgG1, can bind to FcRn at neutral pH on the cell surface, which causes antibody–antigen complexes to be rapidly taken up into the cells in an FcRn-mediated manner and, therefore, degrades more soluble antigens than a recycling antibody (Fig. 2C).

By moderately increasing the binding affinity to FcRn at neutral pH, an *in vivo* study using human FcRn transgenic mice demonstrated that the clearance of soluble antigens can be accelerated approximately 50-fold while the antibody maintains pharmacokinetics similar to a conventional antibody with wild-type IgG1 (Fig. 3). It is important to note that the antigen clearance, not the antibody clearance, was selectively enhanced by simultaneously engineering the pH-dependent antigen binding and moderately increasing the binding affinity to FcRn at neutral pH. For their microscopic assay, Devanaboyina *et al.* used human/mouse chimeric FcRn, which has increased affinity to wild-type human IgG1. In the assay, they reported that, while a soluble antigen remained bound to a non-pH dependent binding antibody in the sorting endosomes, the antigen was dissociated from the pH-dependent binding antibody, and the dissociated antigen was finally transferred to lysosomes [22]. This result also suggests that both pH-dependent antigen binding and enhanced FcRn binding are required to increase antigen uptake into cells. We also observed the same phenomenon in our *in vitro* confocal microscopy assay using the human FcRn-transfected MDCK cell line. While a soluble antigen remained bound to a non-pH-dependent binding antibody in the sorting endosome (Fig. 4A), the soluble antigen was dissociated from a pH-dependent binding antibody with engineered IgG1 and enhanced FcRn binding (Fig. 4B).

It should be noted that previous Fc engineering to increase the binding affinity to FcRn at acidic pH aimed to improve antibody pharmacokinetics by increasing FcRn-mediated IgG recycling [29]. On the other hand, we engineered a pH-dependent antigen-binding antibody to moderately increase its binding affinity at neutral pH to generate a sweeping antibody, which selectively enhanced the clearance of the antigen while maintaining antibody pharmacokinetics similar to wild-type IgG1 (Fig. 3). However, consistent with the report from Vaccaro *et al.* in which they reported that engineering the Fc region of IgG1 to create a strong binding to FcRn at neutral pH modulated *in vivo* concentration of endogenous IgG antibodies [30], a sweeping antibody with strongly increased binding affinity to FcRn at neutral pH resulted in increased antibody clearance, although antigen clearance was more accelerated than that of a sweeping antibody with moderately increased binding affinity to FcRn at neutral pH [23]. An appropriate level of binding affinity to FcRn at neutral pH should be selected, depending on the characteristics of the target soluble antigen.

An alternative approach for enhancing the uptake of antibody–antigen complexes into cells is utilizing uptake mediated by Fc gamma receptor, which has been reported elsewhere as contributing to the clearance of antibody–antigen immune complexes *in vivo* [31]. Especially, the clearance of small immune complexes is dominantly supported by Fc gamma receptor IIb (or CD32b) expressed on liver sinusoidal endothelial cells [32]. Because Fc gamma receptor IIb is reported to be a recycling receptor, enhancing the binding affinity to this receptor could have a similar effect to that observed when the binding affinity to FcRn at neutral pH was enhanced [33]. Previously reported Fc engineering to enhance binding affinity to Fc gamma receptor IIb also enhanced the binding affinity to Fc gamma receptor IIa (or CD32a) with arginine 131 allotype, which represents approximately 70% of the Caucasian population [34]. However, since Fc gamma receptor IIa is not a recycling receptor and is expressed on platelets, enhancing the binding affinity also to Fc gamma receptor IIa could have both pharmacokinetic and safety issues. Recently, we have reported a novel engineered Fc variant that selectively enhances binding to Fc gamma receptor IIb over both the histidine and arginine 131 allotypes of Fc gamma receptor IIa [35]. By combining pH-dependent antigen binding with this novel Fc engineering, we may be able to accelerate the antigen clearance and degrade more soluble antigen than with a recycling antibody.

4. Approaches for generating recycling antibodies

4.1. Experimental approaches

Previously, monoclonal antibodies generated by either animal immunization or *in vitro* antibody library display technology have been selected based on their strong binding affinity to their target antigens at neutral pH and their binding to a functionally appropriate epitope. Therefore, the monoclonal antibodies obtained in such a way seldom had a pH-dependent antigen-binding property. Although most antibodies lose their binding affinity to the antigen at pH 3.0, they do not show

