Studies on factor VIII toward a new therapeutic for hemophilia A

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Abstract: Factor VIII (FVIII) is an essential glycoprotein cofactor that accelerates the generation of factor Xa (FXa) by factor IXa (FIXa). FVIII is transformed into its active form, FVIIIa, after limited proteolytic cleavages by thrombin and FXa at the site of bleeding. My early research, using monoclonal antibodies, recombinant/synthetic polypeptides and anhydro-proteins, identified critical FVIII binding regions for von Willebrand factor (VWF), phosphatidylserine, thrombin, FXa, FIXa, APC and plasmin. The studies led to the generation of a humanized bispecific antibody to FIX(a) and FX as a new therapeutic for hemophilia A patients with and without FVIII inhibitor. The antibody, now termed emicizumab, mimics FVIIIa cofactor function by arranging FIXa and FX in a spatially suitable position, thereby accelerating FIXa-catalyzed FX activation. Phase 1 studies in healthy volunteers and in patients with hemophilia A were performed in Japan. Bleeding rates were remarkably reduced dose-dependently by weekly subcutaneous injection of the antibody irrespective of the presence FVIII inhibitor. The efficacy and tolerability and safety were further confirmed by international phase 3 studies in a total of 400 participants. Emicizumab prophylaxis has now become one of the important therapeutic options for hemophilia A with and without inhibitors.

Key words: factor VIII, haemophilia A, emicizumab, bispecific antibody, inhibitor

1. The role of FVIII in the coagulation process

The traditional cascade theory of blood coagulation helps explain the role of each clotting factor. Current understandings, however, focus on a “cell-based model” of intravascular reactions. In this context, the hemostatic effects of recombinant activated FVII (rFVIIa) in patients with factor VIII (FVIII) or factor IX (FIX) deficiencies (hemophilia A and B), are believed to center on reactions on platelet surfaces. Initially, FVIIa and tissue factor (TF) complexes exposed at sites of bleeding promotes factor X (FX) and FIX activation, and the generated activated factor X (FXa) is incorporated into the prothrombinase complex. The amount of thrombin produced by this reaction is relatively low, however, and is insufficient for effective fibrin formation. Instead, this minimum amount of thrombin activates platelets, factor V (FV), factor XI (FXI) and FVIII. Subsequently, activated factor XI (FXIa) activates factor IX (FIXa), promotes the tenase reaction, and in the presence of FVIIIa results in a thrombin burst that facilitates fibrin formation. FVIII is commonly known as a “cofactor” in the tenase complex. Kinetic expriments have demonstrated, however, that in the presence of FVIIIa the Vmax in FIXa-catalyzed FX activation is enhanced approximately $2.0 \times 10^5$ fold. Consequently, defects in the nature of FVIII would be expected to impair thrombin generation and cause a severe bleeding tendency.

2. Structure and function of FVIII

FVIII circulates in plasma as a non-covalent complex with von Willebrand factor (vWF). VWF stabilizes the synthesis and cofactor activity of FVIII. Mature FVIII is synthesized as a single chain polypeptide with molecular...
mass of 300 kDa consisting of 2,332 amino acid residues. Amino acid homology shows that FVIII has a mosaic domain structure arranged in the order of A1-A2-B-A3-C1-C2 (Fig. 1). In addition, the Lch is cleaved by FXa at Arg1721 producing a 67-kDa Lch fragment. The cleavages at Arg372 and Arg1689 are crucial for the pro-coagulant activity of FVIIIa.

Fig. 1  Domain structure of FIII and thrombin cleavage patterns of FVIII with and without addition of FVIII inhibitor alloantibodies
A: According to the amino acid homology, FVIII has a domain structure arranged in the order of A1-A2-B-A3-C1-C2. There are three highly acidic regions, a1, a2 and a3. Thrombin cleaved FVIII at Arg 372, Arg740 and Arg1689 and transformed to active form of FVIII consisting of 50 kDa and 40 kDa fragments of heavy chain and 72 kDa light chain fragment.
B: FVIII inhibitor alloantibodies, NF, inhibited proteolytic cleavage at Arg1689, but not at Arg372.

