Inhibition of Factor VIIa/Tissue Factor-Induced Blood Coagulation: With Particular Emphasis Upon a Factor Xa-Dependent Inhibitory Mechanism

Samuel I. Rapaport

Three enzyme/cofactor complexes form on cell surfaces in tightly regulated reactions during normal blood coagulation in vivo. The enzymes of these complexes, factor VIIa, factor IXa, and factor Xa, are ancestrally related serine proteases that are members of the vitamin K-dependent group of clotting factors. Each is generated during blood coagulation by limited proteolysis of a zymogen precursor. Highly efficient when associated with its cofactor, each enzyme is physiologically inert in the absence of its cofactor.

The cofactor for factor VIIa is tissue factor, a protein present constitutively in the surface membrane of certain tissue cells, including pericytes and fibroblasts in the adventitia of blood vessels, and appearing transiently, after their activation, in the plasma membrane of two cells to which circulating blood is exposed, vascular endothelium and monocytes. The properties of tissue factor that require association with phospholipid provided in vivo by the lipid bilayer of the surface membrane for its activity, have been summarized in a recent review. A factor VII(a)/tissue factor (TF) complex, which is thought to be the first enzyme/cofactor complex that is formed on cell surfaces during normal blood coagulation in vivo, initiates coagulation through activation of factors IX and X.

The cofactor for factor IXa, which is factor VIIa, and the cofactor for factor Xa, which is factor Va, have many structural and functional similarities. These have also been recently summarized. A factor IXa/factor VIIa/phospholipid vesicle (in soluble systems) or membrane equivalent (on cell surfaces) complex serves as a second activator of factor X. The bleeding of patients with severe hemophilia, who cannot form this second activator, attests to its importance for normal blood coagulation during hemostasis. The third enzyme/cofactor complex, an analogous factor Xa/factor Va/phospholipid vesicle or membrane equivalent complex, is the only known physiologic activator of prothrombin.

Much has been learned about the regulatory mechanisms modulating formation of the second and third complexes. The proteinase inhibitor, antithrombin III, in the presence of negatively charged, sulfated glycosaminoglycans such as heparin or heparan sulfate, can efficiently neutralize both factor IXa and factor Xa. Activated protein C, with the help of protein S, inactivates both factor VIIa and factor Va. Clinical experience, the failure ever to identify a live infant with homozygous antithrombin III deficiency and the fulminating thrombotic disease of the rare infant born totally deficient in protein C teaches us that major break downs of their function are incompatible with life.

Within the last 5 years interest in a mechanism regulating the activity of the factor VIIa/TF complex has reawakened. The mechanism differs basically from the reactions regulating the activity of the other enzyme/cofactor complexes. Antithrombin III inhibits factors IXa and Xa, and activated protein C inhibits factors VIIa and Va more effectively before, rather than after, they form enzyme/cofactor complexes.

In contrast, a factor VII(a)/TF complex must form and catalyze beginning activation of factor X before the reactions inhibiting further factor VIIa/TF catalytic activity are triggered. These involve the participation of a plasma inhibitor that was given the provisional name extrinsic pathway inhibitor (EPI) by one group and the name lipoprotein-associated coagulation inhibitor (LACI) by another. Recently, the inhibitor has been identified as a proteinase inhibitor belonging to the basic proteinase inhibitor gene superfamily.

The term EPI will be used in this review, which should be looked upon as an “interim summary” at an early stage of our understanding of the properties of EPI, of its mechanism of action in inhibiting factor VIIa/TF catalytic activity, and of the potential physiologic significance of EPI-induced inhibition for the regulation of blood coagulation. Other biologic materials reported to be capable of inhibiting factor VIIa/TF catalytic activity will be discussed briefly.
SOME HISTORY

Six years after the first description of hereditary factor VII deficiency, Hjort provided evidence that "activated proconvertin" (factor VIIa) activity decays very slowly in serum. This established that serum does not contain a specific factor VIIa inhibitor capable of functioning effectively in vivo. Later experiments in which antithrombin III failed to inactivate factor VIIa in semi-purified systems provided further evidence for this conclusion. Finally, in experiments from the author’s laboratory, Seligsohn et al found that when hemophilic patients were infused with activated prothrombin complex concentrates containing substantial concentrations of factor VIIa, the factor VIIa persisted in the circulation with a mean half-disappearance time of 144 minutes. It is clear that humans also lack an in vivo mechanism for the rapid clearance of factor VIIa activity from blood.

