The Regulation of Natural Anticoagulant Pathways

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Vascular endothelium plays an active role in preventing blood clot formation in vivo. One mechanism by which prevention is achieved involves a cell surface thrombin-binding protein, thrombomodulin, which converts thrombin into a protein C activator. Activated protein C then functions as an anticoagulant by inactivating two regulatory proteins of the coagulation system, factors Va and VIIIa. The physiological relevance of the protein C anticoagulant pathway is demonstrated by the identification of homozygous protein C–deficient infants with severe thrombotic complications. Recent studies suggest that this pathway provides a link between inflammation and coagulation.

ONE OF THE REMARKABLE FEATURES OF THE COAGULATION system is its ability to respond rapidly to seal a wound site without the clot propagating to occlude the vessel and block blood flow. This property is not manifest in vitro, suggesting that factors existing solely in vivo contribute to this regulation. For years it has been recognized that normal vascular endothelium does not support coagulation. Originally perceived as a passive surface, the endothelium has recently been shown to participate actively in inhibiting clot formation. Both cell surface and intracellular factors contribute to this regulation. Two distinct anticoagulant mechanisms are triggered by contact with the cell surface. One involves cell surface heparin-like molecules that can function to accelerate the inactivation of coagulation proteases by antithrombin III (*1*). The other involves thrombomodulin, a thrombin-binding protein that alters the macromolecular specificity of thrombin, thereby decreasing the ability of thrombin to catalyze clot formation (*2–5*) and at the same time converting thrombin into a potent protein C activator (*4–8*). Activated protein C, a serine protease, then functions as an anticoagulant by inactivating two of the regulatory proteins of the coagulation pathway, factors Va and VIIIa (*9–12*). These two proteins are essential for the function of two of the coagulation proteases, factors IXa and Xa. Hence the cell surface triggers both the inactivation of the proteases and their essential regulatory proteins.

Vascular regulation of the clotting process is not limited to control of the coagulation factors. Intracellular components also contribute to regulation. Prostacyclin (*13*), an inhibitor of platelet activation, and plasminogen activator (*14*) are both synthesized and released from endothelium. Plasminogen activation results in the formation of plasmin, the enzyme responsible for clot lysis. Thus, the endothelial cell contributes to the control of clotting, platelet activation, and clot dissolution. Since the endothelial cell is easily injured (*15*), it is logical to study the influence of endothelial cell injury on these endothelial cell anticoagulant functions. Many factors can alter endothelial cell function by inhibiting at least some of the anticoagulant functions. To explore these changes in greater depth it is useful to focus on one of the anticoagulant systems. For illustrative purposes I have chosen to focus on the protein C anticoagulant pathway (*16–20*). This pathway can be conveniently divided into three steps: protein C activation, expression of the protein C anticoagulant activity, and clearance of activated protein C from the circulation (*21*). Each of the steps involves specific proteins that participate in the control of coagulation. A model representing the interactions of these proteins is shown in Fig. 1.

Protein C Activation

Properties of the protein C activation complex. Rapid activation of protein C occurs on the surface of the vascular endothelium where a reversible high-affinity complex (dissociation constant approximately 0.5 n*M*) is formed between thrombin and thrombomodulin (*7*). Thrombomodulin has the properties of an integral membrane protein (*22–24*) and is located on the surface of all endothelial cells (*25*) except those in the microcirculation of the human brain (*26*). Estimates of surface expression of thrombomodulin suggest that there are approximately 50,000 sites per cell (*27*). On the basis of recovery of the protein in preparative procedures, the concentration of thrombomodulin in the microcirculation, where the surface to volume ratio is high, exceeds 10 n*M* (*22*). This high concentration is important when one considers the potential physiological function of thrombomodulin.

On human endothelial cells, occupancy of thrombomodulin with thrombin appears to lead to internalization of the thrombomodulin (*27*). No known intracellular messages are generated during this internalization process.

Alteration of the thrombin specificity by complex formation with thrombomodulin. Several lines of evidence suggest that thrombomodulin functions by altering the macromolecular specificity of thrombin. When thrombin is complexed with thrombomodulin, there is a >1000-fold increase in the activation of protein C. Insight into the mechanism of protein C activation comes from studying the influence of Ca^{2+} on activation by thrombin in the free and in the complexed form. Ca^{2+} inhibits protein C activation by thrombin alone, but is required for activation by the complex. Both half maximal inhibition and half maximal acceleration occur at the same Ca^{2+} concentration (*28*). Spectroscopic studies show that a Ca^{2+}-dependent conformational transition occurs in the protein C molecule, which correlates well with the Ca^{2+} dependence of the activation process (*29*). The unusual properties of this activation are

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illustrated in Fig. 2. In addition to enhancing protein C activation, complex formation with thrombomodulin also blocks thrombin-catalyzed reactions that lead to clot formation. Specifically, fibrin formation, factor V activation, and platelet activation are all inhibited upon complex formation with thrombomodulin (*16*).

