Thrombomodulin-modified thrombin generation after *in vivo* recombinant factor VIII treatment in severe hemophilia A

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ABSTRACT

Background
Thrombin generation has been shown to reflect coagulation potential and factor VIII (FVIII) levels in patients with hemophilia A. We hypothesize that thrombin generation in the presence of thrombomodulin reflects plasma FVIII levels better.

Design and Methods
Plasma FVIII levels were determined chromogenically and thrombin generation was measured with and without thrombomodulin in 12 patients with severe hemophilia A. Blood was sampled at baseline and 15 min, 1, 3, 6, 24 and 48 hours after recombinant FVIII administration.

Results
FVIII administration restored the decreased baseline thrombin generation (reflected by endogenous thrombin potential, peak height, slope and time to peak). Lag time did not change. All thrombin generation parameters except time to peak returned to baseline within 48 hours, while plasma FVIII concentration was increased and time to peak shortened. Endogenous thrombin potential and peak height showed wide inter-individual variation, with strong intra-individual correlations. Addition of thrombomodulin to the assay shortened time to peak and decreased endogenous thrombin potential and peak height. The decrease in peak height was almost completely offset by FVIII administration. Multiple linear regression analysis revealed thrombomodulin-modified thrombin generation to be a moderately better predictor of plasma FVIII levels than thrombin generation in the absence of thrombomodulin (adjusted R^2 0.79 *vs.* 0.71).

Conclusions
Addition of thrombomodulin has pronounced effects on all parameters of thrombin generation. This thrombomodulin-modified thrombin generation assay better reflects plasma FVIII levels than thrombin generation in the absence of thrombomodulin.

Key words: hemophilia A, thrombin generation, thrombomodulin, coagulation factor VIII.

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Introduction

Hemophilia A is an inherited disorder characterized by decreased levels of coagulation factor VIII (FVIII) in the blood. Upon tissue factor-induced activation of the coagulation system, only small amounts of thrombin are initially formed following activation of FX. Activated FVIII (FVIIIa) acts as a co-factor to FIXa in the formation of the intrinsic tenase complex (FVIIIa, FIXa and FX together with calcium on negatively charged phospholipids), which enhances thrombin formation dramatically through increased formation of FXa. After binding to thrombomodulin, thrombin activates protein C which, together with its co-factor protein S, inactivates both FVIIIa and FVa. At low levels of FVIII or in the absence of FVIII the formation of the tenase complex is diminished and the positive feedback loop is impaired, which decreases the coagulation potential of blood, resulting in a risk of bleeding complications.

Prophylactic treatment of hemophilia A consists of replacing FVIII and nowadays recombinant products are widely used for this purpose. All registered recombinant FVIII products have been shown to be effective and safe in reducing bleeding complications in patients with hemophilia A.[1] Overall, bleeding risk is inversely correlated with FVIII levels, but it is well known that bleeding patterns in patients vary markedly, also at comparable FVIII levels and in the absence of additional abnormalities of hemostasis.[2,3] This raises the question of whether determinants other than FVIII levels contribute to the risk of bleeding, i.e. the total plasma coagulation potential.

Several studies have employed a variety of thrombin generation assays to test the total plasma coagulation potential in hemophilic blood samples and reconstituted plasma models, showing that the endogenous thrombin potential (ETP; area under the thrombin generation curve) reflects hemorrhagic tendency[4-9] and is decreased in patients with severe hemophilia A, while the initiation phase of coagulation is prolonged.[10] The replacement of FVIII, both *in vitro* in FVIII-deficient plasma[11,12] and *in vivo* in treated hemophilia A patients,[13] is able to restore thrombin generation, while increased FVIII levels may reflect a procoagulant state.[14-16] The lag time of the thrombin generation curve was not found to correlate with FVIII levels, and it was suggested recently that slope and time to peak are better predictors of FVIII influences on thrombin generation,[5] tested in hemophilia A patients and healthy controls. Indeed, time to peak was correlated with FVIII levels.[17] The only study available on thrombin generation after i*n vivo* FVIII administration in a platelet-poor plasma assay was recently published by Lewis *et al.*,[18] who used a 1 pM tissue factor stimulus. In this study, the ETP and peak height of the thrombin generation curve show wide inter- and intra-individual variations, indicating that other components of coagulation or inhibition thereof are involved besides FVIII. Alternatively, thrombin generation measurements have been proposed as a way to screen for the effects of inhibitors on blood coagulation in hemophilia A patients.[19-21]

