Blood coagulation dynamics in haemostasis

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Summary
Our studies involve computational simulations, a reconstructed plasma/platelet proteome, whole blood in vitro and blood exuding from microvascular wounds. All studies indicate that in normal haemostasis, the binding of tissue factor (TF) with plasma factor (F) VIIa (extrinsic FXase complex) results in the initiation phase of the procoagulant response. This phase is negatively regulated by tissue factor pathway inhibitor (TFPI) in combination with antithrombin (AT) and the protein C (PC) pathway. The synergy between these inhibitors provides a threshold-limited reaction in which a stimulus of sufficient magnitude must be provided for continuation of the reaction. With sufficient stimulus, the FXa produced activates some prothrombin. This initial thrombin activates the procofactors and platelets required for presentation of the intrinsic FXase (FVIIa-FIXa) and prothrombinase (FVa-FXa) complexes which drive the subsequent propagation phase, continuous down-regulation of which is provided by AT and the thrombin-thrombomodulin-PC complex. TF is required for the initiation phase of the reaction but becomes non-essential once the propagation phase has been achieved. The propagation phase catalysts (FVIIa-FIXa and FVa-FXa) continue to drive the reaction as blood is resupplied to the wound site by flow. Ultimately, the control of the reaction is governed by the pro- and anticoagulant dynamics and the supply of blood reactants to the site of a perforating injury. Our system has been utilized to examine the qualities of hypothetical and novel antithrombotic and anticoagulation agents and in epidemiologic studies of venous and arterial thrombosis and haemorrhagic pathology.

The haemostatic process

The dynamics of the coagulation system and its significance in defining haemorrhagic and thrombotic phenotypes and potential interventions are explored in our laboratory utilizing various techniques (1):
- a synthetic plasma proteome,
- a computational model and
- whole human blood in vitro and in vivo.

Each system has specific advantages and limitations. Numerically based computer models are completely transparent, can be rapidly solved for a given set of conditions but have the lowest level of biological authenticity (Fig. 1). In contrast, studies of blood in vitro and blood exuding from a peripheral wound in vivo are authentic biological models which are technically difficult, time consuming, expensive and, while clearly biologically applicable, maintain a high level of opacity with respect to complete knowledge of what is going on. An intermediate phase is represented by the synthetic plasma proteome which attempts to mimic biological reality in vitro.
- The synthetic coagulation proteome mimics coagulation by utilizing mixtures of the vitamin K dependent zymogens, their (pro)cofactors, and the stoichiometric inhibitors with a membrane source of either platelets or synthetic lipid membranes (2–4). The reaction is started by the addition of relipidated tissue factor (TF) or activated monocytes.
- Computational simulations of the coagulation system based upon individual reaction mechanisms and the concentrations of pro- and anti-coagulants rapidly determine the fate of all protein products (1, 5). These simulations are the least expensive and the most rapid method for analyses. These models provide insights into reaction processes when quantitative and qualitative variables are altered and by the inclusion of pharmacologic or pathophysiologic interventions and are useful in the design of empirical experiments.
- Contact pathway inhibited but otherwise unaltered whole blood induced to clotting by TF is studied using a system of immunooassays which follow pro- and anti-coagulant products generated during the time course of the reaction (6, 7). This technique has been used to study hemorrhagic and thrombotic pathologies and the influence of pharmacologic management in both pathologies (8–10). The vascular component is absent and this is a closed system with no flow.
- The most biologically relevant model is an in vivo bleeding time model of the haemostatic system, in which whole blood exuding from a microvascular wound is sequentially sampled for relevant product formation (11–14). The vascular and extravascular components are present, and an injury, induced by the Simplate device, is the source of coagulation initiation by the perforation of a blood vessel.

The intricate blood coagulation mechanisms that govern normal haemostasis can
be categorized into three overlapping phases:
- the initiation of coagulation,
- its propagation and
- the termination of the procoagulant response.

These phases are analyzed utilizing the models described. Consistency between the results obtained from each model implies that the paradigms used to predict and evaluate the dynamics of the coagulation system are reasonably correct. Discontinuities, when observed, provide insights into new biology/biochemistry associated with this exceedingly complicated system.

**Procoagulant enzymatic complexes**

The dynamics of the blood coagulation system are dominated by three membrane-bound proteolytic procoagulant complexes which assemble at the site of vascular injury on platelets, the damaged endothelium and subvascular tissue (15) (Fig. 2). Their presentation and regulation and their opposing anticoagulant systems dictate the response of the coagulation system.

