Published in final edited form as:

J Thromb Haemost. 2012 November ; 10(11): 2328–2337. doi:10.1111/j.1538-7836.2012.04902.x.

Factor VIII inhibitors: von Willebrand factor makes a difference *in vitro* and *in vivo*

Q. SHI^{*,†,‡}, E. L. KUETHER^{*,†,‡}, J. A. SCHROEDER^{*,†,‡}, C. L. PERRY[†], S. A. FAHS[†], J. COX GILL^{*,†,‡}, and R. R. MONTGOMERY^{*,†,‡}

^{*}Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, USA

[†]Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI, USA

[‡]Children's Research Institute, Children's Hospital of Wisconsin, Milwaukee, WI, USA

Summary

Background—The important association between von Willebrand factor (VWF) and factor VIII (FVIII) has been investigated for decades, but the effect of VWF on the reactivity of FVIII inhibitory antibodies, referred to as inhibitors, is still controversial.

Objective—To investigate the interaction among VWF, FVIII and FVIII inhibitory antibodies.

Methods—Three sources of inhibitors were used for *in vitro* studies, including the plasma from immunized VWF^{null}FVIII^{null} mice, purified plasma IgG from human inhibitor patients, or human monoclonal antibody from inhibitor patients' B-cell clones. Inhibitors were incubated with recombinant human FVIII (rhFVIII) either with or without VWF. The remaining FVIII activity was determined by chromogenic assay and inhibitor titers were determined. For *in vivo* studies, inhibitors and rhFVIII were infused into FVIII^{null} or VWF^{null}FVIII^{null} mice followed by a tail clip survival test.

Results—VWF has a dose-dependent protective effect on FVIII, limiting inhibitor inactivation of FVIII in both mouse and human samples. A preformed complex of VWF with FVIII provides more effective protection from inhibitors than competitive binding of antibodies and VWF to FVIII. The protective effect of VWF against FVIII inactivation by inhibitors was further confirmed *in vivo* by infusing inhibitors and FVIII into FVIII^{null} or VWF^{null}FVIII^{null} mice followed by a tail clip survival test.

Conclusion—Our results demonstrate that VWF exerts a protective effect, reducing inhibitor inactivation of FVIII, both *in vitro* and *in vivo*.

Keywords

FVIII; inhibitory antibody; VWF

Introduction

Factor VIII (FVIII) is a plasma glycoprotein that functions as a cofactor for FIXa in the FX activation complex of the intrinsic pathway of blood coagulation [1]. FVIII circulates in

© 2012 International Society on Thrombosis and Haemostasis

Correspondence: Qizhen Shi, Department of Pediatrics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226. Tel.: +1 414 937 3853; fax: +1 414 937 6811. qizhen.shi@bcw.edu.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

plasma as a complex with its carrier protein, von Willebrand factor (VWF), which is important for FVIII stability and survival [2]. Upon cleavage by thrombin, activated FVIII disassociates from VWF, binds to phospholipid, and facilitates blood clotting [3,4]. Deficiency of FVIII results in hemophilia A, a genetic bleeding disorder that is currently treated with protein replacement therapy using either plasma-derived or recombinant FVIII [5]. Development of inhibitory antibodies against FVIII is a serious complication that occurs in up to 30% of hemophilia A patients after protein replacement therapy [6,7]. These antibodies can inactivate functional FVIII activity (FVIII:C) and make FVIII replacement therapy ineffective. Although the important association between VWF and FVIII has been investigated for decades, the influence of VWF on the Bethesda assay, which is routinely used in the clinic to determine the titer of FVIII-neutralizing inhibitors, is still uncertain [8–10].

Treatment of inhibitor patients is one of the biggest challenges in hemophilia care. The main short-term objective of the treatment of inhibitor patients is to control bleeding episodes, while the long-term goal is to permanently eradicate the inhibitors [11,12]. Currently, aggressive infusion of high-dose FVIII represents the only effective therapeutic approach for eradication of FVIII inhibitors and results in restoration of normal FVIII pharmacokinetics in some inhibitor patients [11,13,14]. Whether the use of FVIII products containing VWF will reduce reactivity of inhibitors against FVIII and enhance the efficacy of the hemostatic clinical effect is one of the hotly debated issues in the treatment of inhibitor patients [13,15].

Taking advantage of the VWF/FVIII association plus time-dependent inactivation of FVIII by inhibitors, we have developed a platelet gene therapy approach for hemophilia A. Our previous studies have demonstrated that targeting FVIII expression to platelets results in a releasable pool of FVIII together with VWF in α -granules and that platelet-derived FVIII can still function even in the presence of inhibitory antibodies in a mouse model. Further studies using tail clip survival tests showed that in the presence of infused inhibitors platelet-derived FVIII was dramatically more effective than equivalent levels of infused FVIII when inhibitors were infused into the circulation prior to FVIII to mimic the clinical scenario of FVIII replacement therapy in an inhibitor patient [16]. Studies done by Gewirtz and co-workers [17] using the FeCl₃ injury model showed that platelet-FVIII provided only modest improvement of clot-forming activity compared with infused FVIII when the infusion procedures were reversed (i.e. FVIII was infused first followed by antibody infusion). We hypothesize that the difference in outcome between our studies and those of Gewirtz can be attributed to the different competitive binding interactions among VWF, FVIII and inhibitory antibodies in the two systems.

