

New Concepts of the Blood Coagulation Reactions

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Everyone is familiar with the traditional cascade hypothesis of blood coagulation in which there is an extrinsic pathway consisting of tissue factor-Factor VIIa activation of Factor X and an intrinsic system consisting of a series of reactions where zymogens are converted to the active enzymes beginning with the activation of Factor XII to XIIa, XI to XIa, IX to IXa, which in the presence of the cofactor, Factor VIIIa, converts Factor X to Xa. This is followed by a common pathway in which Factor Xa in the presence of its co-factor, Factor Va, then rapidly converts prothrombin to thrombin. The traditional cascade has, while extremely helpful in terms of diagnosis of the hemophilias and theoretical considerations of blood clotting reactions, had several problems. One is that the distinction between the intrinsic and extrinsic system does not really exist *in vivo*. The traditional cascade hypothesis was based on coagulation tests which were not cell based and which do not explain the clinical picture Factor XI and Factor XII deficiency (in which patients with Factor XI deficiency have a very mild bleeding disorder while patients with Factor XII deficiency have no bleeding at all). Neither does the traditional cascade explain the efficacy of high-dose Factor VIIa in bypassing inhibitors of Factors IX and VIII.

There have been advances important for revising the coagulation scheme. One has been the isolation and characterization of tissue factor. Another has been the demonstration that factor VIIa and tissue factor initiate coagulation. It has also been demonstrated that tissue factor activates both Factor IX and Factor X. Finally the isolation and characterization of tissue factor pathway inhibitor (TFPI) has been important in revising the coagulation scheme.

In this talk we will examine the blood coagulation reactions based on an *in vitro* cell-based model system to explore the sequence of events leading to cofactor and platelet activation. We will show that the activation of Factor IX to IXa and X to Xa by Factor VIIa tissue factor has very separate and distinct roles in the subsequent blood clotting reactions. We will also show the role of Factor XI in blood coagulation and explain the mechanism of action of high-dose Factor VIIa in bypassing inhibitors of both Factors IX and VIII. Our model system consists of a cell-based source of tissue factor. One source of tissue factor is monocytes, which when treated with lipopolysaccharide will induce tissue factor expression. One can also use fibroblasts and smooth muscle cells and other tissue cells as a source of tissue factor. The model system also has unactivated platelets prepared on density-gradient media followed by gel filtration. And finally, the model consists of the addition of zymogen coagulation factors at their plasma concentration as well as plasma concentrations of the inhibitors Antithrom-

bin III and tissue factor pathway inhibitor. For example, the prothrombin concentration is 100 micrograms per ml; Factor X concentration, 8 micrograms per ml; Factor IX concentration at 5 micrograms per ml; Factor VIII (with von Willebrand factor) at 0.1 microgram per ml; Factor V at 7 micrograms per ml; Antithrombin III at 150 micrograms per ml; tissue factor pathway inhibitor is 0.1 micrograms per ml. The clotting reactions are started by the addition of Factor VIIa at 0.01 micrograms per ml plus the addition of calcium chloride in adequate concentrations. Using this system, in which reactions are carried out in microtiter wells, we can determine the state of platelet activation and quantitate surface-bound proteins using flow cytometry. We can also extract proteins from the surface of activated cells and assess the state of activation of the coagulation factors including Factors IX, VIII, V, and X. We can also determine the quantities of thrombin in Factor X using chromogenic substrate assays. And finally we can examine surface-bound and solution-phase products of thrombin and Factor X.

Data shows that platelet activation requires tissue factor and that platelet activation can occur within a matter of a very few minutes when this system is complete with tissue factor. We can also show that thrombin generation in this system also requires tissue factor initiation. In other experiments we can show the number of monocytes required as a source of tissue factor in five or six thousand per microliter in a microtiter well. This number of monocytes activated by lipopolysaccharide is sufficient to result in platelet activation as well as thrombin generation. Slides depicting these results will be shown.

One of the first things examined with this model of blood coagulation was the question of the mechanics by which Factor VIIa-tissue factor activation of Factor IX and Factor X initiate thrombin generation in this model system. To test this hypothesis, we simply added pre-formed Factor IXa and Factor Xa in the presence of platelets, Factor IX, Factor VIII, Factor X, Factor V, prothrombin, Antithrombin III, and tissue factor pathway inhibitor in microtiter wells with the clotting reactions initiated by Factor VIIa and calcium. Using this system we were able to show that Factor Xa was necessary for platelet activation but not for the subsequent burst of thrombin generation on platelets. On the other hand, Factor IXa was not necessary for the initial platelet activation but was necessary for the subsequent burst of thrombin generation on platelets. The conclusion from this series of events was that the mechanism by which Factor VIIa-tissue factor initiates coagulation involves two distinct signals. One signal is mediated through the Factor VIIa-tissue factor activation of Factor X that leads to platelet activation; the other signal is mediated through the Factor

VIIa-tissue factor activation of Factor IX, leading to the assembly of the procoagulant complex of tenase and prothrombinase on the platelet surface. Platelet prothrombinase then leads to a burst of thrombin generation.

