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Development and Characterization of Recombinant Ovine Coagulation Factor VIII

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Abstract

Animal models of the bleeding disorder, hemophilia A, have been an integral component of the biopharmaceutical development process and have facilitated the development of recombinant coagulation factor VIII (fVIII) products capable of restoring median survival of persons with hemophilia A to that of the general population. However, there remain several limitations to recombinant fVIII as a biotherapeutic, including invasiveness of intravenous infusion, short half-life, immunogenicity, and lack of availability to the majority of the world’s population. The recently described ovine model of hemophilia A is the largest and most accurate phenocopy. Affected sheep die prematurely due to bleeding-related pathogenesis and display robust adaptive humoral immunity to non-ovine fVIII. Herein, we describe the development and characterization of recombinant ovine fVIII (ofVIII) to support further the utility of the ovine hemophilia A model. Full-length and B-domain deleted (BDD) ofVIII cDNAs were generated and demonstrated to facilitate greater biosynthetic rates than their human fVIII counterparts while both BDD constructs showed greater expression rates than the same-species full-length versions. A top recombinant BDD ofVIII producing baby hamster kidney clone was identified and used to biosynthesize raw material for purification and biochemical characterization. Highly purified recombinant BDD ofVIII preparations possess a specific activity nearly 2-fold higher than recombinant BDD human fVIII and display a differential glycosylation pattern. However, binding to the carrier protein, von Willebrand factor, which is critical for stability of fVIII in circulation, is indistinguishable. Decay of thrombin-activated ofVIIIa is 2-fold slower than human fVIII indicating greater intrinsic stability. Furthermore, intravenous administration of ofVIII effectively reverses the bleeding phenotype in the murine model of hemophilia A. Recombinant ofVIII should facilitate the maintenance of the ovine hemophilia A herd and their utilization as a relevant large animal model for the research and development of novel nucleic acid and protein-based therapies for hemophilia A.

Introduction

Factor VIII (fVIII) is an essential glycoprotein procofactor within the intrinsic pathway of the blood coagulation cascade. In blood circulation, fVIII is non-covalently bound to von Willebrand factor (VWF) and is present at relatively low concentration (1 nM). Mutations in the F8 gene often result in diminished or inactive plasma fVIII and are the molecular genetic cause of the monogenic, X-linked, bleeding disorder hemophilia A that affects approximately 1 in 7500 males worldwide. Current treatment is limited to intravenous infusion of plasma-derived or recombinant human fVIII (hfVIII) containing products. This therapy is only available to 30% of the world due to economic factors and requires multi-weekly injections to achieve prophylaxis, which must be maintained for the duration of the patients’ life to avoid debilitating joint disease as well as life-threatening bleeding episodes. While gene therapy is being explored as a potential cure, additional research efforts are aimed at improving the therapeutic utility of recombinant fVIII.

Investigations into the biochemical properties of orthologous fVIII constructs have yielded insight into basic fVIII structure/function as well as translation into novel clinical agents. For example of the former, the characterization of recombinant murine factor VIII (mFVIII) revealed near complete stability at physiologic concentrations following thrombin activation [1]. Porcine fVIII (pFVIII) demonstrates 10 to 100-fold increased expression over hfVIII [2,3], as well as decreased engagement of the endoplasmic reticulum-resident unfolded protein response [4]. Furthermore, Arruda and colleagues described the development and characterization of canine fVIII, which displays 3-fold higher specific activity than that of hfVIII and currently is utilized to manage bleeding in canine hemophilia A colonies [5]. As for the development of novel clinical agents, plasma derived pFVIII has historically been used in the treatment of patients with pre-existing inhibitors to hfVIII and recombinant B-domain deleted (BDD)
pVIII currently is in clinical trials. Likewise, human/porcine (hp) hybrid transgenes with high expression properties are being developed for clinical gene therapy [6].

A line of sheep presenting with hemophilia A recently was re-established and the pathology, clinical profile, and molecular genetics were described [7]. Ovine VIII (oVIII) possesses 96% amino acid sequence homology to hVIII outside of the B-domain and possesses a similar domain structure (A1-A2-B-ap-A3-C1-C2) defined by internal sequence homology. The causative mutation was identified as a single nucleotide insertion resulting in frameshift and a premature stop codon in exon 14 similar to a mutation documented in a human patient with severe hemophilia A [8]. In preliminary studies, administration of hVIII or hpVIII corrected the bleeding phenotype in this model transiently, but invariably induced the formation of high-titer anti-VIII inhibitory antibodies eventually leading to premature mortality. Moreover, transplantation of genetically-modified mesenchymal stem cells expressing a BDD pVIII transgene in this model corrected phenotypic hemarthroses and spontaneous bleeds for several months, thus establishing the potential of the model for the development of novel therapeutics. The utility of this model as a research and development resource hinges on the ability to maintain colonies of these clinically fragile animals. Toward this goal, the cloning, expression, purification and biochemical characterization of recombinant BDD oVIII are described in the current study.