In the present study, we investigate how VWF affects inhibition of FVIII by inhibitors both *in vitro* in a chromogenic- based Bethesda assay and *in vivo* in hemophilia A mouse models. Our results demonstrate that VWF exerts a protective effect, reducing inhibitor inactivation of FVIII, both *in vitro* and *in vivo*.

Materials and Methods

Mice

FVIII knockout (FVIII^{null}) and VWF and FVIII double knockout (VWF^{null}FVIII^{null}) mice used in this study were of a 129/SV × C57BL/6 mixed genetic background. FVIII^{null} mice, which were generated by targeted disruption of exon 17 of the FVIII gene [18], were a kind gift from Haig Kazazian at the University of Pennsylvania School of Medicine. VWF^{null}FVIII^{null} mice, in which both VWF and FVIII are undetectable, were generated by our laboratory by crossing VWF^{null} with FVIII^{null} mice. All mice were maintained in pathogen-free microisolator cages at the animal facilities operated by the Medical College of

Wisconsin. Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin. Isoflurane or 2.5% avertin was used for anesthesia.

Antibodies

Mouse polyclonal antibodies—For mouse anti-FVIII inhibitory antibody generation, VWF^{null}FVIII^{null} mice were immunized by weekly injections of recombinant human B-domain deleted FVIII (rhFVIII, Refacto, Wyeth Pharmaceuticals, Collegeville, PA, USA) at 50 IU kg⁻¹ via the retro-orbital venous plexus for a total of 4 weeks. One week after the last immunization, blood samples were collected into 1/10 volume of 3.8% sodium citrate anticoagulant and plasma was isolated. For inhibitory plasma infusion studies, in which a higher titer of inhibitors was required, donor mice were immunized with a higher dose of 600 IU kg⁻¹ FVIII by intraperitoneal injection in the presence of adjuvant (Sigma, St Louis, MO, USA) twice, with a 3-week interval between injections. Two weeks after the second immunization, blood was drawn from the vena cava and plasma was isolated for infusion studies. A chromogenic FVIII assay-based Bethesda assay was used to determine the titer of inhibitory antibodies.

Human polyclonal antibodies—For human inhibitory antibody studies, plasma polyclonal IgG was purified from four hemophilia A patients with inhibitors using the Montage Antibody Purification Kit (Millipore, Billerica, MA, USA). Plasma IgG was concentrated using Amicon Ultra-15 filters (Millipore), then diluted to the desired volume in phosphate-buffered saline. To remove any remaining plasma VWF from the purified IgG samples, a mixture of mouse anti-human VWF monoclonal antibodies (AVW1, AVW3, AVW5 and 105.4, which were produced by our laboratory) coupled to CNBr-activated Sepharose 4B beads (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) were added to purified IgG samples and incubated at 4 °C for 6 h followed by centrifugation to remove beads and their bound VWF. These antibodies recognize distinct epitopes in mature VWF, and are routinely used by our group for human VWF ELISA [19,20].

Human monoclonal antibodies—Purified human monoclonal antibodies produced by inhibitor patients' B-cell clones BO2C11, BOA8D4 and BOIIB2, which were originally generated by Marc Jacquemin (University of Leuven, Leuven, Belgium), were a joint gift from Marc Jacquemin and GTI Diagnostics (Waukesha, WI, USA). Both BO2C11 [21] and BOA8D4 bind to the C2 domain of FVIII, while BOIIB2 recognizes the A2a2 region (personal communication with Dr Jacquemin).

FVIII assays

FVIII activity (FVIII:C) assay—The levels of FVIII:C were determined by a modified chromogenic assay using the Coatest SP4 FVIII Kit (DiaPharma, Franklin, OH, USA). Briefly, 25-μL samples were added to blocked 96-well microtiter plates in duplicate. Seventy-five microliters of assay components, including FIXa, FX, CaCl₂ and phospholipid, were added to each well, and the plates were incubated for 10 min at 37 °C. Fifty microliters of the chromogenic FXa substrate mixture S-2765/I-2581 was added and the plate was transferred immediately to a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA, USA) preset at 37 °C. A standard curve was constructed by plotting known amounts of rhFVIII (Refacto, which is devoid of VWF) in Coatest buffer against V_{max} (mOD min⁻¹) at 405 nm with a background subtraction at 490 nm. The V_{max} of each reaction was converted to units of FVIII:C (U mL⁻¹) using the instrument manufacturer's software program and the data were averaged.

Inhibitor assay—To determine the inhibitor titer, a modified Bethesda assay was performed. Briefly, 20 μL of serial dilutions of inhibitor samples from the various sources described above were incubated with an equal volume of 1 U mL^{-1} rhFVIII with or without VWF, at 37 °C for 2 h, followed by 1:40 dilution in Coatest buffer, and residual FVIII:C was measured by chromogenic assay as described above. The limit of detection of our chromogenic-based Bethesda assay is 1 BU mL^{-1} . In some experiments the assay was performed without the 2-h incubation. Buffer controls with no added inhibitors were assayed in parallel. Bethesda units were defined by dilution of the inhibitory antibodies until 50% of the initial FVIII:C was neutralized. Recombinant human VWF (rhVWF) (which was kindly provided by Baxter BioScience, Vienna, Austria), plasma from severe hemophilia A patients or plasma from FVIII^{null} mice were used as FVIII-free sources of VWF in this study.

