TITLE: Prolonged activity of factor IX as a monomeric Fc fusion protein

RUNNING TITLE: Long lasting recombinant factor IX-Fc

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Abstract

Treatment of hemophilia B requires frequent infusions of Factor IX (FIX) to prophylax against bleeding episodes. Hemophilia B management would benefit from a FIX protein with an extended half-life. A recombinant fusion protein (rFIXFc) containing a single FIX molecule attached to the Fc region of IgG was administered intravenously and found to have an extended half-life, when compared to recombinant FIX (rFIX) in normal mice, rats, monkeys, and FIX-deficient mice and dogs. Recombinant FIXFc protein concentration was determined in all species, and rFIXFc activity was measured in FIX-deficient animals. The half-life of rFIXFc was approximately 3 to 4-fold longer than that of rFIX in all species. In contrast, in mice in which the neonatal Fc receptor (FcRn) was deleted, the half life of rFIXFc was similar to rFIX, confirming the increased circulatory time was due to protection of the rFIXFc via the Fc/FcRn interaction. Whole blood clotting time in FIX-deficient mice was corrected through 144 hr for rFIXFc, compared to 72 hr for rFIX; similar results were observed in FIX deficient dogs. Taken together, these studies demonstrate the enhanced pharmacodynamic and pharmacokinetic properties of the rFIXFc fusion protein and provide the basis for evaluating rFIXFc in patients with hemophilia B.
Introduction

Hemophilia B is an X chromosome-linked bleeding disorder resulting from a deficiency in functional Factor IX (FIX) and affects 1 in every 25,000 to 30,000 males.\textsuperscript{1,2} Severe hemophilia B patients (FIX < 1 International Units [IU]/dL) in particular suffer from repeated bleeding episodes resulting in hemophilic arthropathy, and other sequelae.\textsuperscript{2,3} Like hemophilia A (Factor VIII deficiency), conventional treatment for hemophilia B has been on-demand, ie, patients are administered concentrates of the deficient factor when hemorrhage occurs or prior to a surgical procedure.\textsuperscript{3} Alternatively, replacement therapy can be given in regular intervals prophylactically to maintain FIX levels > 1%, thereby converting a severe phenotype into one that is milder and to potentially prevent life-threatening hemorrhages and musculo-articular bleeding events.\textsuperscript{3-6}

When treating bleeding episodes on-demand or in the surgical setting, several infusions of FIX may be required to avoid peaks and troughs and maintain minimum coagulation factor levels for a sufficient duration. Similarly, in patients being treated on prophylactic regimens, two to three injections per week are usually required to achieve the necessary FIX plasma levels. This can be attributed, in part, to the relatively short half-lives of approximately 18 hr of currently available products.\textsuperscript{7} In hemophilia B, twice- or thrice-weekly dosing with 40–100 IU/kg of FIX is recommended by the Medical and Scientific Advisory Council of the National Hemophilia Foundation.\textsuperscript{8} Since coagulation factors are administered by intravenous injection, repeat venous access is necessary, which is particularly difficult in a pediatric population and may necessitate the use of ports for central venous access.

A recombinant FIX product with a longer half-life than currently available FIX products would be expected to require fewer injections, thus reducing the need for repeated venous access, potentially improving the acceptance of prophylactic regimens by pediatric patients and their
parents, and possibly decreasing repeated dosing in the treatment of episodic bleeds or in surgical settings. Recombinant Factor IX-Fc fusion protein (rFIXFc) contains a single molecule of FIX recombinantly attached to the constant region (Fc) of immunoglobulin G (IgG) and was developed to address the unmet medical need for a long-acting FIX product. The presence of the Fc domain enables the fusion protein to bind to the neonatal Fc receptor (FcRn), a heterodimer of an MHC class I-like-protein heavy chain with β-2-microglobulin (β2m). FcRn serves a critical role in IgG homeostasis by protecting the Fc-containing molecules from catabolism. We report here the enhanced pharmacokinetics and pharmacodynamics of rFIXFc compared to commercially available recombinant FIX.

Materials and methods

Cloning, expression, and purification of rFIXFc

All molecular biology procedures were performed following standard techniques. The coding sequences for human FIX, the Fc region of the human IgG1 (hinge and CH2 and CH3 domains) and PC5 were obtained by PCR from human liver mRNA, human lymphocyte cDNA, and human liver mRNA, respectively, and cloned into mammalian expression vectors. HEK-293H cells (Invitrogen, Carlsbad, CA) were stably transfected with expression cassettes for both FIXFc (native FIX coding sequence fused to the Fc region with no intervening linker) and Fc alone, leading to the secretion of 3 different protein products: FIXFc dimer, FIXFc monomer (one FIXFc and one Fc chain), and Fc dimer. Cells were cotransfected with the expression cassette for PC5, a processing enzyme, to ensure full cleavage of the FIX propeptide. Cell lines were grown in serum-free suspension media in the presence of vitamin K. FIXFc monomer (hereinafter referred to as rFIXFc) was purified by column chromatography utilizing a Protein A
capture step and 2 anion exchange steps, Fractogel DEAE and Q Sepharose. The last ion exchange step involved pseudo-affinity elution\textsuperscript{13} from a Q Sepharose resin with low ionic strength CaCl\textsubscript{2} to obtain rFIXFc with highest specific activity.

\textbf{Recombinant Factor IX Preparation}

Lyophilized BeneFIX\textsuperscript{®} (coagulation factor IX, recombinant; rFIX; Wyeth, Andover, MA, USA) was reconstituted in the formulation buffer supplied by the manufacturer.