3. The role of the C2 domain in the FVIII light chain

FVIII associates with multiple ligands, including VWF, PL and various clotting and anti-coagulant factors. The functionally important regions of FVIII have been clarified using neutralizing monoclonal antibodies and inhibitor alloantibodies, and we have produced various kinds of monoclonal antibodies against the light chain of FVIII for this purpose. Two types of monoclonal anti-
bodies were developed based on binding regions. Firstly, antibodies designated, NMC-VIII/1 (-VIII/7 and 8–10), recognized the 80 kDa light chain but not the thrombin-cleaved 72 kDa fragment. The amino-terminal region of the light chain is cleaved by thrombin at Arg1689, and therefore, these specific antibodies were expected to recognize the acidic region of the molecule. Hence, competitive assays were devised using synthetic peptides spanning the acidic region in the A3 domain in an attempt to localize the epitopes of these antibodies. The findings demonstrated that all three antibodies completely inhibited the binding of factor VIII to immobilized VWF, and implicated that a peptide containing the fifteen amino acid residues from the Val1670-Glu1684 governed these FVIII binding reactions. The data supported the results of alternative studies suggesting that the acidic region of the amino-terminal A3 domain containing Tyr1680 was an essential region for VWF binding.

The second type of monoclonal antibody, designated NMC-VIII/5 and 6, recognized both the 80 kDa light chain and thrombin-proteolyzed 72 kDa fragments. These antibodies competed with a FVIII inhibitor alloantibody, TK, in binding to FVIII, and we determined that the monoclonal antibodies and the alloantibody inhibited FVIII binding to both VWF and PL. These results indicated that a further VWF binding site was located in the 72 kDa light chain, and that this site was in close proximity to the PL binding region.

Precise epitopes were not identified using these synthetic peptides in competitive assays, however, and our investigations were extended, therefore, utilizing a panel of recombinant C2 domain proteins with fragments deleted from amino-terminal or carboxy-terminal ends. These studies localized the binding sites of NMC-VIII/5 and 6 to amino-acid residues 2170 and 2327. Similarly, we found that the binding region of the alloantibody TK, was contained within amino-acid residues 2248–2312. NMC-VIII/5 and 6, and TK all inhibited FVIII binding to VWF and PL. Since TK also recognized the carboxy-terminus of the C2 domain, we investigated whether other inhibitor alloantibodies have similar binding regions, and demonstrated that residues 2308–2312 were critical for inhibition of VWF binding. These data strongly indicated that the C2 domain contained both VWF and PL binding sites. Subsequently, our collaborators led by Dr Evgueni Saenko, demonstrated that the recombinant C2 domain bound directly to VWF. The three-dimensional structure of the C2 domain has been determined by crystallization, and this molecular segment is now widely recognized as an essential functional region in FVIII.

4. Identification of thrombin and FXa binding sites

As described above, thrombin has an essential role in activating FVIII by limited proteolytic cleavage at Arg372 and Arg1689. The mechanism of FVIII activation by thrombin was not further clarified until 2000, however. Earlier studies were hampered by the lack of reliable methods for assessing direct binding reactions between thrombin and FVIII. We described a FVIII inhibitor obtained from a patient with hemophilia A that recognized the C2 domain and inhibited thrombin cleavage at Arg1689 but not at Arg372 (Fig. 1). These results indicated that the C2 domain contained an essential site for thrombin cleavage at Arg1689, separate from Arg372, and we established direct binding experiments illustrating that the catalytically inactive form of anhydro-thrombin bound to the C2 domain but not to the A1 nor A2 domains within the heavy chain. Furthermore, we demonstrated that the C2 domain alloantibody inhibited thrombin cleavage at Arg1689. In addition, the nature of these interactions was also examined using inhibitor-IgG purified by affinity chromatography with immobilized C2 domain. The affinity-purified F(ab)’2 IgG prevented thrombin cleavage at Arg 1689. These findings were the first to provide conclusive evidence that the C2 domain contains the thrombin binding site responsible for the cleavage at Arg1689.

