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The influence of prophylactic factor VIII in severe hemophilia A

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Abstract

Introduction—Hemophilia A individuals displaying a similar genetic defect have heterogeneous clinical phenotypes.

Aim—To evaluate the underlying effect of exogenous factor (f)VIII on tissue factor (Tf)-initiated blood coagulation in severe hemophilia utilizing both empirical and computational models.

Methods—We investigated twenty-five clinically severe hemophilia A patients. All individuals were on fVIII prophylaxis and had not received fVIII from 0.25 to 4 days prior to phlebotomy. Coagulation was initiated by the addition of Tf to contact-pathway inhibited whole blood ± an anti-fVIII antibody. Aliquots were quenched over 20 min and analyzed for thrombin generation and fibrin formation. Coagulation factor levels were obtained and used to computationally predict thrombin generation with fVIII set to either zero or its value at the time of the draw.

Results—Due to prophylactic fVIII, at the time of the blood draw, the individuals had fVIII levels that ranged from <1% to 22%. Thrombin generation (maximum level and rate) in both empirical and computational systems increased as the level of fVIII increased. FXIII activation rates also increased as the fVIII level increased. Upon suppression of fVIII, thrombin generation became comparable in both systems. Plasma composition analysis showed a negative correlation between bleeding history and computational thrombin generation in the absence of fVIII.

Conclusion—Residual prophylactic fVIII directly causes an increase in thrombin generation and fibrin cross-linking in individuals with clinically severe hemophilia A. The combination of each individual's coagulation factors (outside of fVIII) determine each individual's baseline thrombin potential and may affect bleeding risk.

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Contributions

- MG performed the research, analyzed the data and wrote the paper.
- MFW performed the research.
- LAF performed the research and analyzed the data.
- KGM was involved in discussions regarding hemophilia issues.
- GER recruited the patients and revised the paper.
- KEB designed the study, performed the research, analyzed the data and revised the paper.

Keywords

hemophilia; prophylaxis; factor VIII; thrombin generation; inhibitory antibody; factor XIII

Introduction

Severe hemophilia A is defined by factor (f)VIII deficiency of less than 1% of “normal” levels [1] and is generally manifested by hemarthrosis and soft tissue bleeds [2], although 10 to 15% subjects diagnosed as severe display a clinically mild phenotype [3]. Treatment can either prevent bleeds or minimize their effects so that the patient remains free of disability and complications. Continuous prophylaxis has been shown to reduce bleeding tendency and to result in a higher quality of life for patients compared to those who receive treatment on an on-demand basis [4-7]. However, prophylaxis strategies vary greatly [8].

Although the biological severity is diagnosed by traditional coagulation assays, the hemorrhagic tendency and its clinical management are not always accurately predicted by these assays. The inability to predict bleeding despite knowledge of the functional fVIII level creates a therapeutic dilemma in the optimization of dosing regimens for the treatment and prevention of hemorrhage. Why the level of fVIII does not always correlate with clinical manifestation is a question that remains unanswered. Studies to understand the dynamics of fVIII in severe hemophilia receiving prophylaxis are lacking.

Thrombin generation assays have been shown to be useful in the understanding of the dynamics of global coagulation physiology in hemophilia A [9-13]. In the absence of fVIII, the blood coagulation cascade is defective at the level of fXa generation through the intrinsic fXase complex. Normally, this complex is able to generate fXa at a 50 to 100-fold higher rate than the fVIIa-Tf complex (the extrinsic fXase complex) [14-16]. Thus in hemophilia, less fXa is generated, and consequently, levels of the prothrombinase complex are decreased and prothrombin activation to thrombin is diminished compared to the healthy population [2,10]. In one empirical study, mild, moderate and severe hemophilia A individuals were evaluated utilizing a whole blood model and the results showed that the mean thrombin generation profiles follow the functional classification of the disease [2]. When the severe population alone was evaluated, there existed a wide variation of thrombin generation [2]. To date, the effect of residual fVIII given as prophylaxis on thrombin generation in severe hemophilia A individuals has not been investigated.

In this study, we analyzed thrombin generation utilizing both empirical and computational approaches in a cohort of individuals with severe hemophilia A. In order to understand the individual variation in severe hemophilia A in the context of any empirical global assay, the effects of prophylactic fVIII need to be addressed. The purpose of this study is to understand this influence on thrombin generation. Suppressing the exogenous fVIII will contribute to the understanding of the baseline phenotype in each subject.

