Biochemistry and Physiology of Blood Coagulation

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Introduction

The biochemical investigation of a biological process can be divided into three segments. The first is the development of an inventory of the components involved in the process, the second is the establishment of the connectivity between components, and the third is the establishment of the dynamics of the processes as they occur in the living organism.

Inventory

The biochemical era of blood coagulation research begins toward the end of the 19th century, primarily with the work of Alexander Schmidt[1] and Paul Morawitz,[2] who hypothesized that the fundamental reactions involved in clotting included the activation of prothrombin to thrombin by thromboplastins and the conversion of fibrinogen to fibrin by thrombin. Throughout the first half of the twentieth century, the inventory of blood clotting, which focused principally on procoagulant components, was expanded by the evaluation of human pathology and by laboratory analysis. These studies were greatly advantaged by two factors: (a) blood is easily obtainable, and (b) mothers almost always note when a child is bleeding excessively. As a consequence of the observations of hemorrhagic disease, hemophilia A (factor VIII deficiency)[3,4] hemophilia B, Christmas disease (factor IX deficiency),[5-7] Stuart factor disease (factor X deficiency)[8] SPCA (serum prothrombin conversion accelerator) deficiency (factor VII deficiency),[9] hemophilia C (factor XI deficiency),[10,11] and parahemophillia, factor V (proaccelerin)[12] deficiency were identified. Paul Owren, the discoverer of parahemophilia, named the component missing in his patient as factor V, since it was the fifth component (in addition to prothrombin, calcium, thromboplastin, and fibrinogen) required to produce a clot. He, thus, presaged the subsequent adoption of the array of roman numeral identifications.[13]

The pioneering work of the Smith group in Iowa[14] and the clinical pathology work of Armand J. Quick[15] led to quantitative laboratory evaluations of blood clotting reactions. The length (11-15 seconds) of Quick's prothrombin time assay was, in part, dictated by the fact that blood spontaneously clots, even when removed from the blood vessel under the most carefully controlled conditions. Studies of this "intrinsic" clotting reaction were advanced by the development of the partial thromboplastin time by Langdell et al.[16] This latter assay, exploited in the laboratory as a diagnostic test, led to the identification of deficiencies of factor XII (Hagemann factor),[17] prekallikrein,[18] and high molecular weight kininogen,[18,19] as agents which contributed to the intrinsic clotting potential of blood. However, the deficiency of these "activities" was not associated with clinical bleeding. Factor XIII (plasma transglutaminase) was discovered as the precursor of the agent that produces the insoluble fibrin clot.[20] A number of the antithrombin elements were identified in these early tests, including fibrin (antithrombin I) and antithrombin III.[21] The tissue factor pathway inhibitor, a relatively recent discovery, but an inhibitor of great significance, was initially identified using in vitro clotting assays.[22]

The discovery of gamma carboxylation[23] provided another entre for the expansion of the inventory of blood clotting components and led to the discovery of protein C,[24] protein S,[25] and protein Z.[26] The search for the function of protein C led to the discovery of thrombomodulin[27] and the anticoagulant role of thrombin in the activated protein C pathway, a significant negative-feedback, dynamic regulator of the coagulation process. No function has yet been assigned to protein Z. The inventory of the molecules involved in blood clotting and its regulation, their genes, and plasma lifetimes are presented in Table 1.

Connectivity

The initial connectivity described by Morowitz was expanded as the major paradigm by the hypotheses of McFarland[28] and Davie and Ratanoff:[29]a cascade/waterfall of reactions of intrinsic plasma coagulation reactions leads to the generation of fibrin. This connectivity paradigm has guided investigators in the blood clotting field over the past 35 years. While initially displaying only the plasma coagulation cascade, the mechanism was subsequently expanded to include two arms, corresponding to intrinsic and extrinsic pathways, with two sources of "thromboplastin" leading to the generation of thrombin.

When one combines the component inventory (Table 1) and connectivity provided by biochemistry with the clinical pathology associated with the presentations of hemophilia, one is left with the conclusion that, owing to the absence of bleeding associated with deficiencies of factor XII, prekallikrein, and high molecular weight kininogen, these factors cannot be on the primary route of factor IXa generation during the ordinary hemo-

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Table 1. Blood Clotting Factors and Inhibitors[3]

Protein	Chromosome	Gene (kb)	mRNA (kb)	Plasma Half-life	Plasma Concentration (nM)	Clinical Manifestation[1] H	Clinical Manifestation[1] T
Prothrombin	11	21	2	2.5	1400	+	+
FactorV	1q 21-25	80	7	0.5	20	+	+
Factor VIII	Xq28	186	9	0.3-0.5	0.7	+	+
Factor VII	13	12.8	2.5	0.25	10	+	+
Factor IX	x	34	2.8	1	90	+	
FactorX	13q32-qter	27	1.5	1.25	170	+	
Factor XI	4q35	23	2.1	2.5-3.3	30		
Factor XII	5q33-qter	12	2.6	2-3	375	+	
High molecular weight kinninogen	3q26-qter	27	2.0	5	6000		
Prekallikrein	4q34-q35	22	2.4	NA[2]	450		
Factor XIIIa	6p-24-25	>160	3.8	9-10	70	+	
Factor XIIIb	1q31-q32.1	28	2.3	NA[2]			
Tissue factor	1	12.4	2.3				
Protein C	2q14-21	11	1.7	0.25	60		+
Protein S	3	80	3.5	1.75	300		+
Thrombomodulin	20p12-cen	3.7	3.7	-			
Fibrinogen	4q23-q32	50		3-5	8800	+	+
α chain		5.4	2.2				
β chain		8	1.9				
γ chain		8.5	1.6		2500	+	+
Antithrombin III	1q22-25	14	1.4	2.5-4			
Heparin cofactor II	22q11	16	2.3	2.5	1200		+/-
Tissue factor pathway inhibitor	2q31-32,1		1.4,4	NA[2]	2.5		
Thrombin activated fibrinolysis inhibition	13		1.8	NA[2]	73		

[1]*H - Hemophilia; T - Thrombosis*
[2]*Information not available.*
[3]*From Blood Coagulation in Cardiovascular Disease, K.R. Chien Ed., W.B. Saunders, Philadelphia 1999: 23; 505-535.*

static process that occurs following the perforation of a blood vessel. The absence of pathology with respect to this series of reactions is not coincident with physiological connectivity and, thus, the tissue factor pathway appears to be the ordinary route to the generation of thrombin. The understanding of the intricate relationships between factor VIII, factor IX, and factor XI deficiencies, their associated bleeding diseases, and the seemingly unrelated extrinsic tissue factor pathway were initiated by the seminal observation that factor VIIa-tissue factor could activate factor IX[30] and that thrombin can activate factor XI to factor XIa.[31] The pathology of factor XI deficiency is mild, relative to the pathology of factor VIII and factor IX deficiencies, suggesting that the role of factor XI is principally as an accessory in the generation of thrombin, rather than a fundamental element of the pathway.

