The Dynamics of Thrombin Formation
Kenneth G. Mann, Saulius Butenas, Kathleen Brummel

Abstract—The central event of the hemostatic process is the generation of thrombin through the tissue factor pathway. This is a highly regulated, dynamic process in which thrombin itself plays many roles, positively and negatively its production and destruction. The hemostatic process is essential to normal physiology and is also the Achilles heel of our aging population. The inappropriate generation of thrombin may lead to vascular occlusion with the consequence of myocardial infarction, stroke, pulmonary embolism, or venous thrombosis. In this review, we summarize our present views regarding the tissue factor pathway by which thrombin is generated and the roles played by extrinsic and intrinsic factor Xa generating complexes in hemostasis and the roles of the stoichiometric and dynamic inhibitors that regulate thrombin generation. (Arterioscler Thromb Vasc Biol. 2003;23:17-25.)

Key Words: coagulation ■ fibrinogen ■ aggregation ■ coagulation inhibitors

The inventory of molecular components and the presumed physiology of the hemostatic process and its regulation have been established on the basis of plasma abundance, hemorrhagic or thrombotic pathology, in vitro tests, and chemical signatures. Two in vitro plasma tests, the prothrombin time and the activated partial thromboplastin time, were prominent in the development of the inventory. The former test relies on the addition of an extrinsic tissue factor source (thromboplastin), whereas the latter is based on the introduction of a foreign surface to initiate coagulation using only this surface contact and the biological constituents intrinsic to plasma. Both tests rely on a fibrin formation (clotting) end point. These assays permitted identification of connectivity between the component activities identified as required for plasma coagulation and defined the concept of intrinsic and extrinsic coagulation pathways, which converge at the step of formation of the prothrombinase complex. However, the mechanisms established by in vitro tests are not always mirrored in human pathology associated with bleeding or thrombosis. The primary pathway leading to hemostatic and thrombotic pathologies is associated with the tissue factor–initiated extrinsic coagulation pathway, whereas components unique to the intrinsic or contact pathway (factor XI, factor XII, prekallikrein, HMW kininogen) may have accessory roles in the process. Therefore, in this review, we focus on the dynamics of the reactions associated with the introduction of tissue factor to blood, leading to the formation of thrombin.

An evaluation of the reactions involved in the formation of thrombin leads to the conclusion that the physiologically relevant hemostatic mechanism is composed of 3 procoagulant vitamin K–dependent enzyme complexes (which use the proteases factor IXa, factor Xa, and factor VIIa) and one anticoagulant vitamin K–dependent complex.1 Each complex involves a vitamin K–dependent serine protease and a cofactor protein with the protein-protein complex assembled on a membrane surface provided by activated or damaged cells. The same hemostatic process required for preventing leaks from the vasculature may also be life threatening when responsible for an intravascular occlusion. Thus, nature has elected a system for highly regulated, multiconstituent activity presentation that provides a process that will lead to the local arrest of hemorrhage. The plasma proteins involved in the process require activation to participate in the thrombin-generating process. In addition, platelet adhesion and activation are required to provide membrane binding sites explicitly at the region of vascular damage, because platelet adhesion and activation provides the discrete membrane sites on which all of the plasma-derived procoagulant complexes are assembled.2–7 Equally important are the stoichiometric and dynamic inhibitory systems, which block the presentation of thrombin. The sum of inhibitory functions is far in excess of the potential procoagulant response. These inhibitory processes act in synergy, providing minimal activation thresholds, which must be achieved before significant thrombin generation.8–10

The significance of the components involved in the procoagulant response and its regulation in a genetically homogeneous population is reflected in studies of function-deleted transgenic mice. In mice, elimination of tissue factor, factor VII, tissue factor pathway inhibitor (TFPI), factor X, factor V, prothrombin, and protein C is lethal, whereas deficiencies of factor VIII and factor IX contribute significantly to hemorrhagic risk.11–19 It is instructive to note that in the outbred human population, individuals with equally severe prothrombin, factor X, factor V, and factor VII deficiencies do exist,
and indeed most of these factors were discovered because of the presentation of a living propositus displaying hemorrhagic pathology.20–25 Thus, genetic and environmental events can significantly alter a potentially lethal outcome. It is also interesting to note that congenital fibrinogen deficiency both in mice and man is not lethal and frequently only mildly symptomatic. Therefore, fibrin formation does not seem to be essential for survival.26