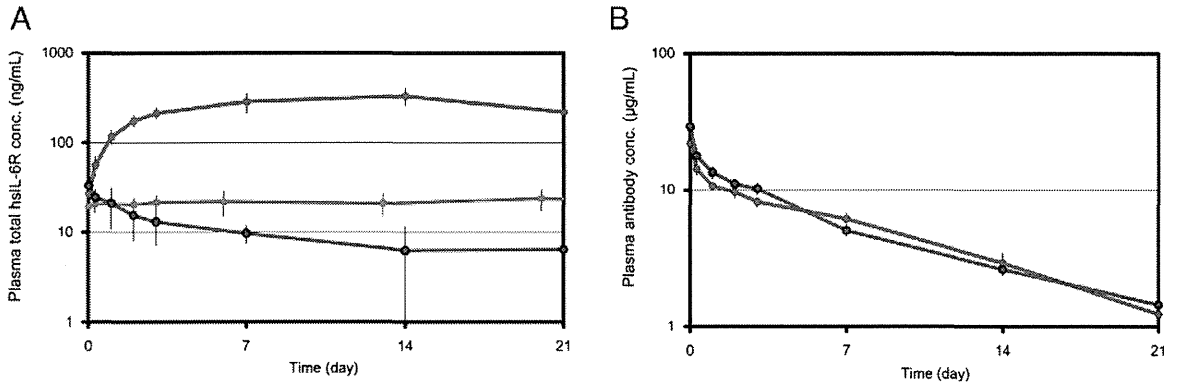

Fig. 3. The effect of a conventional antibody and a sweeping antibody against a soluble antigen in human FcRn transgenic mice. (A) shows the time course of plasma concentration of total soluble antigen, hsIL-6R, after antibodies were administered. (B) shows the plasma antibody concentration. Blue lines indicate a conventional antibody, red lines a sweeping antibody (pH-dependent antigen-binding antibody with enhanced FcRn binding), and gray lines the baseline of the soluble antigen.

rapid enough dissociation of antigens at pH 5.5–6.0 in a sorting endosome.

In our first report of a pH-dependent binding antibody [20], we engineered a conventional antibody against IL-6R, tocilizumab, into a pH-dependent binding antibody, which was identified by mice immunization and subsequent screening of the binding affinity and IL-6–binding neutralizing capability [36]. We took advantage of a natural amino acid, histidine, which has pKa of around 6 (depending on the surrounding environment) and is utilized in naturally occurring pH-dependent protein-protein interactions [37]. We introduced several histidine residues in the CDR or FR of tocilizumab by site-directed mutagenesis. Because of the pKa of the imidazole group, histidine residues are protonated at endosomal acidic pH, which results in destabilizing the antibody–antigen interaction either directly, with histidine residues involved in interacting with the antigen, or indirectly, with histidine residues involved in maintaining the conformation of the CDRs. A crystal structure of tocilizumab Fab fragment in complex with IL-6R suggested that a mutation of the tyrosine residue in the CDR of tocilizumab, which interacts with an arginine residue, into a histidine residue resulted in electrostatic repulsion between the arginine and the protonated histidine residue at acidic pH (unpublished data). A total of three histidine residues and several other mutations were introduced into tocilizumab to compensate for the reduced affinity at neutral pH or to improve its physiochemical properties, and finally histidine-based engineered tocilizumab that rapidly dissociates from IL-6R at pH 6.0 was generated, as we previously reported [20]. The kinetic parameters of tocilizumab and the histidine-based engineered antibody are described in Table 1.

We have applied the same histidine-based engineering approach to several other antibodies against different antigens, and in all cases we have successfully conferred a pH-dependent antigen-binding property. However, in some cases, although introducing histidine(s) in the