FXa also has a role in FVIII activation by limited proteolysis. In addition to the thrombin cleavage sites at Arg372, Arg740 and Arg1689, FXa has a unique cleav-
age site at Arg1721 producing an 80 kDa-derived 67 kDa fragment. FXa-dependent FVIII activation is different from thrombin-dependent FVIII activation in several ways. The magnitude of FXa catalyzed activation is less than that by thrombin, however, and FXa cleaves only the free form of FVIII, and does not catalyze the activation of FVIII in the FVIII/VWF complex. Our experiments showed that a monoclonal antibody, ESH8, recognizing a C2 epitope within Val2246-Gky2285 inhibited FVIII activation by FXa\textsuperscript{20}. Previous studies had demonstrated that neutralizing monoclonal antibodies and FVIII inhibitor IgGs recognizing the C2 domain inhibited binding of FVIII to VWF and to PL, but ESH8 did not inhibit this binding to either ligand. Our studies were extended, therefore, to investigate the inhibitory effect of ESH8 on FXa-catalyzed proteolysis using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). We found that ESH8 completely inhibited FXa-catalyzed cleavage at Arg 1689 and Arg 1721 in the Lch. Furthermore, the antibody also partially inhibited the cleavage at Arg 372 in the Hch in the presence or absence of PL. Another monoclonal antibody recognizing the C2 domain (NMC-VIII5), and other neutralizing antibodies recognizing the amino terminal acidic region of the 80 kDa fragment (NMC-VIII/10), or the A1 (C5) and A2 (JR8) domains had no effect on FXa-related proteolysis. The C2 domain contains a PL binding site, however, and FXa-catalyzed FVIII proteolytic cleavage occurs at a faster rate in the presence of PL. In these circumstances, therefore, indirect inhibition due to inhibition of PL binding by ESH8 could not be ruled out. Further experiments confirmed, however, that ESH8 inhibited FXa-catalyzed FVIII cleavage in the absence of PL. Our results strongly indicated that the C2 domain contained an essential region for FXa binding to FVIII, and therefore, we further developed our direct binding assays using anhydro-FXa. Both 80 kDa and 72 kDa Lch and recombinant C2 dose-dependently bound to anhydro-FXa. This was the first study to identify the FXa binding site in FVIII.

5. Identification of other serine protease binding sites

1) Activated protein C (APC) and VWF

APC is an essential natural anti-coagulant enzyme that inactivates both FVIIIa and FVa by limited proteolysis. Cleavage of FVIIIa by APC occurs at Arg 336, but the procoagulant is protected by complex formation with VWF in the circulation. Our original studies using auto-antibodies developed in three patients with acquired hemophilia A demonstrated that in these circumstances FVIII circulates in plasma as an immune complex\textsuperscript{21}. We found that these three antibodies blocked FVIII binding to anhydro-APC, and consequently inhibited APC catalyzed FVIII inactivation. Furthermore, we illustrated that these antibodies bound to synthetic peptides comprising amino acid residues His2009-Val2018, which contains the APC binding site. Based on these findings, our research then focused on the mechanisms by which VWF protected FVIII from APC inactivation. We hypothesized that VWF inhibited APC binding to FVIII, and we performed binding experiments using anhydro-APC in the presence and absence of VWF. As expected, VWF dose dependently inhibited the binding of anhydro-APC to FVIII\textsuperscript{22}, and we localized the binding region of VWF responsible for the protection from APC activity to amino acid residues 2013–2022. Previous reports had shown that APC binding sites were localized within 2009–2018, and our data were in keeping with the concept that the regions for binding of APC and VWF overlap this essential sequence. Furthermore, our results provided the first evidence that the protective effect of VWF on APC-catalyzed FVIII inactivation is mediated by competitive APC binding to FVIII.

2) Plasmin

Plasmin functions as a key enzyme in fibrinolysis. Our studies revealed that FVIII activity was increased approximately two-fold by plasmin for an initial three minutes before falling to undetectable levels\textsuperscript{23}. SDS-PAGE analysis demonstrated that plasmin cleaved FVIII in a similar manner to that of FXa. In contrast to FXa-
catalyzed proteolytic cleavage of FVIII, however, plasmin-catalyzed cleavage at Arg336 proceeded faster than that at Arg372. Furthermore, the cleavage at Arg 336 and Lys 36 occurred rapidly in the presence of A2 and A3-C1-C2 subunits. This was the first study to highlight the role of plasmin in the negative regulation of coagulation through proteolytic inactivation of FVIII. We later demonstrated by direct binding experiments using anhydro-plasmin that plasmin-interactive sites in the Lch of factor VIII were responsible for proteolytic cleavage at Lys 36.