Infusion studies

FVIII^{null} or VWF^{null}FVIII^{null} mice were infused with inhibitory plasma to 2.5, 25 or 250 BU mL^{-1} and rhFVIII to 0.015 or 0.02 U mL^{-1} either with or without rhVWF (to 1 U mL^{-1}) followed by tail clip survival challenge to assess phenotypic correction as previously described [16,22]. Clot formation evidenced by survival beyond 24 h was used to indicate correction of the murine hemophilia A phenotype.

Statistical analysis

All FVIII:C and inhibitor titer results are presented as mean $\pm$ SD and the differences were evaluated by Student *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

The effect of VWF on the FVIII activity assay

Because our Bethesda assay is based on a chromogenic assay, we first explored whether VWF and/or plasma would affect FVIII activity measured by the chromogenic assay. We diluted rhFVIII to various concentrations in the presence or absence of one unit per ml rhVWF followed by 1:80 dilution in Coatest buffer. FVIII activity in each sample was measured using the chromogenic Coatest assay. The presence of VWF did not significantly affect the apparent FVIII activity in the chromogenic assay although there may be a slight enhancement of activity (Fig. 1A). We also performed similar experiments with addition of various concentrations of rhVWF to either a constant low level of FVIII at 0.1 U mL^{-1} or a physiological level of 1 U mL^{-1} FVIII in Coatest buffer followed by chromogenic assay to determine FVIII activity. There was a small increase of apparent FVIII activity with increasing concentrations of VWF, but this was not found to be significant (Fig. 1B). To determine the effect of plasma on the FVIII:C chromogenic assay, we prepared serial dilutions of rhFVIII using various dilutions of plasma from FVIII^{null} mice, which express endogenous VWF, or VWF^{null}FVIII^{null} mice, which do not express endogenous VWF, as diluent. We found that both FVIII^{null} and VWF^{null}FVIII^{null} mouse plasma cause the depression of apparent levels of FVIII activity, which is overcome by dilution of plasma to at least 1:40 (Fig. 1C,D). According to these data, we conclude that VWF does not significantly affect FVIII activity measured in the chromogenic assay.

The effect of VWF on the measurement of FVIII inhibitor titers

To explore whether VWF would affect *in vitro* measurement of FVIII inhibitors, we used three sources of inhibitors, including (i) plasmas from immunized VWF^{null}FVIII^{null} mice with inhibitor titers ranging from 3 to 8000 BU mL^{-1} , which contained polyclonal antibodies (mPoAb), (ii) purified polyclonal plasma IgG from human hemophilia A patients who developed inhibitory antibodies (hPoAb) with titers ranging from 90 to 2000 BU mL^{-1}

and (iii) purified human monoclonal antibody from hemophilic inhibitor patients' B-cell clones (hMoAb) with inhibitor titers of 24–200 BU μg^{-1} . Dilutions of inhibitory antibody were mixed with rhFVIII in the presence or absence of 1 U mL^{-1} rhVWF followed by incubation at 37 °C for 2 h. The remaining FVIII:C after inactivation was determined by chromogenic assay and inhibitor titers were calculated. In all cases when inhibitor samples were incubated with rhFVIII in the absence of VWF, the residual FVIII activity was lower than in the presence of 1 U mL^{-1} VWF, resulting in higher apparent inhibitor titers. Representative experiments using the chromogenic-based Bethesda assay to determine inhibitor titers are shown in Fig. 2(A). The average ratio of inhibitor titers in the absence versus presence of VWF was 6.8 ± 5.8 (ranging from 1.7 to 26, $n = 27$) for mouse inhibitory plasma (mPoAb), 5.0 ± 3.4 (ranging from 2.2 to 9.7, $n = 4$) for human plasma purified polyclonal inhibitor IgG (hPoAb), and 6.1 ± 1.2 (ranging from 5.0 to 7.3, $n = 3$) for cloned human monoclonal antibodies (hMoAb), with no significant difference between inhibitor sources (Fig. 2B).

To explore whether the dose of VWF affects the protection of FVIII from inhibitor inactivation, various levels of rhVWF were added to rhFVIII before exposure to inhibitors (mPoAb). Subsequent Bethesda assay results demonstrate that the protective effect is VWF dose dependent. As VWF concentration increases, more FVIII is recovered, resulting in lower apparent titers even though the same amount of antibody was present in all samples. The slope between 0 and 0.2 U mL^{-1} VWF is much steeper than the slope between 0.2 and 1.5 U mL^{-1} and there is a plateau when the VWF level reaches 1.5 U mL^{-1} (Fig. 2C).

To investigate whether plasma-derived VWF has the same protective effect as recombinant VWF, we used severe hemophilic patient plasma as a FVIII-free source of VWF, compared with rhVWF diluted in severe VWF-deficient patient plasma (both VWF and FVIII-free) to maintain a consistent level of other plasma constituents. Both plasmas were heat-inactivated to inactivate residual FVIII activity. Human plasma purified polyclonal inhibitor IgG samples were used in these studies. The results demonstrate that both human plasma-derived VWF and recombinant human VWF have a similar protective effect against inhibitor inactivation of FVIII (Fig. 2D). We also performed experiments using plasma from FVIII^{null} mice to investigate the effect of VWF on inhibitor inactivation of FVIII and showed that mouse plasma-derived VWF has a protective effect similar to human recombinant VWF (data not shown).