Previously, we validated the calibrated automated thrombogram assay in our laboratory with platelet-poor plasma activated by 1 pM tissue factor and added thrombomodulin to this assay to study the influence of the protein C system on the initiation and, in particular, the propagation phases of coagulation.[22] Adding thrombomodulin to the calibrated automated thrombogram assay decreases total thrombin generation through inhibition of FV and FVIII. Since FVIII is the varying factor in hemophilia A, the residual thrombin formation still present after addition of thrombomodulin may be a more direct reflection of FVIII levels. We, therefore, hypothesize that addition of thrombomodulin to plasma samples from hemophilia A patients after *in vivo* FVIII replacement therapy (with different intra-individual FVIII levels) may make the thrombin generation assay reflect FVIII levels better.

In this study, we determined thrombin generation with 1 pM tissue factor and FVIII levels using a chromogenic assay in 12 patients before and 48 hours after administration of a recombinant FVIII product, and we assessed changes in thrombin generation upon addition of thrombomodulin.

Design and Methods

Patients

Male patients with severe hemophilia A who had a regular *in vivo* recovery of FVIII upon administration of this clotting factor at the Division of Haematology at the University Hospital Maastricht, The Netherlands, were asked to allow further testing of the remaining amount of their plasma. Inclusion criteria were FVIII levels below 1%, no FVIII administration in the previous 72 hours, and no evidence of active bleeding or inhibitors. Informed consent was obtained from all patients according to our local hospital guidelines.

Study design

All patients received a fixed dose of 3000 U of a recombinant FVIII product through infusion after a wash-out period of at least 72 hours, resulting in a mean dosage of 40.0 U/kg [range, 39.2-43.2].

Normal pooled plasma

Normal pooled plasma was prepared at the Departments of Hematology and Clinical Chemistry of the University Hospital Maastricht, The Netherlands, by pooling plasma from 85 healthy volunteers not using any medication.

Blood collection and preparation

Venous blood (3.2% citrate (w/v)) was collected from an antecubital vein at baseline (before infusion), and 15 min, 1, 3, 6, 24 and 48 hours after infusion. Platelet-poor plasma was prepared by two-step centrifugation: first at 2000 g for 15 min followed by centrifugation at 11000 g for 5 min. Plasma aliquots were stored at -80 °C until use and thawed at 37 °C for 15 min before analysis.

FVIII analysis

FVIII levels were measured according to the manufacturer's instructions in a chromogenic assay on BCS (Dade Behring, Liederbach, Germany) using a chromogenic assay kit. All FVIII assays employed standard human plasma (Dade Behring), calibrated against the World Health Organization-standard.

Thrombin generation measurements

Thrombin generation in tissue factor-triggered platelet-poor plasma was measured with the calibrated automated thrombogram method (Thrombinoscope BV, Maastricht, The Netherlands).[2,3] Measurements were conducted on 80 μL plasma with final concentrations of 1 pM tissue factor (PPP Reagent Low, Thrombinoscope BV) and 4 μM phospholipids in the absence and presence of 1.5 nM recombinant soluble thrombomodulin (Asahi Kasei Pharma Corporation, Tagata, Japan). The concentration of thrombomodulin was chosen such as to inhibit thrombin generation in normal pooled plasma by 50%. Thrombin Calibrator was obtained from Thrombinoscope BV. Fluorescence was read in a Fluoroskan Ascent reader (Thermo Labsystems OY, Helsinki, Finland) equipped with a 390/460 filter set and thrombin generation curves were calculated with the Thrombinoscope software (Thrombinoscope BV).

Five parameters were derived from the thrombin generation curves: lag time (initiation phase of coagulation), ETP, peak height, time to peak and slope (propagation phase of coagulation). The lag time was defined as the time to reach one-sixth of the peak height. The slope was calculated by dividing five-sixths of the peak height by the time to peak minus the lag time.