Overview

The key initiating event in the generation of thrombin depends on the interaction of membrane-bound tissue factor and factor VIIa, the latter of which is preexistent in the plasma milieu at ∼1% to 2% of the total factor VII concentration (10 nmol/L).27,28 The source and presentation of active tissue factor is controversial.29–33 However, its damage-related presentation is essential. The factor VII zymogen is cleaved at arginine 152 by a variety of proteases, including thrombin, factor IXa, factor Xa, and factor VIIa–tissue factor to produce the serine protease factor VIIa.34 However, although this “enzyme” seems to possess all of the appropriate catalytic machinery to display the active site of an effective serine protease, it does not express proteolytic activity unless it is bound to tissue factor. Thus, naked factor VIIa at natural biological concentrations has no significant activity toward either factor IX or factor X before its binding to tissue factor.35 The defective active site also makes factor VIIa impervious to the high concentration of antithrombin-III (AT-III) present in blood, which permits its continued existence.36 The factor VIIa-tissue factor protein–protein interaction increases the $k_{cat}$ of the enzyme for synthetic substrates by two orders of magnitude37,38 and increases the rate of factor X activation by four orders of magnitude.39,40 This latter increase is the result of the aforementioned improvement in catalytic efficiency and the membrane binding of the macromolecular substrates factor IX and factor X. Substrate–membrane binding leads to an effective reduction in $K_m$. Overall, the $k_{cat}$ increase and $K_m$ lowering leads to a $10^4$ increase in the expression rate of the enzyme toward its natural substrates.

The factor VIIa-tissue factor (extrinsic) complex (Figure 1) catalyzes the activation of both factor IX and factor X, the latter initially being the more efficient substrate. Thus, the initial product formed by the extrinsic factor Xase is factor IXa alone and factor-factor VIIa-factor Xa product complex, thus effectively neutralizing the extrinsic factor Xase and eliminating thrombin production.41–43 Once factor VIIa is formed, the factor IXa generated by factor VIIa-tissue factor combines with factor VIIIa on the activated platelet membrane to form the intrinsic factor Xase (Figure 1) that becomes the major activator of factor X. The factor VIIIa-factor Xa complex is $10^4$–$10^6$-fold more active than factor IXa alone and ∼50 times more efficient than factor VIIa–tissue factor in catalyzing factor X activation; thus, the bulk of factor Xa is ultimately produced by factor VIIa-factor IXa.44 Factor Xa combines with factor Va on the activated platelet membrane surface, and this “prothrombinase” catalyst (Figure 1) converts prothrombin to thrombin. Prothrombinase is 300 000-fold more active than factor Xa in catalyzing prothrombin activation.

The coagulation system is under extraordinarily tight regulation by both stoichiometric and dynamic inhibition systems. The tissue factor concentration threshold for reaction initiation is steep, and the ultimate amount of thrombin produced is largely regulated by the stoichiometric inhibitors tissue factor pathway inhibitor (TFPI) and antithrombin III (AT-III) and by the dynamic anticoagulant process of protein C activation and functional expression (Figure 2).45–51

The principal influence of TFPI is to block the tissue factor-factor VIIa-factor Xa product complex, thus effectively neutralizing the extrinsic factor Xase and eliminating this catalyst’s generation of both factor Xa and factor IXa.46 TFPI is present in a low abundance (∼2.5 nmol/L) in blood; it is also releasable from the vasculature by the action of heparin.47 The stoichiometric inhibitor AT-III is normally present in plasma at more than twice the concentration (3.4 μmol/L) of any potential target coagulation enzyme generated by the tissue factor pathway. AT-III is an effective neutralizer of all of the procoagulant serine proteases.45 The targets of AT-III are primarily the mature enzyme products of these reactions. AT-III is a weaker inhibitor of the factor VIIa-tissue factor complex46; thus, the principal influence of this inhibitor is in quenching thrombin production and the thrombin produced.
To initiate the dynamic protein C system, the product enzyme thrombin binds to constitutively present vascular thrombomodulin (Tm) and activates the protein C (PC) to its activated species APC. APC competes with factor Xa and factor IXa for factor Va and factor VIIIa binding and ultimately leads to inactivation of factor Va and factor VIIIa by proteolytic cleavage. Antithrombin III neutralizes all serine proteases present in the reaction mixture with the exception of factor VIIa. The tissue factor-factor VIIa complex is neutralized by the tissue factor pathway inhibitor as the factor Xa product complex. Thrombin binds to thrombomodulin and activates protein C. APC competes with factor Xa and factor IXa for factor Va and factor VIIIa, interfering with the formation of the “prothrombinase” and the “intrinsic Xase,” initially by competition with factor Xa and factor IXa and ultimately by cleaving the cofactor factor Va and factor VIIIa to eliminate these complexes.