3) FIXa
Binding of FVIIIa to FIXa is essential for the PL surface-dependent activation of FX. The C2 domain of FVIII and the Gla domain of FIX are believed to contain PL binding sites, and we hypothesized that the FIXa binding site also locates to the C2 domain. Our experiments demonstrated that Glu-Gly-Arg active site-modified FIXa bound to recombinant C2 dose dependently under physiological conditions. Furthermore, Gla-domainless FIXa failed to bind to the C2 domain. Using V8 protease-cleaved C2, we demonstrated that peptide 2228–2240 inhibited FIXa binding to C2 and FXa generation. These results indicated that residues 2228–2240 in the FVIII C2 domain constitutes an interactive site for the Gla domain of FIX.

4) FVIIa
FVIIa acts as a pivotal clotting factor in the initial stage of the clotting reaction by forming a complex with TF. This complex plays a role in the activation of FVIII by limited proteolysis, and our investigations clarified the mechanism of FVIIa dependent FVIII activation. FVIII was activated approximately 4-fold within an initial 30 seconds incubation with FVIIa/TF. In addition, SDS-PAGE analysis, demonstrated that proteolytic cleavage by FVIIa/TF at Arg 740 and Arg372 was more rapid than that induced by thrombin. Furthermore, we showed that VWF inhibited FVIIa/TF-catalyzed FVIII activation, indicating a further difference between mechanisms involving FVIIa/TF and thrombin, and highlighting the potential role of FVIIa/TF in priming FVIII activation in the very early phase of blood coagulation.

6. Current issues in hemophilia A treatment
Regular prophylaxis using standard recombinant FVIII (rFVIII) or rFVIII with extended half-life (rFVIII-EHL) constitutes the primary treatment for non-inhibitor patients. Several unmet issues and limitations remain with the current prophylactic protocols, however. The need for frequent intravenous infusions may be especially problematic in pediatric patients and older adults who depend on support from caregivers. Moreover, long-term protection from progressive hemorrhosis remains a serious clinical concern. A further critical objective for early prophylactic intervention centers on the risk of intracranial hemorrhage (ICH) in neonates and young infants. Retrospective analyses have demonstrated that the median age at the onset of ICH was two years (IQR: 0.6–7.3) and that 40% of the incidents occurred before the age of two years. Finally, the development of FVIII inhibitors persists as an extremely difficult challenge in the hemostatic management of hemophilia A, due to ineffectiveness of FVIII concentrates in these circumstances.

7. Pre-clinical development of emicizumab
The ideal therapeutics for patients with hemophilia A may be considered to include easier injectability, effectiveness irrespective of the presence of FVIII inhibitor, and higher trough levels of coagulation activity. For these reasons, Chugai Pharmaceutical Company developed a recombinant bispecific antibody recognizing factor IXa and factor Xa. The company had already established the necessary novel technology for producing recombinant bispecific antibodies, and several initial concepts regarding physiological reactions related to FVIII activity prompted investigations into applying these immunological agents for the treatment of hemophilia A. Principally, the central perception was based on the hypotheses that FVIIIa aligns FIXa and FX in a spatially suitable position to accelerate FIXa-catalyzed FX activation, and that the distance between the FIXa-
FX-binding sites in the FVIIIa molecule is similar to that between the two antigen-binding sites of human IgG.

The project developing the antibody commenced around the year 2000. In outline, a total of 460 bispecific antibody clones were examined, leading to the production of 20 non-neutralizing anti-FIXa and 23 anti-FX monoclonal antibodies. Chromogenic assays identified one clone, XB12, which shortened the prolonged APTT of FVIII deficient plasma. Collaborative work with Nara Medical University started in 2003. Unfortunately, however, experiments in vivo were not successful and the project was halted. A number of difficulties became apparent including the critical spatial positioning of FIXa and FX, together with the non-neutralizing properties and the requirement not to affect the functions of FIX and FX at higher concentrations. Nevertheless, we considered that more effective antibodies might be produced by further analyzing antibody clones to assure enough diversity of the variable sequence regions. Hence, the Chugai investigators screened a total of 40,000 clones, and produced approximately 200 anti-FIXa and 200 anti-FX antibodies from various animals. One especially promising antibody, hBS23 was developed that was almost identical to currently used “emicizumab”\textsuperscript{30}. Experiments in vivo using a primate model of acquired hemophilia A produced by the administration of neutralizing anti-FVIII antibodies, demonstrated that hBS23 prevented the fall in hemoglobin levels after blood loss. The effect was similar to that observed by infusions of recombinant porcine FVIII. Further antibody engineering to improve manufacturability led to final version of the biphasic antibody, ACE910, suitable for clinical trials.

