Safety, Stability and Pharmacokinetic Properties of (super)Factor Va, a Novel Engineered Coagulation Factor V for Treatment of Severe Bleeding.

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Safety, stability and pharmacokinetic properties of superFactor Va, a novel engineered coagulation factor V for treatment of severe bleeding

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Abstract

Purpose—Activated superFactor V (superFVa) is a novel engineered FV with excellent prohemostatic efficacy. SuperFVa has three APC cleavage site mutations and an interdomain disulfide bond. Stability, pharmacokinetics, and immunogenic and thrombogenic potential are reported here.

Methods—Stability and circulating half-life were determined after incubation in buffer and human plasma, and after injection into FVIII-deficient mice. Immunogenicity potential was assessed by B- and T-cell specific epitope prediction and structural analysis using surface area and atomic depth computation. Thrombogenic potential was determined by quantification of lung fibrin deposition in wild-type mice after intravenous injection of superFVa (200 U/kg), recombinant human (rh) Tissue Factor (0.4–16 pmol/kg), rhFVIIa (3 mg/kg) or saline.

Results—SuperFVa retained full activity over 30 hours in buffer, the functional half-life in human plasma was 4.9 hours, and circulating half-life in FVIII-deficient mice was ~30 minutes. Predicted immunogenicity was not increased compared to human FV. While rh Tissue Factor, the positive control, resulted in pronounced lung fibrin depositions (mean 121 μg/mL), superFVa did not (6.7

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μg/mL), and results were comparable to fibrin depositions with rhFVIIa (7.6 μg/mL) or saline (5.6 μg/mL).

Conclusion—^{Super}FVa has an appropriate safety and stability profile for further preclinical development as a prohemostatic against severe bleeding.

Keywords

Immunogenicity; thrombogenicity; Factor V; bleeding; hemostasis; hemophilia

Introduction

Activated ^{super}Factor V (^{super}FVa) is a novel coagulation FVa variant with potential for clinical development to treat catastrophic bleeding situations such as bleeding in hemophilia, bleeding associated with direct oral anti-coagulants (DOACs), or traumatic bleeding. The molecule was engineered to be resistant against irreversible proteolytic inactivation by activated protein C (APC) involving mutation of all three APC cleavage sites at Arg506, Arg306 and Arg679. In addition, an engineered interdomain disulfide bond (involving His609Cys-Glu1691Cys) connecting the A2 and A3 domains (A2-SS-A3) was introduced (1), which confers increased specific activity, presumably by preventing domain dissociation as the final step of FVa inactivation (1,2). ^{Super}FVa is a potent molecule to achieve “FVa activity augmentation”, which is emerging as a new concept to enhance hemostasis (2). FVa is required as an essential cofactor in the prothrombinase complex where it enhances the rate of thrombin generation approximately 10,000-fold (3). FVa is generated by proteolytic cleavage of the procofactor, factor V, by thrombin, which removes an internal “B domain” resulting in the active FVa (3). However, since FVa is rapidly inactivated by APC, the principle of “FVa activity augmentation” is to deliver APC-inactivation resistant FVa to enhance the prothrombinase complex. This concept was based on several previous observations implying that the prothrombotic FV_{Leiden} mutation changed phenotypic bleeding in hemophilia patients and mice (4,5), and that recombinant human (rh)FV_{Leiden} (Arg506Gln) and rh$FV_{Cambridge}$(Arg306Thr), which are partially resistant against inactivation by APC, improved thrombin generation in hemophilia plasma (6,7). Although B domain-deleted FV already has some inherent cofactor activity (8), ^{super}FV is being used in fully activated form to remove FV activation as the rate limiting step for its availability in the prothrombinase complex. In addition to APC-resistance, delivery of this activated cofactor appears to be another critical mechanism of the “FVa activity augmentation” strategy, supported by previous observations that clot formation with human plasma FV in FVIII-deficient mice required prior activation of the FV (4). Since FVa is a cofactor in the prothrombinase complex that only accelerates thrombin generation once factor Xa is enzymatically activated at the site of injury, the prothrombotic risk of “FVa activity augmentation” was deemed low. Taken together, these properties were thought to provide a safe and efficient approach to enhance hemostasis in severe bleeding situations.

Towards that end we studied several APC-inactivation resistant FVa variants for their degree of APC-resistance and their hemostatic properties in hemophilic plasma (2). We identified one lead candidate, subsequently denoted ^{super}FVa, that demonstrated near complete APC inactivation resistance and superior ability to enhance *in vitro* hemostasis when compared to

the other FVa variants (2). Subsequent studies of superFVa demonstrated its effectiveness to control bleeding in hemophilic mice (2), in wild-type mice with inhibitors against FVIII (9), in bleeding induced by the direct oral FXa inhibitors (DOACs) (10), and in a mouse trauma model with intensified bleeding by exogenously administered APC (11). In the trauma model superFVa also rescued survival of the bleeding mice (11). Additionally, superFVa improved the coagulation parameters in plasma from hemophilia patients with and without inhibitors in bleeding situations, improved thrombin activatable fibrinolysis inhibitor (TAFI)-dependent fibrinolysis inhibition, and demonstrated cooperative effects with rhFVIIa (9). With respect to hemophilia patients, FVIIa-based clotting factor preparations, e.g., either rhFVIIa (NovoSeven®, Novo Nordisk, Bagsvaerd, Denmark) or a plasma-derived product (FEIBA®, Baxter Biosciences, Westlake Village, USA), are the only available options for patients with inhibitors. Unfortunately, treatment with FVIIa-based agents remains suboptimal and less effective compared to FVIII-based or FIX-based clotting factor concentrates in patients without inhibitors (12–14). Additionally, there are currently no effective targeted treatment options available for traumatic bleeding other than blood component replacement (15), or for bleeding associated with DOACs. Taken together, the void of effective, targeted treatment strategies against severe bleeding creates an unmet clinical need for new prohemostatic agents such as superFVa (16).