To accommodate both of these changes in specificity, we have proposed that a conformational change occurs in thrombin upon complex formation that allows the Ca^{2+}-stabilized conformation of protein C to interact effectively (*29*). Neither complex formation nor the inhibition of the coagulation reactions is dependent on Ca^{2+}. This change in macromolecular specificity is completely reversible by dissociating the bound thrombin from the complex. Although the macromolecular specificity is dramatically altered for most substrates, the ability of the thrombin-thrombomodulin complex to be inhibited by antithrombin III, the major thrombin inhibitor in blood (*1*), is not impaired. In fact, in plasma the rate of inactivation is enhanced because of the ability of thrombomodulin to block interaction of thrombin with alternative substrates, such as fibrinogen, which normally compete with antithrombin III (*4, 5*).

These studies suggest that thrombomodulin exerts two anticoagulant functions: the acceleration of protein C activation and the direct inhibition of thrombin's procoagulant functions. The relative physiological importance of these two processes is not known. Recently, studies with human thrombomodulin have suggested that this protein is not as effective in directly blocking thrombin's coagulant properties as the rabbit or bovine thrombomodulin. Whether this lower capacity to block the coagulant activity is related to the properties acquired during isolation is uncertain, but even with the decreased ability to function as a direct anticoagulant the thrombomodulin concentration in the microcirculation would probably be sufficient to significantly inhibit thrombin's clotting activities.

Effect of cells and thrombomodulin reconstitution into liposomes on protein C activation. Activation of protein C, a vitamin K–dependent protein, on the surface of the vascular endothelium presents special conceptual problems. In general, the interaction of vitamin K–dependent proteins with membrane surfaces requires the presence of negatively charged phospholipids (*30*). If these phospholipids provided the surface for protein C, they could also serve as the site for propagation of coagulation reactions. Since the endothelial cell functions predominantly to prevent coagulation under normal circumstances, it follows that protein C activation must use a different mechanism. From comparisons of the affinity of the soluble thrombomodulin and the cell surface it is clear that the affinity for protein C is ten times higher on the cell surface. This difference in affinity requires the presence of the 4-carboxyglutamic acid (Gla) residues (*7, 22*). Reconstitution of thrombomodulin into neutral phospholipid membranes (liposomes) results in an increase in affinity for the substrate comparable to that observed with the endothelial cell (*24*). Thus, with protein C activation, the requirement for negatively charged phospholipids is circumvented. The role of the membrane appears to be to expose a site on the thrombomodulin molecule that interacts through a Ca^{2+} bridge to the Gla residues of the protein C (see Fig. 2).

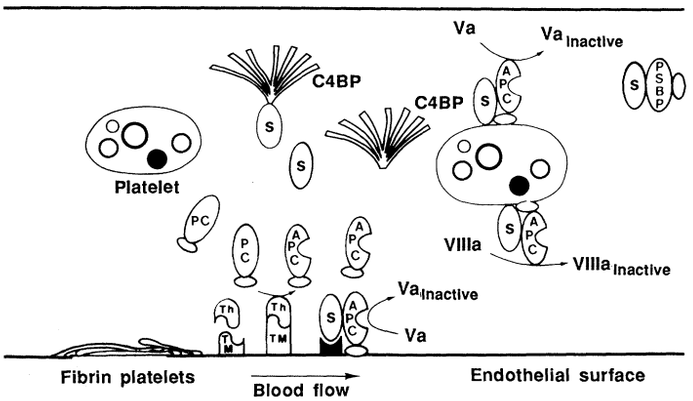

Fig. 1. Schematic representation of the proteins and cell surfaces that participate in the protein C anticoagulant pathway. At the site of injury, platelets adhere and fibrin is formed. Excess thrombin (Th) can bind to thrombomodulin (TM), initiating protein C (PC) activation. Activated protein C (APC) can interact with either the platelet or endothelial cell surface, but optimal interaction requires the presence of protein S (S). Protein S exists free, in complex with C4BP, or in complex with protein S–binding protein (PSBP). Since the endothelial cell binds protein S with higher affinity than the platelet, a cell surface–binding site is hypothesized. Protein S in complex with C4BP is not functional in the anticoagulant pathway, whereas PSBP facilitates assembly on membrane surfaces (*63*). Factors Va and VIIIa serve as the substrates for the anticoagulant complexes. See text for additional discussion.

limited proteolysis, and the inactivated factor Va loses the ability to interact effectively with the enzyme, factor Xa, or the substrate prothrombin (*34*). Since cell surface interaction is critical to the expression of the anticoagulant activity, it is clear that loss of these cell surface binding sites could constitute one mechanism for regulating this anticoagulant pathway.