8. Characterization of emicizumab

Although ACE910 mimics FVIIIa, the mode of action of the antibody is different than that of the natural procoagulant, especially in that it does not depend on activation by thrombin or FXa. Emicizumab exerts cofactor activity at the beginning of coagulation cascade involving FVIIa/TF-derived FIXa (Fig. 2). This early reaction is subsequently maintained and propagated by FIXa promoted by FXa, and results in a thrombin burst. Our studies showed that thrombin generation was substantially decreased in FVIII deficient plasma immunodepleted of FXI, indicating that FXIa-derived FIXa is crucial for full emicizumab potential\textsuperscript{31}. Binding affinity of emicizumab is also different from that of FVIII. Emicizumab binds to FIX/FIXa and FX/FXa with moderate strength with calculated $K_D$ values of 1.58/1.52 and 1.85/0.978 μM, respectively, indicating that the antigen binding affinities of emicizumab were much greater than those of other antibody therapeutics\textsuperscript{32}. Hence, generated FXa is released quickly from the FIXa–emicizumab–FX ternary complex, and is consequently supplied to downstream mechanisms of the coagulation cascade. The enzymatic kinetic potentials of emicizumab in FIXa-catalyzed FXa generation, $K_{cat}$ (μM) and $K_{cat}/K_{m}$ (μM), are 2.88 and 570, respectively. These values are 1/44 and 1/11 of FVIIIa, respectively. Moreover, emicizumab potential is not affected by VWF and is not inhibited by APC. However, emicizumab-triggered coagulation reactions appear to be negatively regulated by TFPI and AT\textsuperscript{33}. Furthermore, FVa is more strongly inhibited by APC in the presence of emicizumab than in the presence of FVIIIa\textsuperscript{34}.

9. Emicizumab prophylaxis and phase 1/2 clinical trials

Our phase 1 studies of emicizumab in healthy individuals confirmed the necessary safety profile, and demonstrated advantageous PK properties with a half-life of approximately 30 days after a single subcutaneous injection\textsuperscript{35}. In this study, the prophylactic use of the antibody was also explored in hemophilia A patients with and without FVIII inhibitor\textsuperscript{36}. Three dosing schedules were examined with weekly subcutaneous injections. Cohort 1 was the lowest dosing arm at 0.3 mg/kg after loading at 1.0 mg/kg. Cohort 2 was the middle dose at 1.0 mg/kg after loading at 3.0 mg/kg, and Cohort 3 was the highest dosing arm at 3.0 mg/kg, again after an initial 3.0 mg/kg.
In each cohort, six patients with or without FVIII inhibitors were enrolled, and observed for 24 weeks. The median annualized bleeding rates (ABR) in cohorts 1, 2, and 3 decreased from 32.5 to 4.4, 18.3 to 0.0, and 15.2 to 0.0, respectively. Plasma mean trough levels of emicizumab (±SD) in cohorts 1, 2 and 3 were 10.3 ± 4.54, 29.9 ± 6.88 and 87.9 ± 20.0 μg/mL, respectively. The FVIII equivalent activity of emicizumab was estimated at approximately 0.3 IU/dL/μg/mL, and the steady state FVIII equivalent level in each cohort, therefore, was estimated to be 3, 9 and 26 IU/dL, respectively. The trough levels of activity in these instances were much higher than those of current regular prophylactic treatment with FVIII concentrates. The overall results of safety and efficacy were satisfying, and therefore, international phase 3 studies were commenced. The data from these phase 3 clinical studies indicated that maintenance at an estimated FVIII equivalent activity of approximately 13–15 IU/Dl resulted in zero treated joint bleeding. Plasma concentrations of 45–50 μg/mL emicizumab were calculated, therefore, to correspond to a FVIII equivalent activity of 12–15 IU/dL. The three dosing regimens had been determined by pharmacometric simulation. Firstly, the loading weekly injection at 3.0 mg/kg for four weeks followed by weekly injections at 1.5 mg/kg (QW). Secondly, infusions every two weeks at 3.0 mg/kg (Q2W) after the same loading dose, and lastly, every four weeks at 6.0 mg/kg, again after loading at 3.0 mg/kg for four weeks. This alternative to conservative dose-finding studies provided sufficient data for undertaking the phase 3 studies without performing orthodox phase 2 investigations.