Materials and methods

Subjects

We enrolled 25 documented clinically severe hemophilia A patients (baseline fVIII:C <1% at the time of diagnosis, age range 16 – 33) who were recruited by Dr. Rivard and advised according to a protocol approved by the Institutional Review Boards at the Centre Hospitalier Universitaire Sainte-Justine (Montréal, QC) and by the University of Vermont Committees of Human Research (Burlington, VT). Informed written consent was obtained from all individuals. All individuals were on different fVIII prophylaxis programs and did

not need to self-infuse with fVIII from 0.25 to 4 days prior to the blood drawing. All patients had normal platelet ($225 \pm 45 \times 10^9/L$, range 147 – 282) and fibrinogen levels (2.8 ± 0.5 mg/mL, range 2.2 – 4.1), as well as prothrombin times (13.7 ± 0.6 sec, range 12.9 – 15.0). Individuals with known inhibitors towards fVIII or were not on fVIII prophylaxis were excluded.

Materials

HEPES, sodium chloride, benzamidine-HCl and EDTA were purchased from Fisher (Pittsburgh, PA). 1-palmitoyl-2-oleoyl-phosphatidyl serine (PS) and 1-palmitoyl-2-oleoyl-phosphatidyl choline (PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Recombinant Tf was a gift from Drs. Lundblad and Liu (Hyland division, Baxter Healthcare Corp, Duarte, CA) and was relipidated in PCPS (75% PC:25% PS) vesicles by a previously described protocol [17,18]. Corn trypsin inhibitor (CTI) was prepared as described [19]. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPR-ck) was a gift from Dr. Jenny (Haematologic Technologies, Essex Junction, VT). A sheep polyclonal -fVIII antibody (#PAHFVIII-S) was purchased from Haematologic Technologies. Enzyme-linked immunosorbent assay (ELISA) kits were used to estimate thrombin-antithrombin (TAT) complex formation (Behring, Westwood, MA).

Bleeding phenotype

A variety of phenotypic bleeding severities were seen in our severe hemophilia population. We used our published bleeding score system [2]. Briefly, each individual's bleeding history included the number of hemarthrosis, soft tissue hematoma and annual fVIII usage. The scoring points were as follows: hemarthrosis, 0/y 0 pts, 1–3/y 3 pts, 4–6/y 6 pts, 7–12/y 9 pts, >12/y 12 pts; soft tissue hematoma, 0/y 0 pts, 1–3/y 2 pts, 4–6/y 4 pts, 7–12/y 6 pts, >12/y 8 pts; annual fVIII unit/kg usage, 0/y 0 pts, <1000/y 3 pts, 1000–3000/y 6 pts, >3000/y 12 pts. Surgery, dental extractions and major accidents were excluded for calculation of annual fVIII usage. Mean scores over the course of the 5 years prior to blood draw are reported.

Whole blood clotting assays

The procedure used was a modification of Brummel *et al.* [20]. Blood was collected by venipuncture on site at the Centre Hospitalier Universitaire Sainte-Justine and immediately aliquoted (1 mL) into rocking tubes at 37 °C containing CTI (100 µg/mL) and Tf (5 pM) relipidated in PCPS (1:2000 protein/lipid). Two series ($\pm$ an inhibitory -fVIII antibody used at 0.3 mg/mL, which prolonged the APTT of normal plasma to a time (70 sec) seen in severe hemophilia A plasma) were studied. Tubes were quenched at defined intervals over a time course to 20 minutes 1:1 with a cocktail of inhibitors (final: 25 mM EDTA, 10 mM benzamidine, 50 µM FPR-ck). All samples were centrifuged (4 °C, 1200 g, 30 min), and both the soluble and clotted material were frozen at –80 °C until further analysis.

Immunoassays and western analyses

FXIII activation and fibrinogen consumption were followed by western blotting analysis. For fXIIIa, the primary antibody was a rabbit polyclonal -fXIII A-chain antibody (Zymogenetics, Seattle, WA). For fibrinogen, the primary was a monoclonal mouse -fibrinogen-2E purified in house. The secondary antibody in both cases was goat -rabbit HRP (Southern Biotech, Birmingham, AL). Fibrinopeptide A was quantitated by HPLC methods [21]. TAT ELISA was performed according to the manufacturer's protocol as previously described [21].

