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Abstract

Introduction:

Altered levels of factor (F)VIII, prothrombin, or antithrombin have been associated with an increased risk for venous thromboembolism (VTE). However, the exact molecular mechanism by which these altered factor levels modulate the risk is incompletely understood. Here we hypothesize that elevated factor levels affect the pro- and anticoagulant balance in coagulation such that even minute amounts of tissue factor (TF) will initiate thrombin formation, thereby contributing to the VTE risk.

Materials and methods: To test this so-called TF-threshold hypothesis, we monitored thrombin generation initiated by very low TF concentrations in FXII-deficient plasma, to avoid any contact pathway-mediated thrombin formation. Furthermore, similar experiments were performed in the presence of increasing concentrations of pro- and anticoagulant proteins.

Results: A TF-threshold was established in the FXII-deficient plasma, which is subject to inter-individual variation. Elevated plasma levels of procoagulant factors, such as FVIII or prothrombin, enhanced thrombin generation and reduced the amount of TF required for the initiation of thrombin formation. Conversely, elevated levels of the coagulation inhibitor antithrombin increased the TF-threshold.

Conclusions: Our findings support a mediating role for the TF-threshold in the association between high procoagulant factor levels and the risk for VTE. Furthermore, elevated levels of anticoagulants may have a protective effect on the development of VTE.

Keywords

- Tissue factor, TF, F3, CD142, TF, TFA
- Factor VIII, FVIII, F8, AHF, DXS1253E, HEMA
- Antithrombin, AT, SerpinC1, AT3, AT3D, ATIII, THPH7
- Venous thromboembolism, VTE, DVT, VT, PE

Highlights

- A minimum amount of tissue factor (TF) is required to initiate coagulation
- This TF-threshold is subject to inter-individual differences
- The TF-threshold decreases with increasing factor VIII and prothrombin concentrations
- The TF-threshold increases with increasing antithrombin concentrations
- The altered sensitivity of plasma for TF may affect the occurrence of VTE

Introduction

Hemostasis is tightly regulated by pro- and anticoagulant pathways. Any irregularity in these regulatory pathways may shift the balance to a hypercoagulable state, which in turn could lead to venous thromboembolism (VTE). Elevated levels of the procoagulant proteins factor VIII (FVIII) and von Willebrand factor (VWF) are associated with a moderate to high risk for VTE [1]. For instance, individuals with FVIII levels ≥150 IU/dL have a 6-fold increased risk for VTE relative to those with FVIII levels lower than 100 IU/dL [1]. Conversely, reduced plasma levels (≤85 IU/dL) of the anticoagulant antithrombin (AT) are also associated with VTE risk [2]. However, the exact molecular mechanism by which these altered pro,- and anticoagulant protein levels modulate the VTE risk is incompletely understood.

Several studies have aimed at uncovering the relationship between factor levels and VTE occurrence. *In vitro* methods in which the plasma conditions can be manipulated are commonly used, such as the calibrated automated thrombogram (CAT). The CAT is utilized to study the plasma potential to form thrombin, thereby demonstrating the coagulant potential and hypo- or hypercoagulability of the plasma sample. Employing CAT analysis, it has been shown that increased procoagulant factor levels including FVIII can induce a hypercoagulable plasma state [3].

Tissue factor (TF) is the physiologically relevant initiator of coagulation. While TF is mainly found in the subendothelial tissue, other sources of TF have been defined [4]. For example, TF is found on microparticles that are released from various cell types such as endothelial cells, monocytes, and cancer cells [5, 6]. The release of these microparticles is associated with cytokine stimulation and various diseases including cancer, myocardial infarction, and thrombosis [5, 6]. However, TF-expressing microparticles also circulate in very low concentrations in the plasma of healthy individuals [7]. Given that the constant exposure to these minute concentrations of TF does not seem to initiate full-blown clotting, this may imply that a specific TF concentration is required to trigger coagulation. This is

supported by previous studies using purified component coagulation systems demonstrating that significant thrombin generation follows when surmounting the factor VIIa-TF treshold [8]. Here, we assess the hypothesis that altered coagulation factor levels affect this so-called TF-threshold, thereby inducing a hypercoagulable state. As such, this might explain the association between altered coagulation factor levels and an increased risk for VTE.