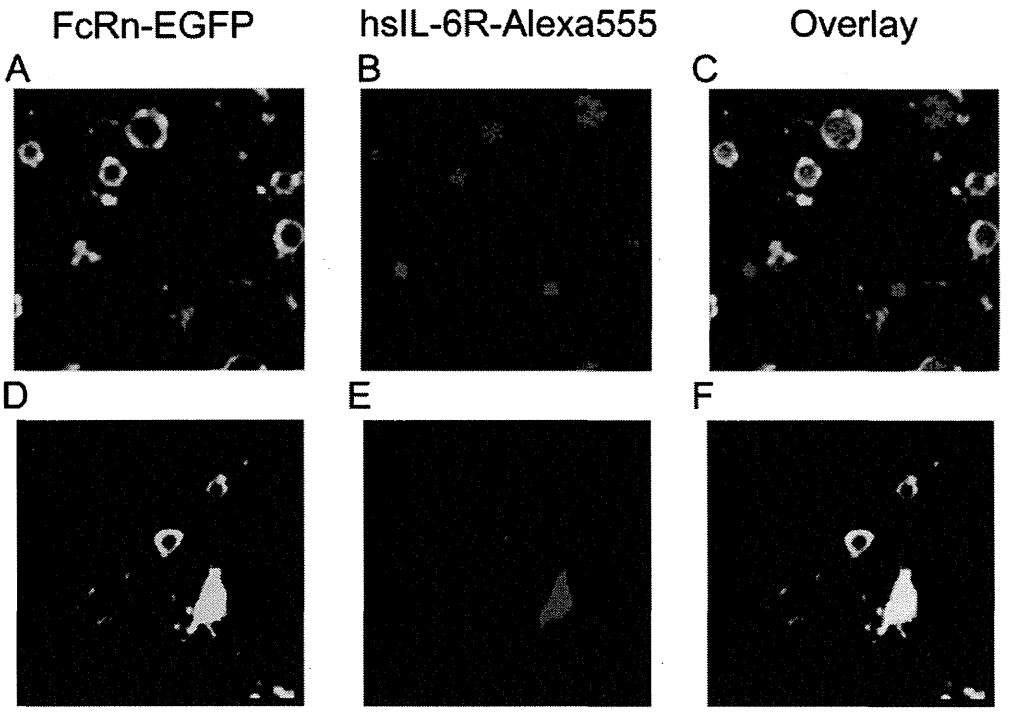

Fig. 4. The intracellular trafficking of an antigen in complex with either a conventional antibody or a pH-dependent antigen-binding antibody in MDCK transfected with human FcRn-EGFP (in green) and beta-2-microglobulin. (A–C) are images of representative endosomes after a pH-dependent antibody with enhanced FcRn binding was incubated in complex with its target antigen, hsIL-6R, which was labeled with Alexa-555 (in red). (D–F) are images of representative endosomes after a conventional antibody with enhanced FcRn binding was incubated in complex with hsIL-6R. (A) and (D) are images of FcRn-EGFP. (B) and (E) are images of hsIL-6R-Alexa555. (C) and (F) are overlays of the FcRn and hsIL-6R images. These are unpublished data.

appropriate position can provide pH dependency (i.e. reduction of the binding affinity at acidic pH), it simultaneously reduces the binding affinity to the antigen at neutral pH to some extent (but less than that at acidic pH). Although such an antibody would elicit a recycling property, the loss of binding affinity at neutral pH would reduce the biological activity of the antibody in plasma. If the biological activity does not meet the therapeutic goal, the binding affinity at neutral pH needs to be improved by the affinity maturation process.

Following our report, Chaparro-Riggers *et al.* reported their pH-dependent binding antibody against PCSK9, which was also generated through histidine-based engineering of the parent antibody [21]. Moreover, Devanaboyina *et al.* recently reported generating a pH-dependent binding antibody against IL-6 in the same way [22]. Changing a single aspartic acid to a histidine residue in HCDR3 conferred pH-dependent binding on IL-6, but the binding affinity to IL-6 at neutral pH was slightly reduced. Other histidine mutations tested in this report either did not display an increased pH dependency or resulted in a substantial loss of the binding affinity at neutral pH.

These reports verify that histidine-based engineering is a general approach for generating pH-dependent binding antibodies that demonstrate a recycling property. However, in some cases, a simultaneous loss of binding affinity at neutral pH may occur. Since the binding affinity at neutral pH is important for the biological activity of the antibody, this loss of binding affinity at neutral pH may need to be recovered by subsequent affinity maturation (discussed further in section 6).

The second approach for generating a pH-dependent binding antibody is to isolate the antibody directly from immunized animals. Our trial to screen a large number of antigen-binding antibodies suggested the feasibility of directly isolating rare pH-dependent antigen-binding antibodies (generally less than 5% of the binders) from immunized animals by using direct B cell-cloning technology, which allows antigen-binding antibodies to be identified more efficiently than conventional mouse hybridoma technology [38].

The third approach for generating a pH-dependent binding antibody is to isolate the antibody directly from a synthetic antibody phage library. Since histidine is rarely used in the CDR of human antibodies (0.5%–0.6%), the possibility of directly isolating a pH-dependent binding antibody from naïve or synthetic antibody libraries that mimic the human repertoire is low. Therefore, a library of synthetic antibodies that use more histidine in the CDR in combination with pH-dependent antigen panning (antigen binding at neutral pH and elution at acidic pH) can be used to directly isolate a pH-dependent antigen-binding antibody.

