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Blood coagulation kinetics: high throughput method for real-time reaction monitoring

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Summary

A high throughput 384-well plate assay of blood function in 60 µl reactions with the fluorogenic thrombin substrate, boc-VPR-MCA, allowed for real-time monitoring of coagulation under a diverse set of reaction conditions. Using recalcified, citrated whole blood diluted 3-fold with corn trypsin inhibitor (to block Factor XIIa), addition of 0 to 13.8 pM of tissue factor (TF) reduced the time of maximal rate of thrombin production (T_{max}) from 45 min to 11 min. Over this range of TF, T_{max} was reduced from 35 min to 6 min by co-addition of 10 nM convulxin to activate platelets via GPVI. The maximal rate of thrombin production at T_{max} was not a function of exogenously-added TF, Va, or reVIIa, but increased 30% with added convulxin. Addition of 0.07 to 0.7 pM TF along with convulxin produced small, but detectable reductions in T_{max}. Addition of up to 0.67 nM reVIIa reduced T_{max} by up to 53% in the range of 0.7 to 7 pM TF. Interestingly, platelet factor 4 (2.7 µM) caused a prolongation of T_{max} from 45 min to 78 min at 0 TF, while protamine (1.8 µM) reduced T_{max} to 30 min at 0 TF. Finally, combinatorial reaction studies with exogenously-added ADP, histamine, fMLP, indomethacin, anti-CD18, and fibrinogen revealed no unusual synergies amongst the agents, but demonstrated a striking procoagulant activity of added fibrinogen, due to protease contaminants in the "purified" fibrinogen. This high throughput approach allowed automated profiling of blood (50 reactions/ml of blood) to generate large data sets for testing cellular-proteomic kinetic models, screening drug interactions, and potentially monitoring subtle changes in the functional phenotype of a patient blood sample.

Keywords

Fluorescence, thrombin, kinetics, platelet factor 4

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Introduction

Advances in robotic liquid handling and fluorescence plate reading allow the assembly of over 10^6 individual reactions for the purposes of high throughput screening (HTS) of combinatorial chemical libraries in well volumes as small as 1 to 10 µL. Similarly, protein microarrays, microfluidic devices, and Systems Biology analytical tools are beginning to find use in clinical research and diagnostics. As a reactive biological fluid, blood (and its fractions) is fully amenable to liquid handling and fluorogenic substrate probes for real-time monitoring of protease function (1, 2, 3, 4), including microarray reactions with nanoliter volumes (5). Additionally, kinetic models of tissue factor-triggered clotting are quite advanced (6-8), but do not incorporate aspects of intracellular metabolism. Unique to blood as a proteomic system, however, is the hemodynamic regulation of transport physics and receptor-mediated adhesion (7, 9, 10).

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Thrombin generation initiated by tissue factor can be divided into two phases (11). The *initiation phase* that occurs after the addition of TF can be characterized by the appearance of sub-nanomolar amounts of Xa and thrombin. This process continues with the accumulation of Va, VIIa, VIIIa, IXa, and XIa. During the initiator phase the main inhibitor is TFPI (12). After the initiation phase, when certain levels of each of the procoagulant proteins are generated, the *propagation phase* is characterized by an explosive increase in thrombin generation to nanomolar (or micromolar) levels at which time fibrin becomes polymerized.

In recent years, the regulatory complexity and triggering mechanisms in blood treated with exogeneously-added tissue factor has been explored and refined (13) without the confounding effects of contact activation (14, 15). However, gel electrophoresis and western blotting of discrete samples taken from clotting reactions do not allow high throughput automation or realtime measurement. Presently, high throughput techniques are starting to be developed (16-18). We have developed an approach of tissue factor titration combined with liquid handling and approaches from combinatorial chemistry and fluorescence high throughput screening, to help scan and identify blood coagulation interactions that have not been distinguished before. The current study evaluated the potential to run 100s (or even 1000s) of reactions with a small individual blood sample using a 384-well plate assay and a fluorogenic thrombin substrate.

Material and methods

Sodium citrate solution, N-Formyl-Met-Leu-Phe (fMLP), adenosine 5'-diphosphate (ADP), indomethacin, and histamine (Sigma-Aldrich, St. Louis, MO), thrombin, platelet factor 4, antibodies for factor XI (PF4, Enzyme Research Labs, South Bend, IN), corn trypsin inhibitor (CTI), factor Va, and antibodies for factor IX (Haematologic Technologies, Essex Junction, VT), recombinant lipidated human tissue factor (TF), fibrinogen, and antibodies for factor VII/VIIa and tissue factor (American Diagnostica, Stamford, CT), calcium chloride (Fisher Scientific, Pittsburgh, PA), convulxin (Centerchem, Norwalk, CT), protamine sulfate (American Pharmaceutical Partners, Los Angeles, CA), recombinant factor VIIa (NovoSeven, Novo Nordisk, Bagsvaerd, Denmark), D-Phe-Pro-Arg-chloromethylketone, HCl (PPACK) and H-Glu-Gly-Arg-CMK (GGACK) (Calbiochem, San Diego, CA) were stored following the manufacturers' recommendations. The fluorogenic substrate for thrombin, boc-Val-Pro-Arg-methylcoumarin-7-amide (boc-VPR-MCA), was obtained from Bachem Bioscience (King of Prussia, PA). Unless otherwise noted, all dilutions were made using Hank's Balanced Salt Solution (HBSS, 1X, without calcium chloride, magnesium chloride, magnesium sulfate, or phenol red; Invitrogen Corp., Carlsbad, CA). Human blood was collected from healthy adult donors and anticoagulated with sodium citrate (9 parts blood to 1 part sodium citrate). Corn trypsin inhibitor was added at a final concentration of 50 µg/ml to block the intrinsic pathway by blocking factor XIIa (19).