Materials and Methods

Materials

The cloning and characterization of full-length ovine VIII in the pUC57 vector has been described previously [7]. Phusion High Fidelity PCR MasterMix, PNGase, and all restriction enzymes were purchased from New England Biolabs (Ipswich, MA). All cell culture materials were purchased from Corning Inc. (Corning, NY). AIM V and DMEM/F12 media was purchased from Invitrogen (Carlsbad, CA). Citrated VIII-deficient plasma and normal pooled human plasma (FACT) were purchased from George King Biomedical (Overland Park, KA). Activated partial thromboplastin reagent (aPTT) was purchased from Organon Teknika (Durham, NC). Monoclonal antibodies were provided by Dr. Pete Lollar (Allac Cancer Center and Blood Disorders Service, Emory University, Atlanta, GA). Recombinant human thrombin was provided by Haematologic Technologies Inc. (Essex, VT). Desulfatothirudin was a generous gift from Dr. R. B. Wallis (Ciba-Geigy Pharmaceuticals) to our colleague Dr. Pete Lollar (Emory University, Atlanta, GA). SDS-PAGE precast gels were purchased from Bio-Rad (Hercules, CA). Polyethyleneimine was purchased from Polysciences, Inc. (Warrington, PA). A colony of exon 16-disrupted hemophilia A mice (E16−/−) was kept and maintained within the Emory Division of Animal Resources Pediatrics Facility.

Generation of BDD oVIII

Replacement of the ovine B-domain with an SQ linker containing a PACE/furin recognition sequence was conducted by SOE mutagenesis as described previously [1]. Primers for heavy chain (HC) and light chain (LC) were manufactured by Integrated DNA Technologies (Coralville, IA). HC forward: 5′-ACA TCA AGC TCT GTA CTT CCT GAG CCA CCA TGC ACA TCA AGC TCT GTA CTT CCT GGC C-3′; HC reverse: 5′-ATT CTG GGA GAA GCT CTT AGG TTC AAT GAC ATT GTT TTC ACT CAG GAG G-3′; LC forward: 5′-GTT ACC GAC TGA CCT GAG AAC CTG CCA CCA GGC-3′; LC reverse: 5′-AGT GCC AGG TGC TGC AGC GCC CGC CCT CAG TAC TGC TGC TGT GCC TCA C-3′. PCR amplification was conducted in the following cycles: 30 s at 98°C, 35 cycles of 10 s at 98°C and 30 s at 58°C, and annealing at 78°C for 13 minutes followed by 25°C hold. Amplified products were digested utilizing NotI, AvrII and XhoI restriction nucleases and separated using SeaKem GTG® Agarose (Lonza; Rockland ME) gel electrophoresis. Digested fragments were purified using a QIAquick Gel Extraction Kit (Qiagen) and ligated using a T4 DNA Quick Ligase (Promega). The final construct was cloned into ReNeo mammalian expression vector using NotI and XhoI restriction sites. BDD oVIII ReNeo was sequenced by Beckman Coulter Genomics (Danvers, MA) using overlapping primers spanning the entire transgene.

Generation and Characterization of Stable OfVIII

Expressing Clones

Naive baby hamster kidney-derived (BHK-M) cells were transduced in 6-well plates with 1.5 μg/10⁶ cells of ReNeo mammalian expression plasmid encoding the respective VIII transgene. Polyethyleneimine was administered at a final concentration of 6 μg/ml in DMEM containing 10% FBS. Media was replaced at 24 h and expanded at 48 h to 10 cm plates in media containing 500 μg/ml G418 (Gibco, Grand Island, NY) and cultured for 10–14 days. Fifty to sixty-eight clones were selected and expanded. For determination of specific VIII production rates, the clones were cultured for 24 h in serum free AIM V media and then counted by hemacytometer for normalization to units/10⁶ cells/24 h. FVIII activity measurements were made by one-stage coagulation assay and linear regression analysis of clotting times against a pooled human plasma standard (FACT) using a ST art Coagulation Instrument (Diagnostica Stago, Asnieres, France). Geneticin-resistant clones that expressed VIII below the limit of assay detection (0.01 units/ml) in 24 h were not included in the statistical analysis. From 35 antibiotic resistant clones with BDD oVIII activity above measurable detection, the highest expression clone was selected for further study. Peak expression from this clone was 6 units/10⁶ cells/24 h.