Evidence was obtained early in this century (summarized by Hjort) that tissue extracts lose coagulant activity on incubation with serum. In 1947, in experiments whose original purpose was to search for a placental toxin responsible for toxemia of pregnancy, Schneider and Thomas showed independently that the material in placental extracts that caused mice to die after infusion of the extracts was thromboplastin (tissue factor) and that its incubation with serum before injection could prevent the mice from dying. These observations and later in vitro experiments of others, independently that the material in placental extracts that caused mice to die after infusion of the extracts was thromboplastin (tissue factor) and that its incubation with serum before injection could prevent the mice from dying. These observations and later in vitro experiments of others, which are explainable today only if the techniques used to adsorb serum were inadequate to deplete the serum of factors VII and X, were taken as evidence that plasma or serum can inhibit tissue factor.

Hjort’s careful and systematic studies were reported in a remarkable monograph in 1957. They provided strong evidence that serum contains an inactivator not of tissue factor itself, but of the reaction product formed on incubating tissue factor with proconvertin (factor VII) and calcium ions. Hjort referred to this reaction product as convertin and to the inactivating moiety in serum as anti-convertin. He showed that anti-convertin formed an inactive complex with proconvertin (factor VIIa) in the presence of calcium in a stoichiometric reaction that was reversible on removal of calcium from the reaction mixtures.

For more than 25 years after the publication of Hjort’s monograph, little was added to our knowledge of the mechanism of the inactivation of factor VIIa/TF in human plasma. In 1983, Sanders et al reported that a plasma protein inhibiting the factor VIIa/TF activation of factor IX required the presence of factor X for its function. This work, fully described in 1985, provided an explanation for the observation made in 1984 by Morrison and Jesty that factor Xa exerts a negative control over its own activation and the activation of factor IX by factor VIIa/TF in human plasma. The existence of a mechanism that requires the participation of both a plasma factor and factor Xa for inactivating factor VIIa/TF catalytic activity was confirmed in several laboratories. It was also established that the factor Xa-dependent reaction inhibits not TF itself but the factor VIIa/TF complex, that inhibition is associated with binding of the inhibitor to the factor VIIa/TF complex, and that removal of calcium from a reaction mixture containing an inhibited factor VIIa/TF complex releases active TF and factor VIIa from the inhibited complex. Since these characteristics first described for inhibition induced by anti-convertin, one may conclude that anti-convertin, whose properties were delineated before factor X was known to exist, is identical to the factor Xa-dependent plasma protein inhibiting factor VIIa/TF (EPI, LACI).
mation of the vitamin K-dependent clotting proteins essential for their functional activity. A calcium-dependent conformation of factor Xa in an EPI/factor Xa complex is apparently also required for EPI/factor Xa to bind to factor VIIa/TF and inhibit its catalytic activity.

Thus, EPI-induced inhibition of factor VIIa/TF may be thought of as occurring in two steps. In the first step, factor Xa binds to EPI in a Ca$^{2+}$-independent reaction requiring the active site of factor Xa and presumably the participation of a reactive site arginyl peptide bond on EPI. In the second step, a putative Ca$^{2+}$-dependent EPI/factor Xa/factor VIIa/TF complex is formed with resultant loss of factor VIIa/TF catalytic activity. Inhibition cannot begin in a biologic system before sufficient factor Xa has been generated by factor VIIa/TF, factor IXa/VIIa/phospholipid, or by both to permit an EPI/factor Xa complex to be formed. Inhibition is presumably stoichiometric and each step is potentially reversible. Benzamidine, by competing for binding to factor Xa’s active site, will cause an EPI/factor Xa complex to dissociate with release of functional EPI. Chelating Ca$^{2+}$ with EDTA will cause an inhibited factor VIIa/TF complex to dissociate with release of active factor VIIa and TF. The mechanism whereby the binding of EPI/factor Xa complex to factor VIIa/TF inhibits the latter’s catalytic activity remains to be elucidated. However, it is known that EPI/factor Xa not only inhibits factor VIIa/TF formed in soluble systems with purified tissue factor reconstituted into phospholipid vesicles but also inhibits factor VIIa/TF expressed on the surface of monolayers of cultured human umbilical vein endothelial cells.

