the knocked-in hIL6R and the *H-2L*^{d}-h*IL6* transgene. These mice were maintained under specific pathogen-free conditions and fed standard laboratory chow (CE-2, CLEA Japan, Inc.) *ad libitum*. Genotypes for these mice were determined by PCRs for knocked-in h*IL6R* allele and *H-2L*^{d}-h*IL6* transgene. Genotyping for knocked-in h*IL6R* allele was performed by the PCR analysis mentioned above. *H-2L*^{d}-h*IL6* transgene was detected by PCR with the forward primer (5′- ACCTCTTCAGA-ACGAATTGACAAA -3′) and the reverse primer (5′- AGCTGCGCAGAATGAG-ATGAGTTGT -3′). After an initial denaturation at 94 degrees C for 4 min, 35 cycles of 94 degrees C for 30 sec, 65 degrees C for 30 sec and 72 degrees C for 30 sec were run with TaKaRa Ex Taq (TaKaRa). *H-2L*^{d}-h*IL6* transgene is detected as a signal at approximately 450 bp.

Treatment with an hIL6R-neutralizing antibody in humanized Castleman's disease model mice. These humanized Castleman's disease model mice were injected with 2 mg/body of humanized mAb to human IL6R (tocilizumab), rat mAb to mouse Il6ra (MR16-1) or physiological saline used for vehicle, intravenously, once at 4 weeks of age. Then from the week after the first injection, mice were given 0.1, 0.25 or 0.5 mg/body of tocilizumab or 0.1 mg/body of MR16-1 subcutaneously twice weekly. In the treatment regimen, first dosing was set at a relatively high amount to attempt to induce tolerance in mice to mAbs originating from other species[29]. Under isoflurane anesthesia, whole blood samples were collected from the inferior vena cava. Spleens were removed, weighed and fixed with 10% neutral buffered formalin for histological examination. *Il6ra*$^{+/+}$-h*IL6* transgenic mice, previously reported by Katsume *et al.* as a Castleman's disease model[8], were used as a disease control. Additionally *Il6ra*$^{+/+}$ and *Il6ra*$^{hIL6R/hIL6R}$ mice were used for healthy control.

Soluble human and mouse IL-6R-specific ELISA. The plasma levels of soluble human and mouse IL-6R were determined by using a commercially available kit (R&D Systems) according to the manufacturer's protocols.

Human and mouse IL-6-specific ELISA. The blood levels of human and mouse IL-6 were determined using a commercially available IL-6-specific ELISA kit (Invitrogen) according to the manufacturer's instruction.

Measurement of antibody titer to drug. Plasma samples were incubated with biotin-labeled tocilizumab and SULFO-TAG-labeled tocilizumab overnight. These mixtures were placed in the wells of MSD SA plates and incubated for 2 hours. After washing and addition of the read buffer, chemiluminescence was determined immediately by *SECTOR PR 400* (Meso Scale Discovery, Maryland).

Statistical analysis. Statistical analysis was performed using JMP 9.02 (SAS Institute Japan, Tokyo, Japan). Statistical significance in spleen weights was determined by nonparametric comparisons with control using Dunn method for joint ranking. $P < 0.05$ was regarded as statistically significant.

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Acknowledgments

We wish to thank Dr. A. Kawamura for critical discussion, Dr. J. Amano for giving biotin-labeled TCZ and MR16-1, Dr. T. Igawa for giving hIL6R, Dr. K. Kamei for important suggestions for determining anti-drug antibody, Mr. A. Takakura for measuring plasma soluble hIL6R, Mr. K. Matsumoto and Mr. K. Satoh for reproduction of the humanized Castleman's disease mouse model, and Dr. S. Shiota, Mrs. S. Uchida and Mrs. Y. Nakajima for skillful technical assistance. We also thank Mrs. S. Matsuura and Editing Services at Chugai Pharmaceutical Co., Ltd. for reviewing the manuscript.

Author contributions

K.J. and O.U. conceived and designed the experiments and coordinated the work presented. O.U., H.T., Y.H., E.F., A.K., Y.K., N.A.W., T.T., M.Kakefuda, C.G. and M.Kawaharada performed the experiments. S.S. and K.H. commented on the manuscript. O.U. and K.J. wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Ueda, O. *et al.* Novel genetically-humanized mouse model established to evaluate efficacy of therapeutic agents to human interleukin-6 receptor. *Sci. Rep.* **3**, 1196; DOI:10.1038/srep01196 (2013).

nature medicine

A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model

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Hemophilia A is a bleeding disorder resulting from coagulation factor VIII (FVIII) deficiency. Exogenously provided FVIII effectively reduces bleeding complications in patients with severe hemophilia A. In approximately 30% of such patients, however, the 'foreignness' of the FVIII molecule causes them to develop inhibitory antibodies against FVIII (inhibitors), precluding FVIII treatment in this set of patients[1–3]. Moreover, the poor pharmacokinetics of FVIII, attributed to low subcutaneous bioavailability and a short half-life of 0.5 d, necessitates frequent intravenous injections[3–5]. To overcome these drawbacks, we generated a humanized bispecific antibody to factor IXa (FIXa) and factor X (FX), termed hBS23, that places these two factors into spatially appropriate positions and mimics the cofactor function of FVIII. hBS23 exerted coagulation activity in FVIII-deficient plasma, even in the presence of inhibitors, and showed *in vivo* hemostatic activity in a nonhuman primate model of acquired hemophilia A. Notably, hBS23 had high subcutaneous bioavailability and a 2-week half-life and would not be expected to elicit the development of FVIII-specific inhibitory antibodies, as its molecular structure, and hence antigenicity, differs from that of FVIII. A long-acting, subcutaneously injectable agent that is unaffected by the presence of inhibitors could markedly reduce the burden of care for the treatment of hemophilia A.