The protective effect of VWF and FVIII in complex

To explore whether a preformed complex of VWF with FVIII provides more effective protection from inhibitors versus unbound VWF, a series of mixing-order experiments were performed using plasmas from immunized VWF^{null}FVIII^{null} mice as the inhibitor source. rhVWF (at 1 U mL^{-1}) was either added to (1 U mL^{-1}) rhFVIII first followed by mixing with inhibitory plasma, allowing VWF and FVIII to pre-associate into a non-covalent complex before exposure to inhibitory antibodies, or VWF was mixed with inhibitory plasma first followed by addition of rhFVIII so VWF and antibodies encounter FVIII at the same time. Control samples with no added VWF were assayed in parallel. The time dependence of inhibition reactions was also assessed by either performing the chromogenic FVIII assay after 2 h incubation of sample mixtures at 37 °C, or assaying samples immediately without incubation. As shown in Fig. 3(A), whenever VWF was present FVIII was protected from inhibitor inactivation, resulting in higher recovery of FVIII activity than when VWF was absent and consequently apparent inhibitor titers were lower. The protective effect was more pronounced when VWF and FVIII were pre-associated with one another prior to exposure to inhibitory antibodies.

When sample mixtures were incubated for 2 h at 37 °C per the usual Bethesda assay procedure, apparent titers averaged 5.6-fold lower for samples containing a preformed FVIII/VWF complex when compared with no-VWF controls. In samples where VWF and inhibitors were mixed first and then added to FVIII at the same time, VWF must compete with antibody for binding sites on FVIII and the protective effect was lessened somewhat, but apparent titers were still 1.9-fold lower than controls (Fig. 3A).

The protective effect of VWF was even more evident when samples were assayed immediately rather than incubated for 2 h, indicating that time is an important consideration when dealing with the multiple association and dissociation interactions that are occurring between VWF and FVIII and inhibitory antibodies. The average inhibitor titers measured in samples containing a preformed FVIII/VWF complex were 38.9-fold lower than in no-VWF controls, while apparent titers were 5.8-fold lower than controls for samples in which VWF and antibody compete for FVIII-binding (Fig. 3A).

To further investigate the timing requirements of VWF/FVIII interaction an extended pre-incubation was added to allow additional time for complex formation before exposure to inhibitory antibody. rhVWF and rhFVIII were mixed at 1 U mL⁻¹ of each, pre-incubated at 37 °C for 2 h, then mixed and incubated with inhibitory plasma for 2 h at 37 °C before the remaining FVIII:C was determined by chromogenic assay. Inhibitor titers following pre-incubation of VWF and FVIII were not significantly different from those obtained in the parallel experiment without pre-incubation (Fig. 3B), indicating that a protective complex of VWF with FVIII forms very quickly.

Effect of VWF present in assay reagents on measured inhibitor titer

Other than the antibody-containing inhibitor sample being assayed there are only two other reagent components added to an inhibitor assay reaction: an exogenous FVIII source, and the diluent used for volume adjustments. Because the choice of diluent, and in particular whether the diluent contains VWF or not, has been reported to affect the apparent inhibitor titers measured using an aPTT-based Bethesda assay[8–10], we performed a series of experiments to determine whether the presence or absence of VWF in various assay reagents would influence titers measured using the chromogenic assay. Antibody-containing plasma pooled from immunized VWF^{null}FVIII^{null} mice, was diluted in VWF^{null}FVIII^{null} mouse plasma to titers ranging from 20 to 2000 BU mL⁻¹ and used as inhibitor samples for these experiments. Because inhibitor patient samples virtually always contain fairly normal levels of VWF, rhVWF was added to all inhibitor samples to 1 U mL⁻¹. The FVIII source for these experiments was rhFVIII, either with added rhVWF at 1 U mL⁻¹ to allow formation of a preformed complex, or without addition of rhVWF, and the diluent used for dilution of inhibitory plasmas was plasma from non-immunized VWF^{null}FVIII^{null} mice, also with or without added rhVWF.

As expected from the results of our previous experiments, the remaining measurable FVIII activity was higher when VWF was present in the assay reagents, resulting in lower apparent inhibitor titers. This was true if VWF was present as a preformed complex with FVIII (and not in diluent), but the protective effect was further enhanced when VWF was also supplemented in the diluent. When VWF was added to FVIII, but was not present in diluent, the final VWF concentration in the inhibition reaction varied from 0.5 to 1 U mL⁻¹ due to the variable dilutions of inhibitory plasmas, which also contained added VWF. A higher final concentration of 1 U mL⁻¹ VWF was present when VWF was also added to diluent. While allowing VWF to pre-complex with FVIII seems to provide the largest effect, additional VWF provided in diluent provides an added incremental measure of protection for FVIII, indicative of the VWF dose-dependency of the effect (Fig. 4A,B).

VWF affects inactivation of FVIII by inhibitors *in vivo*

To confirm the protective effect of VWF against FVIII inactivation by inhibitors *in vivo*, we separately infused rhFVIII and inhibitory plasma at various final titers into FVIII^{null} mice, which express endogenous VWF, followed by tail clip survival tests. In the absence of inhibitors a relatively low level of 0.02 U mL⁻¹ rhFVIII corrected the hemophilic phenotype, resulting in survival following the tail clip challenge. When this amount of FVIII was infused into FVIII^{null} mice first to allow formation of a complex with endogenous plasma VWF, followed by inhibitory plasma infusion, all mice with inhibitor titers of 2.5 BU mL⁻¹ survived tail clipping, and two of four survived with either 25 BU mL⁻¹ or 250 BU mL⁻¹. The protective effect of a preformed complex of VWF/FVIII against FVIII inactivation by inhibitors was still seen in animals infused with a lower dose of rhFVIII first (0.015 U mL⁻¹) followed by inhibitory plasma infusion. However, if inhibitory plasma was infused first followed by rhFVIII infusion to 0.02 U mL⁻¹, only two of six mice with inhibitor titers of 2.5 BU mL⁻¹ survived the tail clip challenge and none survived with 25 BU mL⁻¹ or 250 BU mL⁻¹ (Fig. 5A).