\textbf{Gamma-carboxylation analysis}

Analysis of the gamma-carboxylation (Gla) content of the rFIXFc was performed by total amino acid analysis after base hydrolysis, and the results were compared to those for plasma-derived FIX (pdFIX, Mononine\textsuperscript{®}; CSL Behring, King of Prussia, PA), which has been shown to contain 12 Gla/mol FIX protein.\textsuperscript{14} Gla content was confirmed by mass spectroscopy after digestion with LysC and subsequent peptide mapping.

\textbf{Additional posttranslational modification and other analyses}

\textbeta-hydroxylation, propeptide content, and O-glycosylation were assessed by LysC peptide mapping. Tyrosine sulfation was determined by AAA (base hydrolysis) relative to pdFIX. Serine phosphorylation was determined by Pro-Q Diamond staining quantitation of SDS-PAGE gels relative to pdFIX. N-glycan content was determined by a normal-phase HPLC method profile of N-linked glycans released by PNGase F, followed by 2-aminobenzamide (2-AB) labeling.

Activated FIX (FIXa) was determined by ELISA based on the binding of FIXa to antithrombin III (ATIII) in the presence of heparin,\textsuperscript{15} modified for detection with an anti-human FIX antibody conjugated to horseradish peroxidase (HRP).
Activation by FXIa was assessed by reducing SDS-PAGE analysis after 5 min incubation of either rFIXFc or rFIX with FXIa at 100:1 ratios and quantification by densitometry of the intact protein.

**FIXFc and FIX ELISA**

A sandwich ELISA designed to detect the rFIXFc protein concentration in animal plasma was developed using a goat anti-human FIX IgG capture antibody (Enzyme Research Laboratories, Southbend, IN) and a goat anti-human IgG (Fc-specific)-HRP conjugate (Pierce Biotechnology, Rockford, IL).

The sandwich ELISA to detect rFIX used the same goat anti-human FIX capture antibody as for rFIXFc. The detection antibody for the FIX ELISA was a goat anti-human FIX-HRP conjugate (Enzyme Research Laboratories, Southbend, IN).

Protein concentrations determined by ELISA analysis are expressed as means ± standard deviation calculated from values obtained from multiple, individual animals assayed in triplicate, except in certain cases for rFIX (as indicated in figures) where due to low levels of protein, samples were pooled to provide sufficient material for detection and are expressed as single data points. Data from the 2 individual FIX-deficient dogs are expressed as the mean of triplicate measurements for each data point.
Animals

A FIX knockout strain of mice, produced using selective gene targeting, was acquired from Dr. Darrel Stafford, University of North Carolina (UNC), Chapel Hill, NC. Female Sprague Dawley rats and C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA). FcRn knockout (FcRn KO) mice were derived from C57BL/6J mice, and human FcRn transgenic (Tg32B) mice were originally obtained from Dr. Derry Roopenian (The Jackson Laboratory, Bar Harbor, ME). The FcRn KO mice do not express murine FcRn or murine β2m and are designated mFcRn(-/-)/mβ2m(-/-). FcRn transgenic mice express human FcRn and human β2m on a murine FcRn and β2m knockout background, and are designated muFcRn(-/-)/muβ2m(-/-)/hFcRn(+/-)/hβ2m(+/-).

Two hemophilia B dogs (1 male and 1 female) from the closed colony at the Francis Owen Blood Research Laboratory (FOBRL) at the University of North Carolina at Chapel Hill were infused with rFIXFc intravenously. These experiments were performed at the FOBRL. All cynomolgus monkeys underwent pre-study physical exams; all animals were determined to be in good health prior to dosing.

All animal studies were conducted in compliance with Institutional Animal Care and Use Committee-approved protocols at their respective institutions.

Administration of FIX preparations and blood sampling

Pharmacodynamic studies in FIX-deficient mice

Single and repeat dose studies were performed in FIX-deficient mice to evaluate clotting activity of rFIXFc compared to rFIX. In both studies, FIX-deficient mice received 219 IU/kg rFIXFc or 200 IU/kg rFIX by intravenous administration. In the repeat-dose study, the same dose
of each factor was given on days 0, 4, and 8. To obtain sufficient citrated plasma (0.32% final) free of tissue factor contamination (which has the potential to confound factor IX activity assay results), blood samples were obtained via cardiac puncture following CO2 euthanasia at 0.25, 8, 24, 48, 72, and 96 hr after administration for the single-dose study, and at 0.25 hr and 96 hr after administration for the multiple dose study, to evaluate clotting activity by a FIX activity assay. Citrated plasma was also obtained at 8, 24, 48, and 72 hr after each dose by tail bleeds in the multiple-dose study to evaluate FIX concentration by ELISA. Calculated rFIXFc/rFIX clotting activity was determined by converting rFIXFc/rFIX concentrations from μg/mL to IU/mL values using the specific activity of each protein.

Pharmacokinetic studies in mice

Mice (normal C57BL/6J, FIX-deficient, FcRn KO, FcRn transgenic) were administered a single intravenous dose of 100 or 200 IU/kg of rFIX or rFIXFc. Blood was collected from the tails at 0.25, 8, 24, 48, 72, 96, and 168 hr after dosing in each animal, and citrated plasma (0.32% final) prepared.