Following vascular injury and the exposure of subvascular TF, the TF-FVIIa extrinsic FXase complex is formed which activates FX and FIX to their respective enzymatic products (16, 17) (Fig. 2a). This reaction is tightly regulated by the tissue factor pathway inhibitor (TFPI) which binds the FVIIa-TF-FXa product complex (18) (Fig. 2b). The small amount of FXa initially formed activates prothrombin which activates additional platelets, FVIII and FV to their respective cofactor forms (19). Subsequently, the combination of activated platelets and the formation of the intrinsic FXase (FVIIIa-FIXa) and prothrombinase (FVa-FXa) complexes dominate FXa and thrombin generation respectively (Fig. 2a). Cofactor-protease assembly on membrane surfaces yields enhancements in the rates of substrate processing ranging from $10^5$–$10^9$ fold relative to rates observed when the same reactions are limited to solution phase bimolecular interactions between the individual proteases (FVIIa, FIXa and FXa) and their corresponding substrates (20–22).

The dynamics of procoagulant complex formation are illustrated in the four panels of Figure 3 which show the formation and the life spans of the complexes during the coagulation process after the presentation of TF to blood. The insets on each figure illustrate the concentrations of active complexes which drive thrombin generation (19). Dominant features include the observation that in every case the limiting reactant in complex formation is the protease complex.
Component of the complex. The concentration of each complex is extremely low, relative to its potential, ranging from less than 100 femtomolar (10^{-15} \text{ mol/l}, \sim 2\% of potential total) for the concentration of TF-FVIIa to 0.7 pmol/l (10^{-12} \text{ mol/l}, \sim 1\% of potential total) for FVIIIa-FIXa and approximately 3 nmol/l (10^{-9} \text{ mol/l}, \sim 15\% of potential total) for FVa-FXa. The time-scales of formation and concentrations of the procoagulant complexes are illustrated in Figure 3 panel D. Note that the vertical axis in panel D is on an exponential scale. Functional levels of the extrinsic FXase complex are limited by both the competitive binding of FVII to TF which initially occupies \sim 90\% of the TF pool and by TFPI which efficiently

**Fig. 3** Procoagulant complex formation: The reaction in electronic plasma is started by addition of 5 pmol/l TF; data expressed as metabolite concentration (molar) versus time (s). A) Extrinsic FXase complex: Inert complexes: TF not bound to other proteins (◆); TF-FVII (■); TF-FVIIa-FXa-TFPI (●); also shown TF-FVIIa (▲), inset: the first 200s of TF-FVIIa complex formation (▲) and the generation of the TFPI inhibited complex (●). Note: The extrinsic FXase peak level is \sim 80 fmol/l or \sim 2\% of the available TF cofactor. B) Intrinsic FXase complex: FVIII consumption (+), FVIIa formation (▲), FVIIa inactivation via dissociation (•), and FIXa formation (■), over a 1200 s; inset: time courses for the formation of FVIIa-FIXa complex (▲) and FVIIa-FIXa-FX complex (●). The formation of the intrinsic FXase complex over 1200 s results in sub picomolar levels. C) Prothrombinase complex: FV consumption (+), FV activation (■), FXa formation (●), FXa-FVa complex (◆), and FXa-AT complex (▲); inset: formation of free FXa and the inhibited complex D) active procoagulant complex formation with concentrations of FXa-FVa (◆), FVIIa-FIXa (●), and TF-FVIIa (▲) shown on a logarithmic scale

**Fig. 4** Two pathways to FXa production: A computational estimation of the temporal dependence of the relative FXa production by the extrinsic FXase (◆) and the intrinsic FXase (■) complexes. Clot time (CT, →) is the time at which free thrombin levels are predicted to reach 2 nmol/l. Reprint from Hockin et al. (1) with kind permission from the American Society for Biochemistry and Molecular Biology.
Mann et al. inhibits the extrinsic FXase complex via FXa dependent reaction (23). Levels of the intrinsic FXase complex appear controlled by the instability of FVIIIa and the offset between the timing of peak FVIIIa levels and the production of FIXa.