Purification of Recombinant OfVIII

Recombinant oVIII was purified using a two-step ion exchange chromatography procedure as cited previously [2]. Expressing clones were expanded into 500 cm² flasks in DMEM/F-12 growth media containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells at 60–70% confluency were washed 2× with 50 ml Dulbecco’s phosphate-buffered saline (PBS) (Thermo Scientific) and re-fed with 125–150 ml AIM V serum free media. Media was collected every 24–48 h and replaced with equal volume of fresh AIM V. FVIII containing media was subjected to centrifugation at 2000×g for 10 min and the supernatant was frozen at −80°C in 0.05% sodium azide until time of purification. Media was thawed at 37°C and loaded onto a 5×20 cm SP-Sepharose High Performance column equilibrated to 0.15 M NaCl, 20 mM HEPES, 5 mM CaCl₂, 0.01% Tween-80, pH 7.4 (HBST). The column was washed twice with equilibration buffer followed by 18% NaCl containing buffer prior to elution. Fractions were eluted over a linear 0–0.6 M NaCl gradient in HBST. Fractions containing FVIII were assayed for FVIII activity and activation quotient (AQ) and those with AQ values greater than 20 were pooled. The AQ assay was conducted using both one-stage and two-stage coagulation assays as described previously [1]. The activation quotient is defined as the ratio of FVIII activity measured by two-stage coagulation assay divided by the FVIII activity measured by the one-stage coagulation assay.

Pooled material was diluted to 0.15 M NaCl in the HBST, applied
to a Source Q HR5/5 FPLC column and eluted with a linear 0.2–0.65 M NaCl gradient. Fractions were assayed by one-stage coagulation assay, absorbance at 280 nm, and SDS-PAGE. Final AQ and specific activity measurements of pooled material were recorded following a freeze/thaw cycle at −80°C.

SDS-PAGE Analysis and Mass Spectrometry

Polypeptides were resolved by 4–15% gradient SDS-PAGE and fixed with 30% methanol/10% glacial acetic acid prior to staining with GelCode Blue (Pierce, Rockford, IL). Deglycosylation was conducted according to manufacturer’s directions (NEB). Briefly, 2 μg purified protein was activated with 2 units recombinant human thrombin at 37°C for 10 min, denatured in denaturation buffer supplied by the manufacturer at 95°C for 10 minutes, and deglycosylated by incubation with 500 units PNGase at 37°C for 1 hour. Confirmation of N-linked glycan location was carried out by tandem mass spectrometry. OvFVIII was treated with PNGase as described above, purified via SDS-PAGE, and stained with coomassie blue. Excised bands were subjected to in-gel digestion (12.5 μg/ml trypsin). Extracted peptides were loaded onto a C18 column (100-μm inner diameter, 20 cm long, ~300 nl/min flow rate, 1.9-μm resin by Dr. Maish Gbhm, Ammerburgen, Germany) and eluted during a 10–30% gradient (Buffer A: 0.1% formic acid, 1% ACN; Buffer B: 0.1% formic acid, 99.9% ACN). The eluted peptides were detected by Orbitrap (300–1600 m/z; 1,000,000 automatic gain control target; 500-ms maximum ion time; resolution, 30,000 full-width at half-maximum) followed by ten data-dependent MS/MS scans in the linear ion trap quadrupole (2 m/z isolation width, 35% collision energy, 5,000 automatic gain control target, 150-ms maximum ion time) on a hybrid mass spectrometer, LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA). The acquired tandem mass spectrometer (MS/MS) spectra were searched against and decoy-concatenated Ovis aries database (807 target proteins and 1 customized coagulation factor VIII sequence) from the NCBI RefSeq protein database project (version 54) using the Sorcerer-SEQUEST Algorithm version 4.04 (Sage-N Research, San Jose, CA) with differential modification of +0.984016 Da on Asn and +15.99492 on Met. Search results were filtered to 1% FDR and summarized by in-house programs, as described by Gozal et al [9].

Glycosylation sites were determined by differential analysis of peptide elution profiles and tandem mass spectrum between glycosylated and PNGase-F treated samples. In summary, ion chromatograms for peptides containing a matching asparagine modification mass shift were extracted from the PNGase-F treated sample. Only ions that have a noise-level chromatogram in the glycosylated sample were considered to be confidently matched to a glycopeptide.

