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Functional factor VIII made with von Willebrand factor at high levels in transgenic milk

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Summary

Background—Current manufacturing methods for recombinant human Factor VIII (rFVIII) within mammalian cell cultures are inefficient which limit the abundance needed for affordable, worldwide treatment of hemophilia A. However, rFVIII has been expressed at very high levels by the transgenic mammary gland of mice, rabbits, sheep and pigs. Unfortunately, it is secreted into milk with low specific activity due in part to the labile, heterodimeric structure that results from furin processing of its B domain.

Objectives—To express biologically active rFVIII in the milk of transgenic mice through targeted bioengineering.

Methods—Transgenic mice were made with a mammary specific FVIII gene (226/N6) bioengineered for efficient expression and stability containing a B domain with no furin cleavage sites. 226/N6 was expressed with and without von Willebrand Factor (VWF). 226/N6 was evaluated by ELISA, SDS-PAGE, Western blot, and one- and two-stage clotting assays. Hemostatic activity of immunoaffinity enriched 226/N6 was studied *in vivo* by infusion into hemophilia A knockout mice.

Results and conclusions—With or without co-expression of VWF, 226/N6 was secreted into milk as a biologically active single chain molecule that retained high specific activity similar to therapeutic-grade FVIII. 226/N6 had >450-fold higher IU/ml than previously reported in cell culture for rFVIII. 226/N6 exhibited similar binding to plasma-derived VWF as therapeutic-grade rFVIII and intravenous infusion of transgenic 226/N6 corrected the bleeding phenotype of hemophilia A mice. This provides proof-of-principle to study expression of 226/N6 and perhaps other single chain bioengineered rFVIII in the milk of transgenic livestock.

Keywords

factor VIII; haemophilia; mammary gland; recombinant protein; transgenic animal; von Willebrand factor; modified B domain

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Introduction

Hemophilia A is an X-linked, inherited disorder of blood coagulation primarily caused by deficiency or dysfunction of factor VIII (FVIII) that affects approximately one in every 5,000-10,000 males [1-3]. While occurring at only 100 to 200 ng/ml in blood plasma, FVIII is a post-translationally complex, large glycoprotein of about 260 to 280 kDa [4-6]. FVIII acts as an essential co-factor to Factor IX in the coagulation cascade to amplify the activation of Factor X by >1000-fold. The structural complexity of FVIII [7] remains at the core of therapeutic problems such as abundance and frequently encountered inhibitory immune responses to intravenous therapy. Thus, both the development of universal prophylactic and non-intravenous, low bioavailability treatments in economically developed countries and routine access in developing countries are precluded [1,8,9].

The biosynthesis and secretion of FVIII into blood plasma by hepatocytes and recombinant FVIII (rFVIII) by mammalian cells are greatly and similarly inefficient due to limitations in transcription [10-12], post-translational processing and translocation from the endoplasmic reticulum to the Golgi apparatus [13,14]. The most biosynthetically restricted rFVIII structure is the analogue of native FVIII containing it's full length (907 amino acids,90 kDa) B domain (FL-rFVIII). The B domain is processed by furin at two different sites leading to a complex mixture of labile [15] metal ion stabilized, heterodimers (Fig. S1). Thus, all therapeutic-grade plasma-derived FVIII (FL-FVIII) and FL-rFVIII are heterodimeric and contain no single chain species [7]. von Willebrand Factor (VWF) helps stabilize the labile, heterodimeric structure of both FL-FVIII in blood circulation and FL-rFVIII in cell culture media. Although VWF does not increase the expression levels of FL-rFVIII protein, the presence of VWF in cell culture media increases the accumulation of FVIII activity to about 1-2 IU/ml which is about the same as is in normal human plasma [16].

We and others have previously reported high concentrations of FL-rFVIII in the milk of transgenic mice [17], rabbits [18,19], sheep [20] and pigs [21] showing that the mammary gland can efficiently secrete rFVIII with a full B domain. However, the specific activity of the FL-rFVIII was very low at about 0 to 10% of expected levels (Table S1). The native appearance of the FL-rFVIII heavy and light chains found in these milks suggested that the conformation of the assembled, heterodimer and not necessarily proteolytic degradation was the main cause of the low activity in milk (Fig. S2) [21].

Unlike previous studies which expressed FL-rFVIII in milk, this study expresses a bioengineered rFVIII with a modified B domain (226/N6) having no furin sites in the presence and absence of the potentially stabilizing influence of recombinant VWF (rVWF) co-expressed in mouse milk. The consensus sites for furin cleavage at Arg1313 and Arg1648 were removed by truncating the B domain to the first 226 amino acids (Fig. S1B). We provide the first report of rFVIII made at high levels in transgenic milk with native procoagulant activity.

Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan and Virginia Polytechnic Institute and State University.

DNA constructs

The 226/N6 (WAP7FVIII-226/N6) construct was assembled by altering the Kpn I site of pUCWAP6 [22] by introducing a Sal I site immediately downstream of the 4.1 kbp WAP (whey acidic protein) promoter and ahead of the 1.7 kbp of mouse WAP 3’ UTR containing the coding of the polyadenylation signal to produce pUCWAP7. The sequence for

FVIII-226/N6 was removed by restriction enzyme digest using Sal I and Xho I from $pMT_2226/N6$ as described previously [14] and introduced into the Sal I site of pUCWAP7 yielding the pUCWAP7FVIII-226/N6 plasmid. The plasmid pUCWAP6VWF containing the WAP6VWF expression construct (gift from Dr. Henryk Lubon, American Red Cross, Rockville, MD) contained the cDNA for VWF placed between the 4.1 kbp WAP promoter and the 1.7 kbp 3'UTR. The plasmid designated PPL456 containing the BLG (beta lactoglobulin) driven AAT construct, which has been previously described [23], was a gift from Dr. David Ayares (Revivicor, Blacksburg, Virginia). The WAP7FVIII-226/N6 was released by Not I. The WAP6VWF was released by digestion with Not I and Sfi I. The BLG-AAT was released by digestion with Not I and Sal I. All constructs were purified by extraction prior to microinjection using a Nucleospin Extract kit (Clonetech Laboratories, Inc. Palo Alto, CA).

Transgenic mouse generation, identification and milk collection

Transgenic mice were generated by pronuclear microinjection of purified DNA constructs as previously described [24] at the Transgenic Core facility at the University of Michigan. Southern analysis was performed as previously described [21]. PCR probes (KPL, Inc. Gaithersburg, Maryland) are shown in Figure S3A. 500 bp PCR primers were: for WAP, WAPprobeS1 (gcatgctcacactcaacagg)/ WAPprobeA1 (taagagtgtggaggcgcttg); for AAT, same primers as for PCR, BLG-S1/AAT-A1. Milk was collected and defatted as previously described [24]. Samples were stored at -70°C.

Factor VIII Biochemical Analysis

FVIII activity was measured by one-stage aPTT clotting assay on an MLA Electra 750 fibrinometer by reconstituting human FVIII-deficient plasma (George King Bio-Medical Inc., Overland Park, Kansas) and modified two-stage assay utilizing the COAMATIC Factor VIII (Chromogenix, Milano, Italy) according to the manufacturer's instructions. FVIII antigen levels were determined by ELISA kit (Affinity Biologicals Inc., Ancaster, ON, Canada) which assumed a FVIII concentration of 200 ng/mL per 1 IU/mL of activity (5000 IU/mg). 1 IU/ml is the defined concentration of Factor VIII activity in normal human plasma which contains an average concentration of 100-200 ng per ml.

Immunoaffinity chromatography enrichment of 226/N6

Defatted milk was diluted and incubated overnight with anti-heavy chain FVIII monoclonal antibody-conjugated sepharose beads (kindly provided by Baxter BioScience, Deerfield, IL). The enrichment procedure was carried according to the protocol described previously [25] with and without wash steps with 250 mM $CaCl_2$ and $MgCl_2$ to disrupt FVIII:VWF complexes [26] prior to elution of FVIII. 1 mg/ml AAT was added as a stabilizer after IAC enrichment. 226/N6 from CHO cells was enriched from stably-transfected CHO cell lines as described previously [14].

Immunoblotting

Non-reduced and reduced samples were evaluated by sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel electrophoresis on 4-12% NuPage® Bis-Tris gels (Invitrogen Carlsbad, CA) and then electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). Blots were probed with polyclonal antibodies for FVIII (USBiological, F0016-10, Swampscott, MA), VWF (USBiological, V2700-04B, Swampscott, MA) or AAT (USBiological, A2298-27H, Swampscott, MA).

FVIII-VWF binding ELISA

226/N6 and rFVIII (Advate®, gift from Baxter) were incubated with 1% FVIII-deficient human plasma (George King Bio-Medical). Triplicate samples were incubated with an anti-FVIII antibody (BO2C11, gift from Marc Jacquemin, University of Leuven, Belgium) that inhibits VWF binding[27] prior to incubation with 1% FVIII-deficient human plasma. Samples were loaded into 96-well plates pre-coated with anti-FVIII antibody (F8C-EIAC, Affinity Biologicals). Anti-VWF antibody (p0266, Dako, Carpenteria, CA) was used to detect VWF binding. Absorbance was read at 490 nm.