Models of the Blood Coagulation Reaction

Our laboratory has made use of 4 models in attempts to recapitulate the dynamic mechanism of the coagulation reaction system. These have included the following: (1) synthetic plasma, in which mixtures prepared from purified procoagulants and anticoagulants and a natural or synthetic membrane source are induced to react by the addition of lipid reconstituted tissue factor9,10,41,55–60; (2) paravivo studies of coagulation, in which whole, contact pathway-suppressed blood obtained by phlebotomy and maintained at 37°C is induced to clot by addition of a fixed amount of membrane reconstituted tissue factor52,61–68; (3) numerical (computer) models of the coagulation system, which are based on the ensemble of published (and estimated) rate constants and concentrations of procoagulants and stoichiometric inhibitors and the mechanisms of their interactions89–71 (also referred to as “in silico” simulations); and (4) in vivo studies of the hemostatic reaction, in which whole blood exuding from a microvascular wound is sequentially sampled for relevant product formation.72–75

Each of the model systems used has specific benefits and limitations. The in vivo model, involving a “Simplate” wound, is the least flexible, involves the highest degree of discomfort for the volunteer, and is analytically difficult; however it is the most biologically relevant. The numerical model is the least expensive and the most rapid and convenient method of analysis. It provides insight into the regulation of reaction mechanisms occurring at concentrations of intermediates and products, which may be inferred but not measured with existing technology. It is (obviously) the least biologically relevant.

Thrombin Generation

Regardless of the model system chosen, the display of thrombin generation after tissue factor initiation of the hemostatic reaction is approximately the same.41,42,56,71,73 This behavior is illustrated in Figure 3, which shows the generation of the thrombin-AT-III complex (TAT) as a function of time using the paravivo model. From an operational perspective, thrombin generation may be described as occurring in two phases. Shortly after the addition of tissue factor, tiny amounts of thrombin (nanomolar) are produced in an interval, which we define as the initiation phase of the reaction. Subsequently, the major bolus (>96%) of thrombin is produced during the propagation phase of the reaction. During the initiation phase of the reaction, the factor VIIa-TF complex forms and generates small amounts (subpicomolar) of factor Xa and factor IXa.40,41,55 Factor Xa in collaboration with the membrane surface activates a small amount of prothrombin to thrombin, which serves to generate the platelet membrane and cofactor components required for the major generation of thrombin.

The early events associated with thrombin function during the initiation phase of the reaction are illustrated in Figure 4, which shows the inception points for the detection of thrombin products generated during the reaction measured in
Many of these products are required to provide the catalysts (Figure 1) that generate most of the thrombin produced during the propagation phase of the reaction.

Under normal circumstances, the rate-limiting component of most prothrombinase formation and the generation of thrombin activity is the concentration of factor Xa. Thus, under normal conditions, the activation of factor V and the activation of platelets (probably through thrombin-PAR-1 receptor interactions) occur rapidly to produce surplus factor Va and platelet membrane binding sites. However, under conditions of congenital deficiency, thrombocytopenia, platelet pathology, or pharmacologic intervention, the tissue factor-initiated reaction can become sensitive to factor V or platelets. In studies to assess the influence of preactivation of platelets, we used the thrombin receptor activation peptide to provide for the preexpression of complex binding sites on the platelet surface. Preactivation of the platelets did not change the rate of thrombin generation. However, pharmacologic interventions with agents such as PGE1, ReoPro (Abciximab), and Integrilin do influence the generation of thrombin in the reaction. Similarly, reductions in platelet counts (<10 000/mm³) elicit dependence on platelet concentration.

The end point used in evaluating hemostasis in most bioassays is the generation of a fibrin clot. As illustrated in Figure 4, the formation of a fibrin clot occurs at 10 to 30 nmol/L thrombin or ~3% of the total amount of thrombin produced during the reaction, which is provided by only ~7 pmol/L prothrombinase. Thus, most thrombin formation is ignored using present technology for evaluating clinical hemorrhagic risk or thrombosis.

Figure 4. The generation of thrombin activation products during the initiation phase of the reactions shown in Figure 3. Platelet activation (osteonectin release) is detected at <1 nmol/L thrombin (0.06% of the total thrombin ultimately produced). The clotting time, 4.7 minutes, is observed immediately after the inception of the propagation phase of thrombin generation. The dotted line (●) corresponds with the active thrombin present in the reaction mixture, as determined from fibrinopeptide release. From: Brummel KE, Paradis SG, Butenas S, Mann KG. Thrombin functions during tissue factor-induced blood coagulation. Blood. 2002;100:148–152. Copyright American Society of Hematology, used by permission.
stoichiometric concentrations of factor VIIIa (●) and factor IXa (□) but also the dissociation constant between these two proteins and the instability of factor VIIIa, which spontaneously dissociates, limiting the concentration of factor VIIIa.