10. Phase 3 clinical trials

1) HAVEN 1 study

In the HAVEN 1 study the target patients were over twelve years old. The ABR in the emicizumab prophylaxis group was 2.9 (1.69–5.62). The annualized bleeding rate (ABR) in participants who were randomly assigned to emicizumab prophylaxis was 2.9 events compared with 23.3 events among those assigned to the on-demand group treated with BPAs, representing a significant difference of 87% in favor of emicizumab prophylaxis (P < 0.001). Furthermore, the percentage of zero treated bleeds in the emicizumab-treated group was 62.9%. Intra-individual comparisons between prior BPA prophylaxis and emicizumab prophylaxis indicated that the reduction in ABR was 79%. Most of the adverse events recorded during the observation period were mild,
although three patients developed thrombosis and three developed TMA. All of these patients received high or longer doses of BPA for breakthrough bleeding.

2) HAVEN 2 study

The HAVEN 2 trial was preformed to confirm safety and efficacy of emicizumab prophylaxis in pediatric patients with inhibitor less than 12 years old\(^\text{40}\). The median age of the enrolled patients was 6.0 years, and 11.8% of the patients were less than two years old\(^\text{28}\). The numbers of patients in the three dosing groups (once weekly, every two weeks and every four weeks) were 68, 10 and 10 respectively. In the weekly injection group, the ABR was 0.3 (median 0.0 (0.00–0.00)). The percentage of patients with zero treated bleeding was 49.2%. Intra-individual bleeding events before and after emicizumab prophylaxis were compared in 15 patients receiving BPA prophylaxis. The reduction ABR was 99% compared with that prior to BPA prophylaxis. The data demonstrated, therefore, that the prophylactic efficacy of weekly injections in this cohort was more remarkable than in patients over 12 years old.

3) HAVEN 3 study

The initial phase 1 study had indicated that emicizumab prophylaxis could be effective in patients both with and without FVIII inhibitors, and the results suggested, therefore, that emicizumab would be effective in non-inhibitor patients. Consequently, the HAVEN 3 study was undertaken in adolescent and adult patients over 12 years old\(^\text{41}\). The participants who had received episodic treatment with FVIII (n = 89) were randomly allocated to three groups, including weekly injection (QW) at a maintenance dose 1.5 mg/kg, treatment every two weeks (Q2W) at a maintenance dose of 3.0 mg/kg, and a no-prophylaxis group. The mean ABR of QW and Q2W was 1.5 and 1.3, respectively. The reduction in ABR in the two dosing groups compared with no-prophylaxis cohort was 96 and 97%, respectively. A total of 56% of the participants in QW and 60% of those in Q2W had no treated bleeding events. Forty-eight patients received FVIII prophylaxis prior to participation. In this study group, emicizumab was fixed at the QW dose of 1.5 mg. The ABR in the model group before and after emicizumab treatment was 4.8 and 1.5, respectively, and zero bleeding events were reported in 40% and 54%, respectively. The differences were statistically significant (P < 0.0001). Furthermore, the percent of individuals with 0–3 bleeding events were 73 and 92, respectively. No TMA or thromboembolic events were recoded. The data from the HAVEN 3 study indicated, therefore, that emicizumab offers an alternative to conventional FVIII in non-inhibitor patients for regular prophylaxis.

4) HAVEN 4 study

This study was performed to explore the benefits of emicizumab treatment every four weeks in individuals or caregivers who have difficulties with more frequent injections. The target patients in this study were over 12 years old with or without inhibitors\(^\text{42}\). Participants were initially recruited from Japan and Spain, and subsequently expanded to include 41 patients from 17 sites in Australia, Belgium, Japan, Poland, Spain, and the USA. In the expanded cohort, FVIII inhibitors were identified in 5 and 36 patients respectively. The median (range) number of bleeds in these 41 patients 24 weeks before study entry was 5.0 (0–90), and 25 patients (61.0%) had at least one target joint. Analysis of the trial data demonstrated that the model-based ABR for treated bleeds was 2.4 (95% CI 1.4–4.3). There were no treated bleeds in 23 patients (56.1%;95% CI 39.7–71.5%), and 37 patients (90.2%) had less than 3 treated bleeds. Thirty-four patients (82.9%) reported zero spontaneous bleeds. The most common treatment–related adverse events were injection-site reactions. Thus, the data from the HAVEN 4 study demonstrated that every 4-week dosing was feasible and effective in protecting from bleeding, and that this protocol would be beneficial to patients and caregivers.