In vivo tail clip assay in hemophilia A (HA) mice

Hemophilia A exon 16 knockout mice [28] were subjected to a tail clip bleeding assay. Mice at least 10 weeks of age were injected with immunoaffinity enriched FVIII proteins. C57BL/6 and HA mice littermates were used as controls for injection with lactated Ringer's (LR) solution. Mice were injected with 80 IU/kg body weight of FVIII in 100 μl LR solution via tail vein. Ketamine 85 mg/kg with xylazine 5 mg/kg was injected IP to induce anesthesia. After 5 minutes, tails were cut at 1.5 mm diameter from tail tips then immediately submerged into 14 ml conical tubes filled with saline at 37°C. Blood from the tail was collected 10 minutes. Blood loss was quantified by the difference in tube weight at time 0 and 10 minutes. Mice were subsequently euthanized.

Statistical analysis

Data are presented as mean ± standard deviation except for bleeding data which was presented as median ± standard deviation. A single factor analysis of variance (ANOVA) was performed to compare blood loss results. Student's t-tests (two-tailed) assuming unequal variances were used to compare each treatment group with control mice. An alpha level of 0.05 was used for all statistical analysis.

Results

Production and selection of transgenic mice by expression levels

We made monogenic and bigenic 226/N6 mice using three different transgenes (Fig. S3) to help test the following hypotheses: 1) Single chain rFVIII would be expressed at a range of concentrations in milk similar to or higher than that of FL-rFVIII; 2) Co-expressed rVWF would complex 226/N6 and help stabilize single chain rFVIII made in milk as it does in plasma and cell culture; and 3) Single chain rFVIII would have higher specific activity than FL-rFVIII when expressed in milk. An optimized beta-lactoglobulin, Alpha1 Antitrypsin gene (BLG-AAT) was used to make lineages of bigenic and trigenic 226/N6 mice in an effort to mitigate transcriptional silencing which occurs in >60% of mammary expression in transgenic mice. Silencing was decreased to about 25% in the presence of mice co-injected with the BLG-AAT transgene [29,30]. Twenty different potential founder animal litters were made by outbreeding with control animals. Transgenic mice maintained a natural lactation length of about 17 days and had normal litter growth. About 50 to 100 μl of milk was obtained per milking.

Detectable levels of rFVIII protein and activity occurred in some monogenic mice for 226/N6 alone while no rFVIII protein or activity was detected in milk from mice bigenic for 226/N6 and rVWF (Table 1). In the presence of the BLG-AAT milk gene, the frequency of both 226/N6 protein and activity levels was much higher for bigenic (226/N6 and BLG-AAT) and trigenic (226/N6, rVWF and BLG-AAT) lineages than monogenic 226/N6 mice. For these bigenic and trigenic mice containing the BLG-AAT gene, Alpha1-antitrypsin (AAT) was expressed at >1 mg/ml (Fig. S4). In summary, while transgene silencing likely occurred in some lineages, the concentration range of 226/N6 found in the milk of all mice was

similar to previously reported concentration ranges for FL-rFVIII made in the milk of mice, rabbits, sheep and pigs with the highest concentration of 226/N6 being about 0.2 to 0.3 mg/ml.

Specific clotting activity and analysis by Western blot

We studied the transgenic mice with the highest 226/N6 expression levels and expanded those lineages. The specific activity was determined directly in the whole milk pools by measurement of FVIII antigen by ELISA and coagulation activity by aPTT assay (Table 1). Notably, the 226/N6 specific activities found in the milks from monogenic, bigenic and trigenic mice were all similar and typical of plasma-derived FVIII or FL-rFVIII. Importantly, it was also functional without the presence of AAT or rVWF. Because there are few high molecular weight proteins occurring in the milk of mice near or at the Mr of 226/N6 or VWF under reducing conditions, quantitative Western blotting was possible. The high 226/N6 protein levels determined by ELISA were confirmed using quantitative Western analysis under reducing conditions (Fig. S5).