ASSAY OF EPI

Immunologic assays for EPI antigen have not yet been described but the development of monospecific antibodies for EPI should lead to their future availability. Assays for EPI activity are based upon the ability of a test sample to inhibit factor VIIa/TF activity in the presence but not in the absence of factor Xa. With one exception, reaction mixtures have been designed so that a saturating factor VII concentration results in essentially full factor VII occupancy of limiting TF binding sites; ie, the concentration of TF used in the assay determines the concentration of factor VIIa/TF available to react with the EPI of a test sample. Residual factor VIIa/TF has been measured either by its ability to activate factor IX, as monitored by release of amidolytic assay, or by its ability to activate factor X, as measured in a clotting or amidolytic assay. If the latter is chosen, a multi-stage technique should be used that eliminates error due to carry over of factor Xa activity added or generated initially to support EPI-induced inhibition. Purified clotting factors are used in all current assays, which limits their general availability.

PROPERTIES OF EPI

Broze and Miletich purified EPI from the culture medium of a hepatoma cell line. The purified protein has a molecular weight (mol wt) of 38,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). An anti-body raised to the purified protein inhibited the EPI activity of plasma. EPI purified from the hepatoma cell line neutralized factor Xa activity but failed to neutralize the activity of thrombin, VIIa, IXa, Xla, Xlla, activated protein C, kallikrein, urokinase, tissue plasminogen activator, or elastase. At a concentration of 100 ng/mL, the purified protein had a factor Xa-dependent inhibitory activity against factor VIIa/TF equivalent to that present in serum. Therefore, this value is currently taken as the mean concentration of EPI in plasma. As already mentioned, EPI has been identified as a member of the basic protease inhibitor family and possesses three tandem Kunitz-type inhibitor domains.

EPI activity in plasma exists in two mol wt forms. The mol wts, as originally determined from activity eluted from gel slices after SDS-PAGE, were reported as 42 to 43 kD and 35 to 36 kD. After Western blotting experiments with 125I-factor Xa, these values have been revised to 40 kD and 32 to 33 kD. Thus, the mol wt of the larger plasma form is close to that reported by Broze and Miletich for the protein purified from the medium of cultured hepatoma cells. The reason for the lower mol wt of the second plasma form of EPI is not yet known.

EPI is associated with the lipoprotein fraction of plasma. About one-half of the total EPI activity of plasma can be recovered in the total lipoprotein fraction isolated by ultracentrifugation at density ~ 1.21 g/mL, where it is present in both mol wt forms. Lipoprotein-associated EPI is also relatively resistant to heating with about a 1% loss of EPI activity after 15 minutes at 56°C. Although EPI activity is readily demonstrable in plasma adsorbed with barium sulfate or aluminum hydroxide, such adsorption reduces measurable plasma EPI activity by 40% to 50%.

In addition to its synthesis by a hepatoma cell line, EPI is synthesized by cultured human umbilical vein endothelial cells. It is also synthesized by a cell line derived from a monocytic tumor, U937, particularly after its perturbation by endotoxin or phorbol ester. Bajaj et al reported finding both mol wt forms of EPI in concentrated media from cultured human umbilical vein cells, but others have been able to demonstrate only the higher mol wt form. Both the liver and vascular endothelium may be important sites of synthesis of plasma EPI. Patients with decompensated chronic liver disease have normal plasma EPI levels, but patients with fulminant hepatocellular disease may have reduced levels (T. Warr, personal communication).