High throughput 384-well coagulation assay

Reactions were combined in Costar 384-well plates (Fisher Scientific) using a Perkin-Elmer (Boston, MA) Multiprobe II liquid handling system with 4 variable spacing pipetting heads. Each reaction well of the 384-well plate contained a final reaction volume of 60 µL. To begin, 10 µl of lipidated TF/HBSS with a final concentration of TF from 0 to 14 pM was added to each well. Next, a volume of 10 µl of HBSS containing a defined mixture of procoagulant/anticoagulant agents or buffer alone was added to the wells. Then, 20 µl of CTI-treated, citrated whole blood was delivered into all wells, followed immediately by addition of 20 µl of 30 mM calcium chloride and 30 µM boc-VPR-MCA solution to recalcify each reaction (final concentrations: 10 mM $CaCl_2$, 10 µM boc-VPR-MCA, 16.7 µg/ml of CTI in each 60 µl of 3-fold diluted citrated whole blood). A single loading step of a reagent to the entire 384-well plate required 2.5 min on average. A time of < 1 min was required to transfer the filled plated to a fluorescence plate reader to initiate the first scan. Fluorescence (EX: 390 nm; EM: 460 nm) from cleaved 7-amino-methylcoumarin (AMC) was detected using a ThermoLabsystems Ascent Fluoroskan FL (Franklin, MA) plate reader. The Multiprobe II liquid handling system has a typical operational CV of < 3% for 10 µl manipulations of whole blood. For blood pipetting, the Multiprobe II was set for slow aspiration (10 µl/sec), fast dispensing (900 µL/sec), and blowout (20 µl) with single dispensing for each aspiration to handle the high surface tension of blood. Each single reaction condition was repeated in replicates on the same plate from n = 3 to n = 12 times using a single donor's blood. Any unused wells on the plate were filled with 60 µl of distilled water to help decrease evaporation and thermal gradient effects. To prevent spatial differences between wells, the reactions and their repeats were placed in a spiral conformation from the center of the plate. A volume of 1 ml citrated whole blood allowed 50 reactions with a 10 ml blood draw amply sufficient for 384 reactions on a single plate.

Thrombin detection and data analysis

After calcium chloride and boc-VPR-MCA addition, the plate was moved to the Ascent Fluoroskan FL where it was shaken for 20 sec at 1200 rpm at a 1 mm diameter and scanned at 37 °C every 30 seconds for 3 hr. With full conversion of boc-VPR-MCA after the propagation phase, each well reached maximal fluorescence (F_{max}), which then slowly declined (~ 20% per hr) due to photobleaching and chemical quenching of the cleaved fluorescent product AMC over the scanning period. Using each

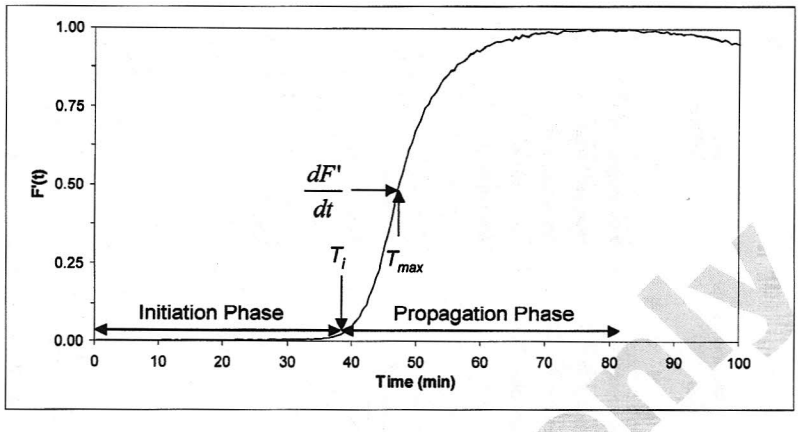

Figure 1: Sample thrombin generation curve for the normalized fluorescence $F'(t) = (F(t)-F_o)/(F_{max}-F_o)$. The initiation time ($T_i$) at $F'(T_i) = 0.03$ separates the initiation phase and the propagation phase. The time of maximum rate (T_{max}) and the maximum rate (dF'/dt_{max}) define the fastest rate of thrombin generation.

well's lowest fluorescence (F_o), the data was normalized between 0 and 1 using $F'(t) = (F(t)-F_o)/(F_{max}-F_o)$ to help control well-to-well liquid handling and detection variation. Analysis of the normalized thrombin substrate conversion traces included extraction of: (a) the *initiation time* T_i defined as the time when $F(T_i) = 1.03(F_o)$ or $F'(T_i) = 0.03$, (b) the time of maximum rate of thrombin production (T_{max}); and (c) the maximum rate of thrombin production (dF'/dt_{max}) (Fig. 1). The rate of thrombin production for each point is determined by fitting a curve to the point in question (excluding the first and last data points) and its adjacent points, and determining the derivative at that point. T_{max} and dF'/dt_{max} are determined when the derivative (dF'/dt) is maximal and does not necessarily correspond to the time when substrate is 50% consumed. The substrate hydrolysis rate is linearly dependent on [thrombin] only at low levels of conversion. T_{max} and dF'/dt_{max} are data analysis tools to define when thrombin production becomes explosive. After T_{max}, the derivative becomes zero due to substrate consumption and ample amounts of active thrombin may reside in the clotting sample. This was proven by addition of fresh substrate at 25 min beyond T_{max} that was rapidly cleaved (data not shown). Statistical comparisons of the data were made using the unpaired Student's t-test.