To understand the biochemistry of the coagulation process, one must integrate physiology and pathology with the biochemical connectivity among the various elements in the coagulation pathway. To focus these elements to the biological level, one must inspect Table 1 for the relative blood concentrations of the plasma components involved in the reaction system. In addition, one must recognize additional features of the reaction, including the cellular compartmentalization of some of the components, the heterogeneity of the vasculature, and the process of flow.

The early work of Seegers identified the phospholipid requirement for coagulation.[32,33] The biological elements contributing to the "phospholipid" include damaged vascular tissue, activated platelets, and inflammatory cells. Tissue factor is ordinarily not exposed to flowing blood, but is exposed as a consequence of the mechanical cellular damage accompanying vessel perforation or as a consequence of inflammatory cytokine activation of either vascular cells or monocytes. In addition, the process of blood clotting is dynamic in nature and occurs in an open system. Therefore, the process of accelerating idling blood clotting reactions to a fulminating state occurs only after an insult to the vascular system of sufficient dimension has occurred.

The contributions of the membrane to the formation and expression of the procoagulant complexes are essential. However, the nature of the related membranes that support procoagulant reactions is poorly understood. Mechanically-damaged cells can provide the anionic membrane bilayer inner leaflet phospholipids, which can support complex formation;

however, more subtle cellular activation events can also generate complex-forming sites on intact cells. The principal contributors, in terms of numbers of sites, are the platelet membranes that express individual binding sites for the factor IXa-factor Vllla and factor Xa-factor Va complexes as a consequence of platelet activation.[34,35] Hemorrhagic pathology is, therefore, associated with thrombocytopenia and is also displayed in a rare disease, Scott syndrome,[36] which appears to result from the improper presentation of these platelet sites. This defect is also associated with reduced shedding of microparticles after platelet activation. Binding sites have also been reported on a number of peripheral blood cells, especially activated monocytes. The vascular endothelium itself can provide sites after stimulation by cytokine growth factors.[37]

Most investigators today believe that the physiologically-relevant hemostatic mechanism is primarily associated with three procoagulant enzyme complexes (Fig. 1), which involve vitamin K-dependent serine proteases associated with membrane-bound cofactors assembled on a membrane surface.[38] Although each of the vitamin K-dependent complexes exhibits discrete substrate and proteolytic specificity, the complexes share many common features. First, the complexes are functionally analogous with structurally homologous constituents. The enzyme complexes exhibit similar requirements for assembly and activity. In each case, the enzyme complex assembly leads to significant enhancement (10^5-10^6-fold) of the localized catalytic rate of substrate activation.

The principal regulatory events associated with the expression of the vitamin K-dependent enzyme complexes are as follows: the conversion of a vitamin K-dependent zymogen to a serine protease; the proteolytic activation of a plasma-derived pro-cofactor to an active cofactor in the case of factors V and VIII or the membrane expression of an integral membrane cofactor in the case of tissue factor or thrombomodulin; and the presentation of the appropriate membrane surface via cellular damage or activation.

Approximately 1% to 2% of the factor VII molecules flowing in blood have been cleaved at position Arg152 to yield the serine protease factor VIIa.[39] However, the active site of this enzyme is not efficiently expressed unless it is bound to tissue factor.[40] Hence, naked factor VIIa cannot express activity toward factor IX or factor X, nor is it subject to inhibition by the antagonist, antithrombin III.

Upon exposure, existing plasma factor VIIa will rapidly bind to tissue factor, leading to the expression of the three procoagulant complexes illustrated in Figure 1.

The initial product of the factor VIIa-tissue factor complex is factor Xa. However, once factor Xa is generated, it may contribute to factor IX activation by a concerted reaction with factor VIIa-tissue factor. Factor Xa can generate a tiny amount of thrombin by an extremely inefficient reaction. However, once produced, thrombin, and potentially the initially formed factor Xa, activate small quantities of factor V to factor Va and factor VIII to factor VIIIa. These two pro-cofactor activations are essential to the formation of the efficient catalysts, factor IXa-factor VIIIa (*intrinsic tenase*), which converts factor X to factor Xa, and factor Xa-factor Va (*prothrombinase*), which converts prothrombin to thrombin. These catalysts are 10^5- 10^6-fold

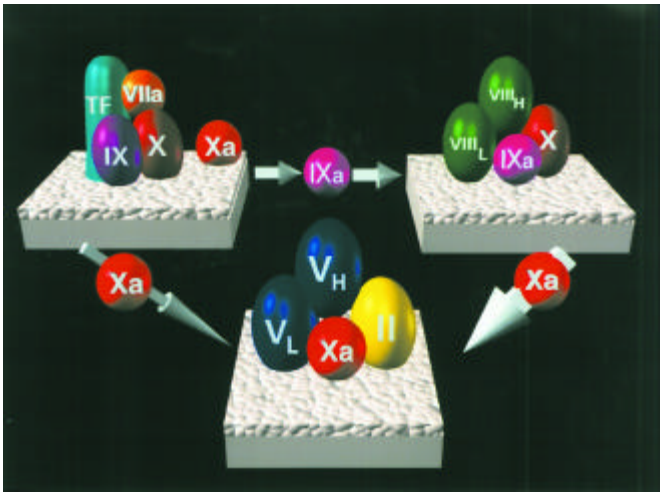


*Figure 1. The pro-coagulant vitamin K-dependent complexes. Blood clotting is initiated when factor VIIa binds to exposed/expressed tissue factor. The factor VIIa-tissue factor complex initiates coagulation by activating factor X and factor IX. Factor Xa will also cleave factor IX to factor IX*α *which will accelerate the process of factor IX activation. The factor IX*aβ*-factor VIIIa complex will activate factor X to factor Xa fifty times more efficiently than the factor VIIa-tissue factor complex. Factor Xa from either source forms a complex with factor Va to convert prothrombin to thrombin (IIa). Used with permission from the "Coagulation Explosion" Vermont Business Graphics, K.G. Mann, 1997.*

more active than their component serine proteases acting independently on their vitamin K-dependent substrates. The factor IXa -factor VIIIa pathway, is approximately 50-fold more efficient than the activation of factor X by factor VIIa-tissue factor. In addition, tissue factor pathway inhibitor (TFPI), a high-affinity, low abundance inhibitor present in plasma and on vascular cells, binds to the factor Xa-tissue factor-VIIa product complex to limit the production of factor Xa and factor IXa by this pathway[41] (Fig. 2). Once this occurs, factor Xa can only be produced by the factor IXa-factor VIIIa complex. Thus, the importance of these two molecules and the pathology accompanying their deficiency states. The pro-coagulant thrombin activates platelets, cleaves fibrinopeptides A and B from fibrinogen, and activates factor XIII. Thrombin also binds to thrombomodulin that is constitutively expressed on the vascular endothelium (Fig. 3). This interaction converts thrombin from a pro-coagulant to an anticoagulant enzyme, activating plasma protein C to the protease activated protein C (APC), which has as its principle substrate factor Va.[42] As a consequence of two cleavages in the factor Va molecule (at Arg506 and Arg306), the function of the prothrombinase complex is terminated.[43] Factor VIIIa is also a substrate for activated protein C. However, the inherent instability of factor VIIIa may make this reaction irrelevant from a biological perspective.[44] Protein S also plays a significant role in the anticoagulation process. However, while many protein S functions have been asserted, none have been unequivocally shown to be the principal role by which protein S exerts its anticoagulant effect. It is clear, however, from the pathology accompanying protein S deficiency that this molecule plays a major regulatory role in blood coagulation. Antithrombin III serves as a scavenger to eliminate any residual