About 1 in 10,000 males suffer from hemophilia A[6]. Approximately half of all patients with hemophilia A are classified as having severe disease[7], defined as <1% of normal FVIII activity, and such individuals typically experience at least one bleeding episode every month without preventive intervention[3,8]. Because patients with moderate hemophilia A (1–5% of normal activity) experience far fewer bleeding episodes, patients with severe disease are routinely given prophylactic supplementation of FVIII to keep the factor's activity to 1% or above, which effectively reduces joint bleeds, leading to better joint status and quality of life[3,8].

Despite these advantages, routine supplementation with FVIII has two major drawbacks aside from its expense: the development of inhibitors and the need for frequent venous access for FVIII injection. Inhibitors precluding the use of FVIII make it difficult to control hemorrhaging because alternative treatment agents (such as recombinant activated factor VII and activated prothrombin complex concentrates) have shorter half-lives, cost more than FVIII and are not always effective[9–11]. Eradication of inhibitors with high doses of FVIII is currently being attempted, but the process is very expensive and does not always work[12]. The need for frequent venous access is also problematic, particularly when treating pediatric patients at home[13], and it negatively affects both the implementation of and adherence to the supplementation routine. Therefore, a new agent that resolves these two drawbacks inherent to the current therapeutic use of FVIII has the potential to markedly improve the treatment possibilities for individuals with severe hemophilia A.

FVIII functions as a cofactor only when activated by either thrombin or factor Xa (FXa). The resultant factor VIIIa (FVIIIa) consists of the A1 subunit, the A2 subunit and the light chain (**Fig. 1a**)[14]. The light chain and the A2 subunit of FVIIIa respectively bind the light chain (K_d = 15 nM) and the heavy chain (K_d = 300 nM) of FIXa[15,16]. The A1 subunit of FVIIIa binds the heavy chain of FX (K_d = 1–3 μM)[17]. These binding properties contribute to FVIII's cofactor activity, enhancing the catalytic rate constant of FIXa and the interaction between FIXa and FX[14].

Recombinant monoclonal antibodies, with not only antagonistic activity but also agonistic[18], catalytic[19] or allosteric activity[20], have been extensively studied for their therapeutic applications[21]. Furthermore, bispecific antibodies, which recognize two different antigens, have been applied not only to simply neutralize two different antigens but also to recruit effector cells against the target cells[22] and to co-ligate two different antigens on the same cell[23]. We hypothesized that a bispecific IgG antibody recognizing FIXa with one arm and FX with the other could place FIXa and FX in spatially appropriate positions, as FVIIIa does, and promote FIXa-catalyzed FX activation (**Fig. 1b**).

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Received 11 May; accepted 16 August; published online 30 September 2012; doi:10.1038/nm.2942

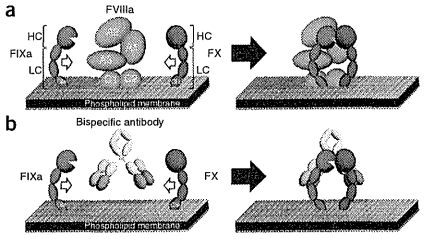

Figure 1 Schematic illustrations of the action of FVIIIa or of a bispecific antibody as a cofactor promoting the interaction between FIXa and FX. (**a**) FVIIIa consists of the A1 subunit, the A2 subunit and the light chain (A3, C1 and C2 subunits). FVIIIa forms a complex with FIXa and supports the interaction between FIXa and FX through its binding to both factors on the phospholipid membrane. HC, heavy chain; LC, light chain. (**b**) Bispecific antibody binding to FIXa and FX would promote the interaction between FIXa and FX on the phospholipid membrane and exert FVIII-mimetic activity. FVIIIa binds the phospholipid membrane via its C1 and C2 subunits; FIXa and FX bind the phospholipid membrane via their Gla domains. The illustrations describe the concept of the study only and do not necessarily indicate precise molecular structures and positions.

In support of this possibility, the distance between the FIXa- and FX-binding sites of FVIIIa[24] is similar to that between the two antigen-binding sites of human IgG[25]. In addition, antibody binding to the appropriate epitope of FIXa would mimic the allosteric properties of FVIIIa and enhance the catalytic activity of FIXa[14,16].

A bispecific antibody mimicking FVIII function could be an ideal agent for overcoming the two major drawbacks of current treatment. First, a bispecific antibody with a completely different molecular structure, or antigenicity, from FVIII would neither elicit inhibitors in patients with hemophilia A nor be neutralized by inhibitors that were already present. Moreover, humanized antibodies recently approved for clinical use have shown a low incidence of immunogenicity (<5%)[26]. Second, IgG antibodies have long plasma half-lives of 2 or 3 weeks and high subcutaneous bioavailability in humans, enabling once-a-month or less frequent treatment without venous access[27].