Rats

Single intravenous doses of 200 IU/kg rFIXFc or rFIX were administered into the lateral tail vein in each of 2 groups of rats. Blood samples were collected at 0.25, 4, 8, 24, 48, 72, 96, and 168 hr after administration of rFIXFc or at 0.25, 8, 24, and 72 hr after administration of rFIX, and citrated plasma (0.32% final) prepared.
FIX-deficient dogs

Intravenous infusions into the cephalic vein were performed with 140 IU/kg rFIXFc. Blood samples were collected pre-dose from the cephalic vein of the opposite leg at 5, 15, and 30 min and at 1, 2, 4, 6, 8, 12, 24, 27, 30, 48, 51, 54, 72, 80, 96, 144, and 168 hr post-dose and analyzed directly for WBCT, or citrated plasma (0.32% final) was prepared for clotting activity and protein concentration measurements using the rFIXFc-specific ELISA.

Cynomolgus monkeys

Animals were fasted a minimum of 12 hr and were anesthetized with ~ 10 mg/kg of ketamine intramuscularly prior to administration of rFIXFc. Single intravenous doses of rFIXFc (0.5, 2, and 10 mg/kg, corresponding to approximately 25, 100, or 500 IU/kg, respectively) were administered through an indwelling cephalic vein catheter in a total infusion volume of 5 mL followed by a 3 mL saline flush. Blood samples were collected by femoral venipuncture into citrated vacutainer tubes pre-dose and at 0.25, 0.5, 1, 8, 24, 48, 72, 96, 120, 144, and 168 hr post-dose, and citrated plasma (0.32% final) was prepared.

Coagulation testing

Factor IX activity assay

An automated Factor IX activity assay was performed using the MLA Electra 1600C (Medical Laboratory Automation/Instrument Labs, Pleasantville, NY) to quantify the ability of the FIX component of the rFIXFc protein to restore the clotting activity of FIX-deficient plasma. This assay also detected the amount of FIX activity in citrated plasma from animals treated with rFIX or rFIXFc. Test samples were mixed with equal volumes of human FIX-deficient plasma.
(Diagnostica Stago, cat. #00724) and cephalin-containing ellagic acid activator (aPTT-soluble activator, Helena Laboratories, cat. #5389), and after 4 min incubation, 5 mM calcium chloride (25 mM stock, Instrumentation Laboratory, cat. #020006910) was added and the time to clot measured. Activity was calculated based on a calibration curve of clotting times versus activity unit concentration (IU/mL) of serial dilutions of a World Health Organization (WHO) FIX standard for purified proteins, dilutions of a calibrated human plasma standard for the mouse experiments, and dilutions of rFIXFc spiked into FIX-deficient dog plasma for the FIX-deficient dog experiments.

Whole blood clotting time (WBCT)

FIX-deficient mice were dosed intravenously with 50 IU/kg rFIXFc or rFIX. Blood samples (30 μL) were collected pre- and post-dose at various times and incubated at 37°C. Samples were visually inspected for the presence of a blood clot once per minute and the time for a clot to form was recorded. Sampling ceased when clotting time returned to baseline (144 hr for rFIXFc and 72 hr for rFIX).

FIX-deficient dogs were infused intravenously with 140 IU/kg rFIXFc and blood samples were drawn to measure the whole blood clotting time (WBCT) as described.19,20
Pharmacokinetic data analyses and statistical methods

Pharmacokinetic analysis was performed using non-compartmental modeling with WinNonlin, version 4.1 or 5.1 software (Pharsight Corporation, Mountain View, CA). The pharmacokinetic parameter estimates derived from ELISA data included maximum concentration ($C_{\text{max}}$), area under the time versus concentration curve (AUC), and elimination half-life ($t_{1/2}$).

Results

Biochemical characterization of rFIXFc

Recombinant FIXFc samples were analyzed by a number of biochemical methods (Table 1). Conditioned media from transfected cells contained 3 species, FIXFc dimer, FIXFc monomer, and free Fc dimer (Figure 1), and were purified to yield FIXFc monomer (hereinafter referred to as rFIXFc) using a 3-column process. Purified rFIXFc was analyzed by both reducing and non-reducing SDS-PAGE. Non-reduced samples ran as single bands of the expected size (~120 kDa), correlating to the intact rFIXFc, with no detectable Fc or FIXFc dimer (Figure 1). Reduced samples ran as 2 bands of expected sizes, corresponding to the rFIXFc (~100 kDa) and Fc (~30 kDa) chain. Protein was found to be >95% pure by size exclusion chromatography (data not shown) and reducing and non-reducing SDS-PAGE.

FIX Western blotting confirmed the identity of the FIX protein and the absence of the propeptide (data not shown). Proper propeptide processing was confirmed by Lys-C peptide mapping (data not shown). The rFIXFc chain contains 641 amino acids, and the Fc chain contains 227 amino acids. FIX is composed of a number of functional domains which undergo extensive post-translational modification. Recombinant FIXFc was found to have 11.2 ± 0.7 Gla.
per molecule, comparable to rFIX which was found to contain 11.6 ± 0.52 Gla per molecule, consistent with literature values of 11.5 Gla per molecule.\textsuperscript{21} Peptide mapping confirmed the gamma-carboxylation pattern to be similar to rFIX, with 10 of the 12 sites fully occupied, and the residues primarily at position 36 and 40 being under-gamma-carboxylated, which has been shown to have no effect on activity.\textsuperscript{14}

In addition to gamma-carboxylation and propeptide processing, a number of other posttranslational modifications were assessed for rFIXFc and compared to rFIX (BeneFIX\textsuperscript{®}) and pdFIX (Mononine\textsuperscript{®}). In general, rFIXFc was found to be comparable to rFIX, with respect to Ser 158 phosphorylation and Tyr 155 sulfation (Table 1). N-linked glycosylation fingerprints indicated that the FIX-derived glycans are not fully sialylated, similar to rFIX (data not shown). O-linked glycosylation isoforms in the first EGF domain were the same as rFIX but present at different relative ratios (data not shown). Recombinant FIXFc possessed a greater degree of \(\beta\)-hydroxylation of Asp 64 than rFIX or pdFIX. The levels of activated FIX were measured in all 3 proteins, and found to be significantly lower in rFIXFc than either rFIX or pdFIX.