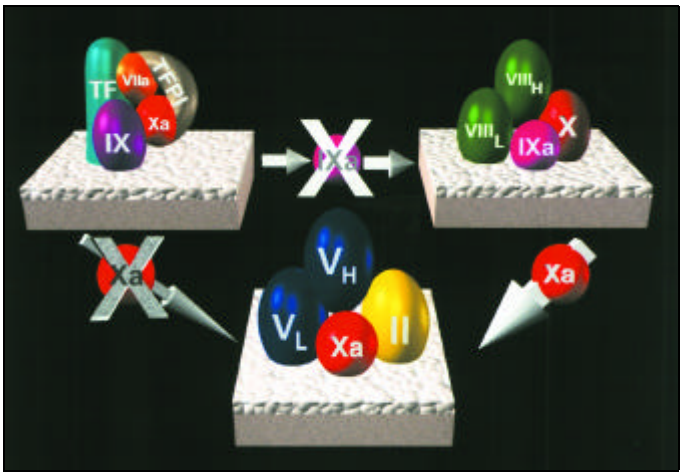


Figure 2. The tissue factor pathway inhibitor, TFPI, interacts with the factor VIIa-tissue factor-factor Xa product complex to block the tissue factor-initiated activation of both factors IX and factorX leaving the factor IXaβ-factor VIIIa complex as the only viable catalyst for factor X activation. Used with permission from the "Coagulation Explosion" Vermont Business Graphics, K.G. Mann, 1997.

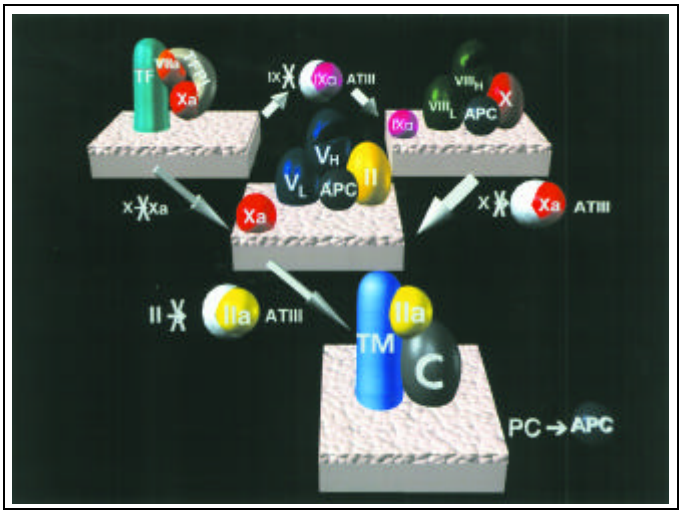


Figure 3. The generation of thrombin (IIa) initiates the dynamic pathway of anticoagulation. Thrombin binds to thrombomodulin exposed on the vasculature, activating plasma protein C to activated protein C (APC). Activated protein C competes with both factor Xa and factor IXaβ for factor Va and factor VIIIa, respectively. Once bound, APC cleaves both cofactor proteins in their A2 domains leading to destruction of these two enzymatic complexes. Antithrombin III forms complexes with factor IXa, thrombin, and factor Xa, effectively scavenging the remaining protease activity. Used with permission from the "Coagulation Explosion" Vermont Business Graphics, K.G. Mann, 1997.

procoagulation enzyme activity.[45] Heparan sulfate on the vascular wall accelerates this process.[46]

Dynamics

Under normal circumstances, the intricate choreography in the reaction systems displayed in Figures 1, 2, and 3 leads to regulated generation of the appropriate amount of thrombin and, hence, a fibrin-platelet clot that is a response to leakage in the vasculature–a response that, in normal circumstances, is quantitatively related to the vascular insult. Vascular pathology, whether hemorrhagic or thrombotic, occurs as a consequence of failure in the choreography of the expression of the procoagulant or anticoagulant functions and leads either to the underexpression or overexpression of pro-coagulant activity.

Our laboratory has been studying the dynamics of the coagulation process using three approaches. The first of these is the development of synthetic plasma models constructed with highly purified natural and recombinant proteins that can be mixed and induced to generate thrombin only upon the addition of tissue factor. The reaction products are analyzed by a collection of enzymatic, immunochemical, and chromatographic assays to assess the nature of products formed during the generation of thrombin and the rates of product formation. In the second approach, theoretical (mathematical) models are generated from the known (or anticipated by homology) reaction parameters for each of the complexes illustrated in Figures 1, 2 and 3. These computer-solved, mathematical models are far easier to manage than empirical models and provide the opportunity for assessment of an extraordinary range of conditions on reaction parameters and outcomes. The third model system involves studying the in vitro coagulation of whole blood obtained both from normal subjects and from individuals with either genetic, acquired, or induced coagulation abnormalities. These "para vivo" assays of the tissue factor-initiated blood clotting system take advantage of corn trypsin inhibitor to suppress the contact pathway of blood clotting. The use of this inhibitor permits the tissue factor reaction to be studied exclusively in minimally-altered whole blood in vitro at 37°C with no other anticoagulants added.

The convergence of results obtained from these three experimental systems, in some circumstances, provides the satisfying conclusion that the models provide an appropriate description of the "para vivo" physiology of the coagulation system in chemical terms. Divergence provides the opportunity for guiding discovery of new contributors to the procoagulation process and its regulation.

Synthetic "Plasma" Coagulation

When thrombin formation is initiated in a synthetic mixture of the pro-coagulant proteins at their average plasma concentrations (Table 1) with tissue factor and phospholipids or platelets, the thrombin-generating reaction can be separated into two phases. Following factor VIIa-tissue factor addition, an initiation phase occurs, which is primarily influenced by the concentration of the factor VIIa-tissue factor complex added to the reaction mixture. During this phase, barely detectable levels (<50 pmol/l) of thrombin are generated, factors V and VIII are nearly quantitatively cleaved, and tiny amounts (<10 pmol/l) of factors Xa and IXa are generated. Subsequently, in the absence of inhibitors, a propagation phase occurs, in which prothrombin is quantitatively converted to thrombin at reaction rates almost independent of the initial tissue factor-factor VIIa concentration.[47] This propagation phase is largely driven by the factor IXa-factor VIIIa activation of factor Xa. When fibrinogen is