To create this type of bispecific antibody, we first generated various FIXa- and FX-specific monoclonal antibodies by immunizing animals with human FIXa and FX, respectively. We next used the genes encoding these monoclonal antibodies to generate bispecific IgG antibodies in which one arm would recognize FIXa and the other would recognize FX, as described in the Online Methods, and screened approximately 40,000 of these antibodies by an enzymatic assay for FX activation in the presence of FIXa and phospholipid. We identified a lead chimeric bispecific antibody with human IgG_4 heavy chain and κ light chain. Subsequent humanization and further engineering of the antibody generated an antibody termed hBS23. Surface plasmon resonance analysis showed that one of the arms of hBS23 recognizes factor IX (FIX) and FIXa, whereas the other arm recognizes FX and FXa (**Supplementary Fig. 1**). Western blotting analysis under reducing conditions showed that the epitope recognized by each arm of hBS23 is located in the FIXa light chain and the FX light chain, respectively (data not shown).

In an enzymatic assay using purified coagulation factors, hBS23 greatly enhanced FX activation, whereas monospecific one-armed antibodies[28] that had either the FIXa- or FX-specific variable region of hBS23 were completely inactive, as was a mixture of the two monospecific one-armed antibodies (**Fig. 2a**). hBS23 did not enhance FX activation in the absence of FIXa (**Fig. 2a**) or phospholipid (**Supplementary Fig. 2**), indicating that hBS23 functions as a cofactor mimicking FVIII and that the reaction required the presence of phospholipid. To compare the mechanistic basis for the cofactor function of hBS23 to that of FVIIIa, we performed a kinetic analysis of FIXa-catalyzed FX activation. Both hBS23 and FVIIIa increased the catalytic rate constant (k_{cat}) and decreased the Michaelis constant (K_m), consequently increasing the catalytic efficiency (k_{cat}/K_m) (**Table 1**). However, the extents to which they each affected k_{cat} and K_m were quite different; compared to FVIIIa, hBS23 showed ten times the effect on decreasing K_m, but 1/140th the effect on increasing k_{cat}, resulting in 1/14th the effect on increasing k_{cat}/K_m.

We next evaluated the activity of hBS23 in human FVIII-deficient plasma. With respect to the time to initiation of clot formation, hBS23 dose-dependently shortened the activated partial thromboplastin time (APTT) in FVIII-deficient plasma both in the presence and absence of inhibitors, whereas the effects of recombinant human FVIII (rhFVIII) on APTT were blocked by the presence of inhibitors (**Fig. 2b**). Doses of hBS23 greater than 30 nM had a greater effect on shortening the APTT compared to 1 U ml^{-1} rhFVIII (100% of normal FVIII activity). The shorter APTT achieved by hBS23 compared to that under normal FVIII activity can be explained by the fact that FVIII requires additional time to be activated by thrombin or FXa, whereas hBS23 does not. To further examine the therapeutic potential of hBS23, we evaluated its effect on thrombin burst in a thrombin generation assay (TGA)[29] in human FVIII-deficient plasma. hBS23 dose-dependently improved thrombin generation parameters, including peak height (defined as the peak of free thrombin concentration) and endogenous thrombin potential (ETP, defined as the area under the curve, a read-out of the overall capacity of plasma to form thrombin) even in the presence of inhibitors that rendered rhFVIII completely ineffective (**Fig. 2c–e** and **Supplementary Fig. 3**). With respect to peak height, hBS23 exerted activity equivalent to 0.01 U ml^{-1} (1%) rhFVIII at a concentration of ~30 nM and equivalent to nearly 0.1 U ml^{-1} (10%) rhFVIII at a concentration of ~300 nM. These results suggest that hBS23 has the potential to exert sufficient FVIII-mimetic activity for routine prophylactic use, even in patients with inhibitors who are nonresponsive to FVIII treatment.

To evaluate the *in vivo* hemostatic activity of hBS23, which is highly species specific in the manner in which it exerts FVIII-mimetic activity, we needed a hemophilia A model in nonhuman primates. As nonhuman primates with inherited hemophilia A were unavailable, we established an acquired hemophilia A model. For this purpose, we identified a neutralizing antibody against FVIII from mice, termed VIII-2236, which was cross-reactive to cynomolgus monkey FVIII but not to porcine FVIII (**Supplementary Fig. 4**). Injection of VIII-2236 into cynomolgus monkeys neutralized endogenous FVIII (data not shown) but not exogenous recombinant porcine FVIII (rpoFVIII). We established hemophilia A status by injecting cynomolgus monkeys with VIII-2236 and then artificially induced bleeding (**Fig. 3a**). The monkeys in the control group showed progressive anemia (as assessed by a decrease in hemoglobin levels) and an expanded bruised area (**Fig. 3b,c**). Compared to the control, bolus intravenous administration of 0.3 mg per kg body weight of hBS23 significantly prevented the decrease in hemoglobin levels ($P = 0.0116$) and tended to reduce the bruised area ($P = 0.0522$). This hemostatic activity of hBS23 was comparable to that of twice-daily intravenous administration of 1 U per kg body weight of rpoFVIII (**Fig. 3b,c**), which would