The fourth approach is to use a combinatorial antibody library to generate a pH-dependent binding antibody from a conventional antibody. Murtaugh *et al.* described a combinatorial histidine scanning library approach to engineer a conventional antibody into a pH-dependent binding antibody [39]. In their study, a VHH antibody against RNase A was used as a model antibody, and a combinatorial library was constructed that consisted of combinations of histidine and wild-type residues in the antibody–antigen interface. Antibodies displayed on phages were coselected for high-affinity binding and pH-dependent binding, which resulted in generating pH-dependent VHH antibody against RNase A. Although there was a slight loss in the binding affinity at neutral pH, this approach may be an efficient way to generate a pH-dependent binding antibody from a conventional antibody. Although a histidine-based pH-dependent binding antibody is a promising type of recycling antibody, we are exploring an alternative approach to designing an antibody that dissociates the antigen within sorting endosomes. While the recycling antibodies discussed above take advantage of the pH difference between plasma and endosomes, another environmental difference between plasma and endosomes is calcium ion concentration. Calcium ion concentration in plasma is reported to be 1.2–2 mM in plasma which drops to 3–30 μM in endosomes after endocytosis [40]. Therefore, a calcium ion-dependent binding antibody, which binds to the antigen at the calcium ion concentration in plasma and dissociates the antigen at the calcium ion concentration in endosomes, may be used to generate a recycling antibody.

4.2. Computational approaches

Significant progress has been achieved in the field of computational approaches that design functional proteins, so identifying a pH-dependent antigen-binding antibody by computational design could be an alternative approach. However, although computational design has already been applied to improve antibody–antigen interaction [4] and to generate pH-dependent binding proteins [41,42], there is no report to date of successfully designing a pH-dependent antigen-binding antibody. Strauch *et al.* computationally designed a pH-dependent protein that binds to the Fc region by utilizing histidine residues in the Fc region [41]. Sarkar *et al.* designed a pH-dependent granulocyte colony-stimulating factor (GCSF) that maintained the affinity to its receptor at neutral pH. To do this, they computationally identified negatively charged residues in the interface of GCSF and the GCSF receptor (GCSFR). If these negatively charged residues are replaced with histidine residues, the GCSFR binding affinity at neutral pH is expected to stay the same but the binding affinity at acidic pH will be reduced. They selected residues to mutate into histidine by comparing the free energy values at neutral and acidic pH of each histidine-mutated GCSF in complex with GCSFR [42]. A similar approach could be applied to provide antibodies with a pH-dependent antigen-binding property.

5. Advantages of pH-dependent antigen-binding antibodies and potential applications

pH-dependent antigen-binding antibodies have an advantage over conventional antibodies, especially when the target molecules are membrane-bound antigens or soluble antigens abundantly present in the body. Antibodies targeting these antigens require either a high dose that cannot be injected subcutaneously or frequent dosing, both of which are not patient-friendly, especially in chronic diseases.

When an antibody targets an antigen that is both membrane-bound and has a high rate of synthesis, such as IL-6R, EGFR, CD4, or CD40, the antibody is rapidly eliminated from plasma through antigen-mediated clearance [11–14]. In the case of tocilizumab, which targets IL-6R, neither improving the binding affinity to IL-6R nor reducing the non-specific clearance by increasing the binding affinity to FcRn at acidic pH significantly improved the duration of IL-6R neutralization *in vivo* [23]. This is because an antibody that targets IL-6R is mainly cleared from plasma by IL-6R-mediated clearance. Modeling and simulation suggested that the molar amount of IL-6R produced in the body within approximately 18 days is the same as 2 mg/kg of antibody. Since a conventional antibody can bind to the antigen only once, it is evident that,

Table 1
Kinetic parameters of parent antibody (tocilizumab) and histidine-engineered antibody (recycling tocilizumab).

	k_a at pH 7.4 (s^{-1} M^{-1})	k_d at pH 7.4 (s^{-1})	K_D at pH 7.4 (M)	k_d at pH 6.0 (s^{-1})	pH dependency (k_d at pH 6.0/k_d at pH 7.4)
Tocilizumab	2.2×10^5	1.1×10^{-3}	5.1×10^{-9}	2.3×10^{-3}	2.3
Recycling tocilizumab	5.3×10^5	1.0×10^{-3}	1.9×10^{-9}	1.5×10^{-2}	15

even if a conventional antibody has infinite binding affinity to IL-6R or very low non-specific clearance, a 2 mg/kg dose cannot be effective for more than 18 days. A pH-dependent binding antibody against IL-6R (SA237) that is recycled back to plasma after IL-6R-mediated uptake into cells, can bind to IL-6R multiple times, which allows 2 mg/kg of SA237 to remain effective for more than one month. Importantly, because the amount of an antibody that can be administered in a single subcutaneous injection is limited (generally 1 mL of 150 mg/mL IgG formulation), a pH-dependent binding antibody would be the only way to achieve monthly subcutaneous dosing for an antibody that targets IL-6R. The same strategy can be applied to other antibodies targeting membrane-bound antigens that are rapidly eliminated from plasma through antigen-mediated clearance.