Results

Tissue factor titration

At each TF concentration, 12 separate wells were used to illustrate the variability in the coagulation reactions, especially when TF was not added. Without added TF (Fig. 2A), the precise time of onset of the propagation phase was highly variable. This extreme variability at 0 TF was not likely the result of small liquid handling errors (< 3%), but rather the nature of the extreme nonlinear sensitivity of amplifying protease cascades to the initial trigger levels (see *Discussion*) and was observed time and again with multiple donors. Similarly, addition of 1.38 pM of TF (Fig. 2B) did not produce any striking enhancement of clotting under these conditions, likely due to the action of antithrombin III or tissue factor pathway inhibitor (TFPI). In contrast, 2.08 pM TF caused a shortening of the initiation phase and the variability of the initiation times was reduced (Fig. 2C). A level of 13.8 pM TF caused a shorter initiation phase of less

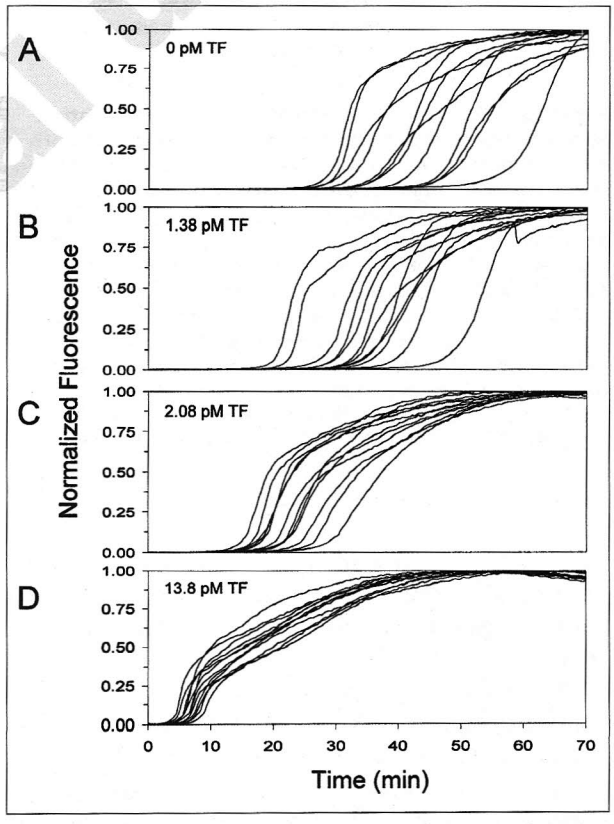

Figure 2: Thrombin generation as indicated by boc-VPR-MCA cleavage as a function of TF addition to CTI-treated 1:3 diluted whole blood. Each plot represents n = 12 experiments under the same conditions using the same donor (for all 48 reactions shown) with 0 nM TF (A), 1.38 pM TF (B), 2.08 pM TF (C), and 13.8 pM TF (D).

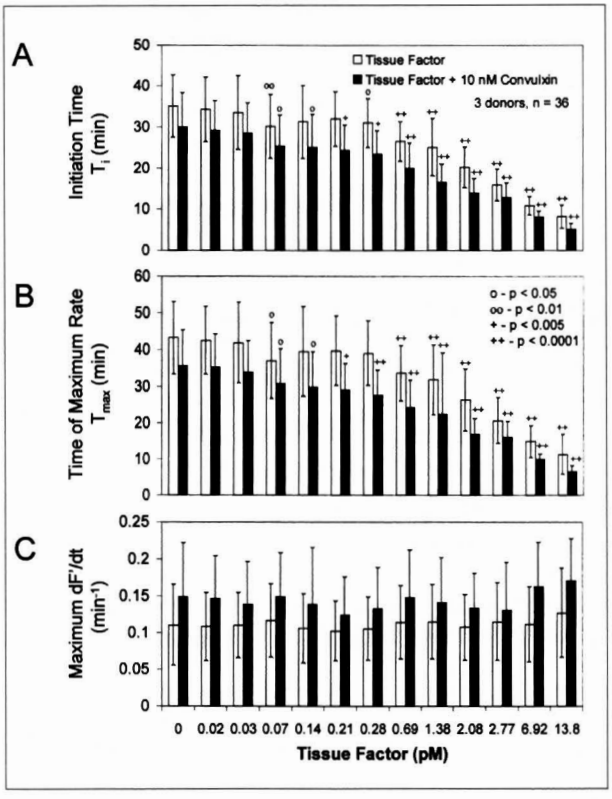

Figure 3: Statistical analysis of tissue factor titration with and without convulxin with n = 36 using 3 donors for each point. The initiation time T_i decreased as more tissue factor was added in the absence (white bars) or presence of 10 nM of convulxin (black bars), a potent platelet GPVI agonist (A). Similar enhancement of coagulation was seen with increasing TF and convulxin for the time of maximal thrombin generation (T_{max}) (B) while the maximal thrombin generation rate (dF'/dt_{max}) was not enhanced by TF but was enhanced by convulxin (C). ° $p<0.05$ for comparison to 0 pM tissue factor concentration for all figures. °° $p < 0.01$; $^{+}p < 0.005$; $^{++}p < 0.0001$. Also, from the calibration curve (Fig. 2), there was ~ 10.4 nM (1.16 U/mL) of thrombin in the absence of convulin and ~ 13.4 nM (1.49 U/mL) of thrombin in the presence of convulxin.