In addition to regulation by controlling receptor density, additional mechanisms for regulating protein S levels have been identified. Protein S circulates both free and bound to C4b binding protein (C4BP), a regulatory protein of the complement system (*16, 35*). Only the free form of protein S functions in the anticoagulant

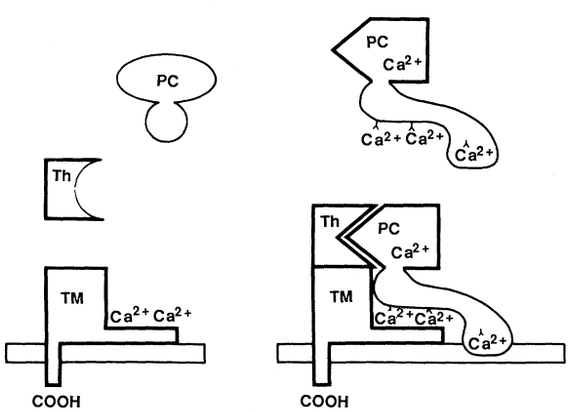

Fig. 2. Schematic representation of protein C activation. This model is based on the following observations. Complex formation between thrombin and thrombomodulin is not dependent on either the membrane surface or calcium ions. Complex formation is associated with a change in macromolecular substrate specificity depicted here as a conformational change in thrombin. Only the Ca^{2+} stabilized conformation of protein C is recognized by the complex, but Ca^{2+} prevents recognition of protein C by free thrombin. Additional Ca^{2+} involvement appears to involve a bridging mechanism between thrombomodulin and Gla residues on the substrate (*24*). Since neutral phospholipids function to enhance activation, a small insertion of the membrane–binding domain into the membrane is hypothesized.

Expression of Protein C Anticoagulant Activity

Once it has been activated, protein C must interact with membrane surfaces to function. High-affinity membrane interaction requires the presence of protein S, also a vitamin K–dependent protein. When bound to negatively charged phospholipid vesicles (*31*), the platelet (*32*), or endothelial cell surface (*33*), activated protein C rapidly inactivates factor Va. Factor Va is inactivated by

Table 1. Modulation of endothelial cell coagulant properties after exposure to endotoxin, IL-1, or TNF (*48–50*).

Agent	Effect on thrombomodulin	Effect on protein S or protein C function	Effect on tissue factor
Endotoxin	Decrease to ≈50% (maximum at 6 to 12 hours)	Not determined	Increase (greatest at 4 hours)
IL-1	Decrease to 33% (maximum at 6 hours)	Decrease to <10% (maximum at 6 to 10 hours)	Increase (greatest at 4 to 6 hours)
TNF	Decrease to 20–30% (maximum at 8 to 12 hours)	Decrease to <10% (maximum at 8 to 12 hours)	Increase (greatest ≈12 hours)

pathway (*36*). Many of the properties of C4BP suggest that this is an acute phase reactant. For instance, the levels rise in patients with infections where other acute phase reactants are elevated. Since C4BP is in reversible equilibrium with protein S (*16*), increased C4BP results in decreased free protein S. Studies in patients suggest that decreases in free protein S to the levels observed during the acute phase response may predispose patients to thrombosis (*20, 37*). At present, the influence of complex formation with protein S on the regulation of the complement system remains unknown.

Influence of activated protein C on clot lysis. In addition to functioning as an inhibitor of coagulation, activated protein C stimulates fibrinolysis at least in part by neutralizing plasminogen activator inhibitor (*14*). Like the anticoagulant activity, stimulation of the fibrinolytic activity is also enhanced by protein S (*38*).

Clearance of Protein C

Two known mechanisms might facilitate the clearance of activated protein C. One involves a slow-acting plasma protease inhibitor (*21*). Alternatively, a plasminogen activator inhibitor has been described that may also inactivate activated protein C (*14*). In either case, the inactivation of protein C appears to be slow in vitro. This is compatible with the available animal studies in which activated protein C appears to have a long half-life in vivo (*16*).