In contrast, when rhFVIII was infused into VWF^{null} FVIII^{null} mice, which do not express endogenous VWF, followed by inhibitory plasma infusion, no animals survived tail clipping at any level of inhibitors. Even though rhFVIII was infused first, there was no VWF to form a protective complex with the infused FVIII in this case. When rhVWF was infused to normal plasma levels (1 U mL⁻¹) and rhFVIII to 0.02 U mL⁻¹ before inhibitor infusion, three of five double knockout mice survived tail clipping with an inhibitor titer of 2.5 BU mL⁻¹ and one of five with 25 BU mL⁻¹ survived tail clipping (Fig. 5B).

Discussion

It is well known that VWF forms a non-covalently linked complex with FVIII to stabilize and protect it from protease degradation in blood circulation. It has been shown that in Bethesda assays that are based on either one-stage aPTT assay or the chromogenic assay anti-FVIII inhibitory antibody inactivation of FVIII activity is time dependent. However, the interactions between VWF and FVIII and inhibitory antibodies have not been thoroughly explored. The current study contributes to a better understanding of these interactions. We showed that for inhibitors, VWF does make a difference both *in vitro* in the Bethesda assay and *in vivo* in hemophilic mouse models.

Previous studies have shown that VWF in the FVIII-deficient plasmas utilized in one-stage aPTT assays appears to depress measurements of apparent FVIII activity [23–25]. We wanted to determine whether similar effects were present when inhibitor titers were calculated using a chromogenic assay to measure residual FVIII activity. To avoid the interference of other plasma proteins that might complicate assessments of the influence of VWF on FVIII:C measurement, we added defined quantities of purified exogenous rhVWF and/or rhFVIII in Coatest buffer in these assays. VWF does not significantly affect the apparent FVIII activity measured by chromogenic assay. However, we did observe that samples containing > 2.5% plasma, regardless of whether they contain VWF or not, interfered with chromogenic measurement of FVIII:C, although the cause is unclear.

In the present study, we found that addition of VWF to otherwise VWF-free inhibitor samples provided a protective effect for FVIII, lessening inactivation by inhibitory antibodies in every inhibitor sample tested, whether of murine or human origin. We observed a fairly large variation among inhibitor samples, but in every case there was a significant protective effect of VWF against inactivation of FVIII by inhibitors. As a 2-h incubation of FVIII with inhibitors has been thought to be critical in the Bethesda assay for determination of inhibitor titers, we compared the effect of VWF on inhibitor inactivation of

FVIII activity either with or without the usual 2-h incubation. We found that the degree of VWF's protective effect following a 2-h incubation is lower than that without incubation. This could be because gradual disassociation of the VWF/FVIII complex over the incubation period sets up a competition between re-association of VWF and FVIII versus binding of inhibitory antibody to the newly dissociated FVIII. We also demonstrated that FVIII that is present in a preformed VWF/FVIII complex is better protected from inhibitory antibody inactivation than FVIII that encounters inhibitors and VWF at the same time. In the latter situation antibodies and VWF appear to compete for binding to FVIII, reducing the ability of VWF to protect FVIII, resulting in lower residual FVIII activity and higher apparent inhibitor titers. More FVIII activity was still recovered than in the absence of VWF, but the protective effect is not as efficient as when VWF and FVIII were already associated with one another before encountering inhibitors.

Verbruggen and co-workers reported that when immunodepleted FVIII-deficient plasmas that lack both FVIII and VWF were used as the diluent for aPTT assays, apparent inhibitor titers were lower compared with assay results using chemically-depleted FVIII-deficient plasma reagents, which contain a normal level of VWF [9]. The presence of unknown and potentially deleterious contaminants carried over from the FVIII immuno- or chemical-depletion process is also of concern, especially in assays designed to measure FVIII inactivation (i.e. Bethesda assays). When we explored the effect of VWF present in assay reagents on measured inhibitor titer (Fig. 4A), we used naturally deficient plasma from VWF and FVIII double knockout mice as the diluent for inhibitory plasmas in inhibition reactions, which allowed us to add controlled amounts of purified VWF and FVIII to investigate their influence on inhibitor titers determined by a chromogenic assay. As such, variations in other plasma components and the potential for undesirable carryover effects from depletion processing were not factors in our system. Invariably, samples lacking VWF resulted in higher apparent inhibitor titers when determined by the chromogenic assay. We showed that VWF has a clear effect on the stability of FVIII in the inhibition reaction preceding the activity assay. The use of reagents lacking VWF significantly affects the amount of residual FVIII activity following exposure to a given amount of inhibitory antibody, resulting in a considerable overestimation of inhibitor titers.