An antibody that targets a soluble antigen with a high baseline plasma concentration, such as IgE or C5, would also require a high dose to neutralize the antigen [15,16]. To use omalizumab, an anti-IgE antibody, in patients with high plasma IgE concentration, subcutaneous injections at two or three sites are required, because plasma IgE concentration (in the range of sub μg/mL) is higher than the concentration of most cytokines (generally in the range of pg/mL) [16]. Similarly, eculizumab, an anti-complement C5 antibody, requires intravenous infusion, because plasma C5 concentration is reported to be 70–140 μg/mL [15]. Although eculizumab requires chronic dosing, such a large amount of antigen cannot be neutralized by a subcutaneous dose. Administering an antibody against a soluble antigen with rapid clearance, such as amyloid beta, MCP1, hepcidin, IL-6, CD23, or VEGF, results in more than 1000-fold increase of plasma antigen concentration from the baseline by the antigen accumulation effect [17,24–28]. Notably, administering an anti-hepcidin antibody resulted in an accumulation of hepcidin by approximately 5000-fold, which required a 300 mg/kg dose to neutralize hepcidin, despite its picomolar binding affinity [17]. Since soluble antigens with a molecular weight below 60,000 g/L are generally very rapidly eliminated through renal excretion [43], an antibody targeting a soluble antigen with a small molecular weight would suffer from the same issue. Taken together, conventional antibodies targeting soluble antigens with a high baseline concentration and/or a large antigen-accumulation effect require a high dose. Since conventional antibodies can bind to antigens only once and cannot accelerate the antigen clearance, pH-dependent binding antibodies would provide a significant advantage over conventional antibodies against these soluble antigens. pH-dependent binding antibodies would enable subcutaneous dosing or less frequent dosing that cannot be achieved by simple high-affinity conventional antibodies with a long half-life.

Recycling antibodies can be engineered into sweeping antibodies by Fc engineering to enhance the binding to Fc gamma receptor or FcRn. The use of a sweeping antibody against a soluble antigen further reduces the dosage compared to a recycling antibody, providing another advantage over a conventional antibody. Moreover, since a sweeping antibody can actively eliminate the target soluble antigen from plasma, a sweeping antibody against soluble IL-6R that targeted a non-neutralizing epitope and showed no neutralizing activity *in vitro* was able to antagonize the activity of soluble IL-6R *in vivo* by directly removing soluble IL-6R from plasma [23]. Therefore, a sweeping antibody can target a soluble ligand with multiple target epitopes, through which the ligand binds to different receptors that cannot be neutralized by a single monoclonal antibody. Administering a conventional antibody may neutralize the binding of a ligand to its receptor through one epitope, but another epitope remains. In addition, the increase of antibody–antigen complexes in plasma may allow increased signaling to the second receptor through the epitope that is not neutralized. The sweeping antibody, on the other hand, could directly remove such antigens from plasma, allowing multiple epitopes to be antagonized *in vivo*. The same strategy could be applied to toxic antigens that do not have a functional epitope. Similarly to an LDL receptor removing LDL or an asiologlycoprotein receptor removing asiologlycoprotein from plasma, a sweeping antibody could directly remove a toxic antigen from plasma to alleviate the disease condition [44,45]. Since these types of soluble antigen could not be therapeutic targets for conventional antibodies, sweeping antibodies may expand the target antigen space of antibody therapeutics.

6. Challenges in developing a pH-dependent antigen-binding antibody as a therapeutic

Although a pH-dependent antigen-binding antibody has attractive features, there are several challenges when generating the antibody for therapeutic application. The following challenges are specific to pH-dependent antigen-binding antibodies and need not be considered when developing conventional monoclonal antibodies.

First, histidine-based engineering to generate a pH-dependent antigen-binding antibody sometimes abrogates the binding affinity at neutral pH. In the case of the anti-IL-6 antibody, histidine mutagenesis conferred a recycling property onto the parental antibody, but at the same time it also reduced the binding affinity against IL-6 at pH 7.4 compared to the parent antibody [22]. If the binding affinity at neutral pH of a histidine-based recycling antibody is not sufficient to elicit the desired therapeutic activity, the binding affinity at neutral pH can be improved by the affinity maturation process, which is commonly applied to generate a potent therapeutic antibody [3,4]. Although a general affinity maturation strategy can be applied to recover the loss of binding affinity at pH 7.4, such mutations tend to also increase the binding affinity at acidic pH, which is undesirable. Nevertheless, we have been able to successfully generate high-affinity recycling antibodies with nanomolar K_D at pH 7.4 and simultaneously have sufficient pH dependency or large k_d at acidic pH by using histidine-based engineering and affinity maturation in all the antibodies targeting various antigens tested to date.