A representative comparison of the proportion of FXa generated by the two FXa activating complexes is illustrated in Figure 4, which shows that at the point of clot formation over 90% of the FXa is produced by the intrinsic FXase complex (1).

**Resupply**

The illustrations and descriptions in the preceding section correspond to the generation of thrombin in closed systems. In contrast, the biological processes of coagulation occur in an open system in which the reaction site is constrained only by protein and membrane binding associated with the region of the vascular wound. We have provided partial insight into the continuity of the process (with respect to thrombin generation) which might occur in an open system by utilizing the three in vitro models and resupplying the quiescent TF-initiated reaction systems with fresh aliquots of electronic, real or synthetic blood. An experimental result of this sort is illustrated in Figure 5.

**Fig. 5** Resupply of a TF-initiated blood coagulation reaction after the cessation of prothrombin consumption: empirical and simulated results. Time courses of TAT formation are shown after addition of 5 pmol/l TF to both electronic plasma and contact pathway inhibited whole blood. The resupply response was evaluated in both electronic plasma and whole blood. Either a fresh aliquot of contact pathway phlebotomy blood from the same individual or an electronic volume of fresh zymogens, cofactors and inhibitors, without additional TF, was added 20 minutes after TF initiation. TAT levels are presented as total moles of product versus time in order to conform to the methodology of the empirical experiments. Computational simulations were performed using the mean values of zymogens, cofactors and inhibitors or the clinically accepted low and high ranges for each of the zymogens, cofactors and inhibitors. The simulated data using mean values are shown for a control (◆) and a haemophilic (■) with the standard deviation of the accepted high and low values for all constituents shown for the control numerical simulation. Empirical TAT formation data were obtained from contact pathway suppressed whole blood collected from one healthy control (●) and one FVIII deficient individual (▲). Reprint from Orfeo et al. (24) with kind permission from the American Society for Biochemistry and Molecular Biology.

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**Fig. 6** Resupply of the synthetic coagulation proteome. A 5 pmol/l TF-initiated reaction mixture was subdivided after 20 min (A), and the seven aliquots resupplied either immediately (20 min → t=0, ◆) or after additional 15 (■), 30 (▲), 45 (●), 60 (▼), 75 (▲) or 95 (■) min of incubation. Resupply was conducted with an equal volume of solution comprised of 1.4 μmol/l FII/3.4 μmol/l AT/2 μmol/l TPCEs. Thrombin levels for the final 5 min of the TF-initiated episode are also shown (◊). Thrombin levels are expressed as total picomoles of active thrombin to normalize for the volume change. Reprint from Orfeo et al. (24) with kind permission from the American Society for Biochemistry and Molecular Biology.

**Table 1** Patient laboratory report Fletcher Allen Health Care (University of Vermont hospital): coagulation factor levels for a healthy volunteer and the normal range values

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Patient Value</th>
<th>Reference Range</th>
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<tr>
<td>Factor X (%)</td>
<td>125</td>
<td>60–140</td>
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<tr>
<td>Factor II (%)</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>Factor V (%)</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Factor VIII (%)</td>
<td>107</td>
<td>64–232</td>
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<td>Factor IX (%)</td>
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<td>69–151</td>
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<td>Antithrombin (%)</td>
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<td>Fibrinogen (mg/dl)</td>
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<td>11.7–13.1</td>
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<td>0.8–1.2</td>
</tr>
<tr>
<td>PTI (s)</td>
<td>27</td>
<td>23–33</td>
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Figure 5 which shows a closed system computational simulation of thrombin-antithrombin (TAT) product accumulation in response to TF-initiation and subsequent resupply (24). The simulated time course of thrombin formation, using the mean values for all coagulation pro- and anticoagulant factors is shown along with the standard deviation reflecting what happens when computational simulations are conducted using clinically accepted low and high values (Tab. 1) for each protein. Data for an actual whole blood experiment conducted utilizing blood from a single healthy individual are superimposed. Also presented is a whole blood experiment and the mean value (minus FVIII) computational simulations for a closed system with hemophilia blood. Consistent with the computational analysis of the temporal dependence of the contributions of the extrinsic and intrinsic FXase complexes (Fig. 4), haemophilia blood (Fig. 5), which lacks the intrinsic FXase complex, does not display a propagation phase of thrombin formation, as this deficiency does not permit the necessary expanded generation of FXa by FVIIa-FIXa.