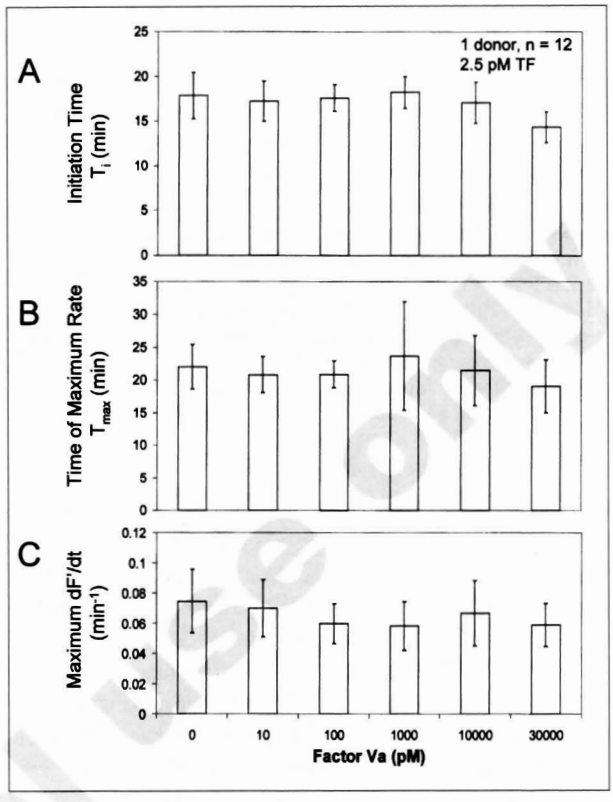

Figure 4: Effect of added factor Va on T_i, T_{max}, and dF'/dt_{max}. Varying levels of Factor Va were added to diluted (1:3) blood activated by 2.5 pM of tissue factor. In each case, Factor Va supplementation did not significantly change initiation time, time of maximum rate, or maximum rate.

than T_i = 10 min compared to T_i = 36 minutes for 0 pM TF. At 13.8 pM TF, there was much smaller variability in T_i (under ± 3 min) compared to the extreme variability seen at 0 pM TF (± 20 min).

Over 3 donors and 936 individual reactions, the average trends in T_i, T_{max}, and dF'/dt_{max} with TF titration (± 10 nM convulxin) demonstrated the role of platelet activation in clotting time analysis when using 3-fold diluted whole blood (Fig. 3). Convulxin is a potent GPVI activator (20). Defects in platelet function (e.g. GPVI signaling) would be detectable by this approach. In thrombin time testing with whole blood, it is commonly assumed that platelet activation occurs promptly after addition of high TF doses. Platelet activation by convulxin decreased T_i and T_{max} as well as increased dF'/dt_{max} over the range of the TF titration. In Figure 3A, the effects of TF can be detected at 0.28 pM ($p < 0.05$) in the absence of convulxin. With co-addition of convulxin, the effects of tissue factor activation can be clearly detected at 0.07 pM (or approximately 1.6 platelets per molecule of TF) demonstrating the sensitivity of the experimental approach as well as the enormous amplifying action of protease cascades in activated blood. Interestingly, the TF concentration did not have a significant effect on the rate of maximal thrombin generation (dF'/dt_{max}), indicative of saturation of prothrombinase formation (XaVa) on platelets activated by thrombin (formed during coagulation) or activated by convulxin.

Supplementation with Va, VIIa, PF4, or protamine

Platelets, upon activation, are known to release stores of factor V and Va (21, 22). To explore the role of various factors on T_i, T_{max}, and dF'/dt_{max}, factor Va was added to the reactions at increasing levels at constant TF concentration (2.5 pM). For each concentration, factor Va supplementation does not signifi-