Second, the extent of pH-dependency required to dissociate the target antigens in acidic endosomes is not clear. An anti-IL-6R antibody with 20-fold larger K_D and 15-fold larger k_d at pH 6.0 than at pH 7.4 showed an improved pharmacokinetic property in cynomolgus monkeys and humans. PCSK9, an antibody with only 2.6-fold larger K_D and 12-fold larger k_d at pH 6.0 than at pH 7.4, showed a prolonged half-life in cynomolgus monkeys, although its binding affinity to cynomolgus monkey PCSK9 at pH 7.4 was also reduced. These results suggest that the pH-dependency in k_d is a more important factor than that in K_D for improving the pharmacokinetics of an antibody, but we assume that the absolute k_d value in acidic pH, not the relative pH-dependency of k_d (the ratio of k_d at pH 7.4 to that at pH 6.0), would also be important to achieve sufficient dissociation of the antigen. However, the absolute k_d value at pH 6.0 required to sufficiently dissociate an antigen from an antibody remains to be elucidated. The absolute K_D at pH 6.0 would also be important if we consider that the internalized antibody–antigen complexes reach a new equilibrium in the acidic endosomes. A detailed kinetic analysis of endosomal trafficking of antibody–antigen complexes would be required to identify the parameters necessary to sufficiently dissociate the antigens in the endosomes. Although these parameters could differ between the pH-dependent binding antibodies that target membrane-bound antigens, those that target soluble antigens, and the Fc-engineered sweeping antibodies, identifying these parameters would be important for generating pH-dependent binding antibodies with optimum *in vivo* properties.

Third, when considering therapeutic application, it would be necessary to predict antibody pharmacokinetics or the rate of antigen clearance in humans by preclinical study. This is often conducted in a pharmacokinetic study using cynomolgus monkeys; therefore, the antibody preferably needs to bind to a cynomolgus monkey antigen with a similar binding affinity as to the human antigen. Although cross-reactivity to cynomolgus monkey antigens is not so challenging for conventional antibodies, it could be more challenging for pH-dependent binding antibodies since the binding kinetics (both K_D and k_d) at pH 6.0 also need to be considered. In the case of the pH-dependent anti-PCSK9 antibody, since pH-dependency of K_D and k_d

and absolute k_d at pH 6.0 is somewhat different between humans and cynomolgus monkeys, predicting human PK/PD from a cynomolgus monkey study could be difficult. Moreover, with sweeping antibodies, since the Fc region is engineered to enhance the binding affinity to an Fc receptor, cross-reactivity of the Fc region also needs to be considered.

7. Conclusion

Recycling antibodies with pH-dependent antigen binding offer significant advantages over conventional antibodies by dissociating the antigens in endosomes and recycling free antibodies back to plasma. Although generating a high-affinity pH-dependent antigen-binding antibody is still challenging, various approaches are currently available and have been successfully applied to various antigens. Because affinity maturation has now become a basic technology for engineering improved antibody therapeutics, pH-dependent binding antibodies should play an important role as a novel modality for antibody therapeutics in the near future.

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Journal of Thrombosis and Haemostasis, **12**: 206–213 DOI: 10.1111/jth.12474

ORIGINAL ARTICLE

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

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To cite this article: Muto A, Yoshihashi K, Takeda M, Kitazawa T, Soeda T, Igawa T, Sakamoto Y, Haraya K, Kawabe Y, Shima M, Yoshioka A, Hattori K. Anti-factor IXa/X bispecific antibody (ACE910): hemostatic potency against ongoing bleeds in a hemophilia A model and the possibility of routine supplementation. *J Thromb Haemost* 2014; **12**: 206–13.

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

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

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Received 25 April 2013
Manuscript handled by: L. Aledort
Final decision: P. H. Reitsma, 27 November 2013