Immunoprecipitation

Purified polypeptide was activated with 1.4 μM thrombin, inactivated with 10 μM desulfatohirudin and incubated with mAb for 45 min at 37°C in M-PER (Thermo Scientific, Rockford, IL) with 150 mM NaCl. Protein G Agarose (KPL, Gaithersburg MD) was added and incubated at 37°C for one hour. Following centrifugation at 14,000 x g, pellets were washed three times in 200 μl HBST followed by centrifugation at 14,000 x g for 5 minutes. Protein G complexes were resuspended in 25 μl HBST and heated for 5 minutes at 95°C. Supernatant was loaded into 4–15% SDS-PAGE gel for analysis.

Activated FVIII Decay Assay

Activated factor VIII (FVIIIa) was measured by chromogenic assay using purified human factor IXa, human factor X, and synthetic phospholipid vesicles as described previously [10]. Briefly, 20 nM ovFVIII or hFVIII was activated with 100 nM human thrombin for 15 seconds at room temperature. Desulfatohirudin (150 nM) was added to stop the reaction and FVIII activity was measured at several time points.

von Willebrand Factor (VWF) Binding

Binding of ovFVIII to human VWF was determined by ELISA. Thermo Scientific Immunulon 1B plates were coated with 50 μl of 6 mg/ml human VWF in buffer A (20 mM HEPES, 150 mM NaCl, 2 mM CaCl2, 0.05% Tween 20, 0.05% sodium azide) overnight at 4°C. Plates were washed twice with buffer A and blocked with 2% BSA in buffer A (blocking buffer). Plates were stored at 4°C until use. Human and ovine FVIII were diluted in blocking buffer and applied to VWF coated wells following two washes with buffer A. Plates were incubated for 1 h at room temperature, washed twice with buffer A, and incubated with 1 μg/ml of biotinylated monoclonal antibody 4F4 1B (generously provided by Dr. Pete Lollar, Emory University) in blocking buffer for 1 h at room temperature. Primary antibody incubation was followed by two washes with buffer A and the addition of streptavidin alkaline phosphatase (Jackson ImmunoResearch Labs, Inc., West Grove, PA) at 1:15,000 in buffer A for 1 h at room temperature. Substrate activation was preceded by two final washes in buffer A. Colorimetric transmission was initiated by the addition of 80 μl para-Nitrophenylphosphate substrate (Bio-Rad; Hercules, CA) and recorded in kinetic mode as the change in A405 s⁻¹. Rates were limited to a maximum optical density of 0.8 OD.

Efficacy of OfVIII In Vivo

Hemostatic challenge was conducted as previously described [11] with alterations. All animal studies were reviewed and approved by the Emory University Institutional Animal Care and Use Committee (IACUC). Briefly, ovFVIII was diluted in saline to a concentration of 300 units/kg mouse body weight. Eight to twelve week old C57/BL6 mice were infused with either saline or ovFVIII via tail vein injection. Immediately following injection, mice were anesthetized with 3.5% isoflurane at a flow rate of 1,000 ml/min for 5 minutes. Tails were placed into a 15 ml conical tube with 15 ml sterile saline at 37°C. Isoflurane was reduced to 2% at a flow rate of 500 ml/min and maintained for the duration of the experiment. At 15 min, tails were transected at 2 mm diameter as measured by wire gauge. In doing so, blood loss between mice is standardized to the diameter of tail vasculature rather than distal length. Blood was collected in a new, pre-weighted 15 ml conical containing 13 ml sterile saline at 37°C for 40 minutes and measured by change in mass and recorded by mg blood loss per gram body weight. The mean evaporative loss of two vials of 13 ml sterile saline at 37°C was used to correct for changes in mass of the efficacy treatments. One mouse injected with ovFVIII displayed blood loss of 7 mg/g body weight, however, this likely was attributed to a technical error in measurement.

Results

Heterologous Expression of Recombinant OfVIII

Recombinant FVIII can be synthesized in two distinct, but functional forms, the endogenous full-length form and the BDD form that displays enhanced expression due to a 1/3 reduction in transcript and transgene product size. Therefore, a recombinant BDD ovFVIII (FVIII) expression plasmid was designed that contained a PACE/turin recognition site (RHQR) within a 14 amino acid linker between A2 and ap-A3 domains similar to those...
biochemical characterization of the final product. BDD ofVIII clone was selected for production, purification and expression of BDD ofVIII measured at 6.12 units/10^6 cells/24 h (median and maximum, respectively). Again, the BDD ofVIII production rate was 2.3 fold difference in expression between test). Deletion of the B domain resulted in increased expression of (median and maximum, respectively) (\(P=0.005\), Mann-Whitney \(U\) test). Deletion of the B domain resulted in increased expression of both BDD ofVIII and BDD hFVIII over their full-length counterparts (\(P<0.001\) for both comparisons, Mann-Whitney \(U\) test) with median expression levels of 0.783 and 0.091 units/10^6 cells/24 h (1% normal FVIII levels) were not included in statistical analysis.