The influence of the activation of the plasma kallikrein-kinin system in conditions of a low tissue factor stimulus (1 pM) was assessed in a separate experiment in which thrombin generation was recorded with and without corn trypsin inhibitor at a final concentration of 40 μg/mL (Haematologic Technologies Inc. Essex, VT, USA). No differences were observed between the two thrombin generation assays. Furthermore, analysis of thrombin generation without the tissue factor trigger showed no thrombin formation within 1 hour, thereby suggesting that the plasma kallikrein-kinin system system was not activated (*data not shown*).

Validation of the calibrated automated thrombogram method in our laboratory showed that it was essential to normalize non-time-dependent parameters in order to obtain acceptable inter-assay variations (*data not shown*). Intra-assay variations for normalized parameters are typically below 6% and inter-assay variations below 8% (*data not shown*). Each thrombin generation measurement therefore includes normal pooled plasma and both the ETP and peak height values are expressed as the ratio of $value_{patient}/value_{normal\ pooled\ plasma}$, as a percentage.

Statistical analysis

Data are expressed as median [interquartile range], unless otherwise specified. Differences over time in thrombin generation parameters and FVIII levels were analyzed using Wilcoxon's signed rank test. Correlations are expressed as Pearson's coefficients. To assess the relation between thrombin generation and FVIII levels, multiple linear regression analysis was performed with FVIII as the dependent variable and the lag time, ETP, peak height, time to peak, slope, age and product dose infused as independent variables. For each model, the adjusted R^2 and the standardized regression coefficients (β) of the independent variables were calculated. The β indicates the change of the dependent variable, expressed in standard deviations (SD), when the independent variable increases one SD and all other variables in the model remain unchanged.

A two-tailed probability value <0.05 was considered statistically significant. Statistics were computed using SPSS for Windows, version 12.0 (SPSS Inc., Chicago, IL, USA), and Prism for Windows, version 5.00 (GraphPad Software Inc., San Diego, CA, USA).

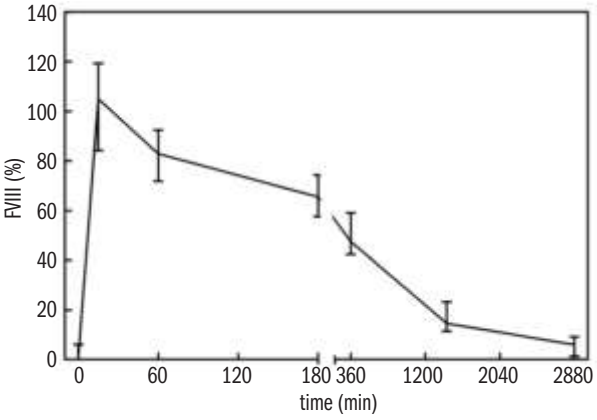

Figure 1. FVIII levels at baseline and upon recombinant FVIII administration. Data are presented as the median [interquartile range].

Results

Patients

Twelve male patients known to have severe hemophilia A formed the study group. Their median age was 34 years (range, 14-66) and their median weight was 72.5 kg [65.0-75.0]. None of the patients had inhibitors. Nine out of the 12 patients had normal liver function, the remaining three patients had slightly abnormal liver function (alanine aminotransferase less than 90 IU/L), two of whom were known to be hepatitis C carriers. No other co-morbidities were known during the period of investigation.

FVIII level measurements

FVIII levels are presented in Table 1(A) and depicted in Figure 1. At baseline, the median FVIII level was 0.9%.

FVIII infusion significantly increased FVIII levels to a peak value of 105.0% after 15 min. Forty-eight hours after administration, the FVIII level was still significantly increased compared to baseline: 6.0% *vs.* 0.9%. Peak FVIII levels correlated with the administered dose, R=0.73 (p<0.01).

Thrombin generation measurements with 1 pM tissue factor trigger in the absence of thrombomodulin

Thrombin generation measurements in the absence of thrombomodulin are presented in Table 1(B), and the ETP and peak height are depicted in Figure 2 (panels A and B, respectively; solid lines). Correlations of the thrombin generation parameters with FVIII levels are presented in Table 2. The effects of FVIII administration and the addition of thrombomodulin on the thrombin generation curve are depicted in Figure 3. The lag time showed no significant changes upon FVIII administration and the lag time and FVIII levels were not correlated.