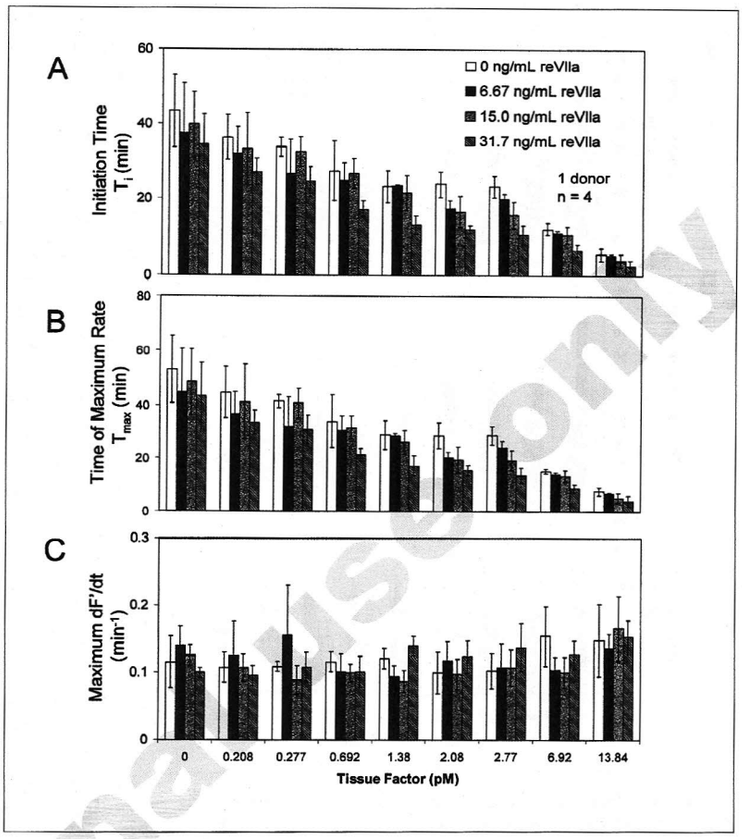

Figure 5: Effect of Factor VIIa supplementation on T_i, T_{max}, and dF'/dt_{max}. Control (white bars) contained only the factor VIIa inherent in normal blood (~ 1.67 ng/mL). Recombinant factor VIIa (reVIIa) was also added to reactions at a final concentration of 6.67 ng/ml (black bars), 15.0 ng/ml (grey bars), and 31.7 ng/ml (striped bars). At low levels of tissue factor, there was little or no change in initiation time or time of maximum rate, but at intermediate tissue factor concentrations (2.08 pM and 2.77 pM, in particular), there was a graded enhancement of clotting with increasing reVIIa.

cantly change initiation time, time of maximum rate, or the maximum rate (Fig. 4). The activity of factor Va was verified by supplementation to factor V deficient plasma (data not shown). From Figure 4, we conclude that the enhancement of dF'/dt_{max} due to convulxin stimulation (Fig. 3C) was not likely due to increased release/generation of Va, but rather reflected the effect of elevated anionic phospholipids exposure on XaVa function.

Recombinant factor VIIa (reVIIa) is used by hemophiliacs to reduce bleeding episodes (23). Normal VII levels are 500 ng/ml with ~ 1% as VIIa (5 ng/mL). Increasing amounts of reVIIa (0, 6.67, 15.0, 31.7 ng/ml final concentration) were added to 3-fold diluted normal whole blood. With 3-fold dilute normal whole blood, the concentrations added correspond to normal (0 ng/ml added), 5X, 10X, and 20X normal VIIa (6.67, 15.0, and 31.7 ng/ml reVIIa, respectively). Procoagulant effects were only detected in the presence of added TF (Fig. 5). At 0 or sub-picomolar concentrations of TF, T_i was not significantly altered by increasing reVIIa. However, at higher levels of TF, significant contributions to initiation time and time of maximum rate were seen at the highest reVIIa concentration (31.7 ng/mL) beginning at 0.277 pM TF. Contributions at the intermediate concentrations (6.67 and 15.0 ng/mL) were visible at 2.08 pM of TF. At the highest TF concentration of 13.8 pM, the procoagulant effects of reVIIa was only statistically significant at the highest concentration of reVIIa (31.7 ng/mL), with high TF concentration triggering strong blood coagulation and masking the effects of the intermediate reVIIa concentrations. For comparison, typical hemophiliac dosages at injection are about 1.26 µg/ml which would yield an equivalence of ~ 420 ng/ml or ~ 251X normal VIIa concentration in the well plate experiment with 3-fold diluted whole blood.

The cationic protein Platelet Factor 4 (PF4) is a major release product of platelets (24) with less defined procoagulant and anticoagulant activities. TF titration demonstrated (Figs. 6A and 6B) that high concentrations of PF4 (black bars) had an overall slowing effect on thrombin production even at high TF concentrations (2.77 pM). Conversely, protamine (grey bars), another cationic protein and known complement activator (25), enhanced the rate of clotting. Similar to reVIIa supplementation, increasing TF to high levels decreased the anticoagulant or procoagulant effects of PF4 and protamine, respectively.

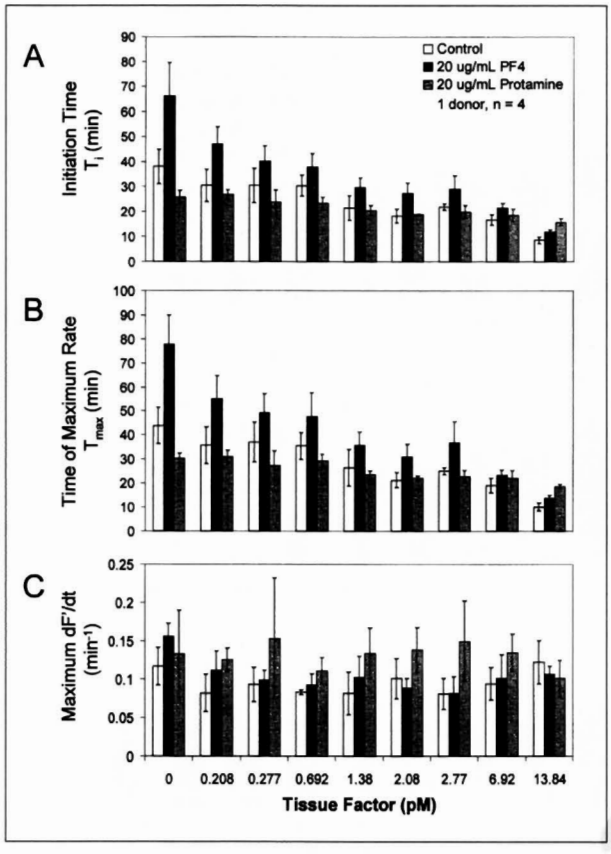

Figure 6: Platelet Factor 4 (PF4, black bars) and protamine (gray bars) supplementation on T_i (A), T_{max} (B), and dF'/dt_{max} (C) during TF titration. PF4 had an overall anticoagulant effect, while protamine promoted the rate of coagulation.