Here, we report the evaluations of superFVa's circulating plasma half-life in the mouse, stability in human plasma and storage buffer, thrombogenic potential in a mouse model of pulmonary embolism and predicted immunogenic potential. Following our demonstration of proof-of-concept that superFVa has superior hemostatic properties compared to wild type FVa and other prohemostatc agents in various bleeding indications (2,9,11), the data presented here indicate that superFVa is suitable to be moved forward into preclinical and clinical development.

Materials and Methods

Materials

The following reagents were used: thrombin, corn trypsin inhibitor, plasmin (all from Enzyme Research Laboratories, South Bend, IN, USA), purified plasma FV (Haematologic Technology, Burlington, VT, USA), hirudin (Calbiochem, San Diego, CA, USA), thrombin calibrator (Synapse BV, Maastricht The Netherlands), Substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland), rh Tissue Factor (TF), Dade Innovin®, (Dade Behring, Newark, DE, USA), aPTT-XL reagent (Pacific Hemostasis/Fisher Scientific, Middletown, VA, USA), heparin (Baxter, Deerfield, IL, USA), rhFVIIa (Novoseven®, Novo Nordisk, Bagsvaerd, Denmark), pooled normal human plasma (NHP) and FV deficient plasma (FVDP) (George King Bio-Medical, Overland Park, KS, USA), phosphatidylcholine (PC) and phosphatidylserine (PS) (Avanti Lipids, Alabaster, AL, USA).

Recombinant wild-type FV and the superFV variant were purified from conditioned media of stable transfected baby hamster kidney cells by a combination of affinity chromatography using anti-FV 3B1 and HV5101 monoclonal antibodies as described (1,17). FV protein concentration was determined by absorbance at 280 nm using FV $\varepsilon_{1\%}$= 16.9 (MW = 174,000 Da) and enzyme linked immunosorbent assay (ELISA) (Enzyme Research

Laboratories, South Bend, IN, USA) according to manufacturer's instructions. FV proteins were activated with 2 nM thrombin for 20 minutes at 37 °C in prothrombinase buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 0.5% bovine serum albumin (BSA), 5 mM $CaCl_2$ and 0.1 mM $MnCl_2$). Activation was terminated by the addition of 1.1 molar equivalent of hirudin (2).

Coagulation and thrombin generation assays

FVa stability in plasma or in storage buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$) with 0.5 % BSA was assayed by activity in the activated partial prothrombin time (aPTT) clotting assay in FVDP. For stability in storage buffer FVa or ^{super}FVa were incubated at 100 nM at 37 °C. Aliquots were removed at indicated time points, diluted 1:200 in plasma, and an aPTT was performed (see below). For plasma stability, FVa or ^{super}FVa (500 pM) were added to plasma at time "zero", and an aPTT was performed at indicated time points as follows. 50 μl plasma with FVa or ^{super}FVa was incubated for three minutes with 50 μL aPTT-XL reagent. Clotting was initiated by addition of 50 μL 25 mM $CaCl_2$ in HEPES buffered saline (HBS) with 0.5% BSA. Clotting was analyzed in a STart coagulation analyzer (Diagnostica Stago, Parsipanny, NJ, USA). Data were fit to single exponential decay (^{super}FVa) or a double exponential decay (FVa) using SlideWrite Plus (San Diego, CA). Half-life was derived from these curve fits.

Thrombin generation assays were performed as described (18). Briefly, corn trypsin inhibitor was added to NHP at 1.5 μM, followed by ^{super}FVa and rhFVIIa at various concentrations as indicated in the Result section. PCPS vesicles, prepared as described, containing 80% PC and 20% PS (19), and rhTF were also added. This mix was incubated at 10 minutes at 37°C before addition of Z-Gly-Gly-Arg-AMC substrate and calcium to initiate the reaction. Each reaction was 100 μL total volume containing 50% plasma. Final concentrations were 0.2 pM rhTF, 4 μM PCPS vesicles, 7.6 mM $CaCl_2$ and 0.5 mM Z-GGR-AMC. Fluorescence was measured at excitation/emission wavelengths of 360/460 nM using the Gemini EM fluorescent plate reader (Molecular Devices Corporation, Menlo Park, CA, USA). The endogenous thrombin potential (ETP), defined as the area under the curve (AUC) and the thrombin peak height were determined as described (20).

Animals

All animal protocols were carried out as approved by the institutional animal care and use committee of The Scripps Research Institute. Several breeding pairs of FVIII-deficient mice (Balb/c background) were a generous gift of Dr. David Lillicrap. Male or female FVIII-deficient mice, aged ≥ 8 weeks, were used to determine the pharmacokinetics of FV variants. Female BALB/c mice, aged ≥ 8 weeks, were used for the lung fibrin deposition assay.

In vivo dosing of FVa variants

In vivo ^{super}FVa dosing was based on units/kilogram (U/kg), determined as prothrombinase cofactor activity, whereby the activity of 20 nM wild-type FVa (FV plasma concentration) was defined as one Unit (2). Dosing by activity is the usual method for clinical administration of clotting factors (such as FVIII), thereby correcting for variations of

specific activity in biological material. For these experiments 200 U/kg corresponded to 0.7 mg/kg ^{super}FVa.

In vivo fibrin deposition in lung thromboembolism

Fibrin deposition in the lungs was measured following retro-orbital injection of the desired agent in a volume of 100 μL into Balb/c mice as described by Hendrickx et al. (21). Briefly, mice were weighed and 110 mg/kg ketamine and 16 mg/kg xylazine were administered intraperitoneally. Mice were maintained in deep anesthesia with 1–2% isoflurane and immobilized on a dissection pad. Approximately 15 minutes after the injection of the desired agent the abdomen was opened, and the inferior vena cava (IVC), heart and lungs were exposed. At 20 minutes 50 μL of 10,000 U/ml heparin was injected into the IVC to stop coagulation. After 10–15 seconds the IVC was cut below the liver, and 10–11 ml of saline with 10 U/mL heparin was injected slowly into the right ventricle to flush out the lungs. Lungs were harvested immediately thereafter and flash frozen on dry ice.