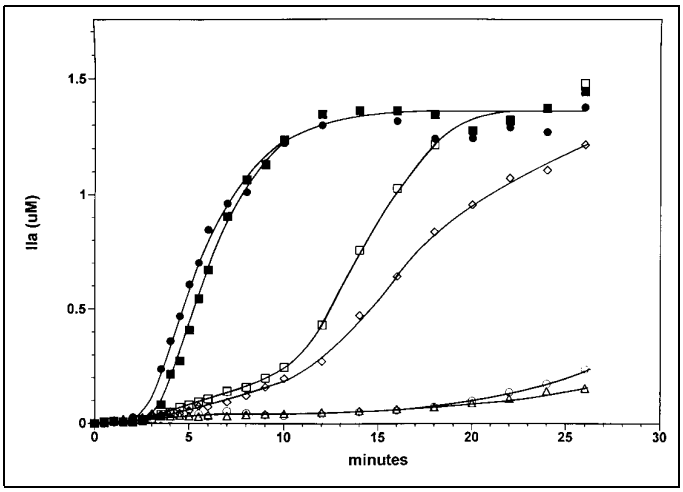
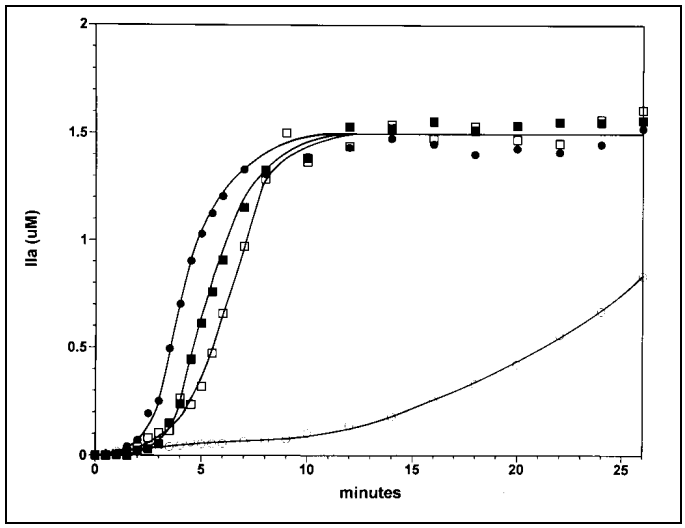


Figure 4A. Thrombin generation by normal factor V and factor V^{Leiden} in the absence and presence of the protein C pathway and TFPI. Thrombin generation is initiated by 1.25 pM factor VIIa-tissue factor in the presence of 2.5 nM TFPI. Closed symbols represent the reactions for normal factor V (●) and factor V^{Leiden} (■) in the absence of the protein C pathway components. Open symbols show the reactions in the presence of the protein C pathway (65 nM protein C plus 10 nM thrombomodulin) with or without protein S (300 nM). Normal factor V (❍), factor V^{LEIDEN}, (❑) normal factor V and protein S (Δ), factor V^{LEIDEN}, protein C pathway, and protein S (◊).



B. Normal factor V and factor V^{Leiden} mutation in the reconstituted model at 1.25 nM TFPI. Curves are shown for reactions in the absence of the protein C pathway for factor V (●) and factor V^{Leiden} (■) and in the presence of 65 nM protein C, 10 nM thrombomodulin, and 300 nM protein S with factor V (❍) or factor V^{Leiden} (❑). Used with permission from van't Veer et al.[50]

included in the synthetic mixture, clotting occurs at the intersection of the initiation and propagation phase rates of thrombin generation.

The influence of the negative regulators of coagulation has been studied by the individual and combined additions of tissue factor pathway inhibitor (TFPI), antithrombin III, and the components of the protein C system (protein C, protein S, and thrombomodulin) to the synthetic procoagulant mixture. The protein C system and antithrombin III are both effective in regulating the propagation phase of thrombin, while TFPI is primarily effective in regulating the initiation phase of the reaction. In the presence of TFPI and antithrombin III and the absence of other inhibitors, the limiting reactant controlling the propagation phase of the reaction is factor Xa. However, when the elements of the dynamic protein C pathway are added to the procoagulant reaction system, the limiting reactant can become factor Va. For experiments conducted at reduced platelet concentrations or with platelet inhibitors, the limiting reactant can become the expression of the platelet pro-coagulant complex assembly sites.

In experiments conducted with mixtures of inhibitors, synergy is observed. For example, when TFPI and antithrombin III are combined, these regulators collectively provide a more effective inhibitory influence on the reaction than would be predicted by the effect of either inhibitor acting alone.[48,49] The observed synergy is a consequence of the reduction in the concentration and rate of factor Xa generation through direct factor Xa inhibition and inhibition of the factor VIIa-tissue factor-factor Xa complex by TFPI, coupled with the slower antithrombin III inhibition of factor Xa, thrombin, and factor IXa. In a similar manner, TFPI and the protein C system behave in a synergistic manner. In this instance, the inhibition of factor VIIa-tissue factor-factor Xa by TFPI leads to the slower generation of factor Xa, which when coupled with the thrombin-thrombomodulin activation of protein C and the resulting destruction of factor Va by the action of APC, leads to the generation of factor Xa in the absence of factor Va. Free factor Xa is rapidly inhibited by antithrombin III. In combination, the interplay of the inhibitors with the initiation and propagation phases of the procoagulant reaction combine to produce a threshold limited response to tissue factor as a stimulus.

Figure 4A illustrates the influence of the combination of TFPI with the dynamic protein C pathway and the resulting influence on thrombin generation in the purified system. The addition of TFPI at normal plasma concentrations (2.5 nM, open circles) to the system containing protein C when thrombomodulin is present leads to a significant attenuation of the expression of thrombin especially during the propagation phase of the reaction. (Compare closed and open circles.) In contrast, at 2.5 nM, TFPI with homozygous factor V^{Leiden} (Arg506→Gln), only a partial attenuation of thrombin generation in the propagation phase occurs (compare open and closed squares). Figure 4B illustrates thrombin generation when the TFPI concentration is at 50% of the normal plasma concentration (1.25 nM) (compare closed and open circles). At half the normal plasma concentration, TFPI is still effective in blunting most of the propagation phase of thrombin generation with normal factor V (open circles). However, this concentration of TFPI (1.25 nM) has virtually no effect in suppressing thrombin generation when factor V^{Leiden} is substituted for normal factor V (compare open and closed squares). Thus, one problem area can be significantly compounded when one adds qualitative or quantitative alterations of other constituents within the normal range.[50]

Studies of the blood coagulation process have been greatly advanced by analyses of the pathology of hemophilia and thrombophilia. However, individuals with congenital hemophilia or thrombophilia represent only the extreme ≥5% of the distribution of effective pro-coagulant and anticoagulant concen-

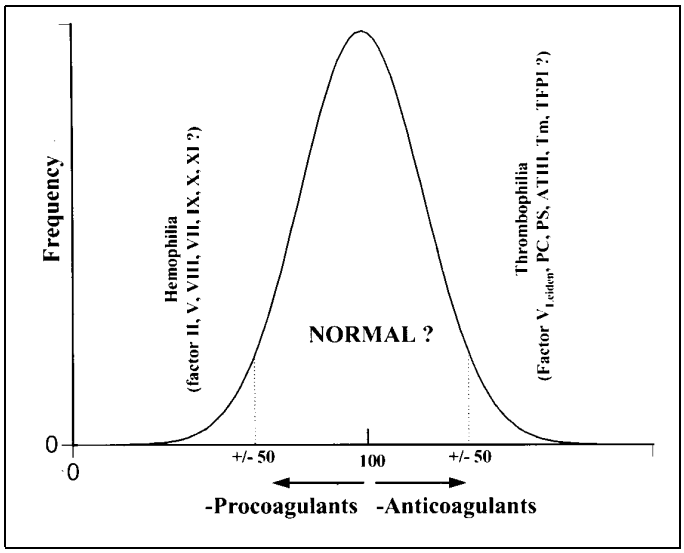


Figure 5. Hypothetical normal population distribution curve of frequency versus the plasma concentrations, of pro-coagulants and anticoagulants. The normal range is identified by the interval between 50% and 150% of mean plasma values. Thrombophilia and hemophilia are represented by the extremes of the curves, in which qualitative or quantitative pro-coagulant or anticoagulant failure occurs.