Materials and Methods

Materials

Calibrator and fluorescent substrate (FluCa) were from Thrombinoscope (Maastricht, the Netherlands). Plasma-derived prothrombin, factor IXa, and antithrombin were from Haematologic Technologies (Essex Junction, VT, US). Recombinant tissue factor (TF) was from Siemens Healthcare Diagnostics (Innovin; Marburg, Germany). The concentration of TF was determined using the ZYMUTEST total tissue factor enzyme-linked immunosorbent assay (ELISA) (Hyphen Biomed, Neuville-sur-Oise, France). Factor VIII (Aafact) was from Sanquin (Amsterdam, the Netherlands). Recombinant wild-type human factor X (FX) and factor Xa (FXa) were obtained as described previously [9]. Antithrombin was from Haematologic Technologies (Essex Junction, VT, USA). Small unilamellar phospholipid vesicles (DOPC:DOPS) composed of 75% (w/w) 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 25% (w/w) 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) (Avanti Polar Lipids, Alabaster, AL, USA) were prepared as described previously [9]. Paired antibodies to determine factors II, IX, X, XI, and antithrombin (AT) antigen were obtained from Cedarlane (Burlington, Canada). Plasma factor V (FV) antigen was measured essentially as described, using the FV light chain-directed monoclonal antibody V-6 as coating antibody and biotinylated V-39 against the FV heavy chain as conjugate [10]. Plasma factor VIII (FVIII) activity was assessed employing the HemosIL FVIII assay using the ACL TOP 700 analyzer (Instrumentation Laboratories, Bedford, MA, USA). Factor VIIIa cofactor activity was determined using a factor X activation assay, in which assay mixtures containing factor IXa (90 nM) and PCPS (20 µM) in 20 mM Hepes, 0.15 M NaCl, 5 mM CaCl2, 0.1% polyethylene glycol 8000, pH 7.5 (assay buffer) were incubated for 5 minutes at 25°C before non-activated FVIII (0.5-1.5 U/ml) was added. The reaction was initiated with FX (170 nM). At various time points (0-5 minutes), aliquots (10 µl) of the reaction mixture were quenched by mixing with 90 µl of 20 mM Hepes, 0.15 M NaCl, 50 mM EDTA, 0.1% polyethylene glycol 8000, pH 7.5. Quenched samples were further diluted (2-fold) in the same buffer and initial

velocities of Spectrozyme FXa (250 μM; Sekisui Diagnostics) hydrolysis were determined. Measured rates were related to the concentration of FXa from the linear dependence of initial velocity on known concentrations of FXa determined in each experiment. Thrombin-specific peptidyl substrate hydrolysis was measured in assay buffer using 100 μM S2238 (Instrumentation Laboratories, Bedford, MA, USA) and initiated with prothrombin (8 nM – 2.33 μM). Molecular weights and extinction coefficients ($E_{0.1\%}$, 280 nm) of the various proteins used were taken as follows: prothrombin, 72,000 and 1.47; FX, 59,000 and 1.16; FXa, 46,000 and 1.16; FIXa, 45,000 and 1.4; and AT, 58,000 and 0.62.

Plasma characteristics

Normal human pooled plasma from 19 healthy donors was obtained from Sanquin and used as standard for coagulation assays and ELISAs. Factor XII (FXII)-deficient plasma of two FXII-deficient individuals was obtained from George King Bio-Medical, Inc. (Overland Park, KS, USA). The plasma levels of factors II (90-93%), V (90-95%), VIII (75%), X (110%), XI (100%), and antithrombin (120-125%) were within the normal range, while those of factor IX (140-150%) were slightly elevated as compared to normal pooled plasma.

Thrombin generation assay

The thrombin generation assay was adapted from protocols earlier described [11]. Briefly, TF was relipidated with phospholipids (DOPC:DOPS 75:25, 20 μM final) for 1 hour at 37°C in a buffer containing 25 mM Hepes, 175 mM NaCl, and 5 mg/ml BSA (pH 7.5). Various concentrations of TF (0-15000 fM final concentration) or calibrator were incubated for 10 minutes at 37°C together with plasma (67% v/v). Coagulation factor levels of the procoagulant FVIII and the anticoagulant AT were elevated by adding an additional 50 U/dL (150%) or 100 U/dL (200%) of the purified proteins to the plasma. Coagulation was initiated