Fibrin was extracted from the lungs as follows: 0.5 ml cold phosphate buffered saline (PBS) was added to the lungs in a 1.5 ml tube. Lungs were diced with dissecting scissors, sonicated on wet ice for 2 minutes, alternating 2 seconds on and 3 seconds off using a microtip sonicator probe. Tubes were centrifuged at 15,000g for 10 minutes at 4°C. The pellet was weighed, washed twice with 500 μL PBS and resuspended in 4 μL PBS per mg of weight. Plasmin (100 nM final concentration) was added, followed by incubation at 37 °C for 4 hours, vortexing every hour. The suspension was then centrifuged for 20 min at 15,000g, and the supernatant was assayed at 1:90 dilution for fibrin degradation products using the mouse fibrinogen ELISA kit (Immunology Consultants Laboratory, Portland, OR, USA). The standard was provided with the kit.

In vivo pharmacokinetics

FVIII-deficient Balb/c mice were anesthetized with 5% isoflurane. ^{Super}FVa, ^{super}FV or plasma FV at the desired concentration were injected retro-orbitally in 100 μL saline. Blood was sampled retro-orbitally at various time points using heparinized micro-capillaries, which were immediately evacuated into ~ 1/10 volume 3 % NaCitrate. Every mouse was sampled at two minutes from the opposite eye as the injection for a “zero” point, and then again at one additional time point from the same eye as the injection. Factor V concentration was determined with FV ELISA using the respective FVa variant as standard.

Prediction of Antigenicity/Immunogenicity of ^{super}FVa

B cell responses were predicted with two methods. First, prediction of β-turns in proteins was used to compute the location of B-cell epitopes (22). Then standard parameters were used for both sequences and the difference between predictions of B-cell epitopes (^{super}FVa – FVa) was directly computed in Bepitope (23). The second method (Bepipred) combines a hidden Markov model with classical propensity scale methods (24). Using Bepipred, the sequences of wild-type FVa and ^{super}FVa were used for predicting linear B-cell epitopes and the difference was computed residue by residue. The NetCTL tool was used for T-cell epitope prediction. NetCTL is available in the IEDB analysis resource (25). NetCTL is a predictor of T cell epitopes based on neural networks (26). Differences in T-cell epitope

prediction were computed by submitting both ^{super}FVa and normal FVa sequences to all the 12 supertypes.

Statistics

All experiments were repeated 2–5 times. Statistical significance between groups was ascertained by one-way ANOVA followed by Dunnett's multiple comparison analysis using GraphPad Prism (San Diego, CA). Error bars represent the standard deviation (calculations performed in Excel).

Results

Stability of ^{super}FVa compared to wild-type FVa in plasma and in storage buffer

The disulfide bond between the A2 and A3 domains confers enhanced stability of ^{super}FVa relative to wild-type FVa, whereas removal of three APC cleavage sites (1,2) provides resistance to proteolysis, most notably resistance to inactivation by APC. Here, we demonstrated notably improved stability of ^{super}FVa compared to wild-type FVa by comparing their retention of activity following incubation in citrated NHP or storage buffer at 37°C for up to 8 or 30 hours, respectively. The remaining activity at various time points was determined by the ability of each FVa variant to shorten the aPTT of FVDP. In NHP at 500 pM ^{super}FVa had greatly enhanced stability with a half-life of 4.9 hours. Wild-type FVa was very unstable in comparison and decayed in a biphasic curve. The first half-life was 0.24 hours with a plateau at 36%, and the second half-life was 2.9 hours with a plateau at zero (Figure 1A).

In storage buffer in the presence of 0.5% BSA to prevent loss of FV through adsorption, ^{super}FVa and wild-type FVa (both 100 nM) were both relatively stable over the tested time period (30 hours). However, ^{super}FVa was more stable compared to wild-type FVa and maintained 100% activity over the 30 hour time course, whereas wild-type FVa lost approximately 40% of its activity (Figure 1B).

In vivo pharmacokinetics of ^{super}FVa

The *in vivo* plasma half-life of ^{super}FVa in comparison to ^{super}FV (not activated) and to plasma FV was determined in FVIII-deficient mice after i.v. injection of 200 U/kg. Blood was sampled at two minutes and at various time points thereafter to determine human FV/FVa concentration by ELISA. ^{Super}FVa and ^{super}FV had similar half-life (26 minutes and 32 minutes, respectively), whereby ^{super}FVa had a greater AUC (3150 nM•min vs 2330 nM•min). However, plasma FV had a much longer half-life (120 minutes, Figure 2). In contrast to plasma FV, which has a full B domain, recombinant super FV is missing most of the B domain, suggesting that presence of a full B domain helps to reduce clearance.

Thrombogenicity of ^{super}FVa

The thrombogenic potential of ^{super}FVa was determined by thrombin generation in NHP *in vitro*, and by fibrin deposition in mouse lungs *in vivo* in response to increasing doses of ^{super}FVa. We demonstrated previously that ^{super}FVa increases thrombin generation in FVIII-deficient plasma (2), and that cooperation between ^{super}FVa and rhFVIIa results in

improved thrombin generations beyond that what can be achieved by either agent alone (9). The effects of ^{super}FVa on thrombin generation in NHP, with and without rhFVIIa, may potentially provide preliminary insights into thrombogenic risk. A substantial increase in thrombin generation may hint towards an increased thrombogenic risk if administered systemically. Thrombin generation following administration of ^{super}FVa to NHP was studied over a concentration range from 6 nM to 400 nM, which exceeded the maximum estimated plasma concentration (~100 nM) that previously achieved complete hemostasis after injury in hemophilic mice or wild-type mice treated with FVIII inhibitors (2,9). Thrombin generation with ^{super}FVa at these same concentrations was also tested in the presence of 40 nM rhFVIIa (approximate therapeutic plasma concentration after infusion of 90 μg/kg) to evaluate cooperative effects (9). Thrombin generation is presented as both maximum thrombin generation peak height and ETP (Figure 3A/B). ^{Super}FVa had only a small effect on thrombin peak height in normal plasma that was less than that of 40 nM rhFVIIa. ^{Super}FVa did increase thrombin peak height in the presence of rhFVIIa but this effect reached a plateau at 120% of rhFVIIa alone (Figure 3A). ^{Super}FVa had a small effect on ETP in the absence and presence of rhFVIIa (Figure 3B), but this effect of ^{super}FVa was saturated at increasing ^{super}FVa concentrations and never exceeded 150% of the ETP in normal plasma regardless of whether rhFVIIa was present or not (Figure 3B). Additionally, in the absence of rhFVIIa the ETP returned to normal as the ^{super}FVa concentration increased (Figure 3B). These findings indicate that thrombin generation in NHP *in vitro*, under experimental conditions where substrates are not continuously replaced, was subject to saturation.