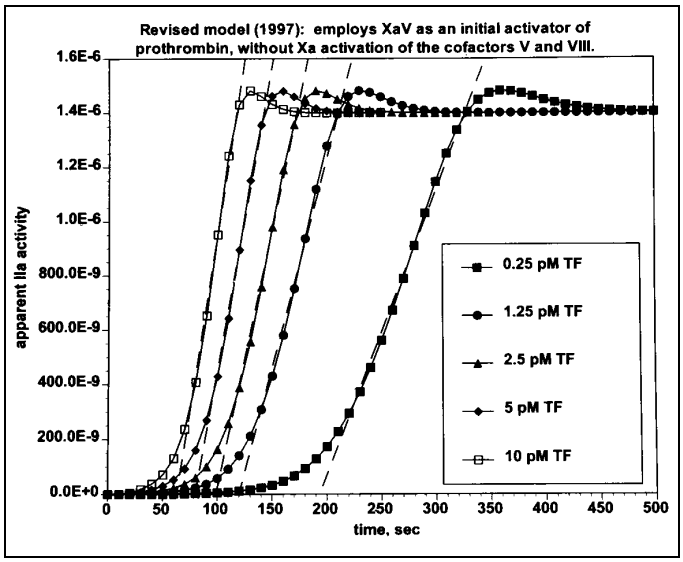


Figure 6. Mathematical model of pro-coagulant reactions initiated by tissue factor-factor VIIa with pro-coagulant proteins at normal plasma concentrations. The initiation phase of the reaction is dependant upon the tissue factor concentration, while the rate of the propagation phase of thrombin generation is almost independent of the initiator concentration.

trations represented in the human population (Fig. 5). The most frequently encountered congenital abnormality associated with thrombophilia, factor V^{Leiden} is present in less than 5% of the Caucasian population. However, most of the "normal" human population will succumb with the generation of clot obstructing blood flow to a major organ. It is likely that the mosaic associated with the expression of pro-coagulants and anticoagulant within "normal" levels contributes to morbid or fatal thrombosis pathology in the human population.

We have conducted in vitro synthetic plasma experiments, in which the concentrations of inhibitors and pro-coagulants (Table 1) are individually and collectively varied over their 'normal' ranges. These data indicate that the balance of pro-coagulant and anticoagulant factors within the 'normal' range can generate extraordinarily different results in terms of thrombin generation, as a result of different tissue factor "insults."

In the most extreme instance, when all pro-coagulants are present in the reaction mixture at 150% of their normal values and all anticoagulants are at 50%, the total amount of thrombin produced in the reaction system is increased by seven-fold over the amount of thrombin produced when all constituents are at 100% of their normal value. Conversely, when all pro-coagulants are present at 50% of their normal plasma values, and the inhibitors are present at 150%, the amount of thrombin produced is only 25% that produced with all reactants at mean value concentrations. The principal contributors to these extremes of "normal" are prothrombin and antithrombin III.

Mathematical Models

Mathematical models have been developed to describe the pro-coagulant reactions of the coagulation system. These models are based upon the thermodynamic, stoichiometric and catalytic parameters of individual pro-coagulant-anticoagulant reactions obtained from experimental measurements or inferred by comparison with analogous reactions. These numerical simulations of the pro-coagulant reactions are consistent with the empirical data obtained for the pro-coagulant mixtures (Fig. 1) in synthetic plasma experiments.[51] Models based upon the individual contributions of antithrombin III and TFPI are reasonably simulated using existing models of inhibitor mechanisms and kinetic models. However, in contrast to the observed synergistic relationships observed in the 'synthetic plasma' models, these outcomes are not predicted with present computer simulations based upon the proposed mechanisms for these inhibition processes.

Our present mathematical models are reasonably accurate in describing, from both a quantitative and qualitative perspective, the expression of pro-coagulant reactions in the generation of thrombin. Computer data generated for simulated pro-coagulant reaction profiles are illustrated in Figure 6. These show the dependence of the initiation phase of the reaction on the concentration of factor VIIa-tissue factor. Conversely, the propagation phase is primarily dependent upon the molar concentrations and qualitative properties of all other constituents in the reaction. These models have been used to explore such questions as the requirements of factor V/factor Va, factor VIII, factor IX, and factor XI in the reaction system and, most recently, to quantify the ability of factor VIIa to overcome the requirement for factor VIII in the generation of factor Xa by the intrinsic factor tenase complex). Our computer models suggest that factor VIIa will be effective in overcoming the requirement for factor VIII or factor IX at concentrations in the range of 5-10 nM. The theoretical simulations, thus, approach those concentrations of recombinant factor VIIa used empirically to manage individuals with hemophilia A and coagulation factor inhibitors.[52]

In contrast to the empirical analyses conducted in vitro with

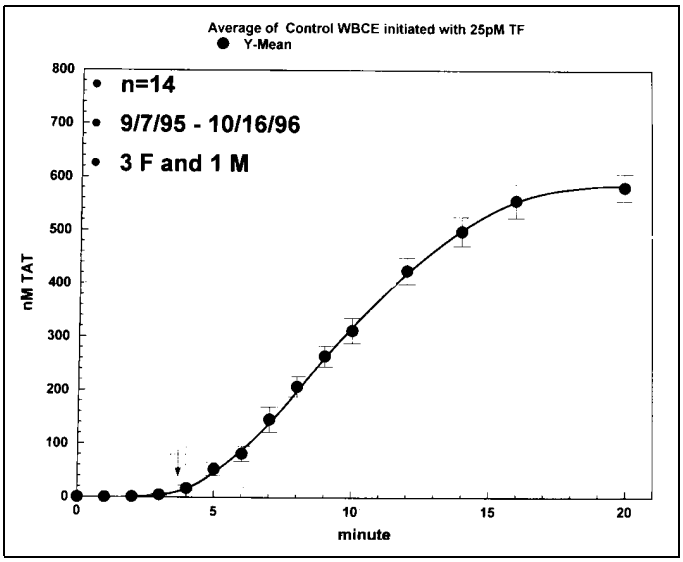


Figure 7. Whole blood clotting experiments initiated with 25 pM tissue factor at 37º in the presence of corn trypsin inhibitor. The data represented are thrombin antithrombin III (TAT) as a function of time (±SEM). The point of clotting is indicated by the arrow at approximately 4 minutes.