Our findings support the premise of our platelet-derived FVIII strategy for gene therapy of hemophilia A with inhibitors, in which FVIII is stored together with endogenous VWF forming a VWF/FVIII complex within the protective confines of platelet α -granules [16,26]. The complex is only released and first encounters inhibitory antibodies upon platelet activation at sites of active coagulation, and our current results indicate that the VWF protective effect extends even into this post-release plasma environment. In our current *in vivo* study, FVIII^{null} mice infused first with FVIII and then with inhibitors were able to form a protective FVIII complex with endogenous VWF before encountering inhibitors. When inhibitors were infused before FVIII, the FVIII was exposed to VWF and pre-infused inhibitory antibodies at the same time, a competitive binding situation that appears to reduce VWF's protective effect. A preformed complex with VWF provides better protection of FVIII from inhibitory antibody inactivation *in vivo* and thus, hemostasis is improved. Indeed, hemostasis was also improved in VWF^{null}FVIII^{null} mice infused with FVIII together with VWF followed by inhibitor infusion, but not by infusion of FVIII alone.

Our current results also explain the difference in outcome between our previous studies comparing the efficacy of platelet-FVIII and plasma-FVIII in the presence of inhibitors [16] and those of Gewirtz and coworkers [17]. In their studies using the FeCl₂-induced carotid artery thrombosis model, the plasma-FVIII model was established by infusion of rhFVIII into FVIII^{null} mice followed 2 min later by infusion of an inhibitor mixture consisting of two monoclonal antibodies. They found that under those conditions transgenic platelet-FVIII

provided only marginally better clot-forming activity than infused FVIII, but it should be noted that the sequence of additions in that system allows pre-association of infused FVIII with endogenous VWF into a protective complex before exposure to inhibitors, while the order of infusions was reversed in our studies, a critical difference. We first pre-infused polyclonal inhibitory plasma – derived by infusion of FVIII protein into deficient individuals, as in a hemophilic inhibitor patient – and then rhFVIII was infused. This sequence of infusions was deliberately chosen, specifically to maintain analogy with the treatment of an inhibitor patient in which FVIII is infused into a milieu of pre-existing inhibitory antibody. We believe that this is the appropriate model, as infusion of FVIII followed by a sudden influx of inhibitors has no real clinical parallel.

Our goal was to contrast two clinically relevant situations for an inhibitor patient, and in that context platelet-FVIII was much more effective than equivalent levels of plasma-FVIII. The relative lack of efficacy of even normal levels of plasma FVIII when faced with an inhibitory challenge was also clearly demonstrated in our earlier studies of transgenic endothelial-specific FVIII expression or in wild-type animals [27], and indeed in inhibitor patients themselves. In the current study we have shown that experimental outcomes are highly dependant on whether FVIII encounters VWF or antibodies first, and our *in vitro* results indicate that the difference is especially dramatic over short time frames. Our results and those of Gewirtz both demonstrate that FVIII is better protected from inhibitors when it is pre-associated with VWF, whether in plasma or in platelets. Rather than implicating differing levels of sensitivity between the tail clip survival test and the FeCl₂ injury model, both provide similar outcomes when the experiments are performed under the same conditions. The critical difference between our previous study and that of Gewirtz is that different interactions and competitions for binding between VWF and FVIII and inhibitory antibodies were allowed in the two systems.

In summary, our studies demonstrate that VWF does not affect FVIII:C measurement in the chromogenic assay. VWF reduces inhibitor inactivation of FVIII, exerting a protective effect, both *in vitro* and *in vivo*. While the role of VWF in stabilizing plasma FVIII has been appreciated for decades, our results indicate that treatment utilizing products containing a complex of FVIII with VWF may be especially beneficial in hemophilia A patients with inhibitors. VWF/FVIII association plus the apparent ability of VWF to delay the time-dependent inactivation of FVIII by inhibitory antibodies provide mechanisms by which platelet-derived FVIII maintains function even in the presence of inhibitors when FVIII is targeted to platelets for gene therapy of hemophilia A with inhibitors.

Acknowledgments

We thank H. H. Kazazian at the University of Pennsylvania School of Medicine for the FVIII^{null} mice. The VWF^{null} mice were developed by D. Wagner at Harvard Medical School and purchased from the Jackson Laboratory. This work was supported by National Institutes of Health grants HL-102035 (QS), HL-44612 (RRM), HL-33721 (RRM) and HL-81588 (RRM), American Heart Association National Center SDG 0730183N (QS), National Hemophilia Foundation CDA (QS), Hemophilia Association of New York grant (QS), and the Midwest Athletes Against Childhood Cancer Fund (QS).

References

1. Mann KG, Nesheim ME, Church WR, Haley P, Krishnaswamy S. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood*. 1990; 76:1–16. [PubMed: 2194585]
2. Wagner DD. Cell biology of von Willebrand factor. *Annu Rev Cell Biol*. 1990; 6:217–46. [PubMed: 2275814]