The *in vivo* thrombogenicity of i.v. injected ^{super}FVa was studied in a murine model of pulmonary embolism that quantifies fibrin deposition in lungs of wild-type Balb/C mice (n = 4–5 per group). Fibrin deposition after i.v. administration of ^{super}FVa (200 U/kg; 100 nM estimated plasma concentration) was compared to fibrin deposition after i.v. administration of rhTF as the positive control. The i.v. doses of rhTF ranged from 0.4–16 pmol/kg, and all resulted in arrested breathing. I.v. injection of saline served as the negative control. We also tested thrombogenicity of rhFVIIa injected i.v. at 3 mg/kg (Figure 4). This dose exceeds the approved dose of rhFVIIa for treatment of hemophilia patients with inhibitors approximately 30-fold and resulted previously in partially corrected hemostasis in Balb/c mice after tail clip (9). While the injection of rhTF resulted in a pronounced increase in fibrin deposition in the lungs relative to saline (mean 120.5 μg/mL vs mean 5.5 μg/mL, one-way ANOVA with Dunnett's multiple comparison test, $p < 0.001$), ^{super}FVa (mean 6.7 μg/mL) and RhFVIIa (mean 7.6 μg/mL) did not significantly increase fibrin deposition relative to saline. Therefore, neither results from the *in vitro* nor *in vivo* assays suggested excessive thrombogenic potential of ^{super}FVa or thrombogenicity higher than observed with high doses of rhFVIIa.

Predicted antigenicity and immunogenicity of ^{super}FVa

Antigenicity is the capacity of an antigen, such as a foreign protein, to be recognized by an antibody, whereas immunogenicity is the capacity of an antigen to elicit immune response (including antibody formation). Exposure to an engineered protein, such as ^{super}FVa, bears

an unknown risk of antibody formation against FV-related molecules that cannot be assessed in animal studies due to species related immunogenicity.

One approach to predict immunogenic risk of a molecule is B- and T-cell specific epitope prediction analysis. Both, B-cell and T-cell responses have to be evaluated for the propensity to produce antigen-specific antibodies and/or to activate cytotoxic pathways to eliminate the antigen.

Regarding B-cell epitope prediction, it is important to determine the difference in antigenicity between wild-type and ^{super}FVa. Two prediction methods were used. In the first, prediction of β-turns was used to compute the location of B-cell epitopes followed by use of Bepitope to calculate the difference between ^{super}FVa and FVa. Depending on the β-turn scale used (named 33 or EE), a modest increase in the putative epitopes located at either position 506 or 1691 was noticed, as well as a consistent decrease in every other sequence change most notably in the C-terminus (replacement of the glycosylation site). In the second method the Bepipred tool was used to predict epitopes and compute differences between B-cell epitopes. Bepipred indicated that the minimum and maximum changes in B-cell epitopes ranged from −0.61 to +0.17. Negative values indicate a decrease in antigenicity. The maximum increase in antigenicity of ^{super}FVa was well below the threshold of the Bepipred protocol (+0.35). Consequently, both methods used to predict B-cell epitope do not suggest increased antigenicity of the ^{super}FVa sequence relative to the wild-type FVa sequence.

NetCTL was used to predict T-cell epitopes and differences in T-cell epitope prediction were computed by submitting both ^{super}FVa and normal FVa sequences to all the 12 supertypes (26). Overall, the maximum range of variation between both sequences was -1.65 and +0.49. As with B-cell epitopes, negative values indicate a reduction in immunogenicity. The maximum value difference (+0.49) was therefore still below the threshold used in NetCTL and did not identify a putative T-cell epitope (+0.75). These results suggest that there are no or very limited changes in T-cell epitopes, predicting no major changes in immunogenicity.

Finally, structural properties of the engineered disulfide bond have been analyzed using surface area with Naccess (27) and atomic depth computation (28) on the complete model of FVa (29). The relative surface accessibility (RSA) calculated is 19 A^2 for Glu1691 and 15 A^2 for His609 whereas the atomic depth of Cβ atoms of Glu1691 and His609 are −3.9 and −6.2 Å, respectively. These values indicate that both residues are buried within the protein and thus should not affect the surface properties of ^{super}FVa.

Discussion

^{Super}FVa is a newly developed, engineered clotting factor molecule to enhance hemostasis in severe bleeding situations. Findings reported here demonstrate that ^{super}FVa is stable in storage buffer and human plasma *ex vivo*. ^{Super}FVa did not exhibit enhanced antigenicity and immunogenicity in B- and T-cell epitope prediction models when compared to the wild-type FVa sequence, nor was thrombogenicity enhanced compared to rhFVIIa in NHP and in a

murine model of pulmonary embolism, where the injection of rh TF served as a positive control.

Despite its activated status, ^{super}FVa was very stable in solution. When incubated at 37°C in storage buffer it retained 100% of activity over the tested 30 hour time course, whereas wild-type FVa lost approximately 40% of its activity. To also test biological stability in human plasma, ^{super}FVa was incubated in citrated NHP at 37°C. Consistent with the findings in storage buffer, ^{super}FVa lost activity slowly with a half-life of ~5 hours, whereby ~20% of activity were still retained at 8 hours. In contrast, wild-type FVa was unstable, rapidly lost activity with a half-life of ~30 minutes, and retained less than 20% of activity at 2 hours. It has been previously suggested that, in addition to APC-resistance, enhanced stability contributes to the superior hemostatic properties of ^{super}FVa. This was supported by observations that FVa variants with the disulfide bond had significantly higher specific activity in purified prothrombinase assays compared to the same variants without the disulfide bond (1,2). The findings reported here confirm that the disulfide bond between the A2 and A3 domains confers enhanced stability of ^{super}FVa relative to wild-type FVa.