Thromboelastography

Venous blood (350 μ L) was obtained at the same time as the whole blood assay. The blood was added to the sample cup, which contained CTI (100 μ g/mL) and Tf (5 pM) relipidated in PCPS (1:2000 protein/lipid). Analysis was carried out on each sample using a Thromboelastograph Hemostasis Analyzer Model 5000 (Haemoscope, Niles, IL) at 37 °C. R, K, A and MA parameters were extracted using TEG V4 software.

Blood collection and coagulation protein analyses

Blood was also drawn into 0.1 volume of 3.2% trisodium citrate, centrifuged within 15 minutes of collection and plasma stored in aliquots at -80°C until further use. Factors II, V, VII, VIII, IX, and X were analyzed by one-stage clotting assays using factor deficient plasmas (Fletcher Allen Health Care, Burlington, VT). AT activity was measured using a Berichrom chromogenic assay (Dade Behring, Deerfield, IL). Free tissue factor pathway inhibitor (TFPI) was determined using an ELISA (Diagnostic Stago, Parsippany, NJ).

Computational modeling

A mathematical model was utilized that describes the Tf pathway of blood coagulation [22,23]. Coagulation factor levels from hemophilia individuals for factors II, V, VII, VIII, IX, X, AT and TFPI were input into the program and simulated to clot upon a 5 pM Tf trigger. The reaction was solved for active thrombin over 20 minutes. To eliminate the effect of fVIII prophylaxis, the level of fVIII was set to zero.

Statistics

Data are expressed as mean $\pm$ SD (in figures only mean+SD is shown). Analysis of variance was used to compare differences in means across groups. If a significant difference was found, Tukey's method was utilized for multiple pairwise comparisons. Associations between two continuous variables were evaluated by Pearson correlation. A two tailed p-value <0.05 was considered to indicate statistical significance. Linear regression was performed on data with a single outcome and possible multiple predictors. Mallows C_p statistic was used to select the best model.

Results

Clinical profile: fVIII levels due to residual prophylaxis and plasma composition

Due to the differing prophylaxis programs, at the time of the blood draw, each individual had differing levels of residual fVIII. The individuals were reclassified according to their fVIII level at the time that the experiment was performed and are shown in Table 1. Of the 25 severe hemophilia patients, 11 individuals had fVIII levels that remained severe (1% fVIII:C), while 14 had residual fVIII levels that were in accordance with a moderate (2 – 5% fVIII:C, $n=5$) or mild (6 – 22% fVIII:C, $n=9$) hemophilia patient. Plasma composition for fII, fV, fVII, fIX, fX, AT and TFPI are also shown as the mean and SD for each reclassified hemophilia group. All other factors were within the normal acceptable range (Table 1). When comparing the groups segregated by fVIII level, no other factor levels were different amongst these reclassified groups.

Thrombin generation: effect of prophylactic fVIII

Several markers of empirical thrombin generation increased as the level of fVIII increased. Maximum TAT was increased (Figure 1A): 159 ± 93 nM for severe; 203 ± 119 nM for moderate; 306 ± 85 nM for mild ($p<0.005$ for mild vs. severe). The maximum rate at which thrombin was generated was similarly increased: 12.6 ± 5.4 nM/min for severe; 14.4 ± 7.4 nM/min for moderate; 22.7 ± 7.6 nM for mild (also $p<0.005$ for mild vs. severe).

Residual fVIII effect on fibrin formation and fXIII activation

When fibrinopeptide A release over time was evaluated in these individuals, there was no change in the maximum level obtained (mild: $9.5 \pm 3.6 \mu\text{M}$, moderate: $9.9 \pm 0.9 \mu\text{M}$, severe: $10.5 \pm 1.3 \mu\text{M}$) or in the initiation of the burst of fibrinopeptide A release (data not shown). Similarly, no difference was observed in the time to complete fibrinogen consumption (mild: $7.1 \pm 2.3 \text{ min}$, moderate: $6.8 \pm 2.8 \text{ min}$, severe: $7.0 \pm 2.2 \text{ min}$). However, fXIII activation rates increased as fVIII increased (Figure 1B): $13.1 \pm 3.5 \text{ nM/min}$ for mild; $9.5 \pm 1.3 \text{ nM/min}$ for moderate; $8.1 \pm 2.4 \text{ nM/min}$ for severe ($p=0.006$ for mild vs. severe).