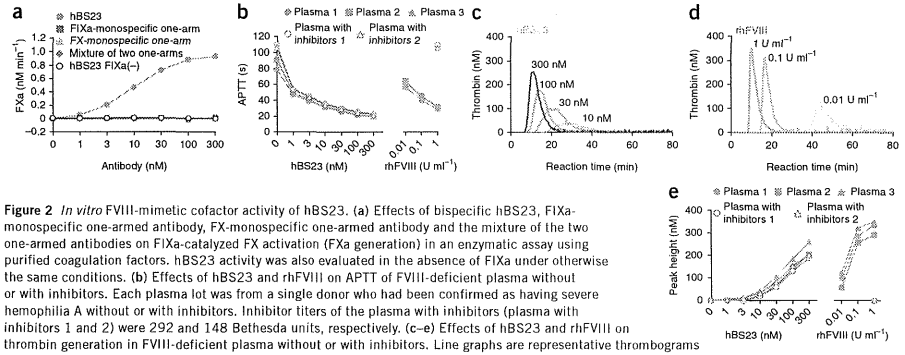

Figure 2 *In vitro* FVIII-mimetic cofactor activity of hBS23. (**a**) Effects of bispecific hBS23, FIXa-monospecific one-armed antibody, FX-monospecific one-armed antibody and the mixture of the two one-armed antibodies on FIXa-catalyzed FX activation (FXa generation) in an enzymatic assay using purified coagulation factors. hBS23 activity was also evaluated in the absence of FIXa under otherwise the same conditions. (**b**) Effects of hBS23 and rhFVIII on APTT of FVIII-deficient plasma without or with inhibitors. Each plasma lot was from a single donor who had been confirmed as having severe hemophilia A without or with inhibitors. Inhibitor titers of the plasma with inhibitors (plasma with inhibitors 1 and 2) were 292 and 148 Bethesda units, respectively. (**c–e**) Effects of hBS23 and rhFVIII on thrombin generation in FVIII-deficient plasma without or with inhibitors. Line graphs are representative thrombograms obtained from FVIII-deficient plasma without inhibitors (plasma 3) in the presence of hBS23 (**c**) or rhFVIII (**d**), indicating the time-course of changes in free thrombin concentration during the reaction triggered by factor XIa, phospholipid and Ca^{2+}. Effects of varying concentrations of hBS23 and rhFVIII on a thrombin generation parameter, peak height, is shown for each plasma lot (**e**). Data were collected in triplicate for each plasma lot and expressed as the means ± s.d., except for the representative thrombograms (in many cases, the bars depicting s.d. are shorter than the height of the symbols).

maintain an rpoFVIII activity of 0.01 U ml^{-1} (1%) or above, according to a simulation of multiple dose injections of rpoFVIII using pharmacokinetic parameters obtained from a single-dose injection study of rpoFVIII in cynomolgus monkeys (A.M., unpublished data). The mean plasma concentration of hBS23 was 5.8 μg ml^{-1} (40 nM) immediately after hBS23 administration and 2.5 μg ml^{-1} (18 nM) at day 3 (the end of the observation period). Neither prothrombin time prolongation nor a decrease in platelet numbers was observed in hBS23-treated monkeys (data not shown), suggesting that hBS23 did not induce consumptive coagulopathy.

We performed a pharmacokinetic study of hBS23 in cynomolgus monkeys by bolus intravenous or subcutaneous injection at a dose of 0.3 mg per kg body weight (**Fig. 3d**). After intravenous injection, hBS23 was cleared from plasma in a biphasic manner (a distribution phase followed by an elimination phase) with a long half-life of 14 d, which is consistent with the general pharmacokinetics of IgG antibodies[27]. The subcutaneous bioavailability of hBS23 was 84%, denoting high subcutaneous bioavailability.

Our data show that a bispecific antibody to FIXa and FX can restore FVIII hemostatic activity by recruiting the two factors into appropriate proximity. We demonstrated that simultaneous recognition of FIXa and FX by a single antibody molecule was required to bring about their activity, as neither a FIXa- or FX-monospecific one-armed antibody, nor a mixture of them, could elicit detectable cofactor activity. Such activity was not detected in the absence of FIXa or phospholipid, indicating that hBS23 exerts its FVIII-mimetic activity only on a tertiary complex consisting of FIXa, FX and phospholipid. Kinetic analysis showed that hBS23 and FVIIIa improved k_{cat} and K_m quite differently: bispecific binding of FIXa and FX by hBS23 improves the interaction between these factors more efficiently than does binding by FVIIIa, but hBS23 has less potential to increase the turnover of the enzyme complex than does FVIIIa. hBS23 was calculated to have 1/14th the catalytic efficiency (k_{cat}/K_m) of FVIIIa. When designing the *in vivo* study, we considered that 0.3 mg per kg body weight of hBS23 would be expected to achieve a plasma concentration that could generate thrombin at a level equivalent to that of 0.01 U ml^{-1} (1%) FVIII *in vitro*. Accordingly, 0.3 mg per kg body weight of hBS23 showed an *in vivo* efficacy comparable to that achieved in a regimen designed to maintain 0.01 U ml^{-1} (1%) or above of FVIII activity, a level sufficient to control hemostasis in patients[3,8]. As the concentration-dependent activity of hBS23 in the TGA in FVIII-neutralized cynomolgus monkey plasma was comparable to that in human FVIII-deficient plasma (**Fig. 2e** and **Supplementary Figs. 3** and **4b,c**), we expect that this *in vivo* efficacy can be translated to the human clinical setting. Although we induced artificial bleeding in this study, the nonhuman primate model that we established has various clinical features of acquired hemophilia, including overt hemorrhage, anemia, bruising and muscle bleeds[30], supporting the extrapolation of this animal study into the clinical setting.