To explore the circulating plasma half-life *in vivo*, half-life studies were performed in mice. FVIII-deficient mice (Balb/c background) were employed for this purpose since many of the *in vivo* bleeding models had been completed in this mouse strain. The half-life of ^{super}FVa was relatively short (~ 30 minutes) and similar to ^{super}FV (non-activated), whereby ^{super}FVa had a greater AUC (3150 nM•min vs 2330 nM•min). In humans, FV has a reported half-life of 12–36 hours and to provide a mean for comparison and extrapolation, the half-life of plasma-derived FV was tested and found to be 120 minutes. The fact that the presence of both molecules, ^{super}FV and ^{super}FVa, could be ascertained in circulating plasma for several hours, and that the half-life of ^{super}FVa was comparable to ^{super}FV is highly encouraging since endogenously produced FVa is rapidly degraded with a half-life believed to be on the order of minutes only (30). These findings also establish that the half-life of ^{super}FVa can be studied in rodents, which is important for further preclinical development. Previous studies demonstrated a wide range of variability concerning the decay of human clotting factors in rodents, which is mostly faster compared to what is observed in human circulation (31–33). Therefore, extrapolating from the measured half-life of human plasma FV in mice (~2 hours) compared to the known half-life of FV in human plasma (~12–36 hours), the half-life of ^{super}FVa in humans could potentially be at least 3 hours. Notwithstanding determination of half-life in human volunteers, half-life estimates at this stage of preclinical development are important to predict feasibility or usefulness of ^{super}FVa in certain indications. Generally, a shorter half-life with the opportunity to rapidly “switch coagulation on and off” is considered advantageous for bleeding associated with DOACs or traumatic bleeding, where patients suffer from underlying prothrombotic conditions or a coagulopathy. In contrast, a longer half-life may be desirable for bleeding in hemophilia to avoid repetitive dosing or afford prophylactic administration. For instance, the circulating half-life of zymogen-like FX currently developed for intracranial bleeding is only ~60 minutes in mice (34), whereas long-acting FVIIa molecules with half-life extension beyond 4–6 hours are in development to afford prophylactic treatment in hemophilia patients with inhibitors to prevent joint bleeding (35,36). Based on current observations, the extrapolated half-life of ^{super}FVa may fall into an intermediate range that may be suitable for a variety of conditions.

There was no finding of thrombogenicity in a mouse model (wild-type Balb/c) of pulmonary embolism, quantifying fibrin depositions in the lung after injection of a single dose of ^{super}FVa (200 U/kg). This dose results in a plasma concentration of ~100 nM based on the assumption that the plasma volume of a 20–25 g mouse is ~1 mL. This dose was the highest dose that previously resulted in complete hemostasis in mouse models of bleeding (2,9,11), and exceeds the plasma concentration of circulating FV (~20 nM) five times. Results with ^{super}FVa were similar compared to what was observed after injection of 3 mg/kg rhFVIIa, which is a ~30-fold higher dose than approved for human use, but necessary to achieve partial hemostasis in mice (9,31). Also, thrombin generation in NHP employing ^{super}FVa at concentrations as high as 400 nM was only marginally increased, even when combined with rhFVIIa at therapeutic concentrations for human use. Taken together, these findings support that ^{super}FVa does not result in undue thrombin generation *in vitro* and *in vivo*, out of proportion to what is experienced with rh FVIIa.

The antigenic and immunogenic potential of recombinant engineered molecules is an important concern for protein replacement therapies. The exposure to an engineered protein, such as ^{super}FVa, bears an unknown risk of antibody formation against FV-related molecules that cannot be assessed in animal studies due to species related immunogenicity. Final assessment of immunogenicity for all such molecules can therefore only be accomplished in human studies. However, the potential for immunogenicity can be predicted to a certain extent by B- and T-cell epitope modelling, whereby a positive signal would substantiate concerns and possibly exclude a molecule from further development. Testing of ^{super}FVa in the B- and T-cell epitope prediction model did not yield increased antigenicity or immunogenicity of the ^{super}FVa sequence relative to the wild-type FVa sequence, thus suggesting a low potential for antibody formation. Of note, antigenicity and immunogenicity are clinically most relevant when exposure to a molecule is frequent and chronic such as in hemophilia to prevent and treat bleeding episodes. There, formation of inhibitory antibodies to exogenous FVIII or FIX preparations is observed at a median of 10 exposure days in ~30% of patients with FVIII-deficiency and ~5% of patients with FIX-deficiency (37,38). Inhibitor formation with rh FVIIa is rare, but has been observed with a modified, higher potency FVIIa product following repeated exposure over many months (39). For single short-term use such as for traumatic bleeding or DOAC-associated bleeding the risk of inhibitor formation appears therefore negligible and less relevant.

Conclusion

^{super}FVa appears to be a stable molecule in buffer solution and plasma, with a favourable pharmacokinetic profile in mice, consistent with its previously observed therapeutic hemostatic effects (2,9,11). The lack of thrombogenicity in mice, and the lack of antigenicity and immunogenicity in B- and T-cell prediction models are critical findings that permit the continuation and advancement of ^{super}FVa in preclinical and clinical development as novel, versatile prohemostatic agent for severe bleeding situations.