Introduction

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

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

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

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

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

Materials and methods

Materials

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

Animals and ethics

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

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

APTT and thrombin generation (TG) assays

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

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

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

Pharmacokinetic study

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

Statistical analysis, pharmacokinetic analysis, and multiple-dosing simulation

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

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

Results

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

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

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

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

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

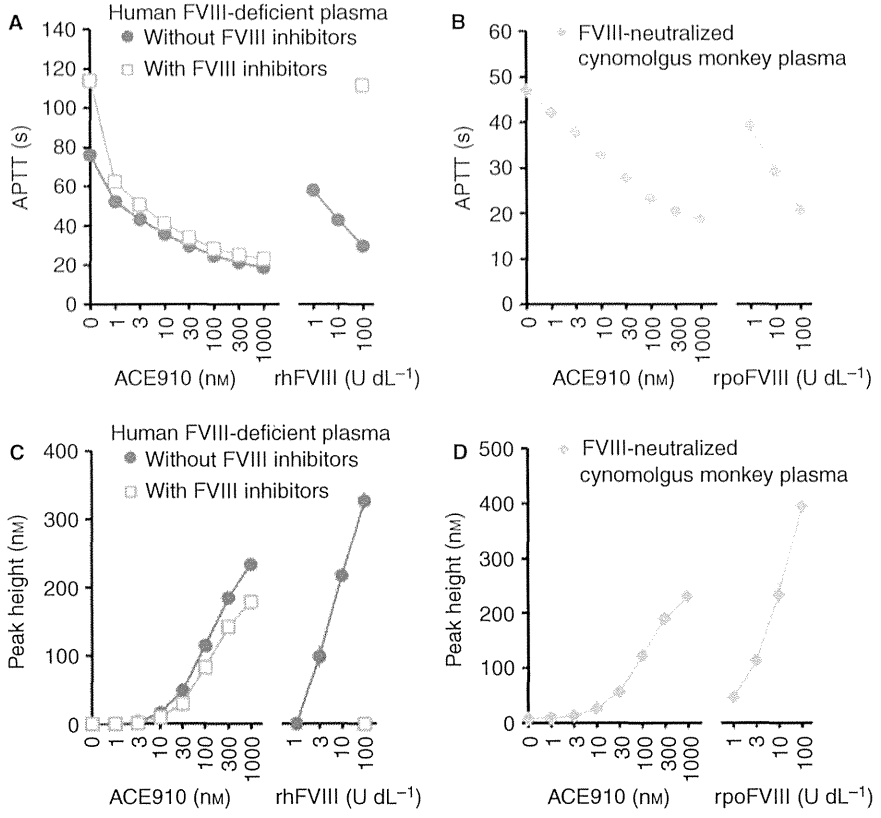

Fig. 1. FVIII-mimetic cofactor activity of ACE910 in human FVIII-deficient plasma without and with FVIII inhibitors and in FVIII-neutralized cynomolgus monkey plasma. Effects of ACE910, recombinant human FVIII (rhFVIII) or recombinant porcine FVIII (rpoFVIII) on activated partial thromboplastin time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