Single-dose pharmacokinetic analysis of hBS23 in cynomolgus monkeys revealed that its half-life (14 d) was slightly longer than that of other humanized or fully human IgG antibodies[31,32]. In addition, whereas the low subcutaneous bioavailability of FVIII necessitates venous access for its injection, hBS23 has high subcutaneous bioavailability (84%) in cynomolgus monkeys. Furthermore, we conducted a simulation study of multiple-dose injections of hBS23 in cynomolgus monkeys using the pharmacokinetic parameters that had been obtained from the pharmacokinetic study. This simulation indicated that once weekly subcutaneous administration of 1 mg per kg body weight of hBS23 would result in a plasma concentration that at any time point would greatly exceed 40 nM (K. Haraya and T. Tachibana, unpublished data), which is the initial plasma concentration in the *in vivo* hemostatic

Table 1 Effects of hBS23 on enzymatic kinetics of FIXa-catalyzed FX activation

Condition	K_m (μM)	V_{max} (nM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m	Fold increase in k_{cat}/K_m
FIXa	0.0558 ± 0.00281	0.0442 ± 0.00332	0.00111	0.0199	—
+hBS23	0.00253 ± 0.000113	0.996 ± 0.0870	0.996	394	19,800
+FVIIIa	0.0249 ± 0.00247	135 ± 6.08	135	5,420	272,000

The K_m and the maximum velocity (V_{max}) are expressed as the means ± s.d. of three separate experiments. The k_{cat} and the k_{cat}/K_m are calculated from the mean values of K_m and V_{max}. The fold increase in the k_{cat}/K_m by hBS23 or FVIIIa compared to FIXa alone is also shown.

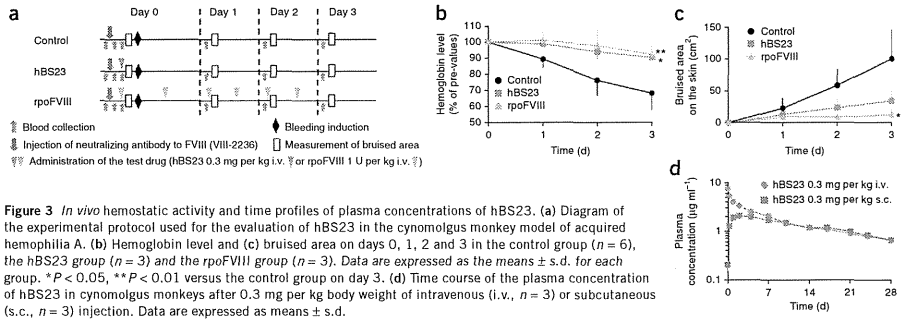

Figure 3 *In vivo* hemostatic activity and time profiles of plasma concentrations of hBS23. (**a**) Diagram of the experimental protocol used for the evaluation of hBS23 in the cynomolgus monkey model of acquired hemophilia A. (**b**) Hemoglobin level and (**c**) bruised area on days 0, 1, 2 and 3 in the control group ($n = 6$), the hBS23 group ($n = 3$) and the rpoFVIII group ($n = 3$). Data are expressed as the means ± s.d. for each group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the control group on day 3. (**d**) Time course of the plasma concentration of hBS23 in cynomolgus monkeys after 0.3 mg per kg body weight of intravenous (i.v., $n = 3$) or subcutaneous (s.c., $n = 3$) injection. Data are expressed as means ± s.d.

study. As the half-life of IgG antibodies in humans is generally longer than that in cynomolgus monkeys[33], hBS23 would be expected to have at least a 14-d half-life in humans, which is substantially longer than that of FVIII (0.5 d)[5]. We therefore expect that once weekly dosing of 1 mg per kg body weight of hBS23 would show a continuous hemostatic effect in humans. Effective prophylaxis by once-weekly subcutaneous administration would be an advantageous feature of our bispecific antibody in view of the frequent venous access required for FVIII treatment. Moreover, although maintenance of 1% of normal FVIII activity should be enough for prophylactic use, dosing of hBS23 above 0.3 mg per kg body weight would be expected to exert higher hemostatic activity, as indicated by the dose-dependent activity of hBS23 above 40 nM (the initial plasma concentration of hBS23 after dosing with 0.3 mg per kg body weight in the *in vivo* hemostatic study) in the TGA (at 300 nM, hBS23 had nearly 10% of normal FVIII activity).

Among the alternative therapeutic approaches for hemophilia A that are being pursued, the bispecific antibody described here has some notable advantages. Modified FVIII agents with longer half-lives than the native form of FVIII, including a FVIII Fc fusion protein ($t_{1/2}$ = 18.8 h), are currently being investigated in clinical trials, but although these modified FVIII agents may achieve a reduced injection frequency, they still require venous access[34,35]. Additionally, although gene therapy is in principle a very attractive therapeutic approach that has been actively explored for the past decade, many issues remain to be resolved before it can be used clinically[36].