by the addition of fluorescent substrate (FluCa) in a calcium-containing buffer. Fluorescence was measured every 20 seconds for 120 minutes at 37°C in a Fluoroskan Ascent fluorometer (ThermoLabsystem, Helsinki, Finland), and the results were corrected for the calibrator using the Thrombinoscope 5.0 software. The lag time, thrombin peak (peak), time to thrombin peak (ttpeak), and the ETP (the area under the thrombin generation curve) were determined in three individual experiments each performed in duplo. Please note that the datasets shown in the figures are derived from experiments performed on the same day. The measurements were considered background when the software-determined cut-off values were not met (cut-off values: lag time <45 minutes, ttpeak <90 minutes, peak >5 nM), and the ETP was set to 0 nM.min. For those analyses in which little to no detectable thrombin was formed, the slope of the crude fluorescence trace at 100-120 min. was determined, which represents the formation of the alpha-2-macroglobulin-thrombin complex and is proportional to the concentration of thrombin, as the substrate consumption and the inner filter effect do not play a significant role under these conditions [12].

Results

Establishing the tissue factor-threshold

While previous research has shown the presence of a TF-threshold in a purified component system [8], here we test the hypothesis that a minimal concentration of TF is required for the initiation of coagulation by direct assessments in plasma. We first set out to determine this TF-threshold under normal conditions. To do so, we monitored thrombin generation initiated by various concentrations of TF (0-15 pM) in FXII-deficient plasma. Factor XII-deficient plasma was used to avoid any contact pathway-mediated thrombin formation, which would hamper data interpretation. Addition of corn trypsin inhibitor (CTI) to exclude effects of traces of activated FXII did not affect the thrombin generation profiles (data not shown). As anticipated, no thrombin generation was observed in the absence of TF (Figure 1). Whereas the addition of minute amounts of TF (50-200 fM) did not result in detectable thrombin formation, substantial thrombin was generated in the presence of higher TF concentrations (300-15000 fM).

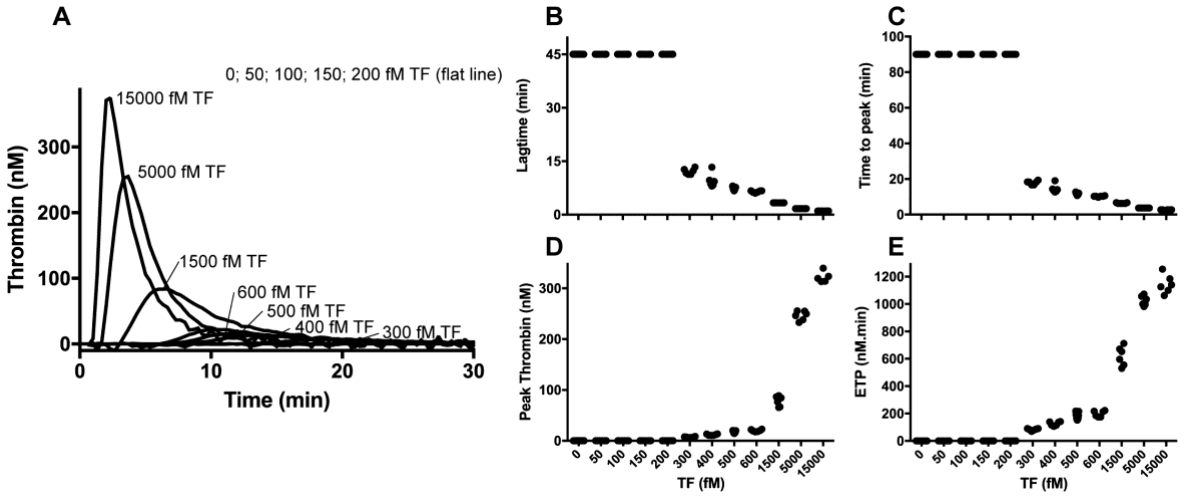

Figure 1. Thrombin generation initiated by increasing concentrations of tissue factor. Thrombin generation was initiated using a TF / DOPC:DOPS (0-15000 fM / 20 µM) mixture in FXII-deficient plasma ('plasma A') and was monitored and analyzed as described in

‘Materials & Methods’. From the thrombin generation profiles (panel A), the lag time (panel B), ttpeak (panel C), peak thrombin (panel D), and ETP (panel E) were determined and plotted versus the TF concentration. The data are derived from individual experiments performed on the same day and represent three experiments each performed in duplo.