Figure 6. The contributions of the intrinsic and extrinsic factor Xase to factor Xa generation over the time course of the reaction. The inset illustrates the relative percentages of factor Xa generated by each catalyst. Reprinted with permission from Hockin MF, Jones KC, Everse SJ, Mann KG. A model for the stoichiometric regulation of blood coagulation. J Biol Chem. 2002;277:18322–18333.

Significance of Intrinsic Factor Xase

The relative factor Xa generation by the factor VIIa-tissue factor and the factor IXa-factor VIIIa complexes is illustrated in Figure 6.71 Initially, the concentration of the factor VIIa-tissue factor complex (∼10⁻¹¹ mol/L) is higher than the concentration of the factor VIIIa-factor IXa complex, which requires activation and assembly. As time progresses, however, the contribution of the latter, more active complex in factor Xa generation exceeds that of the extrinsic factor Xase. As a consequence, most of factor Xa (Figure 6 inset) is ultimately produced by the factor VIIa-factor IXa complex in the tissue factor–initiated hemostatic processes. Clotting would have occurred at 250 to 300 seconds at this tissue factor concentration.

In the absence of factor VIII or factor IX, the intrinsic factor Xase cannot be assembled; thus no propagation phase occurs. This is the principal defect observed in hemophilia A and hemophilia B62,67 in all models, including paravivo blood studies from those affected with these hemorrhagic abnormalities.

Because the presentation of a clot depends only on the generation of 10 to 30 nmol/L thrombin, at high tissue factor concentration, robust generation of factor Xa by factor VIIa-tissue factor can completely mask the contribution of the factor VIIa-factor IXa complex in clot end point assays. This is the case for the prothrombin time in which the concentrations of thromboplastin (tissue factor and phospholipid) are chosen to produce a clot time of 11 to 15 seconds. This corresponds to a tissue factor concentration of >20 nmol/L. For the illustrations of Figures 3 through 6, a concentration of 5 pmol/L TF was used, producing a clotting time of ∼5 minutes. In hemophilia A and B, at these TF concentrations, although the clotting time is prolonged, the major defect is associated with the absence of a propagation phase.62,67

Attenuation of Thrombin Generation

The attenuation of the coagulation system is as important as the procoagulant process and involves both stoichiometric and dynamic regulators. TFPI and AT-III are the principal stoichiometric inhibitors of the process. TFPI is the principal regulator of the initiation phase of thrombin generation, whereas AT-III serves to attenuate thrombin activity and its generation.9 These two agents when combined provide a synergistic regulatory effect by inducing kinetic thresholds such that the initiating tissue factor stimulus must be of significant magnitude to propel thrombin generation. Tissue factor concentrations below the threshold concentration required are ineffective in promoting massive thrombin generation because of the cooperative influence of the inhibitors; concentrations in excess of the threshold yield robust and almost equivalent thrombin generation.9,71 In a similar fashion, TFPI and the PC-Tm-thrombin APC system cooperate to provide a similar, threshold-limited, synergistic inhibition of thrombin production.10 The dynamic activated protein C system is responsive only after thrombin has been generated, because this system depends on activation of the zymogen protein C by the thrombin produced in the procoagulant response. Thus, its influence is mostly associated with quenching the propagation phase of thrombin generation, although the two phases are significantly overlapped.

The activations of the cofactors factor V and factor VIII are multistep processes, and at high thrombomodulin concentrations, the protein C system is an effective neutralizer of the reaction. Factor V activation involves cleavages at arginines 709, 1018, and 1545. The cleavage at arginine 709 occurs first and produces the heavy chain (residues 1 through 709) of the molecule. Factor V activity, however, requires the cleavage at arginine 1545 to produce the light chain (residues 1546 through 2329) of factor Vα.76–79 APC inactivates factor Vα (and intermediates in the activation process) principally by cleavage at arginines 506 and 306. Each of these cleavages is in the heavy chain.53 Thus, the heavy chain can be inactivated before generation of the light chain of factor Vα, eliminating its procoagulant activity.56