5) HOHOEMI study

In the HAVEN 3 study, children less than 12 years old were not included. Consequently, the conclusions are lacking comprehensive data for non-inhibitor pediatric patients. Emicizumab prophylaxis could be especially important in early childhood, however, and the phase 3,
HOHOEMI study was performed in Japan, therefore, to include this category of patient\(^{43}\). Eligible participants were non-inhibitor patients less than 12 years old, and there were two dosing arms. Emicizumab was administered subcutaneously, with four loading doses of 3 mg/kg every week followed by maintenance doses of 3 mg/kg every 2 weeks (Q2W) or 6 mg/kg every 4 weeks (Q4W) in 6 and 7 patients, respectively. The median age (range) was 6.6 (1.5–10.7) and 4.1 (0.3–8.1), respectively. There was one child less than 2 years old in the Q2W cohort, and 2 patients of this age in the Q4W group, including a 4-month-old previously untreated patient (PUP). The model-based treated ABR (95% CI) was 1.3 (0.6–2.9) and 0.7 (0.2–2.6) in Q2W and Q4W respectively, and zero treated bleeding events were recorded in two out of six (Q2W) and five out of seven (Q4W) patients. There were no thromboembolic events nor TMA. The data from this study provided useful information for decisions on early prophylactic treatment designed to protect from ICH and maintain intact orthopedic joints.

11. Long-term observation of emicizumab prophylaxis

Assessments of long-term safety and efficacy of new therapeutics are crucial. Eighteen patients who participated in the phase 1 and phase 1/2 extension trials received emicizumab for up to 5.8 years\(^{36, 44, 45}\). In the phase 1 investigations, six patients were enrolled in each of three dosing groups; 0.3 mg/kg, 1 mg/kg and 3 mg/kg QW groups. Doses were subsequently changed to the approved maintenance dose of 1.5 mg/kg QW, and the studies were continued until enicizumab became commercially available. No new significant adverse events (AE) were reported. FVIII inhibitor titers declined in the majority of patients with pre-existing inhibitors. Treated ABRs decreased from the pre-emicizumab state or remained zero in all patients. Data based on the perceptions of patients and their families indicated that the bleeding phenotype improved, and that the duration of bleeding events decreased in the majority of the patients. Pooled long-term data after almost three years follow-up of phase 3 studies (HAVEN 1-4) have been recently reported\(^\text{46}\). A total of 399 participants were treated with emicizumab with a median (IQR) duration of exposure of 120.4 (89.0–164.4) weeks. The pooled mean ABR for treated bleeds over the entire study period was 1.4. The calculated mean ABR of all HAVEN studies appeared to be relatively higher in the first 24 weeks, but remained within 1.0, except in the HAVEN 4 study when the ABR was 0.8–1.8. The percentage of participants with zero treated bleeds increased over the first 24-week interval, and remained above 80%. Thromboembolic AEs were apparent in 7 cases and five of these were associated with concomitant use of aPCC.

The data from two long-term observations from the phase 1/2 and phase 3 studies are promising. However, future assessment beyond ABR is required to establish the full outcome of emicizumab prophylaxis. For these purposes, assessments of joint function, mental and physical activity will be pivotal, and QOL in caregivers should be considered.

12. Monitoring of emicizumab prophylaxis from the laboratory aspect

1) Measurement of emicizumab potential

As discussed above, emicizumab mimics the procoagulant activity of activated FVIII, and emicizumab-driven FX activation occurs at the initial stages of coagulation by binding to FVIIa/TF-derived FIXa in the absence of thrombin generation\(^\text{47}\). In these circumstances, aPTT-based clotting time assays and one stage clotting assays may overestimate the relative activity of FVIII. Generally, the aPTT is shortened to below the normal range in plasma samples obtained from patients on emicizumab prophylaxis. Moreover, standard one-stage APTT-based assays of FVIII activity using conventional plasma reference products have been shown to be erroneously high. Chromogenic assays (CSA) may also be used to assess emicizumab activity. FVIII CSA using human FIXa and FX are markedly affected by the pres-
ence of emicizumab, and cannot be used directly to measure FVIII activity in these circumstances.