The specific activity of rFIXFc ranged from 4.28 ± 0.52 to 6.12 ± 0.28 IU/nmol for protein lots that had undergone a 3-column purification process, corresponding to 43.8 ± 5.4 to 62.7 ± 2.87 IU/mg based on the Factor IX activity assay using a WHO calibration standard. For some of the earlier in vivo experiments, the specific activity of rFIXFc was lower prior to optimization of the purification process. This however did not have an effect on pharmacokinetic properties of rFIXFc (data not shown).
Coagulation activity in FIX-deficient mice

Fifteen minutes after administration, rFIXFc and rFIX corrected the circulating levels to 1.47 ± 0.32 IU/mL and 2.28 ± 1.01 IU/mL, respectively. At 48 hr, the rFIXFc clotting activity remained high at 0.27 ± 0.15 IU/mL, while clotting activity with rFIX had decreased substantially to 0.07 ± 0.03 IU/mL. Clotting activity for the rFIX-treated mice was undetectable at timepoints later than 48 hr after dosing, while rFIXFc-treated mice continued to retain measurable activity, remaining at 0.10 ± 0.04 IU/mL at 96 hr (Figure 2).

In whole blood samples, 15 min after administration, normal clotting was observed in all mice given rFIXFc or rFIX (Figure 3). Blood clotting times in all of the rFIX-treated animals returned to baseline by 72 hr, whereas blood from all of the rFIXFc-treated mice still clotted throughout this time. Clotting activity in blood from rFIXFc-treated mice returned to baseline by 144 hr in all animals, twice the time observed for the rFIX-treated mice.

In repeat-dose experiments in FIX-deficient mice comparing the activity of rFIXFc and rFIX, 15 min after the first dose, rFIXFc and rFIX demonstrated comparable clotting activity, of 1.96 ± 0.23 IU/mL and 1.09 ± 0.14 IU/mL, respectively. By 96 hr, clotting activity was measurable in all 6 rFIXFc-treated mice (0.12 ± 0.08 IU/mL) but not in rFIX-treated mice (the limit of detection of the assay was approximately 0.01 IU/mL). Similar results were obtained over the next 2 doses, measuring comparable peak activities 15 min after dosing for rFIXFc and rFIX (2.34 ± 0.73 v. 1.94 ± 0.35 IU/ml, respectively, after the second dose, and 2.12 ± 0.20 v. 1.69 ± 0.70 IU/ml, respectively, after the third dose) but only detecting clotting activity in the rFIXFc-treated mice after 96 hours (0.12 ± 0.017 and 0.12 ± 0.034 IU/ml after the second and third dose, respectively) with one exception, in that 1 of 5 animals dosed with rFIX exhibited detectable clotting activity (0.08 IU/ml) at 4 days after the second dose.
Recombinant FIXFc protein levels were also determined by ELISA analysis. In general, the pharmacokinetic profile appears to reflect the activity levels found in the single-dose experiment, possibly with a slight accumulation of drug as the peak levels were found to increase after the first, second, and third doses from 24.05 ± 7.02 to 36.85 ± 10.96 and to 38.19 ± 9.49 µg/mL, respectively. Similarly, the trough levels increased from 1.60 ± 0.60 to 1.88 ± 0.72 to 3.22 ± 0.74 µg/mL, respectively. Based on the specific activity of this lot of rFIXFc (43.8 IU/mg) and the ELISA data (µg/mL), calculated plasma clotting activity level was determined (IU/mL) and compared to measured clotting activity. The results of this comparison are shown in Figure 4a. Calculated clotting activity correlated well with empirical clotting activity data indicating that activity of the rFIXFc molecule is maintained in circulation.

Recombinant FIX was detected at all time points for all 3 doses with each repeat dose resulting in peak and trough values of 3.2–4.2 µg/mL and 0.02–0.03 µg/mL, respectively. Using the measured specific activity for these lots of rFIXFc (43.8 IU/mg) and rFIX (259 IU/mg), it was possible to compare the calculated clotting activity for all plasma samples analyzed by ELISA (Figure 4b).

Pharmacokinetics in mice and rats

In rats, the t½ of rFIXFc was 34.8 ± 5.3 hr compared to an elimination t½ for rFIX of 5.8 hr. The t½ of rFIXFc was 46.2 ± 10.1 hr in FIX-deficient mice compared to 13.2 hr for rFIX, and 47.2 ± 4.8 hr in normal mice compared to 12.3 hr for rFIX (Table 2).

In FcRn KO mice, rFIXFc demonstrated a t½ of 16.9 ± 2.1 hr, similar to the t½ of rFIX in these animals of 16.5 ± 3.0 hr. In human FcRn/human β2m transgenic mice made in FcRn KO
background strain, the t½ of rFIXFc was 53.0 ± 6.6 hr in contrast to rFIX, which had a t½ of 14.2 ± 2.9 hr in these same mice.