The viscoelasticity of fibrin formation was evaluated by TEG and the results are shown in Table 2. There was no difference in the time to clot formation, the rate of clot formation or the maximum amplitude.

Effect on thrombin generation in the presence of an inhibitory fVIII antibody

In the presence of an inhibitory anti-fVIII antibody, Tf-initiated whole blood coagulation in all 25 severe subjects resulted in the thrombin generation profiles that are shown in Figure 1C. The suppression of thrombin generation resulted in the “mild,” “moderate” and “severe” groups to now have maximum levels of $184 \pm 86 \text{ nM}$, $234 \pm 208 \text{ nM}$ and $210 \pm 130 \text{ nM}$, respectively. Maximum rates in the presence of the inhibitory antibody were $16 \pm 6 \text{ nM/min}$, $15 \pm 11 \text{ nM/min}$ and $15 \pm 8 \text{ nM/min}$ respectively. No difference between either empirical thrombin parameter was seen.

Plasma composition effect in severe hemophilia with residual prophylactic fVIII

Using each individual's measured plasma composition (see Table 1) obtained from citrate plasma collected at the time of the blood draw for the whole blood assay was used to simulate thrombin generation in the computational model. When all individuals were segregated by their residual fVIII level as in the empirical experiments (Figure 2A), the maximum level of thrombin was increased: $103 \pm 17 \text{ nM}$ for severe; $145 \pm 23 \text{ nM}$ for moderate; $192 \pm 32 \text{ nM}$ for mild ($p<0.005$ for mild vs. severe). The maximum rate at which thrombin was generated was similarly increased: $0.17 \pm 0.04 \text{ nM/s}$ for severe; $0.33 \pm 0.09 \text{ nM/s}$ for moderate; $0.73 \pm 0.21 \text{ nM/s}$ for mild (also $p<0.005$ for mild vs. severe).

To suppress the residual prophylactic fVIII at the time of the blood draw, fVIII was mathematically set to zero to remove its influence on the plasma compositional thrombin generation profiles. Similar to the empirical experiments, by removing fVIII the previously separated thrombin generation profiles are now overlapping (Figure 2B). Although the differences between groups can be directly explained by the exogenous fVIII, individual differences that illustrate their baseline thrombin profile remain (Figure 2C).

Bleeding score

Bleeding scores for this cohort over the past five years ranged between 7.0 and 30.4 (average: 16.2 ± 6.1). The computational model was used to compare the maximum thrombin generation with fVIII set to zero and the bleeding score (Figure 3). The computational analyses, which provide an integrated assessment of the effect of total compositional differences between individuals on thrombin generation showed a moderate ($r^2=0.34$) negative correlation between bleeding scores and maximum levels in the absence of exogenous fVIII. When the effect of fVIII was not removed (set to the level at the time of the draw), no such correlation was observed. Linear regression showed that only AT (positively) and fII (negatively) were significantly correlated with the bleeding score. The bleeding score was not significantly correlated with any empirical measurement in this severe hemophilia A group, including those in which the -fVIII antibody was used.

Discussion

In this study, we addressed the effect of residual fVIII in severe hemophilia A subjects receiving prophylaxis. Both the empirical and computational studies based upon plasma composition showed that fVIII levels at the time of the blood draw are related to thrombin generation profiles. When fVIII is removed either empirically or computationally, the resulting thrombin profiles are comparable. Upon suppression of fVIII, subtle differences in the remaining plasma components (especially fII and AT) are able to further discriminate individuals via thrombin generation profiles and are related to their bleeding phenotype (Figure 3). This suggests a rationale for why severe hemophilia A patients with the same fVIII level, whether of therapeutic or natural origin, can differ hemostatically.

Measures of fibrin formation via FPA release and fibrinogen consumption were not significantly different between severe hemophilia A individuals separated by prophylactic fVIII levels. Additionally, a wide variation in several TEG parameters were seen within each group, consistent with a study in severe hemophilia A patients by Sørensen and Ingerslev [24]. However, the activation of the thrombin substrate fXIII showed changes reflecting prophylactic fVIII use. These results are consistent with previous findings with the same tissue factor initiated whole blood model, where fXIII activation was shown to play an important role in the discordant formation of fibrin in hemophilia [10]. As well, the use of plasma derived fXIII combined with recombinant fVIIa has recently shown potential as a possible therapeutic agent for severe hemophilia [25].