### Purification and Biochemical Characterization of BDD ofVIII

Three independent expression and purification experiments were conducted although two were at smaller scale. The average specific activity determined from these independent preparations was 2,516±503 U/nmol or 15,130±5,030 units/mg (mean ± standard deviation) polypeptide. Specific activity was calculated by absorbance at 280 nm and an estimation of the molar extinction coefficient based on known tyrosine, tryptophan, and cysteine content [15]. In one experiment, approximately 20,000 units of BDD ofVIII was collected in 5.4 L of serum-free media and purified using a two-step ion-exchange chromatography procedure (Table 1). The process yield was 25% and the final material had a specific activity of 3,050 units per nanomole (18,300 units/mg).

### Table 1. Purification of BDD ofVIII.

| Sample          | Vol. (ml) | A_{280} | Total A_{280} | Activity (U/ml) | Units | Units/A_{280} | AQ | % Yield | Fold Pur. |
|-----------------|-----------|---------|---------------|-----------------|-------|---------------|----|---------|-----------|
| Media           | 5,350     | 1.53    | 8,186         | 3.78            | 20,223| 2.47          | 28 | 100     | 1         |
| SP-Sepharose pool | 35       | 0.415   | 14.5          | 353.4           | 12,370| 853           | 49 | 61      | 345       |
| Source Q pool   | 2.4       | 0.211   | 0.506         | 2.149           | 5,158 | 10,193        | 55 | 25.5    | 4,126     |

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Heterodimeric Structure, Glycan Analysis, and Thrombin Proteolysis of BDD ofVIII

FVIII circulates in plasma as a heterodimer of heavy and light chains associated in a metal ion facilitated, non-covalent manner. Typically, these two large polypeptides readily can be resolved upon visual inspection following SDS-PAGE. Unique to BDD ofVIII, the heavy and light chain polypeptides display similar relative mobility upon SDS-PAGE (Figures 2 and 3). When compared to the respective BDD hFVIII sequence, the ovine A1 and A2 domain sequences contain five and three amino acid residue deletions, respectively. This results in a predicted 1.1 kDa decrease in the overall size of the BDD ofVIII heavy chain. The overall size of both human and ovine light chains is identical based on in silico prediction as well as empirical SDS-PAGE analysis. Eight asparagine residues can be identified as potential sites of N-glycosylation; however, these sites, Asn-41; Asn-213; Asn-239; Asn-582; Asn-1720; Asn-1810; Asn-2118; Asn-2270. Of these sites, Asn-582 and Asn-2270 are not predicted to actually contain oligosaccharides. Consistent with these predictions, in many distinct human FVIII preparations, it has been demonstrated that Asn-582 is not glycosylated [16]. Furthermore, treatment of thrombin-activated oFVIII with PNGase F did not alter the mobility of the ovine A2 domain thus supporting the prediction that Asn-582 is not glycosylated in BDD ofVIII (Figure 2).

Purification and Biochemical Characterization of BDD ofVIII

The activity measured in the one-stage coagulation assay in serum-free media after 24 hr culture. Cell numbers were determined at the time of activity measurement and data was normalized to 10^6 cells. The horizontal lines depict the mean values for each data set.

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Eight asparagine residues can be identified as potential sites of N-glycosylation, Glycan Analysis, and Thrombin Proteolysis of BDD ofVIII

| Source          | Vol. (ml) | A_{280} | Total A_{280} | Activity (U/ml) | Units | Units/A_{280} | AQ | % Yield | Fold Pur. |
|-----------------|-----------|---------|---------------|-----------------|-------|---------------|----|---------|-----------|
| Source Q pool   | 2.4       | 0.211   | 0.506         | 2.149           | 5,158 | 10,193        | 55 | 25.5    | 4,126     |
of additional domain-specific N-linked glycans within both regions. Asn-213 is not present in the human sequence, but is conserved in canine and porcine while Asn-1720 is to-date a uniquely described potential glycosylation site, although this site is not supported by mass spectrometry. MS/MS analysis supports with high confidence the glycosylation of Asn-41, Asn-1810, and Asn-2118. There remains evidence of glycosylation of Asn-213 and Asn-239, however, limitations in the resolution require further investigation. A2 and C2 domain specific MAbs were used to elucidate mobility of heavy and light chains independently. Purified BDD ofVIII was incubated with either 4F4 1B or I14 1B MAbs in a solution known to dissociate the fVIII heavy and light chain polypeptides, independent precipitations of BDD ofVIII material with heavy and light chain-specific MAbs yielded polypeptide species of equal mobility (Figure 3). Immunoprecipitations also were conducted in the presence of thrombin to verify domain-specific MAb interaction.