To study the TF-threshold in further detail, the raw fluorescence traces representing thrombin formation initiated with 0-300 fM of TF were analyzed for the velocity of alpha-2-macroglobulin-thrombin complex formation, which is proportional to the concentration of thrombin generated at these very low TF concentrations [12]. Using this method, thrombin formation was observed upon TF-triggers as low as 50 fM (Figure 2). However, TF concentrations of 50-100 fM triggered thrombin generation in some, but not all of the experiments. This on-off effect shows that at these very low TF concentrations thrombin is not generated in a consistent manner, suggesting that the system is very sensitive to minor changes. Higher TF concentrations (150-300 fM) resulted in thrombin generation in all experiments. Collectively, these raw data indicate that for consistent thrombin formation to occur in this system >100 fM and ≤150 fM of TF is required. A slightly different threshold was found when the processed data was used (>200 fM and ≤300 fM, figure 1). Since both methods result in comparable thresholds and the raw, unprocessed data is not reliable at high TF concentrations (up to 15 pM in this paper), the processed data will be used in the remainder of this paper.

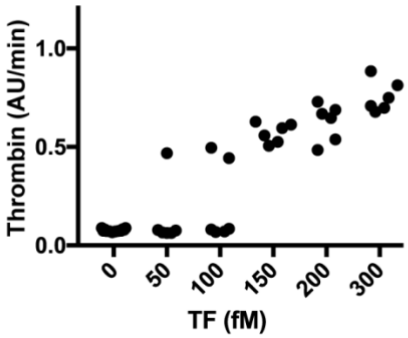

Figure 2. Thrombin generation initiated by 0-300 fM of tissue factor. Thrombin generation was initiated using a TF / DOPC:DOPS (0-300 fM / 20 µM) mixture in FXII-deficient plasma and was monitored as described in 'Materials & Methods'. The slope of the crude fluorescence trace at 100-120 min. was determined and plotted versus the TF concentration. The data are derived from individual experiments performed on the same day and represent three experiments each performed in duplo.

Shifting the tissue factor-threshold with altered coagulation factor levels

The plasma levels of coagulation FVIII are strongly associated with VTE [1]. However, the mechanism by which high FVIII levels might increase the thrombotic risk has not been identified thus far. Therefore, we studied the TF-threshold under conditions with normal and elevated plasma levels of FVIII: 100%, 150%, and 200% of FVIII, corresponding to 75 IU/dL, 125 IU/dL and 175 IU/dL, respectively. These FVIII concentrations are within the same range as those observed in both VTE patients and healthy individuals [13]. While no lag time, ttpeak, thrombin peak, or ETP could be detected in plasma with 100% FVIII following initiation with 200 fM TF, substantial thrombin was generated in the presence of elevated FVIII levels (150 and 200% FVIII; Figure 3A-C) following the same TF trigger, resulting in detectable parameters of thrombin generation. Furthermore, in the presence of higher concentrations of TF (500-600 fM) thrombin generation with FVIII levels of 150% or 200% was hallmarked by a dose-dependent increase in ETP (1.7-fold and 1.9-fold, respectively)

relative to that observed with 100% FVIII present. An even larger increase in peak was observed for higher concentrations of TF in the presence of 150 and 200% FVIII as compared to 100% FVIII (data not shown). These data corroborate with previous findings [3, 14]. The lag times and ttpeak values were not affected by increasing FVIII levels with higher TF triggers (500-600 fM, data not shown). While these findings indicate that elevated plasma levels of FVIII shift the TF-threshold and enhance the sensitivity of plasma coagulation for a TF-trigger, the mechanism explaining this remains elusive. Control experiments using a purified component factor X conversion assay revealed that the non-activated purified plasma-derived FVIII used in these studies was capable of generating 0.4 $\pm$ 0.2 nM factor Xa/min/Unit FVIII, which may be due to trace amounts of partially activated forms of FVIII and/or co-purified proteases. It cannot be ruled out that this contributed to some extent to the observed thrombin generation.