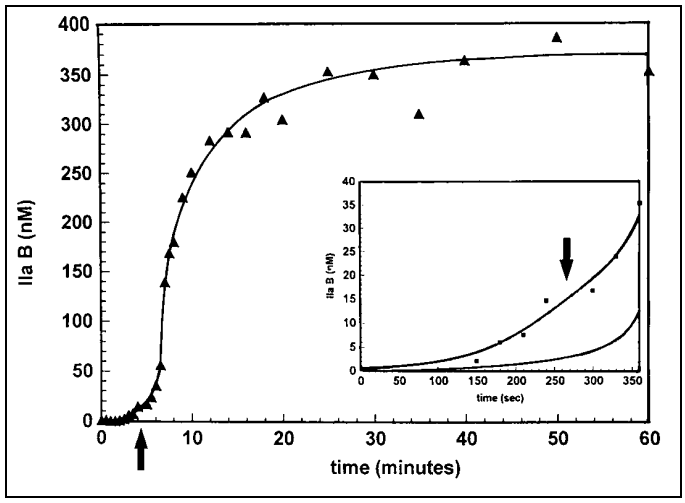


Figure 8. Concentration of thrombin B-chain during the fluid phase of clotting blood. Thrombin B-chain (▲) is seen to appear rapidly after an initial lag phase. The inset shows the relative concentration of B-chain calculated from rates of FPA generation (lower line) and by densitometry (■) over the first 350 seconds. Clot time (4.5 minutes) is indicated by the bold arrow. Used with permission from Rand et al.[53]

purified proteins, which are extremely labor-intensive, computer simulations, such as those illustrated in Figure 6, can be generated in seconds in computation facilities available to most research laboratories. In contrast, empirical experiments involve the production of the exquisitely pure proteins required for the synthetic mixtures and the execution of complex analyses to produce the experimental data.

Minimally-Altered Whole Blood

Our clotting studies involving minimally-altered whole blood attempt to create, in as relevant a process as possible, an in vitro analysis of blood clotting initiated solely by the tissue factor pathway. In the conduct of these experiments, whole blood is collected by phlebotomy, maintained at 37°C, and mixed with corn trypsin inhibitor and a fixed concentration of tissue factor-phospholipid complex.[53] The concentrations of tissue factor used are typically from 25 to 5 pM, producing clotting times from 4 to 15 minutes. The added phospholipid, in which the tissue factor is reconstituted (5-25nM), is insufficient to replace the cellular contribution of blood to the coagulation process. Thus, the reaction is also sensitive to qualitative and quantitative alterations in the cellular population in blood. The reaction is monitored by clotting time, enzyme-linked immunosorbent (ELISA), and immunoblotting assays and by chromatographic evaluation of protein/peptide products.

Experiments have been conducted with blood from normal individuals, individuals with congenital hemophilias, and blood to which platelet and coagulation inhibitors have been added. In general, the observations obtained in whole blood are consistent with those from the synthetic plasma experiments. The most revealing conclusions are that the major defects observed with congenital deficiencies and with clinically effective anticoagulant inhibitors are most significantly reflected in events that occur after clotting, i.e. during the propagation phase of thrombin formation. These experiments suggest that the generation of thrombin beyond the formation of the initial fibrin-platelet clot may be the most significant distinguishing feature associated with the pathology of hemorrhagic diseases and the efficacy of antithrombotic agents.

As illustrated in Figure 7, results obtained using corn trypsin-inhibited-tissue factor initiated whole blood are reproducible. In fourteen experiments conducted with four volunteers over a 13-month interval, the generation of thrombin-antithrombin III (TAT) complexes and the interval required for clotting (arrow) are very consistent. The expression of TAT complex illustrated in Figure 7 is similar to the generation of thrombin in the synthetic system illustrated in Figure 4 and the computer-based models of thrombin generation illustrated in Figure 6. A characteristic initiation phase precedes a propagation phase, in which the majority of thrombin is generated. Visible clotting of blood occurs at the point of transition between these two phases.

The generation of thrombin during the course of whole blood clotting initiated by tissue factor is illustrated in Figure 8. These data were obtained by immunoblots using an antithrombin antibody. The point of clotting, illustrated by the arrow, occurs with the generation of approximately 10-15 nM thrombin. At the time of clot formation, about 2 nM, or 10% of the factor Va (judged by light-chain generation), in this individual had been activated (Fig. 9A). Platelet activation, based upon PF4 release, indicates that, at the point of clotting, approximately 30% of the platelet activation has occurred. An estimation of the concentration of *prothrombinase* present at clot time can be obtained from the rate of the thrombin B-chain generation data. This suggests that, at clotting time, approximately 0.7 nM platelet *prothrombinase* sites are available. As illustrated in Figure 9B, at clot time, only about 10 pM *prothrombinase* is generated, suggesting that the limiting reactant is factor Xa.

Extensive studies conducted with blood from individuals

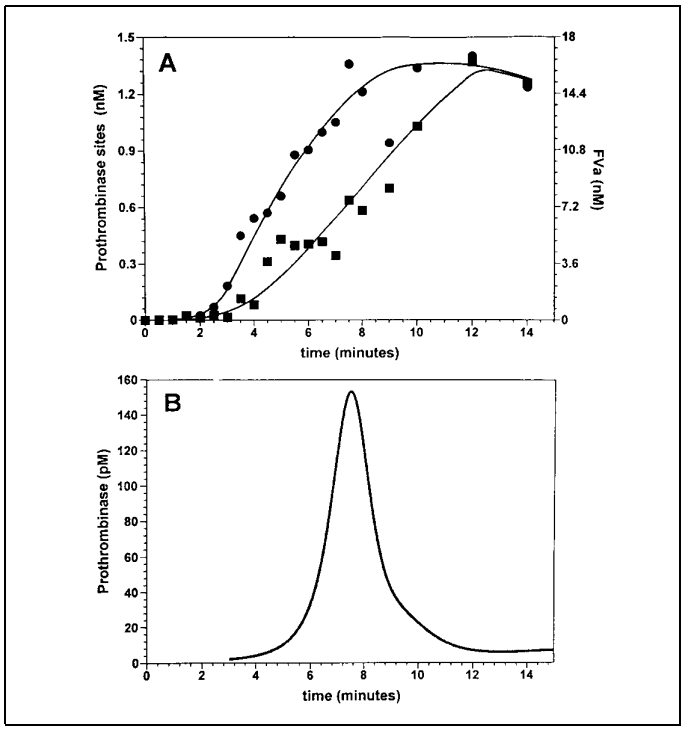


Figure 9A. Prothrombinase expression in whole corn trypsin inhibited (32 μg.ml) blood initiated with 40 pM tissue factor reconstituted in 80 nM phospholipid. (A) (left axis) The concentration of platelet prothrombinase sites formed as a consequence of platelet activation is plotted versus time. (●) (right axis) The concentration of factor Va (❑) (left axis) is plotted versus time. Clot time is at 4.5 minutes. B. The concentration of prothrombinase inferred from the generation of thrombin B-chain (Figure 9A) is plotted versus time. From reference 53 with permission.