The ETP increased significantly after FVIII infusion to a peak value of 114.5% after 15 min. Forty-eight hours after FVIII administration, the ETP returned to the baseline value of 40.0%. FVIII levels correlated with the ETP in the group of patients as a whole (R=0.79, $p<0.0001$). However, when data were analyzed per individual patient, correlations were markedly higher. Despite these correlations, there was a wide range of ETP at higher FVIII levels. Prior to FVIII administration, the peak height was relatively low compared to the ETP (24.0 *vs.* 40.0%), but became greater after 15 min: 126.0%. The peak height returned to baseline by 48 hours (29.0%). Similar to the ETP, the peak height correlated with FVIII levels in the whole group of patients (R=0.79, $p<0.0001$), although stronger correlations were found in individual patients. Administration of FVIII significantly shortened the time to peak (from 16.9 to 10.3 min after 15 min). The time to peak was the only thrombin generation parameter still significantly decreased after 48 hours (15.2 *vs.* 16.9 min at baseline). The time to peak was inversely correlated with FVIII levels (R=-0.75, $p<0.0001$). In contrast to the ETP and peak height, the time to peak values were within a narrow range across all FVIII levels.

The slope was calculated from non-normalized peak height data, a method that yields comparable data to the slope calculated from normalized peak height data (R=0.997, $p<0.0001$, *data not shown*). The slope increased significantly upon FVIII infusion, reaching a peak of 11.0 nM/min after 1 hour. After 48 hours the slope returned to baseline: 1.6 nM/min. The slope correlated with FVIII levels in the group of patients as a whole (R=0.74, $p<0.0001$).The ETP, peak height and slope were strongly correlated in all patients.

Table 1. FVIII levels (A) and thrombin generation at 1 pM tissue factor in the absence (B) and presence (C) of thrombomodulin at baseline and upon recombinant FVIII replacement.

	0 minutes		*15 minutes*		*1 hour*		*3 hours*		*6 hours*		*24 hours*		*48 hours*	
A														
FVIII (%)	0.9	[0.1-5.1]	105.0[a]	[86.0-115.9]	82.9[a]	[74.0-91.1]	65.5[a]	[59.6-73.6]	47.5[a]	[43.2-57.1]	14.5[a]	[11.5-21.8]	6.0[a]	[2.5-8.6]
B														
Lag time (min)	4.5	[3.7-5.5]	4.7	[4.3-5.2]	5.3	[4.2-6.1]	5.4	[4.3-5.9]	5.0	[4.2-5.9]	5.4	[4.3-6.1]	5.7	[4.3-6.2]
ETP (%)	40.0	[30.3-48.5]	114.5[a]	[91.5-147.5]	113.0[a]	[77.3-129.8]	97.5[a]	[82.3-106.8]	84.5[a]	[57.8-104.3]	56.5[a]	[50.8-72.5]	40.0	[37.58-56.0]
Peak height (%)	24.0	[20.0-30.8]	126.0[a]	[89.3-205.3]	119.5[a]	[79.0-148.3]	91.0[a]	[75.0-117.0]	80.5[a]	[50.5-102.3]	45.5[a]	[39.5-58.0]	29.0	[27.0-38.0]
Time to peak (min)	16.9	[15.3-17.5]	10.3[a]	[8.5-11.4]	11.2[a]	[9.3-12.2]	11.8[a]	[9.9-12.5]	11.7[a]	[10.8-13.4]	13.7[a]	[12.7-14.9]	15.2[a]	[14.3-16.9]
Slope (nM/min)	1.0	[0.8-1.2]	10.5[a]	[7.1-22.8]	11.0[a]	[6.1-16.2]	7.5[a]	[5.1-11.3]	5.7[a]	[3.6-8.6]	2.8[a]	[2.2-3.5]	1.6	[1.3-1.9]
C														
Lag time (min)	3.7[b]	[3.1-4.4]	5.0	[4.2-5.4]	5.1[a]	[4.3-5.8]	5.3[a]	[4.6-5.7]	4.8[ab]	[4.2-5.8]	4.9[b]	[3.8-5.4]	4.2[b]	[3.8-4.9]
ETP (%)	16.9[b]	[11.3-19.2]	67.0[ab]	[46.2-101.0]	64.6[ab]	[35.4-73.9]	47.6[ab]	[33.7-59.4]	36.3[ab]	[26.0-62.4]	24.4[ab]	[18.6-33.2]	16.1[b]	[13.3-27.6]
Peak height (%)	13.6[b]	[9.0-17.9]	110.5[ab]	[62.9-190.9]	105.9[ab]	[50.9-136.2]	75.5[ab]	[50.5-98.3]	57.7[ab]	[33.8-94.7]	29.8[ab]	[23.0-37.6]	15.3[b]	[12.7-27.3]
Time to peak (min)	12.8[b]	[10.9-14.6]	9.2[ab]	[7.9-10.2]	10.0[ab]	[8.0-10.3]	10.2[ab]	[8.6-10.8]	10.3[ab]	[8.9-11.0]	11.2[ab]	[9.8-12.0]	12.2[b]	[10.7-13.3]
Slope (nM/min)	0.8	[0.5-1.2]	13.0[ab]	[7.2-25.5]	12.2[ab]	[5.5-18.2]	8.0[ab]	[5.2-11.5]	6.2[a]	[3.3-8.4]	2.5[a]	[2.0-3.0]	1.2[b]	[0.8-2.0]