Combinatorial reaction mixtures

The use of combinatorial addition of coagulation factors, antibodies, and platelet/neutrophil agonists or inhibitors allows the creation of highly constrained kinetic data sets amenable to a full testing of kinetic models of cell and plasma activation during blood coagulation. Automated liquid handling facilitates the pre-programmed assembly of combinatorial mixtures from a set of chemical stock solutions. In a feasibility study, this combinatorial approach (Fig. 7) was conducted on a single donor's blood with no additional tissue factor, and each experimental condition was repeated four times (n = 4). Combinatorial reaction studies with exogenously-added ADP (10 μM), histamine (100 nM), fMLP (10 nM), indomethacin (100 μM), anti-CD18 (10 μg/mL), and fibrinogen (3 mg/mL) revealed no unusual synergies amongst the agents, but demonstrated a striking procoagulant activity of added fibrinogen, shown to be due to protease contaminants in the purified fibrinogen.

Further testing is shown in Figure 8. Antibodies for factors XI, IX, VII/VIIa, and TF were added to investigate the nature of coagulation with no TF added. In the absence of added TF, the addition of anti-XI had no significant effect on initiation time or maximum rate (Fig. 8). This experiment confirmed that CTI was sufficient to block the function of XIIa and that contact initiation of clotting leading to the activation of XIa by XIIa was not likely the initiator of clotting. It is difficult to fully determine the identity of the most proximal trigger(s) of clotting in the assay which could include: extremely low levels of IXa, Xa, IIa, blood-borne TF, phlebotomy-derived TF, cathepsin G, and/or other potential enzymes or cofactors. Addition of anti-IX prolonged of the initiation phase and decreased in the maximum rate. This was expected; at low TF concentrations, thrombin was generated mainly by the intrinsic Xase complex (IXa-VIIIa) (6). Also in Figure 8, data is shown for fibrinogen treated with PPACK (inhibits thrombin, VIIa, and XIa) and GGACK (inhibits Xa) and then dialyzed to remove excess PPACK and GGACK. This showed that commercial "purified" fibrinogen contains procoagulant contaminants.

Discussion

The use of robotic liquid handling and fluorogenic substrates provided for fast and high throughput assay of blood phenotype. Clearly, CTI-treated blood at 3-fold dilution coagulated in the absence of added TF, but with highly varying kinetics under replicate assay conditions (Fig. 2A). Without TF, the initial formation of Xa may be from: trace levels of Xa or IIa in drawn blood, neutrophil protease release to activate Xa via cathepsin G or elastase, vessel wall-derived TF from phlebotomy, blood-borne tissue factor (26) or VIIa-cofactor activity (27), and/or monocyte expression of TF during the incubation. While the signals in Fig. 2A are extremely variable, the initial trigger levels are probably not stochastic (i.e. less than 5 trigger molecules in some microliter-scale macroscopic volume). For example, none of the wells with 0 nM TF failed to initiate coagulation. Rather, the large variability is likely the result of the sensitivity of a highly nonlinear, amplifying system to slight variations in reaction accumulation during the initation phase. Subtle issues of variations in mixing, liquid handling, temperature, and platelet state from well to well can contribute to this high variability. With a higher concentration of trigger at 13.8 pM TF, the variability from reaction to reaction was greatly reduced (Fig. 2D). While the absolute variability in T_i decreased as TF increased, the percent coefficient of variation (%CV) remained relatively constant at ~ 18 to 32%CV

Using recalcified, citrated whole blood diluted 3-fold with corn trypsin inhibitor (to block Factor XIIa), addition of 0 to 13.8 pM of tissue factor (TF) reduced initiation time of thrombin production (T_i) from 35 min to 8 min. These values are expected from other studies (28), which predict initiation times of 4.7 ± 0.2 min for undiluted whole blood with CTI activated by approximately 5 pM TF. The 3-fold diluted whole blood produces an initiation time of approximately 13.2 min at 5 pM TF.