Decay of Activated OfVIII
When activated, fVIIIa serves as a cofactor for factor IXa, which executes the proteolytic cleavage of factor X into its activated form. Dissociation of the A2 domain from the A1/A3-C1-C2 heterodimer results in loss of pro-coagulant fVIII activity and can be measured indirectly by the generation of factor Xa in a purified system [10,15,17,18,19]. BDD ofVIII and BDD hiVIII were activated with thrombin and residual activity was measured over 30 minutes (Figure 4). Similar to previous reports, hiVIIIa displayed a mean (± sample standard deviation) half-life of 1.8±0.087 min [1,2,5] while the ofVIIIa half-life was prolonged significantly to 3.5±0.37 min (P=0.001, Student’s t test).

VWF Binding
VWF is a plasma glycoprotein that performs many roles in the hemostatic system. One of which is to stabilize fVIII in circulation through non-covalent association. VWF circulates as non-uniformly sized multimers composed of individual 270-kDa monomers. Each VWF monomer is capable of 1:1 stoichiometric binding with fVIII. Upon proteolytic activation by thrombin or factor Xa, fVIII dissociates from VWF and is available to participate with factor IXa and Ca2+ in the formation of the tenase complex on a negatively charged phospholipid surface. In the absence of fVIII/VWF association, e.g. due to genetic deficiency of VWF or mutation of the fVIII/VWF binding sites, circulating fVIII levels are severely reduced and pathogenic bleeding often present phenotypically. The ability of ofVIII to bind human VWF, since a source of ovine VWF was unavailable, was assessed using an ELISA developed specifically for this study. Briefly, fVIII was captured by human VWF pre-adsorbed to a plate and detected using a MAb with an A2 domain epitope, which was demonstrated to possess equivalent affinity for human and ovine fVIII (Data not shown). Using this assay, BDD ofVIII

![Figure 2. Biochemical Analysis of BDD OfVIII.](https://www.plosone.org/doi/10.1371/journal.pone.0049481.g002)
Figure 4. Thrombin-Activated Decay Rate of OfVIIIa. Human (closed circle) and ovine (open circle) fVIIIa decay was measured by chromogenic Xase assay in which 20 nM fVIII was activated with thrombin and then stopped with desulfatohirudin. Activated fVIIIa in complex with phospholipid vesicles, activated factor IXa, and factor X was measured at 0.5, 3, 5, 8, 15, and 30 minutes to determine residual fVIIIa activity. Half-lives of 1.8±0.09 and 3.5±0.37 minutes were calculated for human and ovine fVIIIa, respectively. Data shown represents the percent of initial activity by semi-log extrapolation to time = 0. Regression analysis revealed Pearson correlation coefficients of 0.999 for both treatments.

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Figure 5. BDD OfVIII Binding to VWF. Kinetic ELISA was conducted using human VWF to capture human (closed circles) or ovine (open circles) fVIII. Plates were coated with 50 µl of 6 mg/ml human VWF and blocked with 2% BSA. Monoclonal A2 domain fVIII antibody 4F4 1B was added to each well and colorimetric transmission was activated with para-nitrophenylphosphate substrate following streptavidin alkaline phosphatase. Data shown are the mean of three independent experiments ± sample standard deviation.

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and hfVIII displayed indistinguishable binding to VWF at physiologically relevant concentrations (Figure 3).

**In Vivo Efficacy of OfVIII**

In order to demonstrate functionality of BDD ofVIII to restore the blood coagulation in *vivo*, hemophilia A mice were injected with either saline or ofVIII at a dose of 300 units/kg, which was determined previously to restore plasma fVIII activity to near endogenous murine levels (2.9 units/ml for C57Bl/6 mice in the colony at Emory University) [1]. Following fVIII or saline administration, a hemostatic challenge was induced via a tail transection at the 2 mm diameter position of the tail and blood loss was measured over a 40-minute period. Hemophilia A mice injected with saline alone demonstrated a mean (± standard deviation) blood loss of 32.2±9.4 mg/g body weight (Figure 6). In contrast, mice injected with ofVIII demonstrated a mean blood loss of 1.15±2.57 mg/g body weight, which was significantly less than controls (P<0.001; Mann-Whitney Rank Sum Test) and consistent with complete correction of the bleeding phenotype in this model.