Interactions of 226/N6 with rVWF and VWF

Strong interactions between VWF and FVIII result from conformations stabilized by intramolecular disulfide bonding within FVIII and VWF subunits. The interactions between FL-FVIII:VWF in therapeutic-grade preparations are not appreciably destabilized by treatment with SDS under non-reduced conditions as evidenced by the decreased mobility seen in Western blot analysis (Fig. 1). Reduced conditions destabilize these binding interactions resulting in greater mobility that is commensurate with the molecular size of FVIII and VWF monomers, respectively. In contrast, purified preparations of therapeutic-grade FVIII containing no VWF do not show the greatly decreased electrophoretic mobility in SDS-PAGE under non-reduced or reduced conditions. This intramolecular disulfide bridge dependent phenomenon occurring within the VWF and FVIII molecules was used to help determine the extent of 226/N6 complexation with rVWF in milk. The electrophoretic migration of heterodimers under reduced and nonreduced conditions for therapeutic-grade FL-rFVIII made in CHO cells not containing VWF was similar. The Mr of the heterodimers was consistent with the published range of 100-220 kDa (Fig. 1A and 1B, respectively) while possessing no signal for VWF (Fig.1C and 1D, respectively).

A trigenic (226/N6, rVWF and BLG-AAT) mouse milk pool containing 300 μg/ml of both 226/N6 and rVWF, showed a single predominant FVIII signal at about 190 kDa under reduced conditions. The VWF signal had a similar Mr (about 320 kDa) to the plasma derived FVIII:VWF reference under reduced conditions. Importantly, the same shift to a retarded migration of Mr >420 kDa occurred for the trigenic sample under nonreduced condition as occurred with the plasma-derived FVIII:VWF complex. Bigenic mouse milk containing no rVWF migrated similarly to the trigenic sample under reduced conditions, but did not exhibit the greatly retarded migration under nonreduced conditions. These results show that 226/N6 was secreted as a single chain into milk with or without VWF.

Immunoaffinity chromatography (IAC) was applied to a whey phase made from centrifugally clarified and diluted, trigenic mouse milk to provide an infusable preparation enriched in 226/N6. The IAC captures FVIII and FVIII:VWF complexes. Trigenic milk was treated with IAC with loading and wash conditions at which VWF:FVIII complexes were unstable allowing most of the VWF to be efficiently released before and during immunosorption of 226/N6. While the starting milk contained a 1:1 ratio of rVWF to 226/N6, immunoaffinity enrichment reduced the stoichiometric ratio of rVWF to 226/N6 in the FVIII eluate to about 0.1. The yield of 226/N6 from trigenic milk containing high levels of 226/N6 was 10% with an enrichment of 70-fold, and a purity of 39% before addition of

AAT for storage. This IAC preparation was similar to an IAC enriched therapeutic-grade FL-rFVIII made from Recombinate (Table 2). Importantly, the specific activity of the immunoaffinity enriched 226/N6 from trigenic milk was similar to the original milk and also the FL-FVIII and FL-rFVIII references (Table S2).

IAC was also applied to milk from low expressing animals that did not co-express VWF. While the purity was <1% (Fig. S6), a enrichment of 16-fold was achieved for 226/N6 at a similar specific activity to that found in the original milk (Table 2). The yield of 226/N6 captured from milk containing 226/N6:VWF complexes was lower than the yields from non-VWF expressing milk.

Using ELISA, we studied the complexation of human plasma-derived VWF with 226/N6 that could occur in FVIII-deficient plasma derived from a severe hemophilic A donor. We used this model to determine if 226/N6:VWF complexes would form in a context more pertinent to future hemophilia A treatment. The IAC enriched 226/N6 preparations and therapeutic-grade FL-rFVIII were immunocaptured on a microtiter plate. The immobilized anti-FVIII monoclonal antibody has been shown to not interfere with VWF binding. The presence of VWF bound to the immunocaptured 226/N6 was detected by anti-VWF polyclonal antibody. To determine the specificity of the interaction between 226/N6 and VWF in blood plasma, we pre-incubated each immunocaptured rFVIII species with and without a monoclonal antibody (B02C11) that binds to the light chain of FVIII inhibiting FVIII:VWF complexation [27]. In all cases, the B02C11 inhibited the VWF signal arising from incubation in plasma to low background levels. Without pretreatment with B02C11, both immunocaptured FL-rFVIII and 226/N6 exhibited strong VWF binding signals in a similar dose-dependent fashion (Fig. 3). The similar polynomial fits to each of these absorption profiles indicated that FL-rFVIII and 226/N6 have similar avidities for VWF in human plasma.