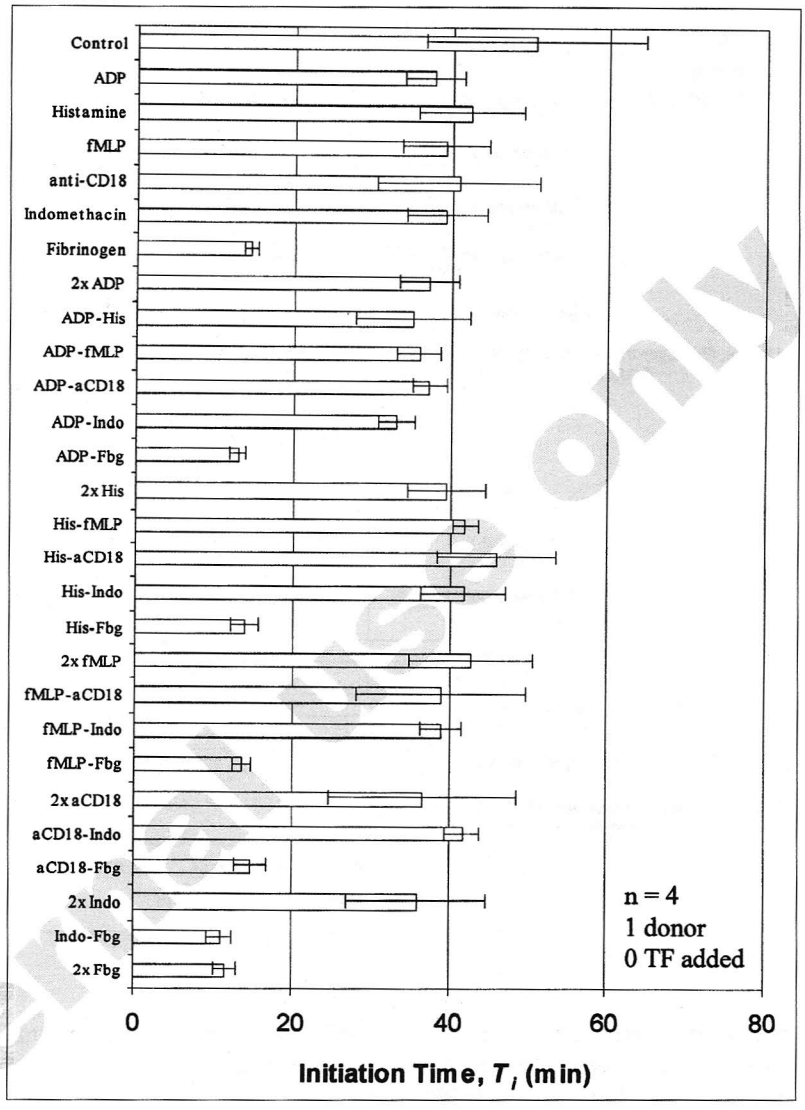

Figure 7: Effect of a combinatorial mixture of reaction modifiers on clotting kinetics without added TF. Individual components had a final concentration of 10 μM ADP, 100 nM histamine, 10 nM fMLP, 100 μM indomethacin, 10 μg/ml anti-CD18, and 3 mg/ml fibrinogen. A procoagulant activity of added fibrinogen was easily detected, potentially due to protease or cofactor contaminants in the original fibrinogen.

This technique demonstrated that the state of platelet activation at t = 0 does have an effect on the initiation phase (T_i), propagation phase (T_{max}), and rate of maximal thrombin generation (dF'/dt_{max}) in a TF-titration assay, although the convulxin effects become small at the highest level of TF added. Activation of platelets with convulxin resulted in initiation times similar to that achieved by adding ~ 0.28 pM TF without convulxin. If platelet activation with convulxin were to result in the exposure of an activity (cofactor or enzyme) and that activity were to be TF or TF-like in function, then the effect of that activity appears to be equivalent to sub-pM levels of TF. However, addition of anti-TF had no observed effect on T_i in the absence of convulxin or TF addition (Fig. 8). The maximal value of dF'/dt is potentially a property set by the activated platelet. Since anti-IX significantly reduced T_i and maximal dF'/dt, we conclude that the activated platelet makes the majority of its thrombin through IXa-VIIIa. From the anti-IX experiment (Fig. 8), it is clear that IXa was a critical "amplifier" in the assay since anti-IX was the only agent to reduce the maximal dF'/dt in our experience.

The action of reVIIa was seen to have little effect on clotting in the absence of added TF, consistent with its use in hemophilia therapy. Also, the cationic PF4 had a significant delaying effect on thrombin generation by an unknown mechanism, potentially involving PF4 complexation with anionic sites on the activated platelet. However, protamine did not mimic the action of PF4 and had a potent enhancing effect on clotting, potentially through complement activation (25). Also, "purified" human fibrinogen is a common reagent in hematological research. However its potential in promoting coagulation via