**Pharmacokinetics and pharmacodynamics in FIX-deficient dogs**

Clotting activity was measured for all time points and demonstrated that the activity versus time curves are similar for the 2 animals (**Figure 5a**), declining in a biphasic manner characteristic of Factor IX.²² The $C_{max}$ at 15 minutes post-infusion was found to be 1.028 IU/mL in dog I08; the earliest time point for which clotting activity was determined for dog I03 was 1 hour, at which time the level was 0.681 IU/mL compared to 0.466 IU/mL for I08. At 144 hours, clotting activity was still detectable, and found to be 0.027 and 0.022 IU/mL for dog I03 and I08, respectively, after which the levels fell below the limit of detection of approximately 0.01 IU/mL at 168 hr. Terminal half-lives of 37.7 hr and 38.8 hr were calculated for dogs I03 and I08, respectively, using the best fit of the data from the 12 to 144 hour time points.

A sandwich ELISA was used to measure the concentration of intact rFIXFc in the Hemophilic B dog plasma samples (**Figure 5b**). The plasma concentration vs time curves were similar for the 2 animals, declining in a biphasic manner and consistent with the activity data (compare **Figures 5a and 5b**). Terminal half-lives of 37.5 hr and 57.4 hr were calculated for dogs I03 and I08, respectively, using the best fit of the data from the 24 hr to 144 hr time points.

Baseline whole blood clotting time (WBCT) is 8–12 min for normal dogs and > 60 min for FIX-deficient dogs. Immediately upon administration of rFIXFc, clotting time was corrected from pre-dose levels to approximately normal levels of 13.5 and 11.5 min for dog I03 and I08, respectively. Whole blood clotting activity remained corrected to approximately normal levels
through 144 hr post-dose before returning to pre-dose levels of > 60 min at 168 hr post-dose (Figure 5c).

Pharmacokinetics in non-human primates

The pharmacokinetics of rFIXFc were evaluated at 3 dose levels (0.5, 2, and 10 mg/kg, approximately 25, 100, and 500 IU/kg), demonstrating consistent levels within each group and showing dose-proportionality between groups with average maximum plasma concentrations ($C_{\text{max}}$) of $6.05 \mu g/mL$, $24.54 \pm 1.59 \mu g/mL$, and $129.82 \pm 3.45 \mu g/mL$, respectively (Figure 6). At 168 hr, rFIXFc protein levels were $0.13, 0.37 \pm 0.03$, and $1.59 \pm 0.07 \mu g/mL$, respectively, for the 3 doses. The area under the time vs concentration curve also demonstrated dose-proportionality, with AUC of $164, 530 \pm 79$, and $2654 \pm 415 \mu g \cdot hr/mL$, at the 0.5, 2, and 10 mg/kg levels, respectively. Pharmacokinetic analysis determined the $t_{1/2}$ to be $47.0 \text{ hr (n = 2), 47.8 } \pm 2 \text{ hr (n = 3), and 47.0 } \pm 13.3 \text{ hr (n = 3), respectively, for the 3 dose groups.}$

Discussion

Recombinant FIXFc, a fusion protein comprised of a single molecule of FIX fused to the Fc domain of human immunoglobulin G1 (IgG1), was developed to address the short half-life of currently available FIX products used to treat patients with hemophilia B. We have demonstrated that the elimination half-life of rFIXFc was consistently 3- to 4-fold longer than rFIX across species (Table 2), including direct comparisons in normal and FIX-deficient mice and normal rats. Half-lives calculated in our studies are markedly longer than published values of 17.7 hr (range 15.6 to 21.6 hr) or 17.3 hr that has been reported for rFIX in FIX-deficient dogs or 12.9 ± 1.5 hr and 13.2 ± 1.6 hr found in normal dogs. Similarly, the half-life of rFIXFc of
approximately 47 hr in cynomolgus monkeys is considerably longer than the previously reported 12.7 hr average for rFIX.23

The mechanism for the extension of half-life is based on the ability of the Fc domain of the fusion protein to bind to the neonatal Fc receptor, FcRn, at acidic pH (< 6.5) but not at physiological pH (7.4).9,10 FcRn is a heterodimer comprised of a major histocompatibility complex (MHC)-class-I-related heavy chain and the β2-microglobulin light chain that is found complexed to all MHC class I molecules.9,10,25 Both components of FcRn have been demonstrated to have a role in IgG homeostasis, as mice deficient in either chain have lower levels of IgG antibodies in circulation, with significantly shortened half lives.17,26-28 Plasma proteins are thought to be internalized by endothelial cells and targeted for degradation by the lysosome, however Fc-containing proteins bind to FcRn present in the acidified endosome in a pH-dependent manner, and are then recycled back to the cell surface where the Fc-containing proteins are released from FcRn at physiologic pH, and thus protected from catabolism.9,10 The data presented herein demonstrate that rFIXFc has a 3- to 4-fold longer terminal half-life in mice expressing human FcRn and β2m compared to rFIX, while both proteins have similar short terminal half-lives in mice lacking FcRn (FcRn/β2m KO). These data confirm that FcRn mediates the longer half-life of rFIXFc, thus utilizing the natural pathway responsible for protecting IgG antibodies from degradation.9,10 Consequently, rFIXFc has a longer circulating half-life than unconjugated FIX molecules such as rFIX in a number of species.