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

Pharmacokinetic study and multiple-dosing simulation

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

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

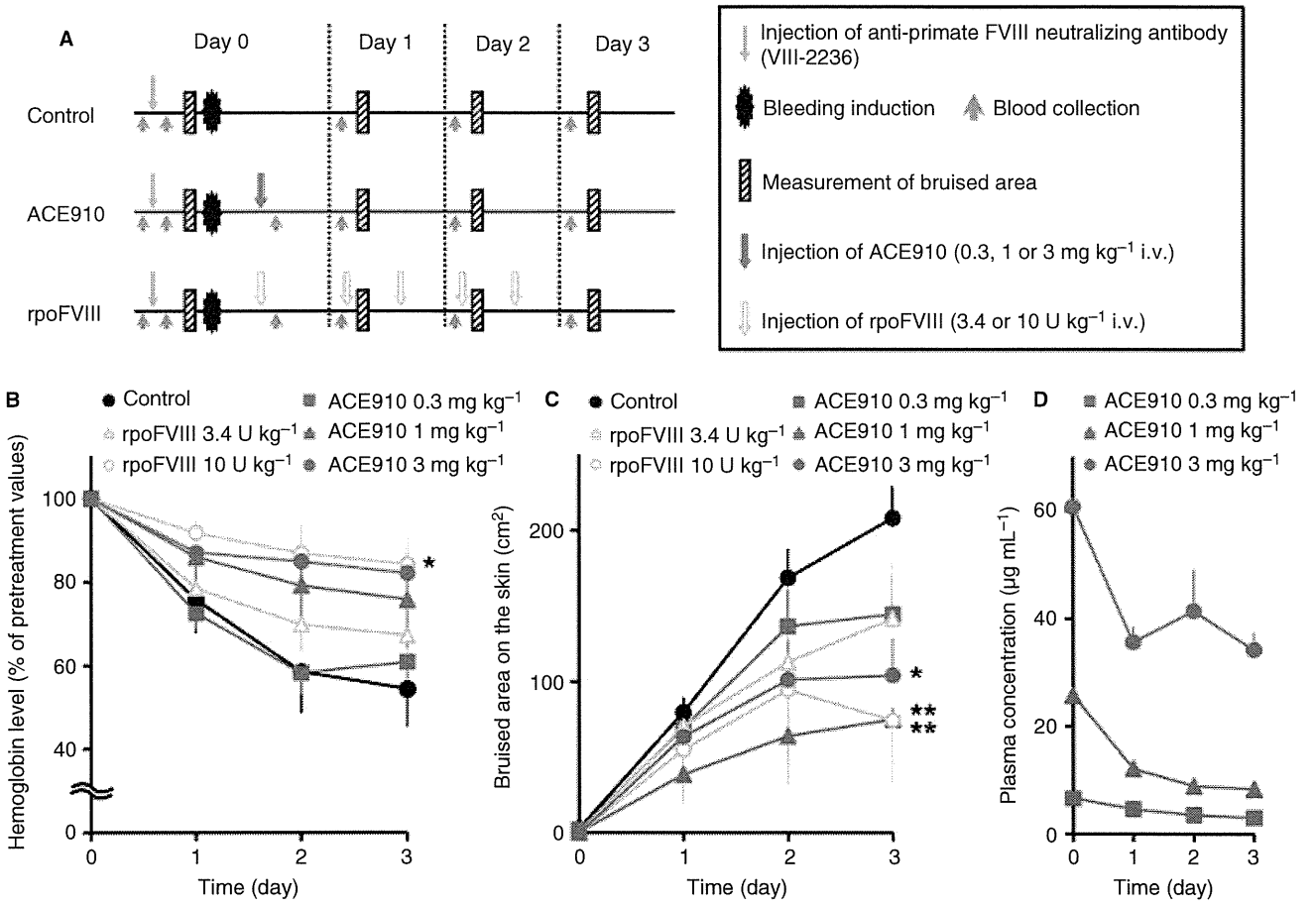

Fig. 2. *In vivo* hemostatic activity of ACE910 against ongoing bleeds in an acquired hemophilia A model. (A) The experimental protocol used. (B, C) Time course changes of (B) hemoglobin level and (C) bruised areas in the control group (no test item; $n = 6$), the ACE910 group (0.3, 1 or 3 mg kg^{-1};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

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

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

Discussion

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

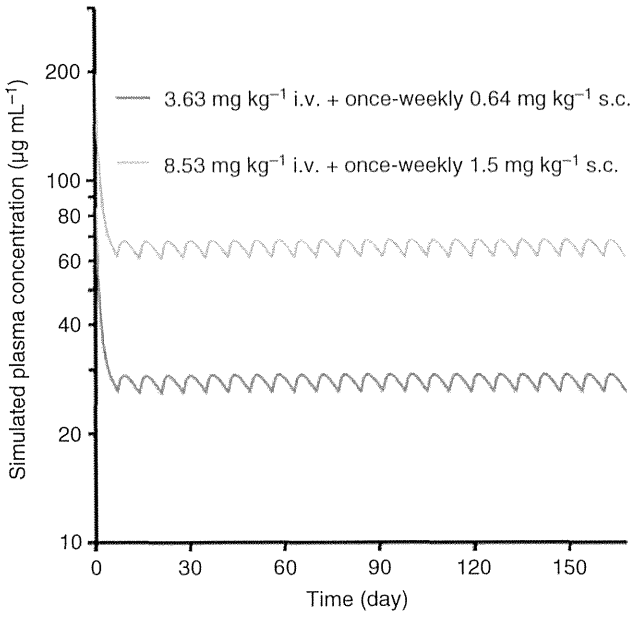

Fig. 3. Examples of simulations; plasma ACE910 concentration after multiple dosing in cynomolgus monkeys. The time course of plasma ACE910 concentration was simulated by use of the pharmacokinetic study data in cynomolgus monkeys for the case of once-weekly subcutaneous (s.c.) administration at 0.64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

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

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

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

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

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

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

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

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

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

Addendum

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

Acknowledgements

We thank our colleagues at Chugai Pharmaceutical – T. Matsuura, M. Hiranuma, R. Takemoto, T. Koike, H. Kitamura and T. Houjo for the *in vivo* pharmacologic experiments; Z. Sampei, T. Kojima, T. Wakabayashi, E. Tanaka, K. Esaki, Y. Kikuchi, A. Sakamoto, M. Wada, M. Goto, H. Tsunoda, T. Suzuki, Y. Okuyama-Nishida, A. Harada, M. Funaki, S. Suzuki, T. Toyoda, Y. Higuchi and M. Ijiri for the preparation of test items and the *in vitro* experiments; T. Tachibana for the pharmacokinetic studies; and H. Saito for providing advice for the various experiments.

Disclosure of Conflict of Interests

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

Supporting Information

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

Table S1. Pharmacokinetic parameters of ACE910 in cynomolgus monkeys.

Fig. S1. Effects of ACE910 or rhFVIII on peak height of thrombin generation triggered by low TF in human FVIII-deficient plasma without FVIII inhibitors.

Fig. S2. SDS-PAGE analysis of rpoFVIII. Purified rpoFVIII (0.95 μg) was analyzed by SDS-PAGE with 4–20% gradient gel under reducing conditions, followed by staining with Coomassie brilliant blue.