Data are presented as medians [interquartile range]. [a]denotes p<0.05 compared to baseline; [b]denotes p<0.05 compared to thrombin generation in the absence of thrombomodulin.

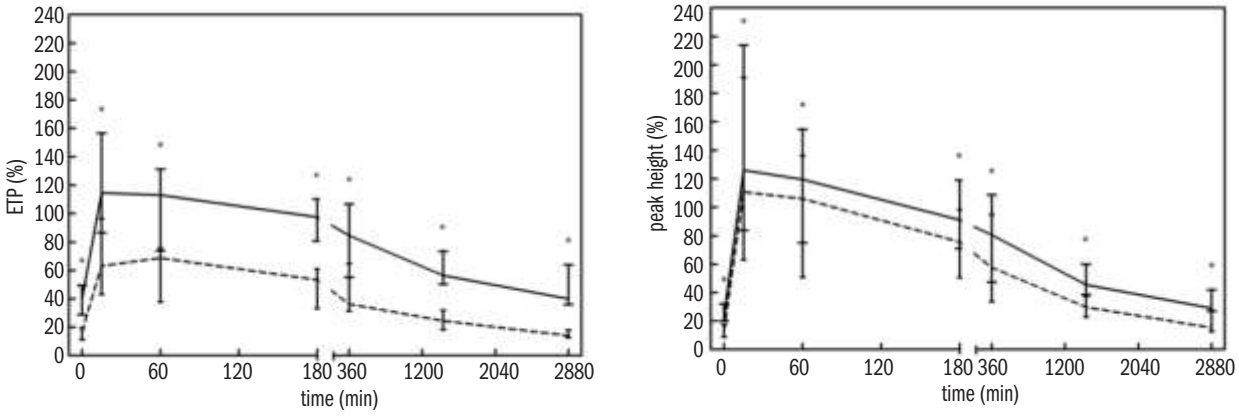

Figure 2. ETP (A) and peak height (B) at 1 pM tissue factor in the absence (solid line) and presence (dashed line) of thrombomodulin at baseline and upon administration of recombinant FVIII. Data are presented as medians [interquartile range]. * denotes $p<0.05$.

Thrombin generation measurements with 1 pM tissue factor trigger in the presence of thrombomodulin

Thrombin generation measurements in the presence of thrombomodulin are presented in Table 1(C), and the ETP and peak height are depicted in Figure 2 (panels A and B, respectively; dashed lines). Correlations of FVIII levels with thrombin generation parameters in the presence of thrombomodulin (calculated from both absolute data and relative reductions) are presented in Table 2. The effects of FVIII administration and the addition of thrombomodulin on the thrombin generation curve are depicted in Figure 3.

The lag time at low FVIII levels was significantly shorter in the presence of thrombomodulin than in its absence. At high FVIII levels, however, the addition of thrombomodulin did not influence the lag time, demonstrating that the addition of FVIII can successfully offset the effect of thrombomodulin. Unlike in the absence of thrombomodulin, in the presence of thrombomodulin the lag time correlated with FVIII levels (R=0.26, $p<0.05$), with a stronger correlation between FVIII levels and the relative reduction in the lag time achieved by thrombomodulin (R=0.71, $p<0.001$).

