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THROMBIN GENERATION AND BLEEDING IN HEMOPHILIA A

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Abstract

Introduction—Hemophilia A displays phenotypic heterogeneity with respect to clinical severity.

Aim—To determine if tissue factor (TF)-initiated thrombin generation profiles in whole blood in the presence of corn trypsin inhibitor (CTI) are predictive of bleeding risk in hemophilia A.

Methods—We studied factor(F) VIII deficient individuals (11 mild, 4 moderate and 12 severe) with a well-characterized five-year bleeding history that included hemarthrosis, soft tissue hematoma and annual FVIII concentrate usage. This clinical information was used to generate a bleeding score. The bleeding scores (range 0–32) were separated into three groups (bleeding score groupings: 0, 0 and 9.6, >9.6), with the higher bleeding tendency having a higher score. Whole blood collected by phlebotomy and contact pathway suppressed by 100µg/mL CTI was stimulated to react by the addition of 5pM TF. Reactions were quenched at 20min by inhibitors. Thrombin generation, determined by ELISA for thrombin – antithrombin was evaluated in terms of clot time (CT), maximum level (MaxL) and maximum rate (MaxR) and compared to the bleeding score.

Results—Data are shown as the mean±SD. MaxL was significantly different ($p<0.001$) between the groups: $504\pm114\text{nM}$, $315\pm117\text{nM}$, and $194\pm91\text{nM}$; with higher thrombin concentrations in the groups with lower bleeding scores. MaxR was higher in the groups with a lower bleeding score; $97\pm51\text{nM/min}$, $86\pm60\text{nM/min}$ and $39\pm16\text{nM/min}$ ($p=0.09$). No significant difference was detected in CT among the groups, $5.6\pm1.3\text{min}$, $4.7\pm0.7\text{min}$, $5.6\pm1.3\text{min}$.

Conclusions—Our empirical study in CTI-inhibited whole blood shows that the MaxL of thrombin generation appears to correlate with the bleeding phenotype of hemophilia A.

Keywords

Thrombin; Hemophilia A; Bleeding; Phenotype

Introduction

Hemophilia A, an X-linked disease, is characterized by the decrease or absence of functional factor VIII with an estimated incidence of 1 in 10,000 males[1]. It is the most extensively evaluated hemorrhagic disorder, with studies extending from clinical observations and treatment regimens[2], to biochemical and molecular analyses[3] and investigative gene therapy[4–7]. The reasons for the intensive efforts directed at hemophilia A include the

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chronic complex clinical management of these patients and cost of intervention both in monetary terms and debility.

In recent years, the treatment outcome of severe hemophilia has improved greatly. With proper diagnosis and treatment using replacement products free of viral transmission, a nearly normal lifespan can be expected. However, the development of inhibitors remains the major threat for subjects with this condition. Clinical screening for hemophilia A is accomplished by measuring factor VIII activity through either clotting (activated partial thromboplastin time (aPTT)) or chromogenic assays[8–11]. Analyses of patients' DNA permits the identification of the gene lesions that cause hemophilia and allows the disease spread to be controlled through carrier detection and antenatal diagnosis[2].

The clinical manifestation, frequency and severity of hemorrhage in hemophilia A is generally related to the functional blood level of factor VIII[1,12]. Three categories of severity have been defined by a consensus committee[13] and are as follows: severe deficiency, classified by factor VIII activity levels <0.01 U/mL (incidence is approximately 50% in affected patients), moderate deficiency classified by factor VIII levels of 0.02 to 0.05 U/mL (incidence is approximately 10% in affected patients), mild deficiency classified by factor VIII levels of 0.06 to 0.40 U/mL (incidence is between 30–40% in affected patients). Severe deficiency defined by factor VIII concentration is manifested clinically by repeated and severe hemarthroses that almost invariably results in crippling arthropathy in the absence of adequate replacement therapy. Moderate deficiency is clinically manifested by less frequent and less severe hemarthroses and seldom results in serious orthopedic disability. In mild deficiency, hemarthroses and other spontaneous bleeding manifestations may be absent altogether, although serious bleeding may follow surgical procedures or traumatic injury[14].

Factor VIII assays have a large potential for error. Extensive studies of the many variables of the factor VIII assay have been reported[15,16]. These discrepancies have been caused by the differences in standards, reagents, procedures, and presumably also by the nature of factor VIII concentrates used for therapy[11,17–20].