Accessory Processes

The zymogen factor XI is a symmetrical two-chain serine protease precursor present in plasma and platelets that has been variably associated with hemorrhagic pathology.80–84 The significance of factor XI as an important procoagulant is established by the bleeding pathology associated with its qualitative or quantitative absence. This zymogen is also a substrate for thrombin and has been invoked in a revised pathway of coagulation.85 Paravivo studies of the clotting of natural hemophilia C blood and synthetic plasma experiments, which mimic factor XI deficiency, illustrate the importance of the feedback activation of factor XI but only at the lowest TF concentrations.62,86 At moderate concentrations of tissue factor (5 to 10 pmol/L), which produce clotting times in the range of 3 to 5 minutes, factor XI has little or no effect on thrombin generation or other procoagulant param-
Pharmacological and Genetic Alterations

Observations from fundamental kinetic studies can be translated back to considerations of the pathophysiology of hemorrhagic and thrombotic disease. Hemorrhagic syndromes are characterized as severe, moderate, or mild on the basis of the relative functional level of a coagulation factor. Severe hemophilia A is associated with factor VIII concentrations <1%.80 Similar observations have been made for other coagulation factor deficiencies and provide paradigms for management of replacement therapy. These subjective assessments derived from clinical experience are consequences of the effective plasma protein concentrations required to generate effective hemostasis. In contrast to expectations, which may be derived from steady-state kinetics, in the pre–steady-state processes relevant to biology, only tiny amounts of the procoagulants upstream from prothrombinase are consumed during the thrombin-generating reaction, and contributions to rate processes rather than stoichiometric considerations predominate.51,55,61,71

The pathophysiologic influence of qualitative and quantitative alterations to the hemostatic system reactants has primarily been explored at the extremes of population distribution curves. These extremes are associated with congenital hemophilias and thrombophilias. In most cases, diagnostic methods are based on evaluations of single analytes with respect to their anticipated influence on the generation of hemorrhagic or thrombotic pathology. Synthetic plasma, numerical, and paravivo analyses show that quantitative differences in coagulation factor levels within the normal range, when combined, can produce significant variability in response to a tissue factor stimulus.59,68 This sort of behavior, as illustrated for prothrombin concentrations in synthetic plasma and numerical models, is presented in Figure 7.71 This figure displays the active thrombin concentration evaluated using the synthetic plasma and numerical systems as a function of time for a variety of prothrombin concentrations ranging from 50% to 150% of the population mean value. It can be seen that the active thrombin concentration present at any point in time as well as the cumulative amount of thrombin produced is significantly different for values of prothrombin concentration, which remain within the normal range. The stimulus-response algorithm that is presented for individuals with different prothrombin concentrations may be significant for predicting hemorrhagic or thrombotic risk, especially when such states are combined with other excursions from mean values. In the future, it may be anticipated that algorithms that combine individual coagulation factor and inhibitor levels will be useful in diagnosis and selection of prophylactic regimens.

The paravivo and in vivo systems have been used to evaluate pharmacologic interventions used to treat hemor-
rhagic and thrombotic diseases, to evaluate congenital abnormalities, and to inspect the influence of common polymorphisms. Studies have been conducted on individuals with hemophilia A, B, and C, factor VII deficiency, the PI41, PI42 platelet receptor polymorphism, and the factor XIII Val34-Leu polymorphism. Pharmacological interventions have included ingestion of aspirin, Simvastatin, and Coumadin anticoagulation. Pharmacological interventions have included ingestion of aspirin, Simvastatin, and Coumadin anticoagulation.

Figure 7. The concentration of active thrombin as a function of time in the presence of prothrombin at 150% (diamonds), 125% (triangles), 100% (squares), 75% (circles), and 50% (asterisk) of its mean physiological concentration. Reprinted with permission from Hockin MF, Jones KC, Everse SJ, Mann KG. A model for the stoichiometric regulation of blood coagulation. J Biol Chem. 2002;277:18322–18333.

Summary

Technical advances in molecular genetics, protein chemistry, bioinformatics, and physical biochemistry provide us with an impressive array of tools and information with respect to normal and pathologic processes leading to hemorrhagic or thrombotic disease. The challenge for the 21st century will be to merge these mechanism-based, quantitative data sets with epidemiologic studies and clinical experience associated with the tendency to bleed or thrombose and with the therapeutic management of individuals with thrombotic or hemorrhagic disease. Our knowledge of the biology of coagulation is incomplete without considerations of genetics, biochemistry, pathology, hematology, and vascular biology. Because the composite of biochemical models needs to become biologically relevant and biological approaches need to become quantitatively enabled, we need to distill and integrate mechanistic data with the vast amount of subjective clinical experience regarding the management of individuals with thrombotic and hemorrhagic disease. Algorithms must be developed that can combine the art of clinical management with the quantitative science available in this field to define the outcome of the challenge or the efficacy of an intervention. Ultimately, we must tailor diagnosis and pharmacologic intervention to the individual.

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References


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