The evaluation of risk for hemorrhage and of an individualized optimal therapeutic strategy could be improved by including a comprehensive integrated analytical system that incorporates thrombin generation to provide predictive information. The major issues are the treatment regimens and their relationships to hemostasis. We demonstrate that levels of thrombin generation in the empirical model appear to be related to overall factor levels through the numerical modeling yielding a similar pattern of thrombin generation. Both models are able to show that the presence of residual fVIII in individuals with severe hemophilia A greatly influences the thrombin generation profiles. These data demonstrate that the contribution of prophylactic fVIII can be measured in severe hemophilia A individuals by either an empirical Tf-initiated whole blood assay or by measuring plasma factor level composition. The removal of the prophylactic fVIII in our assays has revealed considerable individual variation in baseline thrombin potential. Further studies will be needed to investigate variation in severe hemophilia A patients over the complete range of possible fVIII levels (baseline and at various times post-infusion).

Previously, we have shown that it is not one factor alone that contributes to the overall thrombin generation dynamics, but a combination of factors [26,27]. Variations in each subject's other factor levels (excluding fVIII) will influence the thrombin profiles. If subjects possess higher normal procoagulant and anticoagulant factors, they will generate thrombin faster than subjects with the same fVIII levels at low normal anticoagulant and procoagulant levels. Thus, an individual subject could potentially be at a hemostatic advantage over another even though the fVIII levels are equivalent. Although the same fVIII level is achieved with a therapeutic dose, it may be more effective in one patient than another, depending on their overall synergistic coagulation profile.

In summary, the fVIII prophylactic effect in severe hemophilia A can be evaluated by an assessment of thrombin generation by empirical and computational methodologies. The empirical and computational analyses suggest that differences in clinical history between severe hemophilia A individuals with varying bleeding histories, may in part derive from differences in thrombin generation caused by the integrated effect of each subject's

coagulation factors; that is the aggregate influence of normal range variation in factors other than fVIII. Ultimately, this approach may allow for the translation of each individualized baseline thrombin generation potential into a more efficient and effective prophylaxis regimen.

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Figure 1.

TF-initiated whole blood measures of coagulation segregated by fVIII level at the time of the draw: Severe $\square$, Moderate $\blacklozenge$, Mild $\blacktriangle$ **A.** TAT generation profiles. Mean clot times are indicated by arrows. **B.** Factor XIIIa generation profiles. **C.** TAT generation profiles in the presence of an α -fVIII antibody.

Figure 2.

Computational simulation of thrombin generation using plasma factor composition segregated by fVIII level (panel A), or the same groups with fVIII set to zero (panel B). All simulated thrombin generation curves with fVIII set to zero (presented as groups in panel B) are presented individually in panel C. Data in panels A and B are presented as mean + SD at each second.

Figure 3.
Correlation in the computational model between bleeding scores and maximum levels with fVIII set to zero.

Table 1

Plasma factor and inhibitor concentrations of the hemophilia A individuals at the time of the blood draw.

fVIII Level	fII, %	fV, %	fVII, %	fVIII, %	fIX, %	fX, %	TFPI, %	AT, %
Severe (n=11)	97±12	101±19	93±27	1±1	108±23	109±21	76±14	111±12
Moderate (n=5)	113±20	99±22	101±22	4±2	98±15	114±20	80±15	107±13
Mild (n=9)	114±11	103±11	94±15	13±5	92±14	99±14	71±10	101±7
Normal Range	60-140	60-140	60-140	64-232	69-151	60-140	53-147	86-128

* p>0.05 for differences between groups for all factor levels (except fVIII)

Table 2

Influence of residual fVIII on viscoelastic measurements of fibrin formation in severe hemophilia.

TEG Parameters	Severe (n=11)	Moderate (n=5)	Mild (n=9)
R Time (min)	10.9±3.6	8.7±2.7	9.3±4.8
K Time (min)	10.2±3.8	6.7±2.2	11.1±9.2
Angle (deg)	23.5±8.5	36.8±11.2	26.0±15.3
MA (mm)	44.7±6.9	48.4±11.9	43.1±9.8

* categories denote separation by fVIII at the time of the blood draw