A notable feature of our bispecific antibody is that it has the potential to overcome the most crucial drawback of treatment using injected FVIII, which would probably also apply to treatment with modified FVIII: the development of inhibitors. Such inhibitors affect not only 30% of the patients with severe disease but also a small percentage of the patients with moderate and mild disease[1,3]. Our experimental results showed that hBS23 activity was not affected by inhibitors. Moreover, considering the generally low immunogenicity of humanized antibodies[26], we expect the incidence of antibodies to hBS23 to be substantially lower than that of antibodies to FVIII. However, as humanized or fully human antibodies are immunogenic in rare cases[26,37], there is still a small risk for the generation of hBS23-specific antibodies in patients. Were hBS23 to become ineffective because of the generation of hBS23-specific antibodies, FVIII treatment could be a viable alternative as long as the hBS23-specific antibodies do not cross-react with FVIII. To estimate the risk of such cross-reactivity, we scanned the sequences of the variable regions of hBS23 to assess sequence similarity to FVIII. This alignment analysis found no similarity between the two molecules (T. Kitazawa, unpublished data); we therefore consider it unlikely that hBS23 treatment would result in the development of hBS23-specific antibodies cross-reactive to FVIII. Thus, we expect that hBS23 treatment would not lead to inhibitor development and could be used in patients who have already developed inhibitors.

In this study, we have generated an agent with the potential to overcome the two major drawbacks of current treatment modalities. Although hBS23 showed efficacy in an animal study, the molecular structure of hBS23 may require further optimization in several ways before the clinical use of such an agent in humans. The first is molecular engineering to enable large-scale manufacturing of the bispecific antibody at clinical grade. Although technological difficulties have prevented any recombinant bispecific antibody from yet reaching the market, various engineering technologies to overcome them have been reported[38,39]. The second is engineering to further reduce the immunogenicity of the humanized antibody. Even though humanized or fully human antibodies generally have low immunogenicity[26], they can be immunogenic if T cell epitopes are present in the complementarity-determining regions[37]. Various approaches to identify and remove such T cell epitopes have been recently reported[26,39]. The third is engineering to prolong the half-life and improve the physicochemical properties of the antibody, which would enable a subcutaneous formulation with an even longer dosing interval[39,40]. The fourth is engineering to further increase the FVIII-mimetic activity of the bispecific antibody. Mutations in the complementarity-determining regions increased the activity of hBS23 over that of the lead chimeric bispecific antibody (data not shown), and the activity of hBS23 could be improved in a same manner. Although hBS23 may require further optimization, this type of bispecific antibody may be able to overcome current limitations and reduce the burden of care for the treatment of hemophilia A.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We thank T. Matsuura, T. Houjo, K. Kanisawa, R. Takemoto, T. Koike and M. Hiranuma for carrying out the *in vivo* experiments and M. Fujii, Y. Nakata, H. Ishida and F. Isomura for antibody generation and preparation. We also thank S. Ohtsu for carrying out *in vitro* experiments.

AUTHOR CONTRIBUTIONS

T. Kitazawa and T. Igawa led the pharmacological studies and the optimization of the bispecific antibody, respectively, in the program and wrote the manuscript. Z.S. designed the lead chimeric bispecific antibody and hBS23. T. Kojima led the lead identification. H.T. provided ideas on bispecific antibody engineering. T. Suzuki, H.A., T.M., S.I., M.K.-S. and T. Iida generated FVIII-, FIXa- and FX-specific antibodies. T. Soeda, Y.O.-N., A.H., M.E, C.M., E.T., T. Toyoda and A.U. performed the *in vitro* experiments. K.E. and S.S. performed the affinity analyses. Y. Kikuchi, T.W., M.W. and M.G. purified the bispecific antibody and the coagulation factor. A.M. and K.Y. performed the *in vivo* pharmacological study. K. Haraya and T. Tachibana performed the pharmacokinetic study. H.S. and Y. Kawabe provided direction and guidance for the various functional areas. M.S. and A.Y. provided advice on the program from the viewpoints of their medical expertise in hemophilia. K. Hattori provided the hypothesis and directed and organized the program.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

Published online at http://www.nature.com/doifinder/10.1038/nm.2942.
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ONLINE METHODS

The care and use of laboratory animals. Cynomolgus monkey, mouse, rat and rabbit studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical under the approval of the company's Institutional Animal Care and Use Committee and also in compliance with the "Act on Welfare and Management of Animals" in Japan. The company is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, a nonprofit organization that promotes the humane treatment of animals in science through voluntary accreditation and assessment programs (http://www.aaalac.org/). We conducted the cynomolgus monkey research adhering to the principles stated in the US National Research Council's Guide for the Care and Use of Laboratory Animals.

Generation of hBS23, a humanized bispecific antibody to FIXa and FX. We prepared the genes of the variable regions of FIXa- and FX-specific antibodies from 46 female mice, 34 female rats and 12 female rabbits that had been immunized with human FIXa or FX (Enzyme Research Laboratories) and inserted the genes into an expression vector containing the constant region of human IgG. The constant region of all the expressed bispecific antibodies had a S238P (EU numbering) substitution, intended to improve hinge stabilization[41], and 'knobs-into-holes' substitutions, intended to increase the desired bispecific combination by promoting heterodimerization efficiency of the two heavy chains[38]. To express a series of bispecific antibodies in HEK 293 cells, we co-transfected a mixture of four expression vectors encoding the heavy and light chains of FIXa- and FX-specific antibodies. After culturing the transfectants, we individually collected and incubated each of the culture supernatants for 2 h with 10 nM FIXa, 10 nM FX and 62.5 μM synthetic phospholipid in 50 mM Tris-buffered saline (TBS) containing 5 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.6). The phospholipid consisted of 10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine (Avanti Polar Lipids) and was prepared as previously described[42]. After stopping the reaction by the addition of EDTA, we added a chromogenic substrate specific to FXa (S-2222, Chromogenix) and evaluated the ability of each culture supernatant to promote FXa generation by measuring the absorbance at 405 nm. From approximately 40,000 bispecific combinations, we selected a lead chimeric bispecific antibody based on its ability to generate FXa and the feasibility of using only one light chain, the so-called common light chain. The combination of two heavy chains and two light chains results in ten possible combinations, of which only one yields the desired bispecific antibody; however, use of a common light chain for the two arms of the bispecific antibody reduces the number of combinations of heavy and light chains to three[38]. To generate hBS23, we further humanized the lead chimeric bispecific antibody by complementarity-determining region grafting[43] and engineered it to have stronger FVIII-mimetic activity and better pharmaceutical properties. We transiently expressed the one light chain and two heavy chains of hBS23 in HEK 293 cells and purified it with recombinant Protein A and gel permeation chromatography. We also prepared in the same way one-armed monospecific antibodies[28], each of which have one arm containing either the FIXa- or the FX-specific variable region of hBS23, as well as two-armed monospecific antibodies, in which both arms contain the FIXa- or FX-specific variable region of hBS23.