To further study the effect of elevated procoagulant levels on the TF-threshold in thrombin generation, we next focused on prothrombin, given that elevated prothrombin levels are also associated with the risk of developing VTE [15] and affect on thrombin generation to a large extent [16]. Plasma prothrombin levels of 100%, 150%, and 200% were studied in plasma from a second FXII-deficient individual (‘plasma B’) and correspond to 90 IU/dL, 140 IU/dL and 190 IU/dL, respectively. While the TF-threshold identified in this plasma was observed to correspond to lower TF triggers (5 fM > TF ≤ 50 fM; Figure 3D), an on-off effect was observed for thrombin generation initiated with these very low TF concentrations similar to previous findings. Furthermore, higher amounts of thrombin were generated in plasma B relative to plasma A following the same TF trigger. This is in agreement with earlier observations on high inter-individual variability in thrombin generation following (low) TF triggers [17, 18]. Consistent with our findings for FVIII, increasing the plasma levels of prothrombin reduced the lower limit of the TF-threshold range, from 5 fM to 1.5 fM of TF (Figure 3E,F), and thus shifting the TF-threshold. In addition, at higher TF concentrations (150-500 fM TF) an 1.4-fold increase in the ETP was observed for 200% prothrombin

relative to normal prothrombin levels (Figure 3F). At these TF concentrations, peak thrombin was also increased in the presence of higher prothrombin levels, which is in accordance with previous research. [3, 16]. Control experiments using a peptidyl substrate conversion assay confirmed that the plasma-derived prothrombin used in these studies did not contain detectable levels of thrombin. Collectively, these findings indicate that elevated levels of the procoagulants FVIII and prothrombin lower the TF-threshold.

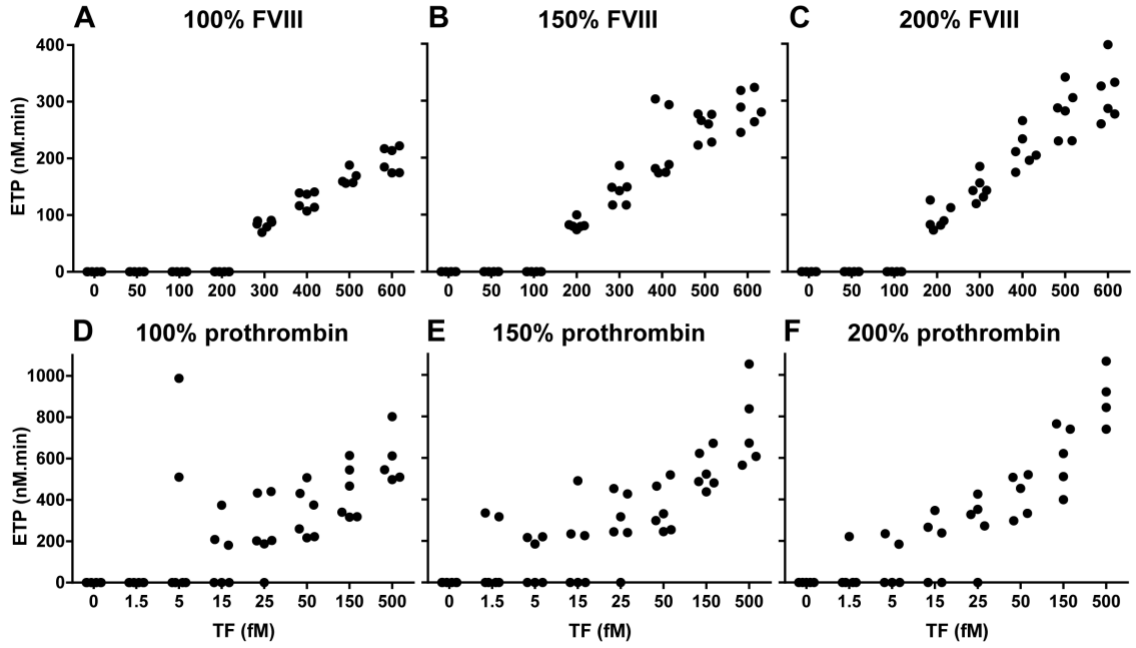

Figure 3. Thrombin generation with increasing plasma levels of procoagulant factors. Panels A-C: Thrombin generation was initiated using a TF / DOPC:DOPS (0-600 fM / 20 µM) mixture in FXII-deficient plasma A in the presence of normal (100% FVIII, panel A) or elevated (150% or 200% FVIII, panels B,C) levels of FVIII and monitored and analyzed as described in 'Materials & Methods'. Panels D-F: Thrombin generation was initiated using a TF / DOPC:DOPS (0-500 fM / 20 µM) mixture in FXII-deficient plasma B in the presence of normal (100% prothrombin, panel D) or elevated (150% or 200% prothrombin, panels E,F) levels of prothrombin and monitored and analyzed as described in 'Materials & Methods'. The ETP was plotted versus the TF concentration. The data are derived from individual

experiments performed on the same day and represent three experiments each performed in duplo.