Several studies utilizing comprehensive assays have evaluated hemophilia blood coagulation and fibrinolytic profiles[21–25] and show different patterns associated with hemophilia severity. The primary focus of these assays has been on thrombin generation. The reasons that thrombin generation in a tissue factor (TF) based plasma assay is a novel approach to investigate hemophilia phenotypes is that the major hemostatic change in the coagulation profiles of individuals lacking factor VIII is in the propagation phase of TF-initiated thrombin generation [21,24–27]. The propagation phase is also depressed in a number of altered hemostatic states[28]; including anticoagulant[21,29] and antiplatelet therapies[21,30]. These observations suggest that the combination of the generation of a sufficient level of thrombin (propagation phase) in a timely fashion (initiation phase) is of importance. In the absence of factor VIIIa (as in hemophilia A), the intrinsic tenase complex (factor VIIIa-factor IXa) is unable to generate the additional factor Xa that is required for the burst (propagation) of thrombin generation through the prothrombinase complex (factor Va-factor Xa)[31]. To date, the understanding of how thrombin generation in hemophilia is related to the phenotype is still an ongoing issue.

In this study we evaluated TF-initiated whole blood coagulation in the presence of corn trypsin inhibitor (CTI) from hemophilia A individuals with varying bleeding clinical phenotype that have either functionally mild, moderate or severe factor VIII deficiency as defined by laboratory analyses.

Materials and Methods

Materials

HEPES, Tris-HCl, EDTA, TFA, 1-palmitoyl-2-oleoyl-phosphatidyl serine (PS), 1-palmitoyl-2-oleoyl-phosphatidyl choline (PC) were purchased from Sigma Chemical Co (St. Louis, MO). HPLC grade H₂O and CH₃CN were purchased from Fischer Scientific (Pittsburgh, PA). Benzamidine-HCl was purchased from Aldrich, Inc (Milwaukee, WI). Recombinant TF was a gift from Drs. Roger Lundblad and Shu-Len Liu (Hyland division, Baxter Healthcare Corp, Duarte, CA) and was relipidated in PCPS (75% PC:25% PS) vesicles by a previously described protocol[32,33]. CTI was prepared as described [34]. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (FPRck) was a gift from Dr. Richard Jenny (Haematologic Technologies, Essex Junction, VT). Enzyme-linked immunosorbant assay (ELISA) kits were used to estimate thrombin-antithrombin (TAT) complex formation (Behring, Westwood, MA).

Subjects

All subjects were recruited and advised according to a protocol approved by the Institutional Review Board at the University of Vermont Human Subjects Committee and the Centre Hospitalier Universitaire Sainte-Justine (Montreal, Canada). Informed written consent was obtained from twenty-seven hemophilia A individuals, comprising 11 mild (factor VIII:C 6-40%), 4 moderate (factor VIII:C 2-5%) and 12 severe (factor VIII:C 1%). Classification of individuals is based upon the clinical diagnoses prior to the blood draw. All hemophilia individuals that required prophylaxis only used factor VIII replacement therapy and did not have any known inhibitors present. All individuals that were included in the study were told not to withhold replacement therapy and did not need to self infuse with factor VIII from 0.25 to 4 days prior to the blood draw. Subjects within the severe population were on different prophylaxis programs.

Bleeding phenotype

A variety of clinical bleeding severities were seen in our hemophilia population. Bleeding tendency was scored independently by two experienced hemophilia nurses and one hematologist (GER) for each individual's bleeding history, and averaged. As there is no internationally accepted and validated scoring system for this type of study, we developed our own, taking in consideration hemarthrosis, soft tissue hematoma and annual FVIII unit/kg usage. The scoring points were as follows: hemarthrosis, 1-3/y 3, 4-6/y 6, 7-12/y 9, > 12/y 12; soft tissue hematoma, 1-3/y 2, 4-6/y 4, 7-12/y 6, > 8/y 8; annual FVIII unit/kg usage, 0/y 0, < 1000/y 3, 1000-3000/y 6, > 3000/y 12. Surgery, dental extractions and major accidents were excluded for calculation of annual FVIII unit usage. Mean scores over the course of five years are reported. The range of scoring was 0-32 and the profile for each subject is presented in Table 1. Scores are reported as means of annual scores for the five year observation period for all 27 hemophilia A individuals.