3. Hamer RJ, Koedam JA, Beeser-Visser NH, Bertina RM, van Mourik JA, Sixma JJ. Factor VIII binds to von Willebrand factor via its Mr-80 000 light chain. *Eur J Biochem.* 1987; 166:37–43. [PubMed: 3109914]
4. Chavin SI. Factor VIII: structure and function in blood clotting. *Am J Hematol.* 1984; 16:297–306. [PubMed: 6424437]
5. Mannucci PM, Mancuso ME, Santagostino E. How we choose factor VIII to treat hemophilia. *Blood.* 2012; 119:4108–14. [PubMed: 22411872]
6. Ehrenforth S, Kreuz W, Scharrer I, Linde R, Funk M, Gungor T, Krackhardt B, Kornhuber B. Incidence of development of factor VIII and factor IX inhibitors in haemophiliacs. *Lancet.* 1992; 339:594–8. [PubMed: 1347102]
7. Iorio A, Halimeh S, Holzhauer S, Goldenberg N, Marchesini E, Marcucci M, Young G, Bidlingmaier C, Brandao LR, Ettingshausen CE, Gringeri A, Kenet G, Knofler R, Kreuz W, Kurnik K, Manner D, Santagostino E, Mannucci PM, Nowak-Gottl U. Rate of inhibitor development in previously untreated hemophilia A patients treated with plasma-derived or recombinant factor VIII concentrates: a systematic review. *J Thromb Haemost.* 2010; 8:1256–65. [PubMed: 20345722]
8. Astermark J, Voorberg J, Lenk H, DiMichele D, Shapiro A, Tjonnfjord G, Berntorp E. Impact of inhibitor epitope profile on the neutralizing effect against plasma-derived and recombinant factor VIII concentrates *in vitro*. *Haemophilia.* 2003; 9:567–72. [PubMed: 14511295]
9. Verbruggen B, Giles A, Samis J, Verbeek K, Mensink E, Novakova I. The type of factor VIII deficient plasma used influences the performance of the Nijmegen modification of the Bethesda assay for factor VIII inhibitors. *Thromb Haemost.* 2001; 86:1435–9. [PubMed: 11776311]
10. Verbruggen B, van Heerde W, Budde U. Methodological shortcomings in assessment of factor VIII concentrate inhibition. *Haemophilia.* 2007; 13:680–1. [PubMed: 17880465]
11. Di Michele DM. Immune tolerance induction in haemophilia: evidence and the way forward. *J Thromb Haemost.* 2011; 9(Suppl 1):216–25. [PubMed: 21781258]
12. Wong T, Recht M. Current options and new developments in the treatment of haemophilia. *Drugs.* 2011; 71:305–20. [PubMed: 21319868]
13. Franchini M, Lippi G. von Willebrand factor-containing factor VIII concentrates and inhibitors in haemophilia A. A critical literature review. *Thromb Haemost.* 2010; 104:931–40. [PubMed: 20838738]
14. Giangrande PL, Escobar MA. Management of difficult-to-treat inhibitor patients. *Haemophilia.* 2010; 16(Suppl 3):52–7. [PubMed: 20586803]
15. Kruse-Jarres R. Current controversies in the formation and treatment of alloantibodies to factor VIII in congenital hemophilia A Hematology. *Am Soc Hematol Educ Program.* 2011; 2011:407–12.
16. Shi Q, Wilcox DA, Fahs SA, Weiler H, Wells CW, Cooley BC, Desai D, Morateck PA, Gorski J, Montgomery RR. Factor VIII ectopically targeted to platelets is therapeutic in hemophilia A with high-titer inhibitory antibodies. *J Clin Invest.* 2006; 116:1974–82. [PubMed: 16823491]
17. Gewirtz J, Thornton MA, Rauova L, Poncz M. Platelet-delivered factor VIII provides limited resistance to anti-factor VIII inhibitors. *J Thromb Haemost.* 2008; 6:1160–6. [PubMed: 18433455]
18. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet.* 1995; 10:119–21. [PubMed: 7647782]
19. Rosenberg JB, Haberichter SL, Jozwiak MA, Vokac EA, Kroner PA, Fahs SA, Kawai Y, Montgomery RR. The role of the D1 domain of the von Willebrand factor propeptide in multimerization of VWF. *Blood.* 2002; 100:699–1706.
20. Jacobi PM, Gill JC, Flood VH, Jakab DA, Friedman KD, Haberichter SL. Intersection of mechanisms of type 2A VWD through defects in VWF multimerization, secretion, ADAMTS-13 susceptibility, and regulated storage. *Blood.* 2012; 119:4543–53. [PubMed: 22431572]
21. Jacquemin MG, Desqueper BG, Benhida A, Vander EL, Hoylaerts MF, Bakkus M, Thielemans K, Arnout J, Peerlinck K, Gilles JG, Vermeylen J, Saint-Remy JM. Mechanism and kinetics of factor VIII inactivation: study with an IgG4 monoclonal antibody derived from a hemophilia A patient with inhibitor. *Blood.* 1998; 92:496–506. [PubMed: 9657749]

22. Connelly S, Andrews JL, Gallo AM, Kayda DB, Qian J, Hoyer L, Kadan MJ, Gorziglia MI, Trapnell BC, McClelland A, Kaleko M. Sustained phenotypic correction of murine hemophilia A by *in vivo* gene therapy. *Blood*. 1998; 91:3273–81. [PubMed: 9558383]
23. Muntean W, Hathaway WE, Montgomery RR. Influence of high molecular weight factor VIII on the measurement of low molecular weight factor VIII procoagulant in different assay systems. *Br J Haematol*. 1982; 51:649–58. [PubMed: 6809034]
24. Butenas S, Parhami-Seren B, Mann KG. The influence of von Willebrand factor on factor VIII activity measurements. *J Thromb Haemost*. 2009; 7:132–7. [PubMed: 18983499]
25. Butenas S, Parhami-Seren B, Undas A, Fass DN, Mann KG. The “normal” factor VIII concentration in plasma. *Thromb Res*. 2010; 126:119–23. [PubMed: 20451961]
26. Shi Q, Fahs SA, Wilcox DA, Kuether EL, Morateck PA, Mareno N, Weiler H, Montgomery RR. Syngeneic transplantation of hematopoietic stem cells that are genetically modified to express factor VIII in platelets restores hemostasis to hemophilia A mice with preexisting FVIII immunity. *Blood*. 2008; 112:2713–21. [PubMed: 18495954]
27. Shi Q, Fahs SA, Kuether EL, Cooley BC, Weiler H, Montgomery RR. Targeting FVIII expression to endothelial cells regenerates a releasable pool of FVIII and restores hemostasis in amousemodel of hemophilia A. *Blood*. 2010; 116:3049–57. [PubMed: 20606161]

Fig 1.