The resupply protocol involves the addition of a fresh aliquot of normal or haemophilia blood or its electronic equivalent, after the TF-initiated reaction becomes completely quiescent with respect to further thrombin production (20 minutes). In the normal resupply case, there is rapid accumulation of thrombin-antithrombin with the rate and extent of accumulation being larger than that initially observed over the first 20 minutes. The source of this additional thrombin generating activity is due to the prothrombinase complex which remains active over the initial 20 minute time course. This is illustrated by a series of resupply reactions using the synthetic plasma proteome model(24) (Fig. 6) in which sequential additions of only prothrombin, anti-thrombin and phospholipid are provided to the reaction system. A new burst of thrombin is observed with each new addition with the intensity of thrombin production reduced with each sequential aliquot, reflecting the slow decay of prothrombinase activity in the presence of antithrombin. Were a complete proteome mixture used for resupply instead of only prothrombin and antithrombin added, the intensity of the response would not show this decrease because of embedded and expanding intrinsic prothrombinase produced by regenerating the intrinsic FXase complex in the reaction system.

The overall interpretation of these resupply experimental systems is illustrated in Figure 7 in which the flow regulation component of the coagulation system is hypothesized. Upon disruption of the vasculature, platelets adhere to extravascular matrix proteins and the extrinsic FXase complex is formed after the presentation of plasma FVIIa to extravascular TF (Fig. 7a). Platelets and the procoagulant complexes and fibrin are accumulated in the breach (Fig. 7b) with the reaction continuing as long as flow is maintained to the extravascular space. Once a plug has been completely formed (Fig. 7c), blood and reactant supply is denied to the accumulated complexes on the extravascular side of the barrier plugging the wound causing the reaction to stop. The abundant anticoagulants present in the vasculature and in plasma will consume any residual procoagulants on the intravascular face of the plug during the nor...
Phenotypic heterogeneity in the blood coagulation system

Laboratory tests for the defects in the coagulation system are largely confined to the contributions by various proteins to haemostasis while a limited ensemble of genetic defects is associated with thrombophilia (25). However, in the majority of the healthy population the concentrations of pro- and anticoagulants vary significantly. This is illustrated in Table 1 which shows clinical coagulation laboratory values for the reference ranges of the healthy population. The basis for this heterogeneity is probably a consequence of genetic and environmental factors which influence the biosynthesis, utilization and pharmacokinetics of the coagulation proteins (12, 26, 27). The molecules in blood which deal with coagulation are extremely complicated and require a significant variety of posttranslational and proteolytic processing steps in their overall lifetime. As a consequence of these multiple alterations, the dynamics of the coagulation proteome can be anticipated to be highly phenotypically variable.

The consequences of this variety of reference values is illustrated in numerical and empirical model assessments of thrombin generation for the range of prothrombin concentrations from 50–150% (1, 28) (Fig. 8). Over this normal range of prothrombin concentrations one would observe a substantially different amount of thrombin produced for the same initial TF stimulus. It should also be noted that the numerical model (left side of figure) reasonably represents the empirical proteome model on the right with far lower personnel and reagent costs in a much shorter time period.

Heterogeneity is further illustrated in a numerical simulation of the compositional data for five healthy individuals (INR ~ 1) in terms of active thrombin anticipated to be generated following a challenge by the equivalent concentration of electronic TF
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(29) (Fig. 9). This hypothetical variety of thrombin generating responses in the healthy population has been verified by the examination of TF-induced thrombin accumulation in blood samples from healthy male individuals over a six-month period. These data, illustrated in Figure 10, show that the phenotypic presentation of thrombin generation is fairly constant within any given individual, but highly variable between individuals, suggesting that the stimulus-coagulation coupling response in the healthy population may have utility in predicting risk following a prothrombotic challenge.

Potential predisposing effects of the compositions of the coagulation proteome and propensity for thrombotic disease have been evaluated. We performed a computational evaluation of the controls and cases from the Leiden Thrombophilia Study (LETS).

For the control population (472 individuals), significant variations in hypothetical thrombin generation with a TF insult were evaluated. We performed a computational evaluation of the controls and cases from the Leiden Thrombophilia Study (LETS).

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**Fig. 11** Oral contraceptive (OC) effect on theoretical and empirical thrombin generation; simulations shown as the mean and the 95% confidence interval; mean factor levels of the selected populations recapitulated in a synthetic plasma model (C and D); reprint from Brummel-Ziedins et al. (31) with kind permission from Wiley-Blackwell.