Hemostatic potential of infused IAC enriched 226/N6

We studied the hemostatic efficacy of 226/N6 *in vivo* using a terminal tail transection bleeding model in hemophilia A mice. Figure 4 shows the median total blood loss observed for normal C57/BL mice and FVIII knockout hemophilia A (HA) mice resulting from a terminal tail transection. The HA mice were infused with lactated Ringer's (LR), immunoaffinity enriched 226/N6 made in CHO cells or immunoaffinity enriched 226/N6 from milk. 226/N6 from CHO cells and 226/N6 from transgenic milk were each infused at 80 IU/kg body weight. Normal C57/BL mice showed a cessation of bleeding (4 of 4) at about 2 to 5 minutes with a total weight of blood loss over 10 minutes ranging from 80 to 165 mg. HA mice infused with LR typically did not show a cessation of bleeding (10 of 12) with total weight of blood loss ranging from 115 to 725 mg. HA mice infused with 226/N6 from CHO cells showed a cessation of bleeding (5 of 5) at about 2 to 5 minutes with a total weight of blood loss ranging from 60 to 230 mg. HA mice infused with 226/N6 from transgenic milk showed a cessation of bleeding (5 of 6) typically at about 2 to 5 minutes with most mice having a total weight of blood loss ranging from 10 to 175 mg. Post hoc analysis (two-tailed t-tests) determined that LR-treated hemophilia A mice bled significantly more than LR-treated C57/BL mice ($p = <0.001$, $\alpha = 0.05$), whereas both 226/N6 from CHO cells and 226/N6 from transgenic milk treated HA mice had similar blood losses as LR-treated C57/BL mice ($p = 0.574$ and 0.491, respectively, $\alpha = 0.05$).

Discussion

Production of rFVIII in the milk of transgenic livestock is a promising approach to making rFVIII therapy significantly more abundant. Producing a greater abundance of rFVIII will help advance immunotolerance and non-intravenous therapy [31,32]. Towards that goal, our

previous work showed that FL-rFVIII was efficiently secreted into milk at 10 to 1000-fold higher concentrations than is seen in blood plasma or cell culture (Table S1). However, the specific activity of the FL-rFVIII was very low. The broad range of FL-rFVIII levels expressed in milk is likely caused by transcriptional inefficiency that often occurs as a result of transgene integration into less transcriptionally active chromosomal sites [29,30]. In founder lineages with favorable integration sites for expression, this work shows that 226/N6 can be made at high concentrations similar to that of FL-rFVIII. Therefore, 226/N6 is at least as efficiently secreted by the mammary gland as FL-rFVIII where additional improvements in transcriptional efficiency might enable even higher levels. The 226/N6, which has a modified B domain lacking furin sites, yielded a specific activity similar to therapeutic-grade reference FL-FVIII and FL-rFVIII. Importantly, the infusion of IAC enriched 226/N6 from milk or CHO cells corrected the hemostatic defect of FVIII knockout mice in response to a tail transection trauma challenge.

Co-expressed AAT did not appear to impact the specific activity of the milk-borne 226/N6 since the specific activities of 226/N6 produced by monogenic, bigenic and trigenic mice were similar. AAT was added to enriched preparations to inhibit plasmin generation during storage because plasmin is a naturally occurring, broadly acting protease in milk [33,34]. The general co-expression of milk protease inhibitors in order to protect the recombinant protein product is still a future developmental goal.

This is the first report of any rFVIII co-expressed in milk with rVWF. FVIII circulates as a complex with VWF which is a prerequisite for its stability and thus survival in circulation [13,35]. Therapeutic-grade FL-FVIII:VWF complex and 226/N6 in the presence of co-expressed rVWF had similarly reduced electrophoretic mobility. This, in addition to the similar *in vitro* binding of plasma-derived VWF by 226/N6, suggests that co-expressed VWF:226/N6 complexes existed in the milk. The native binding behavior of 226/N6 with plasma-derived VWF indirectly shows that the proper post-translational sulfation of tyrosine 1680 in the A3 domain of 226/N6 likely occurred as it is required for complexation [36]. 226/N6 expressed with and without rVWF in milk had >450-fold higher activity levels than the analogous bioreactor production of FL-rFVIII and >40-fold higher than expression of 226/N6 in CHO cell culture without rVWF [14]. The high activity levels accumulating in the milk in the presence or absence of rVWF is likely a reflection of the inherent stability of 226/N6. This contrasts the beneficial effects of co-expressed VWF in cell culture which stabilizes FL-rFVIII and increases its accumulated activity levels [13,15,16]. The result of this study has been our first proof that VWF would bind to this 226/N6 design and that a co-expressed FVIII:VWF product may be feasible in milk.