PLASMA EPI LEVELS IN NORMAL SUBJECTS AND PATIENTS

Plasma levels of EPI in normal adults range from about 70% to 150% of a normal pooled plasma reference stan-
and do not appear to vary when measured at different times in the same individual.\(^3\) The mean level in plasma from cord blood of normal full-term infants is about 65% of an adult plasma reference standard.\(^3\) Mean EPI levels in the first and second trimesters of pregnancy do not differ from the mean level for non-pregnant women, but the mean level for women in the third trimester is slightly higher than that for non-pregnant women.\(^3\) Neither intravenous (IV) administration of 1-desamino-8-D-arginine vasopressin (DDAVP)\(^3\) nor venous occlusion of a limb\(^4\) increases plasma EPI levels. Thus, vascular endothelium does not possess stores of EPI releasable by stimuli that cause release from vascular endothelium of stored von Willebrand factor (vWF). However, plasma EPI levels rise sharply within seconds after a bolus IV injection of heparin.\(^4\) The rise cannot be accounted for by an effect of heparin upon the assay for EPI and Sandset et al\(^4\) concluded that heparin causes the release of EPI from vascular endothelium into plasma.

As already mentioned, patients with decompensated cirrhosis have normal plasma EPI levels.\(^3\) Bajaj et al\(^5\) reported that EPI levels are reduced in patients with disseminated intravascular coagulation. Nine of the ten patients studied had disseminated intravascular coagulation secondary to sepsis. However, Andersson et al\(^5\) reported levels of 60% to 190% in patients with disseminated intravascular coagulation. Warr et al\(^4\) found an occasional low plasma EPI level in multiple samples from 24 patients with disseminated intravascular coagulation from a variety of causes including sepsis in 13 patients. Normal plasma EPI levels were reported in patients with pneumonia,\(^5\) in patients with deep venous thrombosis,\(^5\) in patients with the lupus anticoagulant,\(^5\) and in patients receiving therapy with warfarin.\(^5\) Whereas fibrinogen, a known acute phase protein, rises after surgical procedures, EPI levels do not rise after surgery and indeed may fall slightly to a varying extent depending upon the procedure.\(^3\) It is unlikely, therefore, that EPI is an acute phase protein.

**MATERIALS OTHER THAN EPI THAT INHIBIT FACTOR VIIa/TF ACTIVITY**

In 1981, Carson\(^7\) reported that plasma high-density lipoproteins (HDL) inhibit the factor VIIa/TF activation of factor X. Although others have reported that HDL lacks EPI activity,\(^5\) HDL as prepared and tested in the author’s laboratory has consistently possessed EPI activity. Therefore, the author believes that EPI activity in HDL could partly account for Carson’s initial observation. However, Carson has recently shown\(^13\) that purified apoprotein AII (apo-AII), a major constituent of HDL, inhibits factor VIIa/TF activation of factor X, and Kondo and Kisiel\(^13\) have confirmed this finding.

Inhibition of factor VIIa/TF activity by apo-AII differs from inhibition of factor VIIa/TF by EPI in at least three important respects. First, apo-AII appears to inhibit TF by preventing its appropriate association with factor VIIa; increasing the factor VIIa concentration of reaction mixtures partially protects TF from the effects of apo-AII.\(^13\) In contrast, an EPI/factor Xa complex inhibits the activity not of TF but of the factor VIIa/TF complex. Second, the concentrations of apo-AII required to demonstrate apo-AII inhibition of TF, 200 to 400 nmol/L, are very much higher than the concentration of EPI required to inhibit factor VIIa/TF. A final 10% dilution of human plasma will shut off factor VIIa/TF activation of factor X in a reaction mixture containing final concentrations of 0.25 μg/mL factor VII and 10 ng/mL of purified, reconstituted TF.\(^5\) If one assumes a plasma EPI concentration of 100 ng/mL and a mean mol wt for EPI in plasma of 36 kD, then the EPI concentration in a reaction mixture containing 10% plasma would be <300 pmol/L. Third, whereas apo-AII inhibits factor VIIa/TF-mediated activation of factor X, apo-AII apparently does not inhibit factor VIIa/TF-mediated activation of factor IX.\(^13\) As first established by Sanders et al in 1985,\(^58\) EPI inhibits the catalytic activity of factor VIIa/TF upon both of its substrates.