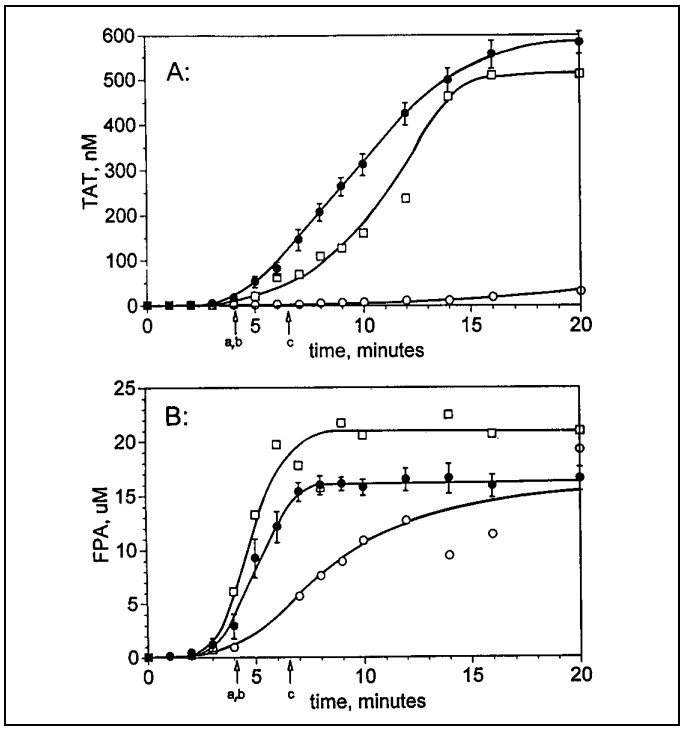


Figure 10 A. Coagulation in normal and hemophila A corn trypsin inhibited (50 μg/ml) blood, with and without factor VIII (0.7 nM) replacement initiated with 25 pmol/l TF. Time courses for TAT In normal blood (●), hemophilia A blood (❍), and hemophilia A blood with recombinant factor VIII (1 U/ml) (❑). Clot time for the normal curve is 4.0 ± 0.2 minutes ("a"). Clotting in factor VIII-deficient blood occurred at 6.5 minutes ("c") and 4.1 minutes with factor VIII replacement ("b"). B. FPA generation in normal blood (●), factor VIII-deficient blood (0), and factor VIII-deficient blood with replacement.(❑) (See Figure 10A for detail). Used with permission from Cawthern et al.[54]

with hemophilia A and hemophilia B have led to a somewhat surprising observation.[54] As anticipated, clotting is delayed for the blood of an individual with severe hemophilia A (<1% factor VIII) (Fig. 10A,B, compare "a" with "c"). Fibrinopeptide generation (Fig. 10B) is delayed with severe hemophilia A; however, the generation of fibrinopeptide A proceeds to completion. In contrast, thrombin generation in hemophilia A is almost totally suppressed (compare closed and open circles) during the propagation phase (Fig. 10A). Thus, the major quantitative differences seen in severe hemophilia A (and reproduced in hemophilia B) are a moderate extension in the duration of the initiation phase of the reaction and a moderate reduction in the rate of fibrinopeptide A generation accompanied by major suppression of the propagation phase of thrombin generation. Thus, clotting is not the most distinctive endpoint in assessing the pathology of hemophilia A.

When challenged with a similar concentration of tissue factor as that used in the studies of hemophilia A and B, the blood of individuals with severe hemophilia C (factor XI deficiency) is not distinguishable from normal.[54] However, when the reaction is initiated at tissue factor concentrations that would clot normal blood in approximately 15 minutes (5 pM), one can see the influence of factor XI deficiency on both thrombin generation and fibrinopeptide A generation. These observations are consistent with the observed severity of the pathology associated with hemophilia A and hemophilia B, as compared to hemophilia C.

Conclusion

The accomplishments of many colleagues in the field of coagulation over the past 150 years portens a bright future for advances in the intervention for hemorrhagic and thrombotic diseases in the 21st century. The principal limitation will be the attraction of new basic and clinical scientists into the relevant fields of biochemistry, molecular genetics, hematology, cardiology, pathology, and surgery. Future research will exploit existing advances in technology in the pursuit of better techniques for diagnosis, prophylaxis, and intervention in cardiovascular diseases.