A) control women with (n = 47) and without (n = 90) OC use; B) women with deep venous thrombosis (DVT) with (n = 30) and without (n = 40) OC use.

**Fig. 12** Computationally derived thrombin generation shown as the mean for the acute coronary syndrome (ACS, n = 28) and coronary artery disease (CAD, n = 25) populations; positive standard deviation in grey; reprint from Brummel-Ziedins et al. (32) with kind permission from Wiley-Blackwell.
observed (30). This variability was not explained by any plasma factor. In a companion paper studying the thrombosis population (n = 472) from LETS, the risk associations obtained using the maximum rate of thrombin generation gave odds ratios of 3.9 in men, 2.1 in women and 2.9 in women on oral contraceptives (OC) (31). As shown in Figure 11A and 11B, OC use created extreme shifts in thrombin generation in both the control and thrombosis populations indicating an interaction of OC use in individuals with an underlying prothrombotic abnormality, consistent with the known risk of OC use. These observations also suggest that a multiplicity of events presage thrombosis of which blood chemistry is but one, as both cases and controls on OC showed similar enhanced thrombin generation in blood.

We have also shown that TF-initiated computational blood coagulation was able to distinguish between acute and stable coronary artery disease by evaluating thrombin generation (32) (Fig. 12).

**Anticoagulant targeting**

The search for new anticoagulants has been a frustrating and expensive adventure for many pharmaceutical companies. An inspection of Figure 5 illustrates resupply in an already activated coagulation system. The data in this figure suggest that the choice and intensity of an anticoagulant might be different when one is using the drug in a prophylactic sense i.e. before the initiating thrombotic event compared to the treatment of an ongoing thrombotic complication with thrombin generation already occurring within the vasculature (24). For the purposes of discussion, we have defined the latter case as a therapeutic intervention.

Figure 13 illustrates the circumstance (33) in which a family of inhibitors all with hypothetical Kd of –nmol/l, but characterized by different on rates, targets either free FXa or free FXa in complex with FVa. Because of the large excess of FVa in the reaction system, virtually all FXa is in complex with FVa (Fig. 4). As a consequence, an inhibitor selective only for FXa would not be very effective regardless of the on rate.

For a therapeutic intervention, the enzyme complex is already formed (24) and able to generate more product as more blood is supplied. Figure 14 illustrates targeting FIXa with an inhibitory antibody in a therapeutic intervention during resupply. These data are in contrast to antibody inhibition of FXa (Fig. 15). Even better inhibition is obtained with a small molecule protrombinase and FXa inhibitor.

**Conclusion**

All in vitro blood tests of the coagulation system are severely limited because of the absence of contributions of the vasculature and surrounding tissue to hemostasis and
thrombosis. In contrast, in vivo tests in humans employ all of the physiology impacting the coagulation system. However, there is an inherent pathophysiologic risk in such studies and the resulting mechanistic interpretations are often superficial. The partnership of carefully constructed numerical, in vitro and in vivo evaluations of the blood coagulation physiology and pathophysiology provides a useful marriage of technologies which assist in decision making in altering the blood coagulation system.

Acknowledgement

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References

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Fig. 15 Resupply of the synthetic coagulation proteome: effect of inhibitors targeting FXa and FXa in the prothrombinase complex; thrombin generation presented for reactions initiated with 5 pmol/l TF reagent and then resupplied at 20 min with an equal volume of synthetic coagulation proteome/2 µmol/l PCPS without TF: control resupply (◊), 320 nmol/l C291–78 included in the synthetic coagulation proteome/2 µmol/l PCPS resupply mixture (◊◊). 0.1 mg/ml α2-PI-2 added 2 min prior to resupply with synthetic coagulation proteome/2 µmol/l PCPS (♦), and 0.1 mg/ml α2-PI-2 added 2 min prior to resupply with 1.4 µmol/l FIIa/3.4 µmol/l AT3/2 µmol/l PCPS (♣). The arrow indicates the time of α2-PI-2 addition. Thrombin generation over the initial 20 min presented as the mean ± SD of 4 determinations; thrombin concentration expressed as total moles of active thrombin to normalize for the volume change; reprint from Orfeo et al. (24) with kind permission from the American Society for Biochemistry and Molecular Biology.


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