In addition to the pharmacokinetics of rFIXFc, we have examined the pharmacodynamics in two FIX-deficient species. In FIX-deficient mice, rFIXFc and rFIX, administered at approximately the same dose, corrected the clotting deficiency to comparable levels; however the effect of rFIXFc was significantly prolonged to at least 96 hr after infusion. This trough
activity of rFIXFc corresponds to approximately 10% of its normal clotting activity. Current prophylactic FIX replacement therapy for patients with hemophilia B aims to maintain plasma levels of 1–2% normal clotting activity.29

The whole blood clotting time (WBCT) assay is an ex vivo test that measures the ability of whole blood to form a clot and is an alternate confirmatory test for the restoration of clotting activity. In response to infusion with rFIXFc, the WBCT of the hemophilic dogs was corrected from > 60 min to approximately the normal range of 10–12 min and remained in this range for 144 hr before returning to pre-dose levels at 168 hr. In a direct comparison in FIX-deficient mice, although both rFIXFc and rFIX led to whole blood clotting immediately upon infusion, only rFIXFc-treated animals demonstrated sustained clotting activity.

FIX is a complex protein that contains a number of functional domains which undergo extensive post-translational modifications.21,30 One of the essential post-translational modifications for FIX activity is gamma-carboxylation of the first 12 glutamic acids in the Gla domain by vitamin K-dependent γ-glutamyl carboxylase. This modification facilitates the binding of FIX to phospholipid membranes and, thus, is critical to its function. FIX that is not gamma-carboxylated is not functional, and hence gamma-carboxylation is a rate-limiting step during FIX production.31,32 HEK-293 cells have proven to be an efficient expression system since they are capable of high-level expression and produce post-translational modifications, in particular, for gamma-carboxylation superior to CHO or BHK cells.33 HEK-293 cells are of human, rather than rodent, origin and therefore may result in posttranslational modifications more representative of human proteins. For these reasons, we chose to express rFIXFc in HEK-293 cells. The Gla content of rFIXFc was found to be comparable to that of rFIX by both amino acid analysis and peptide mapping. The rFIXFc protein was extensively characterized and found
to be of high purity and comparable to recombinant FIX in a number of posttranslational modifications, including serine phosphorylation, tyrosine sulfation, and glycosylation (Table 1). Although one or more of these characteristics have been proposed to be linked to the lower recovery of rFIX as compared to pdFIX, it is unclear from the current data if this will be the case for rFIXFc as well, or if fusion with Fc will alter the recovery. One difference we have found is in the level of activated Factor IX, which is 10-fold lower in rFIXFc preparations as compared to rFIX or pdFIX.

Another key difference observed between rFIXFc and other FIX products has been in the specific activity as assessed by aPTT-based assays. As noted above, the specific activity of rFIXFc ranges from 4.41 ± 0.22 to 6.12 ± 0.28 IU/nmol for protein lots that had undergone 3-column purification, corresponding to 45.2 ± 2.25 to 62.7 ± 2.87 IU/mg, with more recent lots having a specific activity of approximately 60 IU/mg (unpublished data). In comparison, rFIX has a specific activity in aPTT-based assays of approximately 260 IU/mg, or 12.1 IU/nmol. Due to the presence of the Fc domain in rFIXFc, the more appropriate comparison for specific activity is on a molar basis, where rFIXFc is approximately 2-fold lower than rFIX. Extensive biochemical characterization of rFIXFc indicated that the difference in specific activity was not due to any deficiency in critical post-translational modifications, such as gamma-carboxylation or propeptide processing.

Further studies have been performed examining the interactions of rFIXFc in the context of the tenase complex on a variety of phospholipid sources, including cephalin, synthetic phospholipid vesicles, and nonactivated and activated platelets and found them to be comparable to rFIX (Toby et al, unpublished data). Though the exact mechanistic basis for the difference in specific activity remains under investigation, this difference is unlikely to be therapeutically
relevant, as rFIXFc is dosed on an IU basis, and such a dosing regimen has been found to result in similar initial blood levels of activity as compared to rFIX in experiments performed in FIX-deficient mice.

Traditionally, Fc fusion proteins have been made as dimers, with respect to the effector molecule and the Fc region.\textsuperscript{34} Previously we have generated a novel fusion protein which has a monomeric effector molecule conjugated to the Fc domain.\textsuperscript{35} We had also demonstrated that such a configuration of EpoFc enhanced its pharmacodynamic and pharmacokinetic properties. Similarly, we have found that the FIXFc monomer has enhanced pharmacokinetic properties as compared to the FIXFc dimer, in particular, in significantly higher $C_{\text{max}}$ and AUC.\textsuperscript{36}

In conclusion, data from these studies indicate that rFIXFc has enhanced pharmacokinetic parameters compared to rFIX, particularly in its terminal half-life. Clinical studies with this long-acting rFIXFc coagulation factor are in progress to investigate its potential to reduce the frequency of injections in patients receiving routine (prophylactic) doses of factor replacement. Whether rFIXFc can confer prolonged protection from bleeding following each dose of factor, decrease the overall units of factor needed to treat bleeding episodes, or maintain adequate hemostasis during surgical procedures with fewer injections are of considerable clinical interest.

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Authorship Contributions

R.T.P. designed research, performed research, analyzed and interpreted data, and wrote the manuscript. S.C.L. designed research, performed research, and analyzed and interpreted data. J.A.D. and A.J.B. designed research, analyzed and interpreted data. G.D.K., J.V.A. and Q.L. contributed vital analytical tools, performed research, analyzed and interpreted data. G. Z-P. and T.J.R. developed purification method, and performed research. E.P.M. performed research. T.C.N. performed research, analyzed and interpreted data.