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References

1. Schmidt A. Neue Untersuchungen ueber die Fasserstoffesgerinnung. *Pflueger's Arch.f.ges.Physiol*. 1872;6:413-538.
2. Morawitz P. Die Chemie der Blutgerrinnung. *Ergebn Physiol*. 1905;4:307-423.
3. Patek AJ, Taylor FHL. Hemophilia II: some properties of a substance obtained from normal plasma effective in accelerating the clotting of hemophilic blood. *J Clin Invest.* 1937;113-124.
4. Brinkhous KM. Clotting defect in hemophilia: deficiency in a plasma factor required for platelet utilization. *Proc Soc Exp Biol Med*. 1947;66:117-120.
5. Aggeler PM, White SG, Glendenning MB. Plasma thromboplastin component (PTC) deficiency: a new disease resembling hemophilia. *Proc Soc Exp Bio Med*. 1952;79:692-694.
6. Schulman ISCH: Hemorrhagic disease in an infant due to deficiency of a previously undescribed clotting factor. *Blood*. 1952;7:794-807.
7. Biggs R, Douglas AS, MacFarlane RG, et al. Christmas disease: a condition previously mistaken for hemophilia. *Br Med J*. 1952;2:1378-1382.
8. Bachmann F, Duckert F, Koller K. The Stuart-Prower factor assay and its clinical significance. *Thromb Diath Haemorrh.* 1999;2:24-38.
9. Alexander B, Goldstein RA, Landweher G, et al. Congenital SPCA deficiency: a hither to unrecognized coagulation defect with hemorrhage rectified by serm and serum fractions. *J Clin Invest*. 1951;30:596-608.
10. Soulier JP, Wartell O, Menache D. Caracteres differentiels des facteurs hageman et P.T.A.; role due contact dans la phase initiate de la coagulation. *Rev Franc Etudes Clin Biol.* 1958;3:363
11. Botti RE, Ratnoff OD. The clot-promoting effect of soaps of long-chain saturated fatty acids. *J.Clin.Invest.* 1963;42:1569-1577.
12. Owren PA. Parahaemophilia. Haemorrhagic diathesis due to absence of a previously unknown clotting factor. *Lancet*. 1947;1:446-448.
13. Wright IS. The nomenclature of blood clotting factors. *Thromb Diath Haemost*. 1962;7:381-388.
14. Warner ED, Brinkhous KM, Smith HP. A quantitative study of blood clotting. *Am J Physiol.* 1936;114:667-675.
15. Quick AJ. On the Constitution of Prothrombin. *Am J Physiol*. 1999;140:212-220.
16. Langdell RD, Wagner RH, Brinkhous KM. Effect of antihemophilic factor on one-stage clotting tests: a presumptive test for hemophilia and a single one-stage anti-hemophilic factor assay procedure. *J Lab Clin Med.* 1953;41:7637-7647.
17. Ratnoff OD, Colpy JE. A familial hemorrhagic trait associated with a deficiency of a clot-promoting fraction of plasma. *J Clin Invest.* 1955;34:602-613.
18. Jacobsen SFM. Some data on two purified kininogens from human plasma. *Br.J.Pharmacol*. 1967;29:25
19. Mandle RJr, Coleman RW, Kaplan AP. Identification of prekallikrein and high molecular weight kininogen complex in plasma. *Proc Natl Acad Sci USA.* 1976;F3:4179-4183.
20. Duckert F, Jung SH, Schmerling DH. A hitherto undescribed congenital haemorrhagic diathesis probably due to fibrin stabilizing factor deficiency. *Thromb Diath Haemorrh.* 1961;5:179-186.
21. Howell WH. The role of antithrombin and thromboplastin (thromboplastic substance) in the coagulation of blood. *Am J Physiol.* 1911;29:187-209.
22. Broze GJ, Jr., Miletich JP. Isolation of the tissue factor inhibitor produced by HepG2 hepatoma cells. *Proc Natl Acad Sci USA.* 1987;84:1886-1890.
23. Stenflo J, Fernlund P, Egan W, Roepstorff P. Vitamin K dependent modification of glutamic acid residues in prothrombin. *Proc Natl Acad Sci USA.* 1974;71:2730-2733.
24. Kisiel W. Human plasma protein C. Isolation, characterization and mechanism of activation by alpha-thrombin. *J Clin Invest*. 1979;64:761-769.
25. DiScipio RG, Davie EW. Characterization of protein S, a gamma-carboxy-glutamic acid containing protein from bovine and human plasma. *Biochem*. 1979;18:899-904.
26. Broze GJ, Miletich J. Human Protein Z. *J Clin Invest*. 1984;73:933.
27. Esmon CT, Owen WG. Identification of an endothelial cell cofactor for thrombin catalyzed activation of protein C. *Proc Natl Acad Sci USA.* 1981;78:2249-2252.
28. MacFarlane RG. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature.* 1964;202:498-499.
29. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. *Science.* 1964;145:1310-1312.
30. Osterud B, Rapaport SI. Activation of Factor IX by the reaction product of tissue Factor and Factor VII: Additional pathway for initiating blood coagulation. *Proc Natl Acad Sci*. 1977;74:5260-5260.
31. Gailani D, Broze GJ, Jr.. Factor XI activation in a revised model of blood coagulation. *Science.* 1991;253:909-912.
32. Ware AG, Seegers WH. Two Stage Procedure for the Quantitative Determination of Prothrombin Concentration. *Am J Clin Path.* 1949;19:471-482.
33. Seegers WH. Activation of Purified Prothrombin. *Proc Soc Exptl Biol Med*. 1949;72:677-680.
34. Tracy PB, Eide LL, Mann KG. Human prothrombinase complex assembly and function on isolated peripheral blood and cell populations. *J Biol Chem*. 1985;260:2119-2124.
35. Nesheim ME, Pittman DD, Wang JH, Slonosky D, Giles AR, Kaufman RJ. The binding of 35S-labeled recombinant factor VIII to activated and unactivated human platelets. *J Biol Chem*. 1988;263:16467-16470.
36. Weiss HJ, Turitto VT, Baumgartner HR. The role of shear rate and platelets in promoting fibrin formation on rabbit subendothelium:Studies utilizing patients with quantitative and qualitative platelet defects. *J Clin Invest*. 1986;78:1072
37. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MA Jr.. Interleukin-1 (IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *J Exp Med*. 1984;160:618-623.
38. Mann K, Nesheim M, Church W, Haley P, Krishnaswamy S. Surface dependent reactions of the vitamin K-dependent enzyme complexes. *Blood.* 1990;76:1-16.
39. Morrissey JH, Macik BG, Neuenschwander PF, Comp PC. Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood.* 1993;81:734-744.
40. Lawson JH, Butenas S, Mann KG. The evaluation of complex-dependent alterations in human factor VIIa. *J Biol Chem*. 1992;267:4834-4843.
41. Broze GJ, Jr., Girard TJ, Novotny WF. Regulation of coagulation by a multivalent Kunitz-type inhibitor. *Biochemistry*. 1990;29:7539-7546.
42. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J Biol Chem*. 1989;264:4743-4746.
43. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem*. 1994;269:31869-31880.
44. Fay PJ, Smudzin TM. Characterization of the Interaction of the A2 Subunit and AI/A3-C1-C2 Dimer in Human Factor VIII. *J Biol Chem*. 1992;267:13246-13250.
45. Rosenberg RD, Damus PS. The Purification and Action of Human Antithrombin-Heparin Cofactor. *J Biol Chem*. 1973;248:6490-6505.
46. Marcum JA, Rosenberg RD. Anticoagulantly active heparinlike molecules from vascular tissue. *Biochemistry.* 1984;23:1730-1730.
47. Lawson JH, Kalafatis M, Stram S, Mann KG. A model for the tissue factor pathway to thrombin: 1. an empirical study. *J Biol Chem*. 1994;269(37):23357-23366.
48. van 't Veer C, Golden NJ, Kalafatis M, Mann KG. Inhibitory mechanism of the protein C pathway on tissue factor-induced thrombin generation: synergistic effect in combination with tissue factor pathway inhibitor. *J Biol Chem*. 1997;272:7983-7994.
49. van 't Veer C, Mann KG. Regulation of tissue factor initiated thrombin generation by the stoichiometric inhibitors tissue factor pathway inhibitor, antithrombin-III, and heparin cofactor II. *J Biol Chem*. 1997;272:4367-4377.
50. van 'tVeer C, Kalafatis M, Bertina RM, Simioni P, Mann KG. Increased tissue factor-initiated prothrombin activation as a result of the Arg^{506}-Gln Mutation in factor V^{Leiden}. *J Biol Chem*. 1997;272:20721-20729.
51. Jones KC, Mann KG. A model for the tissue factor pathway to thrombin: 2. a mathematical simulation. *J Biol Chem*. 1994;269(37):23367-23373.

52. Shafi T, Jeha JM, Black L, Al Douri M. Severe acquired haemophilia A treated with recombinant factor VIIa. *Br J Haematol*. 1997;98:910-912.
53. Rand MD, Lock JB, van 't Veer C, et al. Blood clotting in minimally altered whole blood. *Blood*. 1997;88:3432-3445.
54. Cawthern KM, van 't Veer C, Locke JB, DiLorenzo ME, Branda RF, Mann KG: Blood Coagulation in Hemophilia A and Hemophilia C. *Blood*. 1998;91:4581-4592