The ETP decreased significantly in the presence of thrombomodulin (titrated at a reduction in ETP of 50% in normal pooled plasma), although this decrease was less pronounced at high FVIII levels (41-51% compared to 57-60% at low FVIII levels). FVIII levels correlated with the ETP to a similar extent with or without addition of thrombomodulin, although the relative reduction in ETP correlated less with FVIII levels.

The addition of thrombomodulin significantly decreased the peak height at low FVIII levels, although this decrease was almost completely offset by FVIII administration, resulting in a peak height comparable to that in normal pooled plasma (Figure 2B). Correlations with FVIII levels were similar to those of the ETP and FVIII. The time to peak was significantly shorter at all time points upon addition of thrombomodulin. After FVIII infusion, the time to peak decreased significantly to 9.2 min after 15 min, and returned to baseline (12.2 min) after 48 hours. Similar to the lag time, time to peak in the presence of thrombomodulin showed a poor inverse correlation with FVIII levels (R=-0.56, $p<0.0001$), while the relative reduction correlated strongly with FVIII levels (R=0.72, $p<0.0001$).

Addition of thrombomodulin to the thrombin generation assay significantly increased the slope, while recombinant FVIII infusion resulted in a maximum of 13 nM/min within 15 min, returning to baseline after 24 hours. The slope correlated with FVIII levels in all but one patient.

In all patients, the ETP, peak height and slope in the presence of thrombomodulin were strongly correlated.

Thrombin generation and product dose

Overall, FVIII levels correlated weakly with the dose of product infused (R=0.25, $p<0.05$), although correlations between 15 min to 6 hours after infusion were markedly stronger (R=0.73, $p<0.01$ at 15 min decreasing to R=0.59, $p<0.05$ at 6 hours). The ETP and peak height at 6 and 24 hours after FVIII administration correlated with the FVIII dose (ETP: R=0.58 and R=0.75, $p<0.05$; peak height: R=0.59 and R=0.81, $p<0.05$), while all parameters (except the slope) of the thrombin generation curve at all other time points were significantly but weakly correlated with the dose infused.

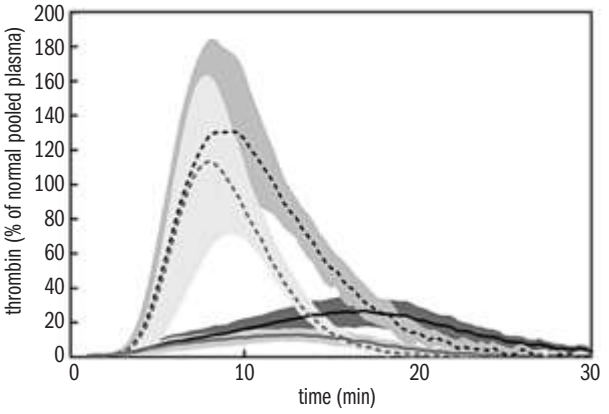

Figure 3. Thrombin generation curves at baseline (solid lines) and after 15 min (dashed lines), in the absence (black line) and presence (gray line) of thrombomodulin. Gray areas represent the 95% confidence intervals.

Table 2. Correlation between FVIII levels and thrombin generation at 1 pM tissue factor in the absence of thrombomodulin (-TM) and its presence (absolute: +TMa; relative reduction: +TMr).

Patient	Lag time		ETP		Peak height		Time to peak		Slope	
	-TM	+TMr	-TM	+TMa	-TM	+TMa	-TM	+TMa	-TM	+TMa
1	-0.46	0.89*	0.84*	0.92*	0.86*	0.92*	-0.99*	0.82*	0.82*	0.84*
2	0.56	0.58	0.96*	0.94*	0.97*	0.97*	-0.89*	0.92*	0.97*	0.97*
3	-0.42	0.89*	0.89*	0.92*	0.94*	0.95*	-0.94*	0.85*	0.92*	0.94*
4	0.17	0.94*	0.93*	0.94*	0.95*	0.94*	-0.91*	0.94*	0.96*	0.95*
5	0.62	0.49	0.72	0.75	0.73	0.78*	-0.79*	0.29	0.75	0.78*
6	0.46	0.76*	0.99*	0.96*	0.98*	0.95*	-0.95*	0.94*	0.96*	0.94*
7	0.49	0.76*	0.96*	0.97*	0.99*	0.99*	-0.87*	0.94*	0.99*	0.99*
8	-0.02	0.84*	0.93*	0.95*	0.97*	0.97*	-0.84*	0.64	0.99*	0.97*
9	0.13	0.90*	0.94*	0.90*	0.97*	0.92*	-0.92*	0.94*	0.97*	0.92*
10	0.82*	0.59	0.90*	0.94*	0.91*	0.94*	-0.88*	0.74	0.85*	0.90*
11	-0.69	0.94*	0.72	0.58	0.70	0.61	-0.78	0.92*	0.63	0.61
12	-0.26	0.88*	0.80*	0.90*	0.84*	0.89*	-0.96*	0.86*	0.85*	0.91*
total group	-0.06	0.71*	0.79*	0.78*	0.79*	0.78*	-0.75*	0.72*	0.74*	0.74*