Disclosure of Conflicts of Interest

Reference List


8. National Hemophilia Foundation #179. MASAC recommendation concerning prophylaxis (regular administration of clotting factor concentrate to prevent bleeding). November 2007. Available at:
http://www.hemophilia.org/NHFWeb/MainPgs/MainNHF.aspx?menuid=57&contentid=1007


Table 1. Biochemical characterization of recombinant FIXFc, recombinant and plasma derived FIX

<table>
<thead>
<tr>
<th></th>
<th>rFIXFc</th>
<th>rFIX</th>
<th>pdFIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma-carboxylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aa 1-23 (K1K2 peptide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 6 Gla</td>
<td>97.8</td>
<td>96.9</td>
<td>99.6</td>
</tr>
<tr>
<td>% 5 Gla</td>
<td>2.2</td>
<td>3.1</td>
<td>0.4</td>
</tr>
<tr>
<td>% 4 Gla</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>aa 24-43 (K3 peptide)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 6 Gla</td>
<td>61.3</td>
<td>63.7</td>
<td>98.9</td>
</tr>
<tr>
<td>% 5 Gla</td>
<td>26.3</td>
<td>30.9</td>
<td>1.1</td>
</tr>
<tr>
<td>% 4 Gla</td>
<td>12.5</td>
<td>5.4</td>
<td>0</td>
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<td>Total Gla/mol, peptide map</td>
<td>11.5</td>
<td>11.6</td>
<td>12.0</td>
</tr>
<tr>
<td>Total Gla/mol, AAA</td>
<td>11.2 ± 0.7</td>
<td>11.6 ± 0.5</td>
<td>(12)</td>
</tr>
<tr>
<td>Propeptide content</td>
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<td>none detected</td>
<td>none detected</td>
</tr>
<tr>
<td>β-hydroxylation Asp 64</td>
<td>70%</td>
<td>49%</td>
<td>37%</td>
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<tr>
<td>Sulfation of Tyr 155</td>
<td>4%</td>
<td>5%</td>
<td>(&gt;90%)</td>
</tr>
<tr>
<td>Phosphorylation of Ser 158</td>
<td>&lt;10%</td>
<td>&lt;10%</td>
<td>(&gt;90%)</td>
</tr>
<tr>
<td>Ala 148/Thr 148</td>
<td>Thr</td>
<td>Ala</td>
<td>30% Ala / 70% Thr</td>
</tr>
<tr>
<td>Activated FIX</td>
<td>&lt;0.013%</td>
<td>0.11 ± 0.0019%</td>
<td>0.21 ± 0.010%</td>
</tr>
<tr>
<td>FXIa Activation</td>
<td>94.8 ± 2.4%</td>
<td>96.6 ± 1.8%</td>
<td>Not done</td>
</tr>
</tbody>
</table>
Table 2. Summary of terminal half-lives of rFIXFc and rFIX after a single intravenous dose

<table>
<thead>
<tr>
<th>Species</th>
<th>rFIX</th>
<th>rFIXFc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mice</td>
<td>12.3 hr</td>
<td>47.2 ± 4.8 hr</td>
</tr>
<tr>
<td>FIX-deficient mice</td>
<td>13.2 hr</td>
<td>46.2 ± 10.1 hr (47 hr)</td>
</tr>
<tr>
<td>FcRn/β2m KO mice</td>
<td>16.5 ± 3.0 hr</td>
<td>16.9 ± 2.1 hr</td>
</tr>
<tr>
<td>hFcRn/hβ2m transgenic Tg32b mice</td>
<td>14.2 ± 2.9 hr</td>
<td>53.0 ± 6.6 hr</td>
</tr>
<tr>
<td>Rats</td>
<td>5.8 hr</td>
<td>34.8 ± 5.3 hr</td>
</tr>
<tr>
<td>FIX-deficient dogs</td>
<td>(17–18 hr)*</td>
<td>47.5 hr (38.3 hr)</td>
</tr>
<tr>
<td>Monkey</td>
<td>12.7 hr ‡</td>
<td>47.3 ± 9.1 hr</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. SDS-PAGE gel of purification intermediates and purified rFIXFc monomer.
Samples from different steps in the purification of rFIXFc were analyzed by non-reducing SDS-PAGE. Lane 1: SeeBlue Plus Molecular Weight Markers (Invitrogen). Lane 2: Empty lane. Lane 3: Protein A Load. Lane 4: Protein A Eluate. Lane 5: Fractogel DEAE Eluate. Lane 6: Q Seph FF Eluate. Lane 7: Final Bulk Material: rFIXFc. Lane 8: Empty lane. The Final Bulk Material was also analyzed by SDS-PAGE after reduction with 2-mercaptoethanol in Lane 9.

Figure 2. Functional activity of rFIXFc and rFIX in FIX-deficient mice.
FIX-deficient mice were dosed intravenously with 219 IU/kg rFIXFc (3 or 4 per group, 6 groups, n = 23) or 200 IU/kg rFIX (3 or 4 per group, 5 groups, n = 23) at time = 0. Blood samples were collected at various times after dosing (0.25 hr to 96 hr) and analyzed for clotting activity using FIX activity assay.
* rFIX activity is undetectable in all of the mice at time points later than 48 hr after dosing.

Figure 3. Whole blood clotting time of rFIXFc versus rFIX in FIX-deficient mice.
FIX-deficient mice (6 per group) were dosed intravenously with 50 IU/kg rFIXFc or 50 IU/kg rFIX. Blood samples were collected before dosing and at various times after dosing. Blood samples were incubated at 37°C and were visually inspected for the presence of a blood clot once per minute. The time needed for a clot to form was recorded and once the clotting activity returned to baseline (ie, no clot formation), no additional samples were obtained (samples collected 15 min to 144 hr for rFIXFc or 15 min to 72 hr for rFIX).
Figure 4. Pharmacodynamics of rFIXFc and rFIX in FIX-deficient mice.