Influence of VWF and/or plasma on the chromogenic FVIII activity assay. (A) The effect of 1 U mL⁻¹ VWF on measurement of FVIII activity. Various levels of rhFVIII were tested. (B) Influence of VWF on measurement of low or physiological levels of FVIII activity. (C) Influence of plasma with VWF on the FVIII chromogenic assay. Various dilutions of plasma from FVIII^{null} mice, which express endogenous VWF, were used as diluent. Data shown are from two repeats of each experiment. (D) Influence of plasma without VWF on the FVIII chromogenic assay. Various dilutions of plasma from VWF^{null}FVIII^{null} mice, which do not express endogenous VWF, were used as diluent. Data shown are from two repeats of each experiment. Apparent FVIII:C denotes the measurable FVIII activity measured.

Fig 2.

Effect of VWF on measurement of FVIII inhibitor titers. Three sources of anti-human FVIII inhibitory antibodies were used including: (1) plasmas from immunized VWF^{null}FVIII^{null} mice (mPoAb) with inhibitor titers ranging from 3 to 8000 BU mL⁻¹; (2) purified IgG from human hemophilic inhibitor patients (hPoAb) with inhibitor titers of 90–2000 BU mL⁻¹; and (3) human monoclonal antibody clones (hMoAb) with inhibitor titers of 24–200 BU μg⁻¹. Inhibitors were incubated with rhFVIII at 37 °C for 2 h either with or without 1 U mL⁻¹ rhVWF. The remaining FVIII:C was determined by chromogenic assay and inhibitor titers were calculated. (A) Determination of inhibitor titers. The residual FVIII:C from representative experiments is shown. Inhibitor titers were defined by dilution of inhibitor samples until 50% of the initial FVIII:C was neutralized. (B) Ratio of inhibitor titers measured in the absence versus presence of VWF for individual inhibitor samples. (C) VWF dose response of FVIII protection from inhibitor inactivation. Inhibitory plasma from an immunized VWF^{null}FVIII^{null} mouse was used in this study. Data points are the mean value of three to five independent experiments. (D) Plasma-derived and recombinant VWF have a similar protective effect on FVIII. Inhibitor titers were normalized by defining the titer without VWF as 100%. The source of plasma-derived VWF was severe hemophilia A patients' plasma, and rhVWF was diluted into VWF-deficient patients' plasma.

Fig 3.

Protective effect of VWF and FVIII in complex. To investigate whether a preformed complex of VWF with FVIII provides more effective protection from inhibitors than unbound VWF, mixing-order experiments were performed. Inhibitory plasmas from immunized VWF^{null}FVIII^{null} mice were used in these experiments. Adding VWF to rhFVIII before mixing with inhibitory plasma allows association into a preformed FVIII/VWF complex. Adding VWF to inhibitory plasma followed by mixing with rhFVIII did not allow a preformed complex to form. (A) Normalized inhibitor titers measured with or without the usual Bethesda assay incubation at 37 °C. Data presented are the mean values of five inhibitory plasma samples assayed without incubation and six samples with incubation. Inhibitor titers were normalized by defining the titer without VWF as 100%. (B) Effect of pre-incubation to allow FVIII/VWF complex formation before the inhibitor assay. Inhibitor titers were normalized by defining the titer without pre-incubation as 100%.

Fig 4.

Additive influence of VWF in the FVIII source and diluent on measurement of FVIII inhibitor titers. Inhibitory plasmas pooled from immunized VWF^{null}FVIII^{null} mice were diluted in VWF^{null}FVIII^{null} mouse plasma to titers ranging from 20 to 2000 BU mL⁻¹ and used as inhibitor samples for these experiments. rhVWF was added to all inhibitory plasmas before dilution. rhVWF was either added to or omitted from the other assay reagents. (A) Effect of VWF in various components of the reaction on measurement of FVIII inhibitors. (B) Statistical comparison of normalized FVIII inhibitor titers measured under the various conditions. Titters were normalized by defining the titer with VWF present only in inhibitory plasma as 100%.

Fig 5.

In vivo effect of VWF on inactivation of FVIII by inhibitors. (A) Infusion studies in FVIII^{null} mice, which have endogenous VWF. rhFVIII was infused to 0.02 or 0.015 U mL⁻¹ either before or after infusion of inhibitory mouse plasma to various levels of inhibitor titers, followed by tail clip challenge. *n* = 4–7 mice for each group. (B) Infusion studies in VWF^{null}FVIII^{null} mice, which lack endogenous VWF. rhFVIII was infused to 0.02 U mL⁻¹ with or without co-infusion of rhVWF to 1 U mL⁻¹ before infusion of inhibitory plasma to various levels of inhibitors, followed by tail clipping. *n* = 4 to 5 mice for each group.