We acknowledge that utilization of a bioengineered rFVIII could result in neoantigenicity; however, the advantage of the 226/N6 design is that its bioengineering strategy targets modifications to the B domain where inhibitor antibodies have infrequently been described. In addition, after thrombin activation, 226/N6 assumes a active FVIII structure that is identical to full-length wild type FVIII [14]. There is also evidence that VWF complexation with FVIII increases the effectiveness of immune tolerance therapy [37]. Thus, the ability of 226/N6 to complex with co-expressed rVWF and plasma-derived VWF may be a beneficial attribute in the development of future applications of transgenically produced rFVIII.

The safety and efficacy of transgenic milk-derived products has crossed an important threshold as the US FDA and EMEA have approved recombinant anti-thrombin III made in goat milk (ATRYN®) [38]. Our study in mice provides the fundamental understanding needed for the future expression of biologically active, single chain rFVIII molecules of different designs in the milk of livestock. Pigs are a likely choice of transgenic livestock for future production of rFVIII [32] because they combine the advantages of milk volume and

hepatocyte-like post-translational biochemistry such as glycosylation [39]. With respect to 226/N6, which retains six of the 19 potential glycosylation sites in the B domain, the glycosylation may impact pharmacokinetics. While ruminants also provide ample milk volume, their mammary biochemistry produces higher mannose, low sialic acid glycoforms which may affect circulation residence times due to interactions with asialyo receptors found in the human liver [40]. Given the fundamental knowledge about rFVIII stability provided by our studies in mice and pigs, we have begun to make transgenic pigs with various modified B domain FVIII constructions. In contrast with mice, the amount of rFVIII that will be produced in pig milk will permit bioengineering studies on purification and formulation. The advantage of pig milk over other FVIII sources such as cell culture bioreactors and human plasma is evident in the greater than two orders of magnitude reduction in source volume estimated to meet global FVIII demand [38] (Table S3).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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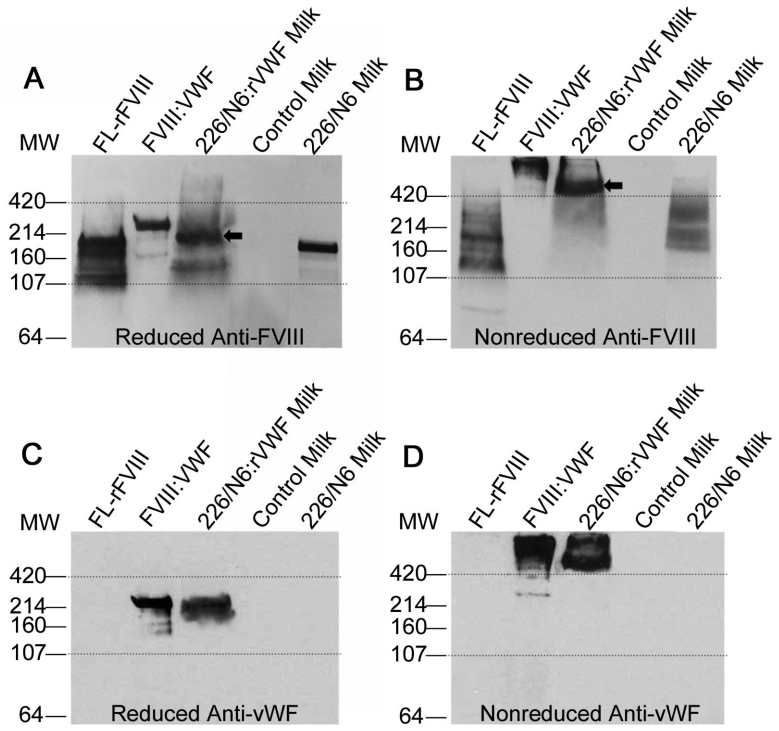

Fig. 1.

SDS-PAGE of FL-rFVIII and milk-borne 226/N6 in the presence and absence of VWF under reduced and non-reduced conditions. Assessment of VWF binding by (A) reduced anti-FVIII, (B) non-reduced anti-FVIII, (C) reduced anti-VWF, and (D) non-reduced anti-VWF SDS-PAGE Western Blots. All blots contain a molecular weight marker (MW), recombinant FVIII (FL-rFVIII), plasma-derived FVIII:VWF concentrate (FVIII:VWF), diluted trigenic mouse milk equivalent to 0.07 μl undiluted milk (226/N6:rVWF:AAT Milk), diluted non-transgenic mouse milk equivalent to 0.07 μl undiluted milk (Control Milk) and diluted bigenic mouse milk equivalent to 0.07 μl undiluted milk (226/N6:AAT Milk). Arrows indicate primary single chain 226/N6 species.