*Data are presented as Pearson's R. *denotes p<0.05.*

Multiple linear regression models

Multiple linear regression models for FVIII incorporating thrombin generation parameters both in the absence and presence of thrombomodulin, age and product dose infused are presented in Table 3.

Inclusion of age and parameters of thrombin generation in the absence of thrombomodulin in a regression model resulted in a high adjusted R^2 of 0.71. Determinants in this model were age, the lag time and time to peak, while the ETP and peak height were not significant predictors of FVIII levels. Inclusion of parameters of thrombin generation in the presence of thrombomodulin in a regression model resulted in a higher adjusted R^2 of 0.79, with all thrombin generation param-

eters and age as significant predictors. Instead of including the absolute parameters, inclusion of the relative reduction in the lag time and time to peak increased linear regression results due to better correlations with FVIII (Table 2). In this model, the peak height showed a very strong positive association, while the ETP and slope were strong negative predictors, contrary to their positive correlation with FVIII levels. This effect is likely due to very strong correlations between the ETP, peak height and slope. The inclusion of only one of these three parameters in the model together with the lag time, time to peak and age did not result in a change of the adjusted R^2, and the included parameter had a positive β between 0.45 and 0.50 (*data not shown*).

Discussion

In this study we compared thrombin generation parameters at 1 pM tissue factor in the calibrated automated thrombogram assay with chromogenically determined FVIII levels during 48 hours after FVIII administration in patients with severe hemophilia A. In addition, we studied whether a modification of the calibrated automated thrombogram (by adding thrombomodulin, a component of the activated protein C pathway, inhibiting FVIIIa and FVa) results in a thrombin generation assay that reflects FVIII levels better.

FVIII levels increased to normal after administration, then declined by approximately 40% within the first 3 hours, followed by a more gradual decay up to 48 hours, when the FVIII levels were still higher than at baseline. All assessed thrombin generation parameters (the ETP, peak height, time to peak and slope), except lag time, showed concomitant changes towards an increased coagulation potential, i.e. shorter times and higher total thrombin. The lack of shortening of the lag time emphasizes the importance of FVIII in the propagation rather than in the initiation phase of coagulation. Unlike all other parameters the time to peak was still shortened by 1.7 min after 48 hours, indicating that, although the total amount of thrombin formed was comparable to that at baseline, the propagation phase of coagulation was still enhanced.

Although the ETP and peak height generally correlated with FVIII levels,[17] there was a very large variation in these parameters at higher FVIII levels, with ETP ranging from 50 to 160% at FVIII levels of approximately 100%. While correlations were around R=0.79 for the group as a whole, patients showed much narrower intra-individual variation with very high correlations when studied individually. Theoretically, this may coincide with the known inter-individual variations of bleeding risk, but this study was not designed to assess this relationship. In our assay, the slope of the thrombin generation curve strongly correlated with the ETP and peak height, but correlated less well with the other parameters it is calculated from (the lag time and time to peak). In contrast to the results of Dargaud *et al.*,[6] in our 1 pM tissue factor setup, calculation of the slope does not seem to reveal information additional to that yielded by either the ETP or peak height, although the actual calculation methods

Table 3. Multiple linear regression models for FVIII, incorporating thrombin generation parameters at 1 pM tissue factor in the absence (A) and presence (B) of thrombomodulin, age and dose of product infused.