**Discussion**

Innovation in hemophilia A treatment has benefited significantly from the use of animal models of the disease and the study of orthologous IVIII molecules. For example, hemophilia A biopharmaceuticals continue to be hindered by low-level cellular production whether they be produced endogeneously (plasma-derived IVIII), heterologously (recombinant IVIII), or following gene transfer into the patient (gene therapy). Secretion of IVIII is observed at 2–3 orders of magnitude lower than glycoproteins of similar size, including homologous factor V [20]. This is attributed to inefficient mRNA expression, protein mis-folding, and engagement of the unfolded protein response, and subsequent inefficient rate-limiting transport from the endoplasmic reticulum to the Golgi apparatus. Recently, we identified or generated orthologous and bioengineered IVIII constructs, respectively, that display enhanced secretion efficiency over BDD hfVIII. Specifically, certain pfVIII sequences were identified and shown to improve the efficiency of IVIII secretion by 10–100-fold. IVIII constructs containing these high expression porcine IVIII sequences now have been demonstrated to outperform BDD hfVIII in heterologous expression systems [21,22,23] and in preclinical gene therapy studies [6,14,24]. Therefore, a rational design approach for novel hemophilia A therapeutics has arisen out of the discovery of species-specific differentials in certain properties such as the rate of biosynthesis, half-life, antigenicity and immunogenecity [1,2,3,6,10,25,26].

Disregarding the B-domain, the majority of IVIII orthologs contain greater than 80% amino acid identity to human IVIII. Despite this primary sequence similarity, numerous unique properties have been characterized and utilized toward bioengineering improved IVIII constructs. During re-establishment of a line of hemophilia A sheep, a new IVIII ortholog was cloned and characterized with the potential for discovering novel biochemical characteristics, while additionally providing a life-saving therapeutic for the ovine hemophilia A colony. In the current study, bioengineering, heterologous expression and biochemical characterization of ofVIII are described. Both recombinant full length and BDD ofVIII were shown to be expressed at greater levels than the equivalent hfVIII constructs in a BHK-M based heterologous expression system. These constructs were not modified beyond deletion of the B domain and the inclusion of a PACE/furin linker between the A2 and A3 domains as previously described [12,13]. The biosynthesis levels observed for full length and hfVIII are comparable to previously published reports [2,6,14]. It was possible to purify significant quantities of BDD ofVIII using the same two-step ion exchange purification procedure that previously was described for recombinant BDD human, porcine and murine IVIII. OfVIII was purified to near homogeneity and shown to harbor specific activity higher than has been described previously for BDD human or murine IVIII [1,2]. The purified product displayed AQ values indicative of very little to no contamination of the product with IVIIIa, which would artificially inflate the specific activity measurement. Therefore, it is concluded that the specific activity of BDD ofVIII is approximately 1.5 and 3 fold higher than recombinant BDD human and murine IVIII, respectively.

BDD ofVIII shares 86% amino acid identity to BDD hfVIII with eight amino acid deletions in the heavy chain. Analysis of N-linked glycosylation patterns with the use of PNGase F endoglycosidase revealed a greater mobility shift in the activated light chain and A1 domain of ofVIII than was observed for BDD hfVIII. Both the A1 and A3 domains of ofVIII have predicted N-linked glycosylation sites not present in hfVIII, the former also being present in canine, porcine, and murine IVIII. The results of mass spectrometry analysis of BDD ofVIII do not support the presence of N-linked glycosylation at Asn-1720, and an explanation for the differential mobility of the ovine IVIII light chain remains elusive. However, one possibility is that the processed glycans vary in size and composition thus accounting for the observed discrepancy. Similar to what was described for recombinant canine IVIII by Arruda and colleagues, the majority of secreted BDD ofVIII is maintained as single chain despite the presence of a consensus RHQR PACE/furin recognition site [5]. Entirely unique to ofVIII is the observation that there is not clear separation of the heterodimeric heavy and light chains observed upon SDS-PAGE. The chains are resolved upon immunoprecipitation of each polypeptide independently using domain-specific MAbs under conditions where the heterodimeric IVIII is dissociated. Following thrombin proteolysis, the A1 domain, A2 domain, and the activated light chain could be immunoprecipi-
tated specifically. The biochemical basis of this observation is not yet understood. It seems likely that the amino acid deletions in the heavy chain coupled with potentially larger glycans bound to the light chain alter the relative mobilities in opposite, but converging directions. However, the predicted relative changes in molecular mass for these structural disparities (approximately 1 kDa for missing residues), may not be sufficient to account for this observation entirely. The differences in other post-translational modifications (e.g. O-linked glycosylation and tyrosine sulfation) may contribute to the diminished mobility of ovine light chain, but have not yet been characterized.