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Abbreviations

FVa	activated factor V
SuperFVa	activated super factor V
DOAC	direct oral anticoagulant
APC	activated protein C
FVIII	factor VIII
rh	recombinant human
ETP	endogenous thrombin potential
FVDP	factor V deficient plasma
PC	phosphatidyl choline
PS	phosphatidyl serine
rhTF	recombinant human tissue factor

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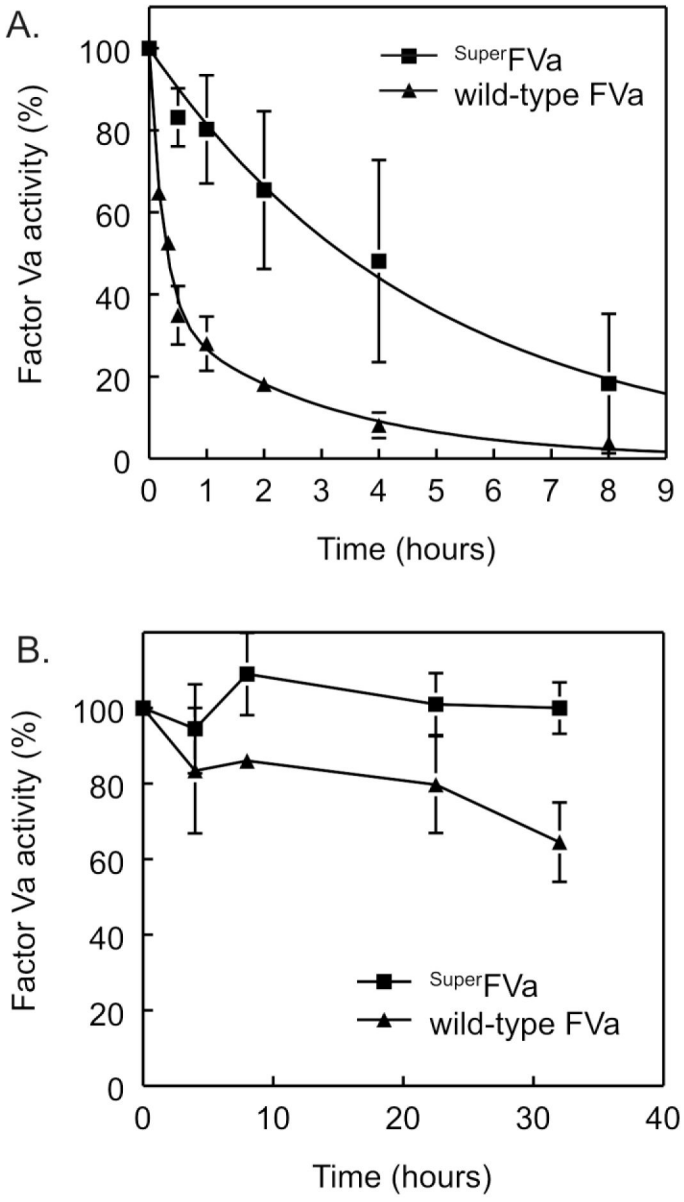

Figure 1. Stability of ^{super}FVa in plasma and in storage buffer at 37 °C

(A) ^{Super}FVa or wild-type FVa were added to citrated FVDP at 500 pM. This mix was incubated at 37 °C and aliquots were assayed at specified time points for remaining FVa variant activity in an aPTT coagulation assay (n=4). **(B)** ^{Super}FVa (squares) or wild-type FVa (triangles) were incubated at 100 nM in storage buffer + Bovine Serum Albumin at 37 °C. At specified time points aliquots were removed, diluted into FVDP 1:200 and assayed in an aPTT with FVDP (n=3). Error bars are standard deviations. FVa, activated Factor V. FVDP, Factor V deficient plasma. NHP, normal human plasma.

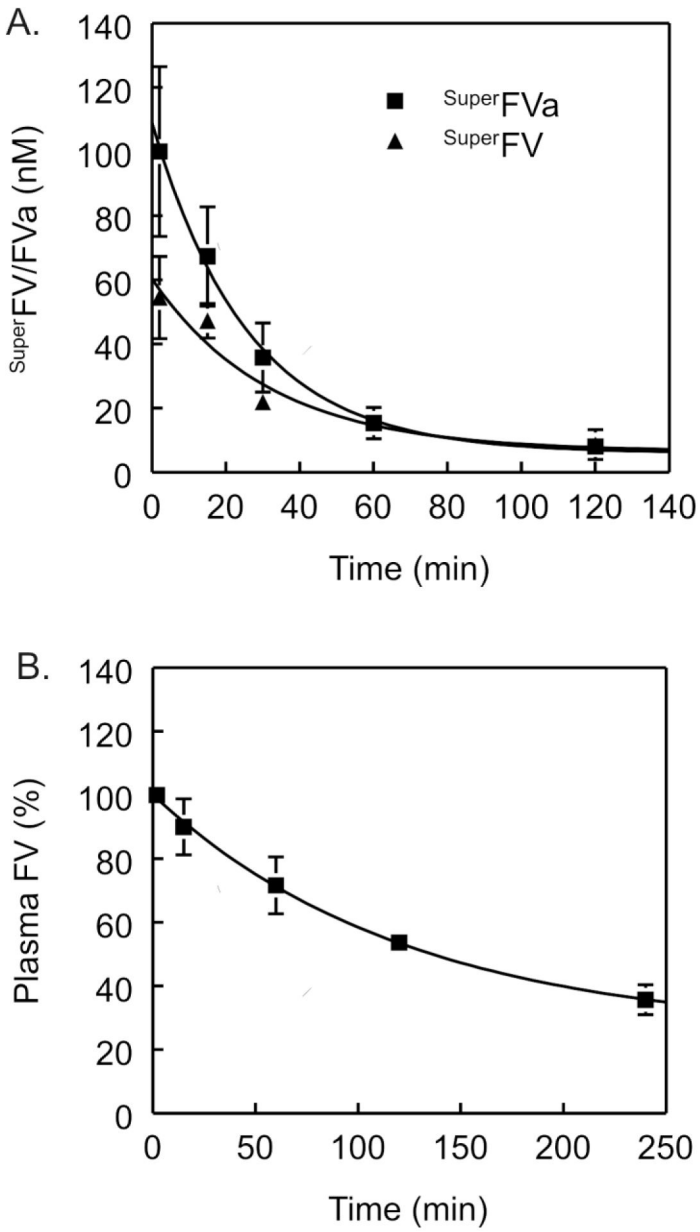

Figure 2. Pharmacokinetics of ^{super}FVa and ^{super}FV in mice relative to human plasma-derived FV

FV and FVa molecules were injected retro-orbitally at 200 U/kg. Blood was sampled at various time points and FV was quantified in plasma by ELISA. Data were fit to an exponential decay using SlideWrite Plus. **(A)** ^{Super}FV and ^{super}FVa pharmacokinetics in mice. Half-life of ^{super}FVa = 26 minutes. Half-life of ^{super}FV = 32 minutes. **(B)** Plasma FV pharmacokinetics in mice. Half-life of plasma FV = 124 minutes. Error bars represent the standard deviation (n = 2–4, except for T=2 min for ^{super}FV/^{super}FVa where n = 9 and 14 respectively). FVa, activated FV. ELISA, enzyme linked immunosorbent assay.