After studying the effect of elevated procoagulant factors, the TF-threshold was assessed in the presence of normal (100%; 185 µg/mL) and increased (150 and 200%; 260 µg/mL and 335 µg/mL, respectively) levels of the anticoagulant antithrombin (AT), which has been demonstrated to largely mediate thrombin generation [19]. Here we observed a dose-dependent increase in the amount of TF required to initiate thrombin generation upon increasing the plasma concentrations of AT (Figure 4A-C). Furthermore, at the highest TF concentrations employed (5000-15000 fM) the elevated plasma levels of AT resulted in an up to 3- to 4-fold decrease in ETP and peak, respectively, which is consistent with previous findings [19]. The lag times and the ttpeak values were slightly prolonged relative to normal AT levels (data not shown). The same assessment was performed in FXII-deficient plasma B and resulted in similar findings (Figure 4D-F), further supporting the observation that increased plasma levels of AT attenuate the sensitivity of plasma to the TF-dependent initiation of thrombin generation.

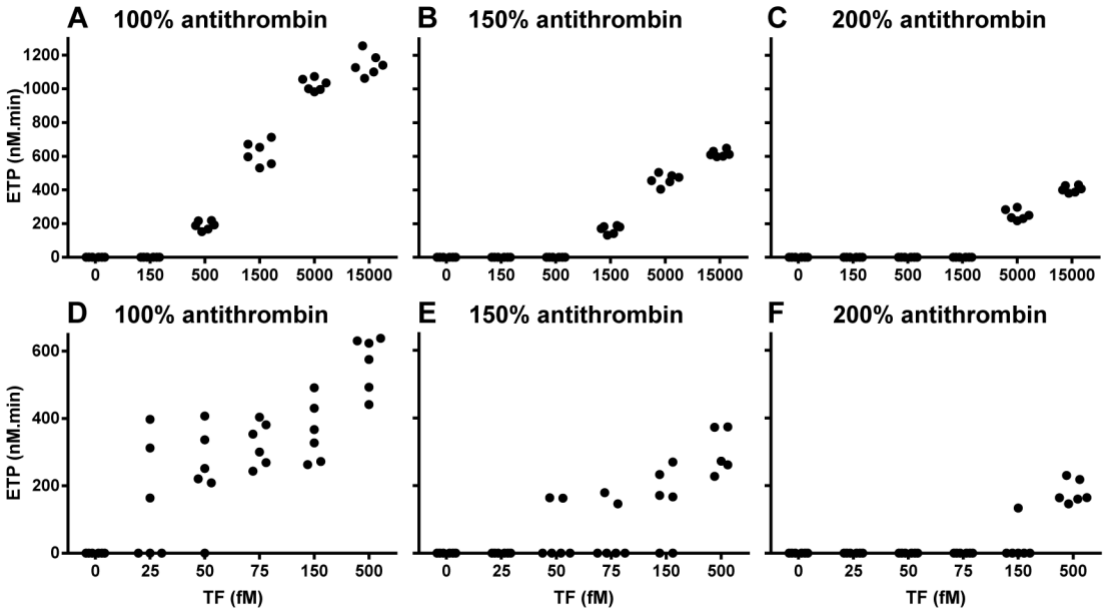

Figure 4. Thrombin generation with increasing plasma antithrombin levels. Thrombin generation was initiated using a TF / DOPC:DOPS (0-15000 fM / 20 µM) mixture in FXII-deficient plasma A (panels A-C) or plasma B (panels D-F) in the presence of normal AT levels (100% AT, panel A) or with elevated AT levels (150% AT, panel B; or 200% AT, panel C) and monitored and analyzed as described in 'Materials & Methods'. The ETP was plotted versus the TF concentration. The data are derived from individual experiments performed on the same day and represent three experiments each performed in duplo.