Whole blood coagulation

The procedure used was a modification of Rand *et al.* [35]. Experiments were performed on a rocking platform enclosed in a 37°C temperature controlled chamber. Venous blood obtained by antecubital phlebotomy from 24 hemophilia A subjects, was added (1mL) to tubes that were preloaded with CTI (100 µg/mL) and relipidated TF (5 pM, PCPS 1:2000 protein:lipid). Three out of the original twenty seven hemophilia A subjects, whose bleeding scores were evaluated, were unavailable for the whole blood experiment. Up to 16 tubes were used for each experiment, including a control tube that contained CTI and no TF. A time course was set up over the time period from 0-20 minutes. The control tube in all

experiments did not clot in the time frame of the 20 minute experiment. Clot time was determined visually by two observers: mean of the two observations was reported. At the appropriate times, samples were quenched with the inhibitors: EDTA (25 mM final); benzamidine-HCl (10 mM); and FPRck (50 μ M) at pH 7.4. The zero time point contained the inhibitors prior to the addition of blood.

After quenching, the clotted material was separated from the serum by centrifugation (15 min at 1200 x g). Both the clots and the aliquoted serum were stored at -80°C for further analysis.

Thrombin generation determination

TAT ELISA was performed according to the manufacture protocol in duplicate or triplicate using a minimum of 5 standards as previously described[36]. Results were analyzed on a Vmax microtiter plate reader (Molecular Devices, Menlo Park, CA) equipped with Softmax version 2.35. The detection limit of the assay for TAT complex is 40 pM.

Statistical analyses

The mean total bleeding score over the years 2002–2006 was calculated for each subject. The distribution of the mean scores was then divided into 3 groups with the 33.3% of subjects with a mean score of 0 assigned to score group 1, subjects with a mean score greater than 0 and less than or equal to 9.6 (29.6%) assigned to score group 2 and subjects with a mean score greater than 9.6 (37%) assigned to score group 3.

Data were analyzed using an analysis of variance with score as a grouping factor. When the overall F-statistic from the analysis of variance was significant ($p < 0.05$), pairwise comparisons of groups were examined for significant differences using the LSD method with $\alpha = 0.05$.

Results

Thrombin generation

A time course of TF-initiated thrombin generation was developed and analyzed for each hemophilia subject. Thrombin generation was evaluated for all individuals by parameters that reflect the onset and maximum concentration of thrombin that is generated during the whole blood experiment of 20 minutes. These parameters include the clot time (CT), which is a reflection of the time it takes 10 nM TAT to form, maximum rate of thrombin generation (MaxR, representative of the extent of the propagation phase of thrombin generation) and the maximum level of thrombin generation (MaxL) at 20 minutes (representative of the TAT propagation and termination phase of thrombin generation[36]). The MaxL in this whole blood model has previously been shown to be a phenotypic characteristic in healthy individuals[37]. Individual thrombin generation curves within the entire hemophilia population showed wide variability with the identical TF challenge. CT varied from 3.7 to 8.3 minutes with a mean of 5.3 ± 1.2 min. MaxL of thrombin generation varied from 75 to 694 nM with a mean of 323 ± 163 nM and MaxR of thrombin generation varied from 16 to 232 nM/min with a mean of 71 ± 58 nM/min. As a comparison, mean thrombin parameters from historical data on 35 healthy individuals[36] were: CT of 4.7 ± 1.1 min; MaxL of 851 ± 311 nM; and MaxR of 84 ± 23 nM/min. Compared to the healthy control, the hemophilia A population, comprising mild, moderate and severe individuals, had slower CTs, slower rates of thrombin generation and an overall lower level of thrombin generated.

Phenotype evaluation

We separated the twenty-four hemophilia A individuals that we obtained a CTI-inhibited whole blood thrombin profile on, into their respective bleeding score group. The first group being all individuals having a score of “0” or no bleeding complications (n= 7). The second group reflected the bleeding score range of >0 and ≤9.6 (n=8). The third group comprised individuals having the most bleeding complications, with a score >9.6 (n=9). The first group “0” included 64% of the mild population and 50% of the moderate hemophilia population. The second bleeding score group of “>0 and ≤9.6” contained 36% of the mild population, 25% of the moderate population and 25% of the severe population. The most severe bleeding score group “>9.6” encompassed 25% of the moderate and 75% of the severe population.

The mean of each group’s thrombin generation curve is compared to a historic control of 35 healthy individuals [36] (Figure 1, panel A). The graphical representation of the data is shown as the mean±SEM to illustrate the confidence in the mean of the groups. TF-initiated thrombin generation was highest in the first group, followed by the second and the third group having the lowest levels of thrombin generated. Individual thrombin generation curves within each group are presented in Figure 1 for group 1 (panel B), group 2 (panel C) and group 3 (panel D).