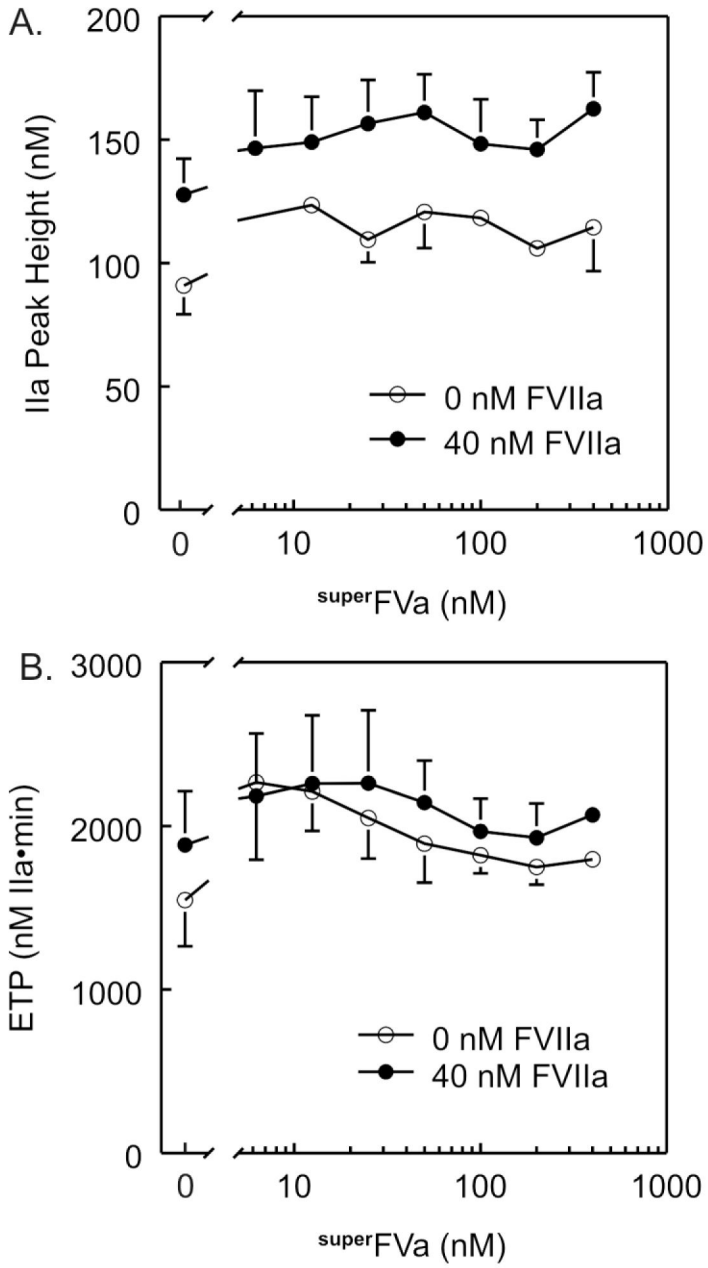

Figure 3. *In vitro* thrombogenicity of ^{super}FVa in NHP with and without rhFVIIa

Thrombin generation was determined as **(A)** peak height or **(B)** ETP. ^{Super}FVa was titrated into NHP from 0 to 400 nM either in the absence (open circles) or presence (closed circles) of 40 nM rhFVIIa (n=3). Error bars represent standard deviation (n=3). ETP, endogenous thrombin potential. FVa, activated FV. rhFVIIa, activated recombinant human Factor VII. NHP, normal human plasma.