Dependent variable: FVIII	*A*		*B*	
Independent variable	*β*	*Adjusted R^2*	*β*	*Adjusted R^2*
Product dose infused	0.06	0.71	0.58	0.79
Age	0.16*		0.22*	
Lag time	0.22*		-0.29*	
ETP	-0.31		-1.45*	
Peak height	1.13		3.37*	
Time to peak	-0.47*		-0.16*	
Slope	-0.40	-1.44*		

**denotes p<0.05.*

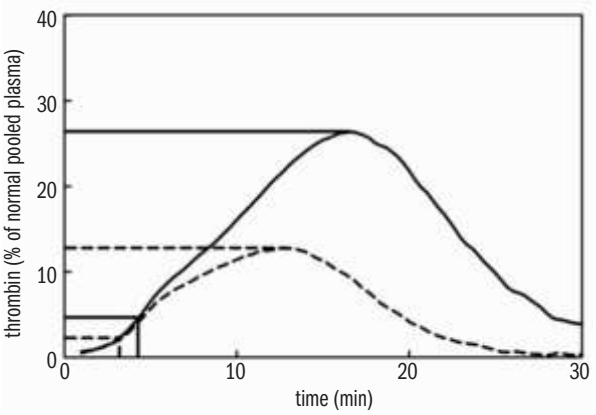

Figure 4. Thrombin generation curves at baseline in the absence (solid line) and presence (dashed line) of thrombomodulin. Horizontal lines indicate the peak height and one-sixth of the peak height of their respective curves.

may differ. This was confirmed in the multiple linear regression analysis, in which the inclusion of either the slope, ETP or peak height as an independent variable alongside the lag time, time to peak and age did not change the adjusted R^2 of the model.

Addition of thrombomodulin to the thrombin generation assay reduced the coagulation potential at baseline, reflected by the lower ETP and peak height. The significant shortening of the time to peak compared to that in the assay conducted in the absence of thrombomodulin may seem in contradiction to the overall decreased thrombin generation, but this is probably due to the decreased feedback loop involving FVIII. In the presence of thrombomodulin, the first thrombin that is generated is unable to activate FVIII and promote further thrombin generation over a prolonged period. This results in a decreased thrombin generation curve in which the peak (although lower) is reached earlier. This effect is illustrated in Figure 3, in which the gray curves (in the presence of thrombomodulin) reflect lower thrombin generation with shorter time parameters.

At low FVIII levels, addition of thrombomodulin to the thrombin generation assay resulted in a shortening of the

lag time. This effect may seem paradoxical since no change in lag time was described by Dargaud *et al.* in a 5 pM tissue factor thrombin generation assay upon addition of thrombomodulin[23] and even a prolongation at low FVIII levels could be expected. However, the observed effect is due to the calculation method used by the Thrombinoscope software to define lag time (i.e. the time to reach one-sixth of the peak height). Thrombin generation curves at low FVIII levels have a distinctive form in which one-sixth of the peak height is reached at an earlier time in the presence of thrombomodulin. This effect is illustrated in Figure 4. In this figure it can be appreciated that addition of thrombomodulin (dashed line) shortens the lag time compared to that of thrombin generation in the absence of thrombomodulin (solid line). Although this paradoxical effect may be regarded as a technical artifact, we show that the reduction in lag time achieved by addition of thrombomodulin does correlate with FVIII levels (R=0.71, $p<0.001$) and may, therefore, be a useful variable of the thrombin generation curve.

The relative reductions in both the lag time and time to peak that are achieved by addition of thrombomodulin correlated well with FVIII levels, and the fact that the ETP, peak height and slope were interchangeable in the multiple regression analysis indicates that the focus should not be on a single thrombin generation parameter. Instead, the total curve should be regarded as a measure of (FVIII-dependent) coagulation potential (the effects on all parameters of the thrombin generation curve are especially clear in Figure 3). Taking all thrombin generation parameters into account, modification of the thrombin generation assay by addition of thrombomodulin results in measurements that better reflect FVIII status in patients with hemophilia A.

Authorship and Disclosures

KH, HtC, WTH and WMRB contributed to the conception and design of the study; AWJHD, WTH, RvO and HMHS analyzed and interpreted the data; AWJHD wrote the article, which was critically revised by Henri MHS, KH and HtC.

The authors reported no potential conflicts of interest.

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