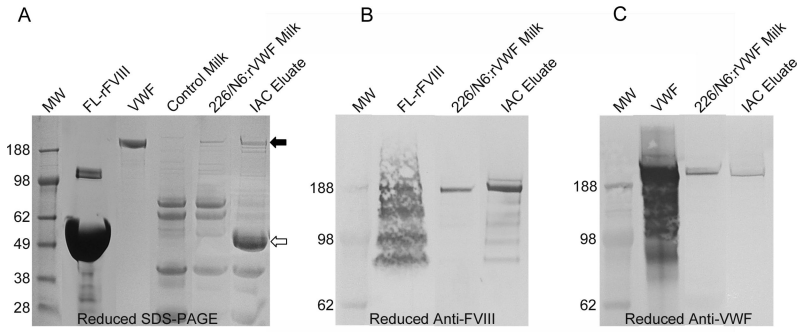

Fig. 2.

SDS-PAGE of IAC enriched 226/N6. 226/N6 and rVWF were immunoaffinity enriched from transgenic mouse milk and separated during the enrichment process. Enrichment of the 226/N6 and rVWF was performed on clarified, diluted milk pooled from six F3 trigenic mice from the 415-101-2 line and analyzed by reduced SDS PAGE (A) and anti-FVIII (B) and anti-VWF (C) Western blots. The SDS-PAGE gel contains a molecular weight marker (MW), a FVIII reference (FL-rFVIII; Recombinate), a VWF reference (VWF), diluted non-transgenic mouse milk equivalent to 0.07 μl undiluted milk (Control Milk), diluted trigenic mouse milk equivalent to 0.07 μl undiluted milk (226/N6:rVWF Milk), and trigenic milk product enriched by immunoaffinity chromatography (IAC Eluate). The enriched 226/N6 is shown by the closed arrow (⬅). The open arrow (⇦) points to the AAT that was added at 1 mg/ml to the IAC eluate for storage formulation. Each blot contains a molecular weight marker (MW), a reference (FL-rFVIII and/or VWF), trigenic transgenic mouse milk (226/N6:rVWF Milk), and immunoaffinity enriched trigenic milk product (IAC Eluate).

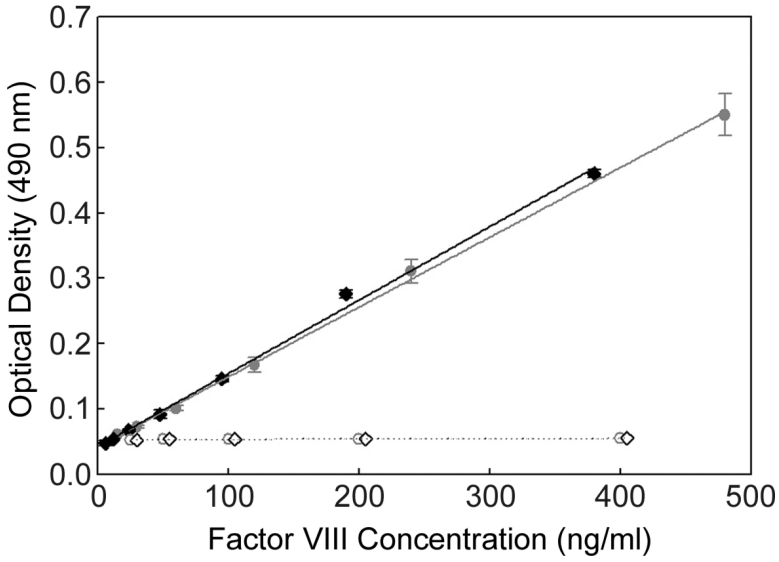

Fig. 3.

Plasma VWF binding of IAC enriched 226/N6. The relative affinity of VWF for 226/N6 immunoaffinity enriched from milk from trigenic mice (◆) and FL-rFVIII (●) were compared by ELISA. The specificity of VWF binding was also evaluated by preincubating 226/N6 immunoaffinity enriched from milk from trigenic mice (◇) and FL-rFVIII (○) with a monoclonal antibody (BO2C11) which binds to FVIII and inhibits VWF binding. Polynomial trendlines are shown for the reference FL-rFVIII (gray line) and 226/N6 (black line) in the absence of the inhibitor and the reference FL-rFVIII (dotted gray line) and 226/N6 (dotted black line) in the presence of the inhibitor. Error bars represent standard deviation.