Kondo and Kisiel\(^13\) also reported that HDL, low density lipoprotein (LDL), and very low density lipoprotein (VLDL), at or below their plasma concentrations, each selectively inhibited factor VIIa/TF activation of factor X in a reaction that antibodies to EPI could not prevent. Since LDL should not contain apo-AII,\(^58\) this implies that other materials associated with plasma lipoproteins may also selectively inhibit factor VIIa/TF-mediated activation of factor X. Indeed, Carson and Ross\(^4\) recently reported that an additional lipid binding protein, C-reactive protein, inhibits factor VIIa/TF activation of factor X by a mechanism that appears similar to inhibition induced by apo-AII. As Kondo and Kisiel\(^13\) have pointed out, plasma lipoproteins that selectively inhibit factor VIIa/TF-mediated activation of factor X would promote the activation of factor IX by factor VIIa/TF in vivo at low tissue factor concentrations. However, there is as yet no evidence that materials associated with plasma lipoproteins other than EPI\(^58\) can inhibit TF activity expressed on the surface membrane of cells.

Funakoshi et al\(^4\) isolated and characterized an anticoagulant protein from human placenta that appears to be a member of the lipocortin family. This placental anticoagulant protein (PAP) binds to phospholipid vesicles and inhibits clotting reactions in which phospholipid participates, including the clotting of plasma initiated with tissue factor. There is as yet no evidence that PAP is normally present in plasma.

**SPECULATIONS ON THE INITIATION AND REGULATION OF THE TF PATHWAY OF COAGULATION IN VIVO**

A concept of the reactions initiating and regulating the TF pathway of blood coagulation is presented schematically in Fig 1 and summarized as follows.

1. When blood comes into contact with cells possessing surface TF activity, factor VII, which is present in plasma in a concentration far exceeding a saturating concentration, will fully occupy a limiting number of TF sites. Since the binding affinities of factor VII and factor VIIa for TF are similar,\(^58\) factor VII will occupy almost all of the TF sites,
but if, as has been reported, circulating blood normally contains traces of factor VIIa, an infrequent factor VIIa/TF complex will also be formed.

2. It is not yet settled whether a human factor VII/TF complex possesses a minimal enzymatic activity capable of generating the initial factor Xa that forms. Nemerson has recently summarized the evidence from his laboratory supporting this view. However, Rao et al have been unable to demonstrate a physiologically meaningful factor VII/TF-mediated activation of either factor IX or factor X. Thus, the alternative possibility exists that the presence of a trace concentration of factor VIIa in circulating plasma allows the formation of a very small number of factor VIIa/TF complexes that activate the first factor X molecules. This hypothesis begs the question of how such traces of circulating factor VIIa are formed. Nevertheless, the approximately two-hour intravascular half-time of plasma factor VIIa makes it clear that many minutes may elapse after factor Xa has started to form before the full inhibitory effect of EPI is evident. A similar lag in EPI/factor Xa-induced inhibition of factor VIIa/TF in vivo could explain why a continuing exposure of blood to TF would allow disseminated intravascular coagulation to continue despite a normal level of plasma EPI in clinical disorders such as gram-negative sepsis and malignancy.

Whether hereditary EPI deficiency states exist that increase a person’s risk for thrombotic disease is as yet unknown. The use of immunologic markers of hemostatic system activation has provided evidence of a continuous minimal activation of blood coagulation in normal subjects, which is held in check by natural anticoagulant mechanisms. The sites of activation undoubtedly include the extravascular tissues, where transcapillary passage of clotting proteins from the plasma into extracellular fluid would bring them into contact with cells possessing constitutively expressed surface TF activity. Thus, EPI could well play a major role in maintaining a biochemical balance between procoagulant and anticoagulant reactions in the extravascular tissues.

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SI Rapaport