Thrombin generation versus phenotype

Variability in each individual hemophiliac’s thrombin generation progress curve was assessed by evaluating the parameters of CT, MaxL and MaxR. To show the range of results within the bleeding score groupings, the data are presented as the mean±SD. Within the first group of no bleeding complications (bleeding score “0”), the CT was 5.6±1.3 min, the MaxL was 504±114nM and the MaxR was 97±51 nM/min. Within the second group (bleeding score “>0 and ≤9.6”), thrombin parameters were: CT, 4.7±0.7 min; MaxL, 315±117 nM; MaxR, 86±80 nM/min. Thrombin parameters within the third group (bleeding score “>9.6”) of hemophilia individuals with the most severe bleeding complications were: CT, 5.6±1.3 min; MaxL, 194±91 nM and MaxR, 39±16nM/min. For comparison to the historic control[36], the mean±SD for the thrombin parameters were: CT, 4.7±1.1 min; MaxL, 851±311 nM; MaxR, 84±23 nM/min. Group 1 individuals with a bleeding score of “0”, were at the low extreme of normal for the MaxL of thrombin generation.

When the thrombin generation parameters are compared to the bleeding phenotype, the MaxL of thrombin generation was significantly different ($p<0.001$) between the bleeding score groupings (Table 2). The MaxR of thrombin generation showed a trend with a faster rate in individuals without a bleeding problem and a slower rate for individuals with a bleeding problem ($p=0.09$). No difference between the groups and CT was detected.

Conclusion

These data show whole blood TF-initiated thrombin generation profiles for a group of mild, moderate and severe hemophilia individuals classified by aPTT laboratory parameters with varying clinical bleeding phenotypes. When assessed by the CT, MaxL and MaxR of thrombin generation, a broad range of thrombin generation patterns was seen. When individual hemophilia clinical bleeding score profile algorithms were compared to thrombin generation, the MaxL of thrombin generated was related to the bleeding score ($p<0.001$). An increase in clinical bleeding was associated with less thrombin being generated in those individuals. Accordingly, hemophilia individuals producing higher levels of thrombin were protected from bleeding. These data suggest that *in vitro* quantitative estimates of TF-induced thrombin generation in contact pathway inhibited whole blood may be a parameter

useful for classifying the clinical bleeding risk in hemophilia. This measure may provide a better method for evaluating hemophilia management relative to bleeding pathology.

As expected the CT, which only requires ~10nM thrombin, is not reflective of any change between healthy control individuals and individuals with bleeding disorders. This subjective CT as well as standard clotting assays (PT and aPTT) is useful in showing a gross hemostatic defect, but they do not encompass the magnitude of the hemostatic response that is seen in the total level of thrombin generated.

Methods that profile thrombin generation (either directly or indirectly) have potential utility in the realm of clinical testing. Thrombin generation assays have proven able to discriminate among specific major hemostatic defects[38–42]. However, results have been mixed regarding the ability to evaluate the individual phenotype. For example, a recent study using a thrombographic method to analyze the relationship between clinical severity and thrombin generation in hemophilia concluded that the technique identified differences among individuals but that these differences did not correlate with clinical phenotype[43]. On the other hand, Trossaert *et al.*[44] suggest that endogenous thrombin potential measurement may be clinically relevant in moderate/mild hemophilic patients with discrepant factor VIII:C results. These studies, along with most thrombin generation studies to date, have employed citrated plasma for analyses. The complexity of the cycle of events set in motion by chelation/recalcification may serve to mask or minimize important differences between thrombin generation profiles in a group of individuals[45].

In our system, thrombin is generated with pure TF activation through the use of CTI, a contact pathway blockade. An advantage of using CTI is that it removes the need to use citrate in experimental settings. The addition of chelators can influence cellular metabolism and numerous plasma protein functions ranging from vitamin K dependent zymogens and the cross-linking of fibrinogen. Consequently, during reactions initiated by the addition of Ca^{++} into either citrate treated whole blood, platelet rich or platelet poor plasma, many Ca^{++} dependent events occur. Thus, biological interpretation should be evaluated with caution.