Following activation by thrombin, IVIII assumes a heterotrimetric structure with the A2 subunit being in weak association with the A1/A3-C1-C2 heterodimer, the latter of which is stabilized by coordination of a metal ion [18,27]. Under physiologic concentrations of approximately 1 nM, the IVIII heterotrimer is thermodynamically unstable and the A2 subunit dissociates from the molecule with a half-life of 2 min for hFVIIIa. Disassociation of the A2 subunit results in the loss of IVIII coagulant function as demonstrated through identification of specific hemophilia A mutations that operate through this mechanism [28]. Although the physical factors directly attributing to A2 subunit dissociation are unclear, it has been shown that instability of the A2 domain association does not factor into one-stage coagulation assay IVIII activity measurements. As a result, measures of specific activity are independent of decay and must be due to factors other than A2 domain stability. However, in the presence of thrombin, mutations in the A1 or A2 domain resulting in diminished stability will show a reduced activity in the two-stage coagulation as compared to the one-stage assay and reduced ability to achieve hemostasis. Previously, we characterized the thrombin-activated decay of recombinant human, porcine, murine and hybrid IVIII molecules [1,2]. Compared to those orthologs, ovFVIII displays an intermediate half-life of 3.7 min, which is almost twice that of hFVIIIa but 0.5-fold that of pFVIIIa and <0.1-fold that of mFVIIIa. Increases in animal size and severity of potential thrombotic effects due to pressure differentials or vascular characteristics may provide an explanation for evolutionarily altered stability of IVIIIa within these species. Tight regulation of IVIII activity must be maintained to prevent unwarranted thrombotic events, as well as to allow cessation of bleeding events upon vessel injury.

In order to test the efficacy of BDD ofVIII, E16+/− mice were challenged with a tail transection after the intravenous administration of 300 units/kg ofVIII compared to saline. In an attempt to normalize physical hemodynamic-related properties due to vasculature size, transections were made at 2 mm diameter as opposed to a fixed distance from the distal end of the tail [29,30,31]. Furthermore, to prevent the false appearance of phenotypic recovery at low time points due to non-fibrinogenic platelet aggregation at the site of transection, mice were observed for 40 minutes post-challenge. Blood loss was reduced dramatically and phenotypic correction was observed through recombinant BDD ofVIII administration. Based on this result, we believe it practical to test the efficacy of BDD ofVIII in hemophilia A sheep and further assess the propensity for BDD ofVIII induced inhibitor formation (i.e., immunogenicity).

Assuming a typical weight of 73 kg, an adult hemophilia A sheep would require approximately 1.5 mg ofVIII per administration using an estimated therapeutic dose of 300 units/kg translated from the data obtained in the current study using hemophilia A mice. Due to the demonstrated bio-production characteristics of ovFVIII shown herein, prophylactic treatment of hemophilia A sheep would be similar in product requirements to that of humans with severe hemophilia and may not be practical due to manufacturing and economic constraints, e.g. the typical cost of prophylactic severe hemophilia A care in the U.S. is $200,000–300,000 per patient per yr. However, a 5 kg neonatal lamb would require only 80 μg per administration. If effective, this post-natal treatment regimen may be complimented by gene therapy trials to measure the safety, efficacy, and immunogenicity of novel gene therapy strategies in neonatal sheep, which would greatly enhance the value of this model in biomedical research.

As has been observed in some canine hemophilia A lines [3,32], hemophilia A sheep develop inhibitors to recombinant human IVIII following parenteral infusion [7]. Hemophilia A sheep possess a premature stop codon in exon 14 caused by a frameshift mutation and are believed to be an accurate phenocopy of severe hemophilia A in humans. If different in any demonstrable manner, the sheep model may display a more severe bleeding phenotype as well as higher inhibitor incidence. The physiology and clinical phenotype is mirrored in ovine and human, and the former model eliminates the requirement of scale up estimation of treatment dosages. Maintaining the ovine hemophilia A colony requires intensive effort and extensive resources. Thus, without adequate validation, this model likely will be lost as a testing ground for the efficacy and immunogenicity of novel hemophilia A biotherapeutics and gene therapy applications. The development and characterization of recombinant ovFVIII should facilitate the validation which in turn will enhance the value and utility of this unique large animal disease model.

**Author Contributions**

Conceived and designed the experiments: CBD PZ HTS GA CP. Performed the experiments: PZ BG. Analyzed the data: CBD PZ HTS. Contributed reagents/materials/analysis tools: GA CP. Wrote the paper: PZ CBD.

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