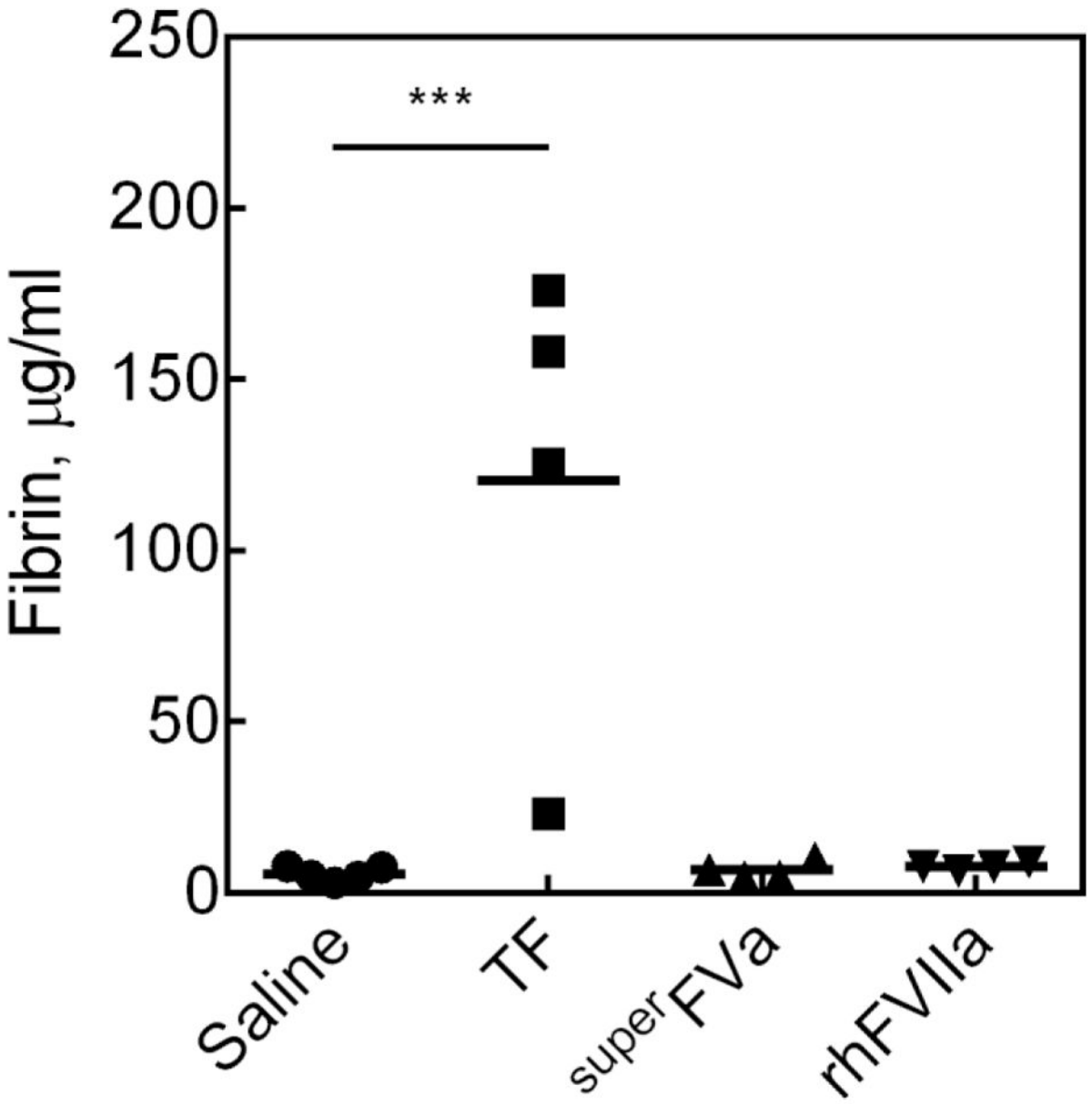

Figure 4. ***In vivo*** **thrombogenicity of ^{super}FVa in mice**

Lung fibrin deposition was measured following injection of saline (n = 5), various doses of recombinant soluble TF (0.4–16 pmol/kg), ^{super}FV (200 U/kg) or rhFVIIa (3 mg/kg (n = 4). Fibrin was quantified in lung homogenates by ELISA. *** = p-value < 0.001, shown for TF relative to control saline, calculated by one-way ANOVA and Dunnett's multiple comparisons test. ^{Super}FVa and rhFVIIa were not significantly different from saline. FVa, activated FV. rhFVIIa, recombinant human FVIIa. TF, tissue factor. ELISA, enzyme linked immunosorbent assay.

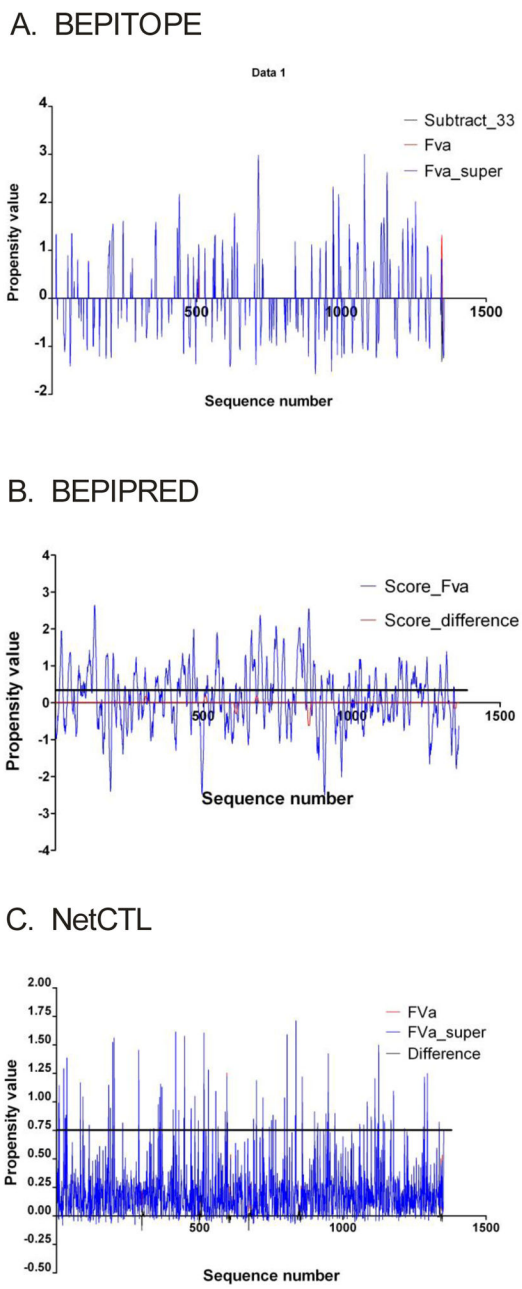

Figure 5.

Difference mapping of B cell and T cell epitope predictions of ^{super}FVa relative to FVa. **(A)** Differences in the detection of B-cell epitope between the ^{super}FVa and FVa sequences using BEPITOPE. Predictions are in blue and red colors whereas the subtraction result is shown in black. Almost no visible changes can be observed. **(B)** Differences in the detection of B-cell epitope between the ^{super}FVa and FVa sequences using BEPIPRED. For clarity, only the prediction for FVa sequence is shown in blue. The resulting differences between prediction in ^{super}FVa and FVa sequences is shown in red. Again, almost no visible changes can be observed. The default threshold (0.35) used to identify linear B-cell epitope is drawn with a

thick black line. **(C)** Differences in the detection of T-cell epitope between the ^{super}FVa and FVa sequences using NetCTL. Predictions are in blue and red colors whereas the subtraction result is shown in black. The default threshold (0.75) used to identify T-cell epitope is drawn with a thick black line. No differences can be observed outside the limit of the threshold.