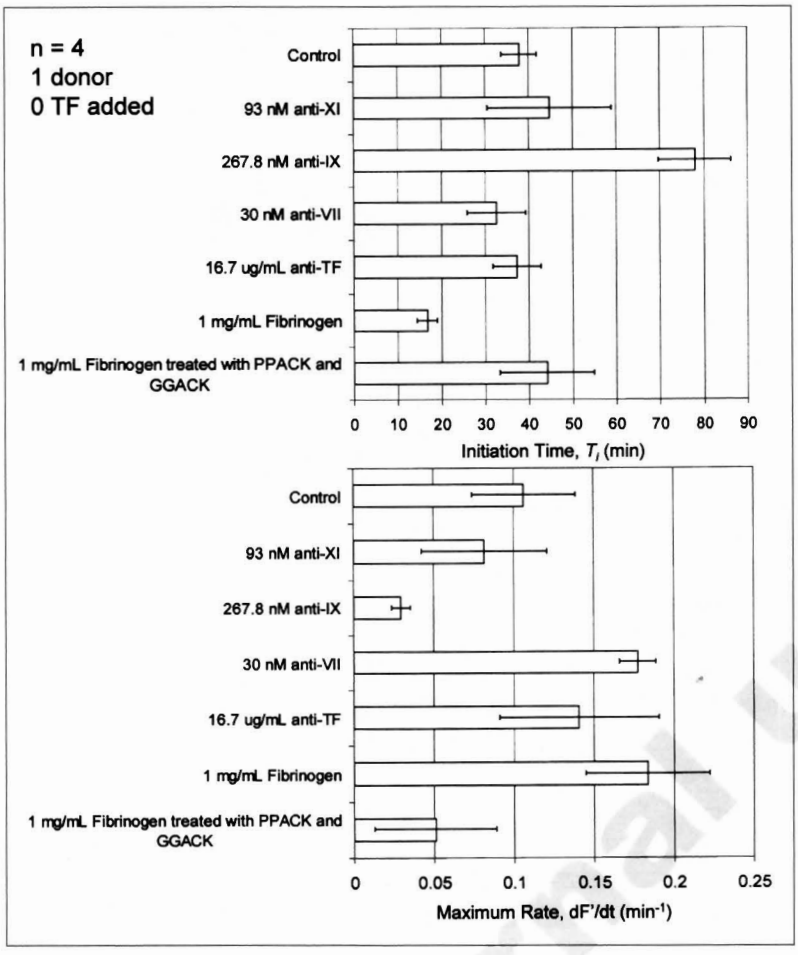

Figure 8: Inhibiting parts of the intrinsic and extrinsic pathway without adding TF and further fibrinogen analysis. Previous data (Figs. 3 to 7) leads to questions of blood coagulation with CTI but without addition of TF and contaminated fibrinogen. Adding antibodies to factor XI does not significantly change the initiation time or maximum rate. Therefore, CTI properly inhibits the intrinsic pathway. Addition of antibodies for VII and TF also do not significantly change the initiation time, but they do affect the maximum rate. When an antibody for IX is added, there is significant increase in initiation time and a significant decrease in maximum rate. This is consistent with the intrinsic factor tenase complex being the principle producer factor Xa (6). Previously (Fig. 7) "purified" fibrinogen, as provided, was shown to be procoagulant. After treatment with PPACK (inhibits IIa, VIIa, and XIa) and GGACK (inhibits Xa), the "purified" fibrinogen is shown to contain procoagulant impurities.

procoagulant contaminants should be considered. We demonstrated that commercial purified fibrinogen contains active enzymes that can be inhibited with PPACK (inhibits factors IIa, VIIa, and XIa) and GGACK (inhibits Xa). Pretreatment of fibrinogen with PPACK/GGACK (followed by dialysis) resulted in a fibrinogen preparation that by itself was not procoagulant (Fig. 8). While elevated fibrinogen is a known risk factor for cardiovascular disease (29) and it is known that thrombin bound to fibrin is resistant to heparin (30), it should be recognized that fibrinogen as provided, contains trace amounts of procoagulant activity.

This method exhibits similarities to a method published by Hemker et al. (16). However differences do exist. Hemker's method uses plasma (either platelet rich or platelet poor). This eliminates the contribution of red and white blood cells to coagulation. Also a calibrator is used to convert the fluorescence data to thrombin concentration. Our method uses fluorescence normalization in an attempt to eliminate some of the variations between wells. Also, conversion of fluorescence velocity to thrombin concentration is extremely complex as noted by Hemker. Thrombin cleaves the substrate in the presence of other competitive substrates (including fibrinogen). That competition changes with time as both fibrinogen and substrate are converted. At low substrate concentrations as in both studies, zero fluorescence velocity (dF/dt) at longer times does not coincide with zero thrombin. It coincides with substrate depletion and photobleaching of the fluorescent product. This means the peak of fluorescence intensity is simply full conversion of substrate, not the maximum thrombin concentration. Although the substrate is consumed (dF/dt = 0), much thrombin remains present in the clot, and addition of fresh substrate after clotting easily reveals this thrombin (data not shown).

This study provides a tool to explore reaction mechanisms where a given molecule, protein, or receptor (e.g. thrombin, GPIb, PARs) may have multiple modulatory roles in clotting, depending on the level of input TF. The use of combinatorial reaction analysis provides for highly constrained data sets where certain reactions are greatly activated or inhibited while other variables are also adjusted. Such data sets become useful for testing mathematical models. For example, the behaviors

seen in Fig. 3 are not adequately described by current models. Additionally, an automated algorithm in coagulation monitoring may have some future role in blood phenotyping as a patient ages or undergoes a therapeutic regimen. Another approach is to analyze the data via multifactor analysis to reveal crucial reactions, compounds, and reaction sets. This leads to the analysis of drug-drug interactions since drugs, new and old, can be combined and examined for their effects experimentally.

Future work in this area will study the effect of donor variability on the critical TF threshold as well as examine the effect of tissue factor pathway inhibitor (TFPI) concentration on the critical TF concentration. More specifically, we hope to gain a better understanding through TF-titration of the competition between TFPI inhibition of factor Xa and the protection from inhibition that factor Va provides when it complexes with factor Xa.

The objective of this research was to establish a quantitative understanding of blood stability, and then determine how interactions in blood affect this stability. We have developed a technique to quickly scan and profile coagulation interactions. The power of this technique lies in the number of experiments that can be run in parallel using a single donor's blood. Different combinations of chemical species can be scanned against blood over a range of tissue factor concentrations and convulxin levels.

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