Previously, we have shown that TF-initiated thrombin generation in whole blood containing CTI, was an individual phenotypic characteristic in healthy individuals [37]. Healthy individuals with lower levels of thrombin are also potentially more at risk for developing a bleeding complication when undergoing resuscitative fluid replacement[46]. In this study, we have shown that the MaxL of thrombin in hemophilia individuals with a bleeding score of 0 have thrombin generation curves that fall at the low end of the standard deviation of normal controls. Thus, they appear hemostatically better off than individuals within groups 2 and 3 which have greater bleeding complications. Ultimately, thrombin generation appears to be a phenotypic marker in the diagnosis and stratification of patients with hemorrhagic disease. The next steps are to evaluate individual thrombin profiles in relationship to factor VIII levels, genotype and phenotype and begin individualized explorations.

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Figure 1.

Tissue factor-initiated whole blood thrombin-antithrombin (TAT) generation profiles of a hemophilia A population separated by bleeding phenotype. Thrombin generation profiles are categorized by bleeding score: 0 (group 1), >0 and ≤9.6 (group 2), >9.6 (group 3). The mean $\pm$ SEM of the thrombin generation profiles in the groups are compared to control individuals (n=35) (**panel A**). Individual thrombin generation curves are shown for group 1 (**panel B**), group 2 (**panel C**) and group 3 (**panel D**).

Table 1

Bleeding score phenotype

Subject	Hemarthrosis score Mean(SD)	Soft tissue score Mean(SD)	Factor VIII replacement score Mean(SD)	Total score Mean(SD)
Subject 1	2.4 (1.3)	4.8 (1.1)	12 (0)	19.2 (1.1)
Subject 2	8.4 (2.5)	5.2 (2.3)	12 (0)	25.6 (4.7)
Subject 3	1.2 (1.6)	2.4 (0.9)	6 (0)	9.6 (1.5)
Subject 4	4.8 (1.6)	1.2 (1.8)	8.4 (3.3)	14.4 (5.4)
Subject 5	3 (0)	8 (0)	5.4 (1.3)	16.4 (1.3)
Subject 6	0.6 (1.3)	1.6 (1.7)	6 (0)	8.2 (2.9)
Subject 7	3.6 (2.5)	2.4 (1.7)	10.8 (2.7)	16.8 (3.1)
Subject 8	3.6 (1.3)	4 (1.4)	9.6 (3.3)	17.2 (3.5)
Subject 9	7.8 (2.7)	3.2 (1.8)	6 (0)	17 (1.4)
Subject 10	12 (0)	6.4 (2.2)	12 (0)	30.4 (2.2)
Subject 11	4.8 (4.0)	7.2 (1.1)	5.4 (1.3)	17.4 (2.5)
Subject 12	2.6 (2.6)	1.6 (0.9)	3 (2.1)	7.2 (3.9)
Subject 13	0	0	0	0
Subject 14	8.4 (1.3)	3.6 (1.7)	3.6 (1.3)	15.6 (2.7)
Subject 15	0	0	0	0
Subject 16	3 (0)	2 (0)	3.6 (1.3)	8.6 (1.3)
Subject 17	0	0	0	0
Subject 18	1.2 (1.6)	0.8 (1.1)	1.8 (1.6)	3.8 (3.6)
Subject 19	0	0	0	0
Subject 20	0	0	0	0
Subject 21	0	0	0	0
Subject 22	0	0	0	0
Subject 23	0	0	0	0
Subject 24	1.2 (1.6)	0	0	1.2 (1.6)
Subject 25	2.4 (2.5)	0.4 (0.9)	1.8 (1.6)	4.6 (4.3)
Subject 26	0	0	0	0
Subject 27	3 (0)	2 (0)	3 (0)	8 (0)

Severe hemophilia: subjects 1–12; moderate hemophilia: subjects 13–16; mild hemophilia: subjects 17–27.

Table 2

Comparison of bleeding score with thrombin parameters

Thrombin Parameters	Bleeding Score			
	Group 1 “0” (N=7)	Group 2 “>0 and 9.6” (N=8)	Group 3 “>9.6” (N=9)	Control (n=35)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD
CT (min)	5.6±1.3	4.7±0.7	5.6±1.3	4.7±1.1
MaxL (nM)*	504±114	315±117	194±91	851±311
MaxR (nM/min)	97±51	86±80	39±16	84±23

Data shown as mean±standard deviation

CT: Clot time; MaxL: Maximum level of thrombin generated; MaxR: Maximum rate of thrombin generation *p<0.001 based on analysis of variance. Pairwise comparisons (Fisher’s LSD) indicates all three bleeding score groups differed significantly from each other (α=.05).