Structure of the Proteins in the Regulatory Pathway

Protein C and protein S are vitamin K–dependent proteins with relative molecular masses (M_r) of 62,000 (*39*) and 69,000 (*40*), respectively. Both proteins have been cloned (*41, 42*). The sequence of protein C is very homologous to the sequences of factors X and IX, two clotting factors that are precursors to serine proteases. The sequence of each of these proteins has (i) a Gla domain, which is the site of posttranslational modification by the vitamin K carboxylase and is responsible for the Ca^{2+}-dependent membrane interactions (*30*); (ii) a region with homology to the epidermal growth factor precursor (*42*); and (iii) a region that is approximately 65% homologous to chymotrypsin and contains the active site. Protein S is similar to protein C through the first two domains, but is not at all related to protein C or trypsin in the third domain. This structure is consistent with the observation that protein S functions as a binding protein.

When examined under the electron microscope, C4BP (M_r 570,000) appears as a spider-like molecule with seven filamentous subunits projecting from a small central globular domain (*16, 43*). Protein S binds to this globular domain. Given the structure, it is not surprising that the complex cannot function in the anticoagulant pathway.

Thrombomodulin is a glycoprotein (M_r 74,000). A partial structure has recently been inferred from a complementary DNA clone of the bovine thrombomodulin (*23*). From this work the authors inferred that thrombomodulin has a limited cytosolic domain at the carboxyl terminus, a short membrane-spanning domain, and then a region immediately above the membrane composed of several growth factor domains. From the available sequence, it appears that this region of thrombomodulin shares many structural properties in common with the receptor for low density lipoprotein (*44*). Phospholipid reconstitution studies suggest the presence of an additional lipid-binding domain distinct from this region (*24*).

Regulation of the protein C anticoagulant pathway. The above properties of the proteins and receptors involved in the control of the coagulation cascade suggest that they could serve as key regulatory components subject to perturbation during disease processes. Interest in this possibility stems from the observation that healthy individuals usually can withstand major trauma without a high frequency of either deep venous thrombosis or disseminated intravascular coagulation. A hallmark of the processes that alter this natural protective status can easily be observed in patients with severe infections. The inflammatory response is often associated with the onset of disseminated intravascular coagulation and many patients experience their first thrombotic episode after an infection.

Insight into the mechanisms responsible for this association came initially from observations that endotoxin could initiate the expression of tissue factor on monocytes (*45*) and endothelial cells (*46*) that in turn triggers the coagulation response. A more general link between inflammation and coagulation became apparent when Gimbrone's group demonstrated that the immune mediator interleukin-1 (IL-1) also initiated tissue factor formation on endothelium (*47*) and, in addition, induced the cells to form leukocyte-binding sites on the cell surface (*15*). These observations combined with our emerging understanding of the protein C anticoagulant system led us to investigate the possibility that exposure of endothelial cells to these mediators results not only in the formation of factors initiating the coagulation response but also in the loss of the natural anticoagulant properties of the endothelial cell surface. When endothelium is exposed to endotoxin (*48*), IL-1 (*49*), or tumor necrosis factor (*50*), thrombomodulin function and the capacity of the cell surface to bind protein S and stimulate factor Va inactivation are lost (Table 1). One interesting difference in the response is that the thrombomodulin activity remains low long after the tissue factor and the leukocyte adherence molecules (*15*) have returned to baseline. When this information is combined with the recent observation that these injury models also lead to an increase in plasminogen activator inhibitor (*51*), and hence impaired capacity to lyse blood clots, a picture begins to emerge in which the response to endothelial cell injury facilitates the overall process of blood clot formation at the expense of many of the processes that control the clotting process and clear the clot. These cell-mediated changes in receptor expression and endothelial cell properties, coupled with the observation that protein S function is impaired because of increased C4BP levels in these conditions, provide new insights into the mechanisms by which the inflammatory pathways may modulate the coagulation responses.

One question that arises from these observations is why an organism would develop these coagulation responses during an inflammatory response. Although purely speculative, it is interesting to note that in certain invertebrates, such as the crab *Limulus*, the

coagulation system is apparently used directly as a defense against invading bacteria that are walled off by a gelation process similar to fibrin polymer formation (*52*). From this perspective, it is likely that mammals retained the link between coagulation and the host defense mechanisms. It is ironic that during acute infections this alteration in control of the coagulation cascade apparently leads to disseminated intravascular coagulation, which decreases the probability of survival. With the advent of antibiotics, the response is probably more injurious than it is protective, at least within advanced countries.