Characterization of antigen-antibody interaction of hBS23 using surface plasmon resonance. We analyzed the interactions of FIX, FIXa, FX and FXa with the corresponding variable regions of hBS23 by surface plasmon resonance using a Biacore T200 system (GE Healthcare). First, we immobilized MabSelect SuRe Ligand (recombinant Protein A; GE Healthcare) onto a CM4 sensor chip (GE Healthcare) that had been preactivated with NHS/EDC and predeactivated with ethanolamine (Amine Coupling Kit; GE Healthcare). To capture the test antibodies on the sensor chip, we injected each of the FIXa- or the FX-monospecific two-armed IgG antibodies into flow cell 2 and natalizumab (Biogen-Idec) as control human IgG_4 antibody into flow cell 1. We next injected each analyte (0 as baseline, 80, 160, 320, 640, or 1,280 nM human FIX, FIXa, FX, or FXa), which had been dissolved in running buffer (10 mM HEPES, 150 mM NaCl, 0.05% (vol/vol) Surfactant P20, 2.5 mM $CaCl_2$ (pH 7.4); GE Healthcare), into both flow cells on the sensor surface at a flow rate of 30 μl min^{-1} to monitor the association phase for 120 s and the dissociation phase for 30 s. The data were analyzed by the 1:1 binding model in the Biacore T200 Evaluation software (version 1.0, GE Healthcare).

Enzymatic assays. We evaluated the conversion rate of FX to FXa in an enzymatic assay using purified coagulation factors. The assay system consisted of 1 nM human FIXa, 140 nM human FX, 20 μM phospholipid and either hBS23, FIXa- or FX-monospecific one-armed antibodies, or a mixture of two monospecific one-armed antibodies, and measured FXa generation at room temperature for 2 min in TBS containing 5 mM $CaCl_2$ and 0.1% (wt/vol) BSA. We also performed the experiments in the absence of FIXa or phospholipid under otherwise the same conditions. We stopped the reaction by adding EDTA at various time points. After adding S-2222 chromogenic substrate, we measured absorbance at 405 nm to determine the rate of FXa generation and quantified it by extrapolation from a standard curve prepared using known amounts of human FXa (Enzyme Research Laboratories). Data were collected in triplicate.

Kinetic analyses. We monitored the rate of FIXa-catalyzed FX activation by incubating 1 nM human FIXa, various concentrations of human FX and 20 μM phospholipid with either 300 nM hBS23 or 30 U ml^{-1} rhFVIII (Bayer Healthcare) that had been preactivated by thrombin and converted into FVIIIa in TBS containing 5 mM $CaCl_2$ and 0.1% (wt/vol) BSA (pH 7.6) at room temperature. We also performed the experiments in the absence of hBS23 and FVIIIa under the same conditions, except that we altered the FIXa concentration to 40 nM. We stopped the FXa generation reaction by adding EDTA at various time points. The rates of FXa generation were determined in the same way as described in the previous section. The values of K_m and V_{max} were calculated using a nonlinear regression data analysis program (GraphPad PRISM version 5.04).

Preparation of rpoFVIII. We expressed a B domain–deleted form of rpoFVIII in Chinese hamster ovary cells by stable transfection. The vector employed for the transfection was designed on the basis of a previously reported sequence of the *F8* (*Sus scrofa*) gene (GenBank: NM_214167) and the sequence of B domain–deleted human FVIII (refs. 44,45). After diluting the supernatant of rpoFVIII-containing medium with 20 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.6), we loaded it onto a Q Sepharose Fast Flow column (GE Healthcare) equilibrated with the same buffer. We then eluted rpoFVIII with a linear 0 to 1 M NaCl gradient in the same buffer. We pooled and concentrated the fractions containing rpoFVIII and then subjected them to gel permeation chromatography on a HiLoad 26/60 Superdex200pg column (GE Healthcare) equilibrated in 150 mM NaCl, 10 mM histidine-HCl, 5 mM $CaCl_2$, 0.1% (wt/vol) sucrose and 0.02% (wt/vol) polysorbate 80 (Junsei Chemical) (pH 7.0). We again pooled the fractions containing rpoFVIII and aseptically filtered the mixture. We assessed the FVIII activity (U ml^{-1}) of the purified rpoFVIII in a one-stage coagulation assay, one of the standard assays for determining FVIII activity, using normal human plasma (Siemens Healthcare) as the standard.