Discussion

In the present study we confirmed the existence of a TF-threshold for the formation of thrombin. Whereas this threshold was previously identified at 10-20 pM TF [8], we observed that very low levels of TF, in the fM range, were required to trigger thrombin generation. The differences between these findings might be explained by the definition of the threshold: we defined this as the change from no thrombin formation to the start of thrombin formation, while van ‘t Veer and Mann determined the change from slow to explosive thrombin generation [8]. Furthermore, the use of a purified component-based system versus our studies in plasma might also have affected the results. While our experiments in FXII-deficient plasma imply the presence of this so-called TF-threshold, the exact concentration of TF needed to initiate thrombin generation varies between individual plasmas, from 5 fM > TF ≤ 50 fM to 100 > TF ≤ 150 fM (Figure 2, Figure 3D). We speculate that this, among others, may be due to the varied presence of TF-expressing microparticles in the plasma. Furthermore, our findings corroborate previously observed substantial inter-individual variability in TF-initiated thrombin generation [17, 18].

It is important to note that the TF-threshold observed here will not be reached under normal conditions, since the concentration of blood-borne active TF in healthy individuals is estimated to be lower than 20 fM [20]. In disease states, however, the TF-threshold may be surpassed since levels up to 0.9 pM TF activity were measured in patients with acute coronary syndrome [21].

To assess how altered coagulation factor levels affect this so-called TF-threshold, we demonstrated that elevated plasma levels (up to 200%) of either the procoagulant FVIII or prothrombin decrease the TF-threshold (Figure 3). Very low TF concentrations that were not able to generate thrombin at physiological FVIII or prothrombin levels initiated detectable thrombin formation in the presence of 150 of 200% FVIII or prothrombin. At higher TF concentrations, the increase in FVIII and prothrombin resulted in an increase in the ETP and

peak thrombin parameters, which is in agreement with findings of Machlus and colleagues [3]. Allen and co-workers also observed an increase in ETP and thrombin peak with high prothrombin, while elevated FVIII resulted in an increase in thrombin peak only [16]. This discrepancy might be due to the use of a purified component system without addition of anticoagulation factors by Allen *et al.*, versus plasma assays in our set-up. The shift in TF-threshold may indicate that conditions of high FVIII and/or prothrombin prime the plasma towards the action of TF, which may render it more susceptible to VTE. Interestingly, plasma concentrations of FVIII or prothrombin similar to or higher than 200% have been detected in individuals. More specifically, levels up to 552 IU/dL FVIII and up to 190 U/dL prothrombin have been described for patients with VTE [13, 22]. Conversely, increased plasma levels of the anticoagulant AT increased the TF-threshold, thereby reducing the plasma sensitivity to the actions of TF. As such, these findings would support a mechanism for the link between altered coagulation factor levels and an increased risk for VTE, as the former impact the TF-threshold thereby potentially inducing a hypercoagulable state. Further studies are required to examine whether this mechanism contributes to the development of VTE.

A limitation of this study is that not all experiments could be repeated in plasma from both individuals due to limited plasma availability. However, we observed the same trends for both plasmas, although the TF-threshold varied between the individual plasmas. Whether this is due to the presence of varying levels of TF-bearing microparticles remains to be determined. Furthermore, in this study we used platelet-poor plasma, which may affect the results. However, a systematic comparison of platelet-free and platelet-rich plasma by Wolberg and colleagues demonstrated no difference in the peak height and ETP [3]. Furthermore, the protein C pathway was not included in our set-up. While the group of Wolberg further demonstrated that thrombomodulin slightly prolonged the lag time and reduced both the thrombin peak and ETP following triggering with 1 pM TF [3], the effect of thrombomodulin and the action of the protein C pathway at very low TF concentrations remains to be determined.

Based on our observations, we conclude that in order for thrombin generation to be initiated, the TF-threshold needs to be met. The exact TF concentration that is required to meet this threshold seems to be subject to inter-individual variation. Elevated plasma levels of procoagulant factors, such as FVIII or prothrombin, enhance thrombin generation, thereby reducing the amount of TF required for the initiation of thrombin formation. Conversely, elevated levels of the coagulation inhibitor AT increase the TF-threshold. As such, our findings support a mediating role for the TF-threshold in the association between high procoagulant factor levels and the risk for VTE. The results also indicate that elevated levels of anticoagulants may have a protective effect in the development of VTE.

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