The question then arises as to how Factor Xa in the milieu of tissue factor cells effects platelet activation. Using the model system to answer this question, we found that very small amounts of thrombin were formed in the milieu of the tissue-factor bearing cell, and this small amount of thrombin was essential for the following reactions. This small amount of thrombin activates platelets; dissociates Factor VIII from von Willebrand's factor and activates Factor VIII; it also activates Factor V to Factor Va; and it activates Factor Xa. When platelets are activated, the cofactors VIIIa and Va bind immediately to binding sites on the activated platelet surface. Therefore, this small amount of thrombin in the milieu of the tissue-factor bearing cell serves as an important signal for subsequent blood-clotting reactions.

The activation of Factor IX to IXa by VIIa-tissue factor, which is the other distinct signal, is different from the activation of Factor X on the tissue Factor VIIa-bearing cell. While the Xa is turned off by the tissue factor pathway inhibitor, the IXa formed does not remain in the milieu or environment of the tissue-factor bearing cell, but rather it moves to the activated platelet surface, where it combines with Factor VIIIa to form the tenase complex, thereby recruiting more Factor X from the solution and activating Factor X. Factor Xa in the presence of its co-factor, Va, forms the tenase complex and results in an explosion of thrombin generation on the activated platelet surface.

Next we examined the activation of Factor XI in our system. Whereas the traditional cascade considers that Factor XI is activated by Factor XII, which requires prekallikrein and high molecular weight kininogen and a surface, our studies indicate that the Factor XI does not require this type of activation *in vivo*, but rather is simply activated by thrombin formed on the surface of the platelet. The Factor XIa then binds to the platelet surface and then converts more Factor IX to IXa. Thus Factor XI simply enhances thrombin generation by converting more IX to IXa. The reason why Factor XI deficiency causes a mild bleeding deficiency in patients is that it is not required for tenase or prothrombinase formation, but is simply present to enhance tenase generation. Experiments demonstrating the role of Factor XI will be presented.

Using our model system, we can also show that high dose Factor VIIa bypasses inhibitors of Factor VIII and IX because high doses of VIIa will bind to the platelet surface with a K_d in the micromolecular range. When added in sufficiently high doses, therefore, Factor VIIa on the surface of platelets can activate Factor X to Xa even in the absence of tissue factor, and thus bypass the need for Factor IX and IXa. Factor VIIa, in these concentrations, can actually enhance thrombin generation but never quite to normal limits. Nevertheless, it can enhance thrombin generation to the extent that hemostasis in patients with inhibitors to Factors VIII and IX is improved. Experiments using our model system to demonstrate the mechanism of how high-dose Factor VIIa bypasses inhibitors to Factors VIII and IX will also be demonstrated.

In summary, using a cell-based model of tissue factor and unactivated platelets, in the presence of plasma concentrations of the procoagulants and normal inhibitors, one can show that blood coagulation is initiated by tissue factor- Factor VIIa, which does two things. One is to activate Factor Xa and the other is to activate Factor IXa. The Factor Xa and Factor IXa formed in the environment of the tissue-factor bearing cell have very distinct and separate functions. The Xa formed on the surface of the tissue-factor bearing cell simply combines with Factor Va in that environment and converts small amounts of prothrombin to thrombin. This small amount of thrombin formed in the environment of the tissue-factor bearing cell can then activate platelets; separate Factor VIII from von Willebrand factor and activate Factor VIII; activate Factor V; and activate Factor XI. Thus the stage is set for a burst of thrombin generation on the activated platelets. The second signal, that is IXa formed in the environment of the tissue-factor bearing cell, does not remain in the environment of that cell, but rather moves to the platelet surface, where it recruits more X from solution to activate Factor X to Xa, which in the presence of the co-factor Va then results in a burst of thrombin generation. The Factor XIa formed by the small amount of thrombin from the tissue-factor bearing cell also binds with the platelets and enhances the formation of IXa on the platelet surface, which simply enhances the degree of thrombin generation.