Global assays of coagulation, including thromboelastography (TEG), Rotating thromboelastometry (ROTEM), thrombin generation assays (TGA) and clot waveform analysis (CWA) provide a different approach for monitoring of emicizumab prophylaxis. TEG/ROTEM using whole blood samples have been used for controlling bypassing therapy in hemophilia patients with FVIII inhibitors, and non-activated ROTEM (NATEM) appeared especially to be more useful than ROTEM activated by ellagic acid (INTEM) or tissue factor (EXTEM) for monitoring of emicizumab.

The ROTEM parameter defined as Clotting Time plus Clot Formation Time (CT + CFT), is inversely correlated with FVIII levels, and this relationship was used to develop a NATEM-based grading of coagulation potential; ‘T1’ (FVIII < 1 IU/dL), ‘T2’ (1 ≤, < 12 IU/dL) and ‘T3’ (> 12 ≤ IU/dL). Using FVIII for calibration, the minimum concentration of emicizumab required to maintain the T3 level was 20 μg/mL, and an Improvement of NATEM-based grades corresponded with a significant reduction in bleeding episodes. These findings demonstrated, therefore, that NATEM may be useful for intra- and inter-individual evaluation of coagulation function in emicizumab-treated patients.

Thrombin generation assays (TGA) may also be considered for monitoring emicizumab activity. The plasma TGA parameter, Peak Height, reflects the intensity of thrombin burst, and demonstrates a bell-shaped concentration-dependency similar to that observed with a simulated conformation of the FIX–emicizumab–FX ternary complex. TGA measurements using an intrinsic trigger, therefore reflect cofactor activity of the ternary complex.

In a phase 1 study, thrombin generation using FXIa as a trigger showed a dose-response curve with both in vivo and in vitro spiked samples.

Another option for assessing the efficacy of emicizumab is provided by the quantitative CWA parameter, maximum coagulation velocity (Min1). This measurement is based on the continuous recording of transmittance or absorbance during the performance of aPTT assays. Analyses of this type have been shown to be useful for the prediction of clinical severity and for monitoring of bypassing therapy. Modified aPTT-CWA methods, using trigger reagents comprising a mixture of ellagic acid and TF designed to reflect both intrinsic and extrinsic coagulation pathways, confirmed that Min1 increased dose-dependently in association with the concentration of emicizumab in FVIII deficient plasmas, irrespective of the presence of an inhibitor. Furthermore, the effects of emicizumab and FVIII on Min1 were additive. These results provided strong support for the use of mixture-triggered aPTT-CWA for monitoring emicizumab.

2) Measurement of FVIII activity and FVIII inhibitor levels during emicizumab prophylaxis

Notwithstanding the advantages of emicizumab in the modern era, there are several clinical circumstances where estimates of FVIII or FVIII inhibitor are required. In particular, hemostatic therapy for major surgery and severe bleeding including intracranial hemorrhage (ICH) remains especially challenging, and although the FVIII equivalent activity of emicizumab is reported to be 12–15 IU/dL at the post-marketing approved dose, the true emicizumab-derived cofactor function in vivo has not been clearly demonstrated. As discussed above, conventional aPTT-based assays may be adversely influenced by the presence of emicizumab, and CSA methods are being adapted using appropriate components for this purpose, especially in European countries. In particular, a novel CSA using human FIXa with bovine FX has been described as capable of measuring FVIII in the presence of emicizumab. Since CSA methods are not widely used in the world, we established a new method with standard clotting assays with addition of anti-emicizumab neutralizing antibodies.

In conclusion

Accomplishments of long-standing collaborative research between industry and academia on basic FVIII
structure and function, together with advances in recombinant and immunological technologies, have facilitated the production of emicizumab as a novel therapeutic for hemophilia A patients. The results of these projects are now widely regarded as providing a breakthrough innovation in the modern history of hemophilia treatment. It is recognized, however, that emicizumab exerts only 15% of FVIII function, suggesting that more extensive experiments are required to fully understand the remaining complications of FVIII deficiency. In this context, more potent next generation bispecific antibodies are currently under clinical investigation, and these trials may provide some further clarification.

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