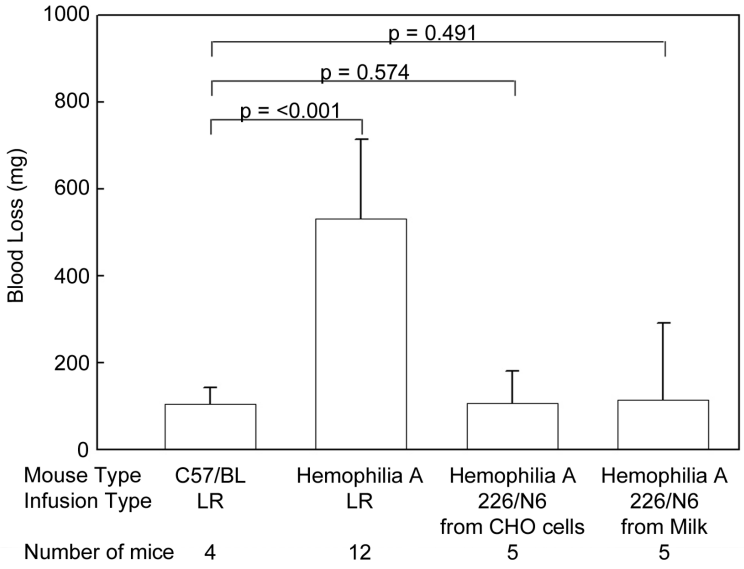

Fig. 4.

Hemostatic efficacy *in* vivo of 226/N6 within the hemophilia A mouse model. Total blood loss (in mg) over 10 min was observed for normal C57/BL mice and FVIII knockout hemophilia A (HA) mice resulting from a terminal tail transection. The HA mice were infused with either lactated Ringer's (LR), immunoaffinity enriched 226/N6 from CHO cells or immunoaffinity enriched 226/N6 from transgenic milk. An analysis of variance indicated that the effect of treatment was significant ($p < 0.001$). Post hoc analysis determined that on LR-treated hemophilic A mice bled significantly more than LR-treated control mice ($p = <0.001$). An α of 0.05 was used as a significance criterion for all statistics. Bars represent the median blood loss and the error bars the standard deviation.

Table 1

The specific activity of 226/N6 in milk.

Mouse Genotype Mouse ID	226/N6 Concentration (μg/ml)	aPTT Activity Level (IU/ml)	Specific Activity (IU/mg)
Monogenic mice: 226/N6			
1960-1702	0.144	0.6	4,167
1926-1742	0.093	0.1	1,075
46-1247	0.066	0.4	6,061
Bigenic mice: 226/N6-rVWF			
376-8	Not Detected	Not Detected	
376-12	Not Detected	Not Detected	
383-49	Not Detected	Not Detected	
Bigenic mice: 226/N6-BLG-AAT			
875-234	34	25	735
875-233	6.2	44	7,097
516-46	7.3	13	1,781
406-128-10	83	122	1,470
Trigenic mice: 226/N6-rVWF-BLG-AAT			
415-101-2	183	678	3,705
415-101-1-2	122	555	4,549

1 IU/ml is the defined concentration of FVIII activity in normal human plasma. An average concentration of 200 nanograms per ml normal human plasma is assumed yielding a theoretical specific activity of 5,000 IU/mg.

Table 2

The specific activity of IAC enriched 226/N6 from clarified, diluted milk.

Sample	Total Protein (mg/ml)	FVIII Antigen (μg/ml)*	FVIII Activity (IU/ml)†	1-stage Activity Level (IU/mg)	% Purity	% Antigen Yield	IAC Enrichment
Therapeutic-grade FL-rFVIII (Recombinate®)	21.95 ± 3.57	26.35 ± 2.64	90.09 ± 9.22	3,419 ± 634	<1	N/A	N/A
IAC Enriched FL-rFVIII (Recombinate®)	0.08 ± 0.13	35.58 ± 0.71	75.84 ± 2.18	2,132 ± 112	44	96	367-fold
IAC Enriched 226/N6 (Bigenic Milk)	0.66 ± 0.25	6.34 ± 0.64	22.10 ± 1.10	2,506 ± 267	<1	63	16-fold
IAC Enriched 226/N6 (Trigenic Milk)	0.03 ± 0.01‡	10.32 ± 1.92	43.21 ± 2.75	4,187 ± 1,425	39	10	70-fold

IAC = immunoaffinity chromatography

*determined by ELISA

†determined by aPTT

‡total protein before addition of 1 mg/ml AAT to IAC eluate; IAC enriched Recombinate used as a control. IAC enrichment calculated by FVIII antigen to total protein ratio.