Generation of VIII-2236, a neutralizing antibody against FVIII. We established hybridomas producing an antibody against human FVIII from eight female mice that had been immunized with rhFVIII. Using APTT measurements and the TGA, we identified VIII-2236 as a neutralizing antibody against FVIII with cross-reactivity to cynomolgus monkey FVIII but not to porcine FVIII.

APTT measurements. We measured APTT with standard techniques using Thrombocheck APTT-SLA (Sysmex). We employed three lots of commercially available human FVIII-deficient plasma without inhibitors (<1% normal FVIII activity) and two lots with inhibitors (George King Bio-Medical). Each lot contained frozen fresh plasma from a single donor with severe hemophilia A. We also used the pooled citrated plasma of nine male cynomolgus monkeys, which contained 300 μg ml^{-1} VIII-2236 (2.6% by volume for VIII-2236 solution). Data were collected in triplicate.

Thrombin generation assays. We obtained thrombograms by calibrated automated thrombography[46] using a 96-well plate fluorometer (Thermo Fisher Scientific Instruments) equipped with a 390/460 filter set, a dispenser and analyzing software (Thrombinoscope software version 3.0.0.29, Thrombinoscope BV). Briefly, into each well we dispensed 80 μl of a plasma solution containing hBS23, rhFVIII or rpoFVIII added to the human FVIII-deficient plasma or the FVIII-neutralized pooled cynomolgus monkey plasma (<1% by volume). We then added 20 μl of the triggering solution containing 0.47 nM human factor XIa (Enzyme Research Laboratories) and 20 μM phospholipid but no Ca^{2+}. For calibration, we added 20 μl of Thrombin Calibrator (Thrombinoscope BV) instead of the triggering solution. To initiate the reaction, 20 μl of FluCa reagent prepared from FluCa kit (Thrombinoscope BV) was dispensed by the instrument as programmed. The instrument's software analyzed the thrombograms, peak height and ETP. Data were collected in triplicate.

***In vivo* experiments in the acquired hemophilia A model.** We used 12 male cynomolgus monkeys (2.50–3.60 kg and 3–4 years of age), housed individually, which were divided into three groups, receiving hBS23 (n = 3), rpoFVIII (n = 3) or neither agent (control group; n = 6). The experimental protocol is diagrammed in **Figure 3a**. On day 0, each monkey intravenously received an adjusted dose of VIII-2236 that would result in an APTT twice as long as the baseline APTT for that monkey. The means ± s.d. of the adjusted VIII-2236 dose was 3.6 ± 3.8 mg per kg body weight in the control group, 3.3 ± 2.3 mg per kg body weight in the hBS23 group and 4.5 ± 1.9 mg per kg body weight in the rpoFVIII group. Two hours thereafter, we anesthetized each monkey by isoflurane inhalation and measured the area of purple-colored bruising on the skin of the entire body of monkeys whose pelage had been closely cropped. We next intravenously administered the test drug to each monkey in the hBS23 or the rpoFVIII group at 0.3 mg per kg or 1 U per kg body weight, respectively. We then induced bleeding in the monkeys by performing an injury procedure: 1-cm-deep insertions of an 18-G needle into muscles at 16 sites (four sites in each thigh, two sites in each upper arm and two sites in each forearm). We maintained the anesthesia until the completion of this injury procedure. In our previous experience, more severe injury procedures did not decrease hemoglobin levels in normal monkeys, so we did not expect this procedure to decrease hemoglobin in the absence of FVIII neutralization. On days 1, 2 and 3, we again anesthetized the monkeys and measured the bruised area. After completing the evaluation of the bruised area on day 3, the monkeys were killed humanely. To each of the monkeys in the rpoFVIII group, we administered additional intravenous injections of rpoFVIII (1 U per kg body weight) in the mornings of days 1 and 2 just before bruise measurement and also in the evenings of days 0, 1 and 2 (twice a day, total of six administrations of rpoFVIII). We collected blood samples before and after injecting VIII-2236, after the first administration of the test drug on day 0 and before measuring the bruised area on days 1, 2 and 3 to confirm the neutralizing effect of VIII-2236 against endogenous FVIII and to determine the concentration of hBS23 and the hemoglobin level. We expressed the change of hemoglobin level as a percentage of the value on day 0 (after the injection of VIII-2236) for the respective monkey.

Pharmacokinetic study. We intravenously or subcutaneously administered a single dose of 0.3 mg per kg body weight of hBS23 to male cynomolgus monkeys (2.57–3.10 kg and 2–3 years of age, n = 3 for the intravenous administration group and n = 3 for the subcutaneous administration group). We collected blood samples before the administration and 0.5 h (intravenous administration only), 2 h (subcutaneous administration only), 8 h, 1 d, 2 d, 4 d, 7 d, 10 d, 14 d, 17 d, 21 d, 24 d and 28 d after the administration. Pharmacokinetic parameters were calculated by WinNonlin Professional software (version 4.0.1, Pharsight). The subcutaneous bioavailability was calculated by dividing the AUC_{inf} for the subcutaneous administration by the AUC_{inf} for intravenous administration.

Statistical analyses. Data are presented as means ± s.d. In the *in vivo* experiments employing the acquired hemophilia A model, significant differences in the data of day 3 were determined by the parametric Dunnett's multiple comparison test (two-tailed) compared to the control group (SAS preclinical package version 5.00, SAS Institute Japan).

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