FIX-deficient mice were dosed with 219 IU/kg rFIXFc (5 per group, 6 groups, n = 30) or 200 IU/kg rFIX (4 or 5 per group, 6 groups, n = 28) on Day 0, 4, and 8. Plasma samples were collected by cardiac puncture at 15 min and 96 hr after each dose and clotting activity was measured using a FIX activity assay. Plasma was also collected by tail bleeds at 8, 24, 48, and 72 hr after each dose. Recombinant FIXFc levels were measured in all of the samples using an ELISA specific for rFIXFc. (a) Measured vs Calculated Activity. Clotting activity for rFIXFc was measured using FIX activity assay 15 min and 96 h after 3 doses. The specific activity for this lot of rFIXFc was determined to be 43.8 ± 5.4 IU/mg. Based on this activity (IU/mg) and the measured protein levels, a calculated plasma clotting activity level was determined for time points at 15 min, 8, 24, 48, 72 and 96 h after each dose. (b) In FIX-deficient mice treated with up to 3 doses of 200 IU/kg rFIX, FIX levels were measured using FIX-specific ELISA. Using the measured specific activities of rFIXFc and rFIX, it was possible to compare calculated clotting activity for all samples analyzed by ELISA.

Figure 5. Pharmacokinetics and pharmacodynamics of rFIXFc in FIX-deficient dogs.

Two dogs with hemophilia B were intravenously infused with 140 IU/kg rFIXFc. Blood samples were collected at 5, 15, and 30 min, and at 1, 2, 4, 6, 8, 12, 24, 27, 30, 48, 51, 54, 72, 80, 96, 126, 144, and 168 hr. (a) FIX clotting activity was measured for all time points with respect to a standard curve generated with rFIXFc. (b) A sandwich ELISA utilizing a FIX capture antibody and Fc-HRP detection antibody was used to measure the concentration of intact rFIXFc in the hemophilic B dog plasma samples. (c) Blood collected from animals was immediately analyzed.
for whole blood clotting time. Blood samples were incubated at 28°C and were visually inspected for the presence of a clot once per minute, and the time in which a clot formed was recorded.

**Figure 6. Pharmacokinetics of rFIXFc in cynomolgus monkeys.**

Monkeys were administered a single dose (0.5, 2, and 10 mg/kg, corresponding to approximately 25, 100, or 500 IU/kg) of rFIXFc (n = 2, 3, and 3, respectively). Blood samples were collected at 0.25, 0.5, 1, 8, 24, 48, 72, 96, 120, 144, and 168 hr post-dose and plasma prepared for analysis of protein concentration by FIXFc-specific ELISA.

**Table 1. Biochemical characterization of rFIXFc**

Posttranslational modifications and other analyses of rFIXFc, rFIX, and pdFIX were assessed in a variety of assays as described in the methods. Numbers in parentheses taken from published values.⁷

**Table 2. Summary of terminal half-lives of rFIXFc and rFIX after a single intravenous dose**

Animals were administered a single dose of rFIXFc (~ 200 IU/kg in normal mice [n = 8], FIX-deficient mice [n = 11], FcRn KO mice [n = 4], FcRn Tg32B mice [n = 4], and rats [n = 9]; 140 IU/kg for FIX-deficient dogs [n = 2]; and 25, 100, or 500 IU/kg for monkeys [n = 2, 3, or 3, respectively]) or rFIX (100 IU/kg in normal mice [n = 5] and FIX-deficient mice [n = 5] and 200 IU/kg in FcRn KO mice [n = 4], FcRn Tg32B mice [n = 4], and rats [n = 5]). Blood samples were collected at various time points between 0.25 hr and 168 hr and plasma prepared for
analysis of protein concentration by rFIXFc or FIX-specific ELISA. Data were then analyzed using WinNonLin to generate terminal half-life, utilizing the best fit of data from all time points. FIX activity data was also obtained from FIX-deficient mice (n = 4 / timepoint) and FIX-deficient dogs (n = 2) and terminal half-life based on activity calculated (listed in parentheses). *Represents published values of t$_{1/2}$ for recombinant FIX, based on activity data.22,23 † Represents published values of t$_{1/2}$ for recombinant FIX, based on ELISA data.23
Figure 1

Lane 1: Molecular Weight Markers
Lane 2: Empty lane
Lane 3: Protein A Load
Lane 4: Protein A Eluate
Lane 5: DEAE Eluate
Lane 6: Q Seph FF Eluate
Lane 7: Final Bulk Material: rFIXFc
Lane 8: Empty lane
Lane 9: Final Bulk Reduced: rFIXFc

188 kDa
62 kDa
49 kDa
28 kDa
6 kDa

rFIXFc Dimer
rFIXFc Monomer
reduced rFIXFc chain
reduced Fc chain
Figure 3

% mice with blood clotting

Time (hr)
Figure 4

(A) rFIXFc in plasma (IU/ml) over time (hr) with dose intervals marked.

(B) Calculated FIX Activity in Plasma (IU/ml) over time (hr) with dose intervals marked.

Legend:
- Green circles: Calculated Clotting
- Pink squares: Measured Clotting
- Orange circles: rFIXFc
- Blue triangles: rFIX
Prolonged activity of factor IX as a monomeric Fc fusion protein