Clinical Data on Protein C Anticoagulant Pathway and Thrombotic Disease

Recent studies have established a severe thrombotic tendency in all reported cases of homozygous protein C deficiency (*16, 19, 53*) and apparently a somewhat less severe situation in patients essentially lacking protein S antigen in the plasma (*19*). The homozygous protein C–deficient patients develop purpura fulminans, a disease involving clotting in the microvasculature of the skin, and the uncontrolled coagulation generally leads to death in infancy unless treated. Treatment with concentrates rich in protein C can correct the coagulation defect (*54*), suggesting that the thrombotic tendency is related directly to the protein C deficiency.

The first patients to be described with a thrombotic tendency and protein C deficiency had protein C levels approximately 50% of normal (*55*). The three affected family members suffered their first thrombotic complications near age 20. Since this initial report, many extended families with heterozygous protein C deficiency and recurrent thrombosis have been described. Within these families the thrombotic complications are restricted almost entirely to the affected family members (*16, 20, 56*). Approximately 5 to 15% of the families with recurrent thrombosis have protein C levels at or near 50% of the normal range. Despite the association between the deficiency and recurrent thrombosis, the clinical picture is complex. Many heterozygous family members of homozygous protein C–deficient infants are clinically unaffected (*53*), and in a large population of blood donors approximately 0.3% have low protein C levels without any history of overt clinical thrombotic problems (*57*).

The observation that both protein C and protein S are vitamin K–dependent proteins, and lose their functional activity when oral anticoagulants are administered, raises an interesting paradox. Oral anticoagulants, which function by blocking formation of Gla residues, are commonly used to treat patients with recurrent thrombosis. When oral anticoagulants are first administered, protein C activity declines more rapidly than most of the other coagulation factors (*58*). This suggests that under rare circumstances, oral anticoagulants might cause clotting problems. One rare complication of oral anticoagulant therapy is warfarin-induced skin necrosis, which involves clotting in the microcirculation of the skin. It appears that this complication is significantly more common in patients with reduced protein C levels than in the normal population (*16, 59*).

Animal Studies with Activated Protein C

The properties of activated protein C outlined above suggested that this anticoagulant enzyme might have interesting properties in vivo. The baboon model of septic shock was our initial focus since it had the two critical characteristics of interest: (i) A coagulation response occurred rapidly, and (ii) coagulation was initiated by an inflammatory response mediated at least in part by the formation of monokines, including tumor necrosis factor (TNF).

When 100% lethal doses (LD_{100}) of *Escherichia coli* are infused into baboons, leukocytes, platelets, fibrinogen, and protein C all decrease in the circulation. After approximately 6 hours, significant liver damage is detected and death occurs in about 36 hours. When activated protein C is infused prior to the *E. coli*, the platelet and the fibrinogen consumption are blocked, liver damage is not observed, and the animals survive (normal life-span). When endogenous protein C activation is blocked with a monoclonal antibody to protein C, the animals respond to sublethal infusions of *E. coli* (10% of the LD_{100} level, which itself causes no coagulation disorder or overt liver damage) as in the lethal model and again are protected by infusion of human activated protein C (*60*). These studies demonstrate that activated protein C can function as an effective anticoagulant in vivo and suggest potential therapeutic utility. These studies also support the conclusion inferred from the clinical studies that protein C is a critical regulatory protein of the coagulation system.

The mechanisms by which protein C prevents the lethal effects of *E. coli* are not clear. Although it is apparent that the activated protein C blocks the coagulation response, studies by Cerami's group suggest that a critical mediator leading to death in septic shock involves the release of TNF (*61*). Preliminary experiments indicate that animals receiving activated protein C have little or no circulating TNF in their plasma compared to controls without activated protein C (*62*). Even though the mechanism is uncertain, it is apparent that activated protein C can decrease the overproduction of this potentially lethal mediator, and the data suggest that the coagulation system and the inflammatory system may be more intimately associated than previously appreciated.

Prospects

The intimate interaction between the coagulation and inflammatory systems appears to be mediated predominantly through the vascular endothelium. Hence, traditional assays of the coagulation system fail to detect abnormalities in these regulatory pathways, and thus assays of coagulation factors and platelet function very seldom provide a basis for the diagnosis of patients with thrombotic complications. New proteins involved in regulating these systems, such as the protein S binding protein (*63*), are still being discovered, and will lead to a more complete understanding of the complex interplay among the components. Development of new assays, such as the one recently described by Bauer *et al.* (*64*) that assesses protein C activation in vivo, may permit a better appreciation of cellular changes predisposing to thrombosis.

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