The Protein C Anticoagulant Pathway

Charles T. Esmon

Historical Background

Although protein C was described as an anticoagulant in 1960 by Dr. Seegers and colleagues,1 widespread appreciation of the complexity of its activation, function, and physiological roles did not begin to develop until Stenflo independently characterized protein C as a vitamin K-dependent zymogen2 that could be converted to a serine protease capable of membrane binding (Esmon et al1). The subsequent demonstrations by Kisiel et al4 that activated protein C was an anticoagulant that inactivated factor V and by Walker et al5 that the enzyme showed a marked specificity for factor Va provided information on the mechanism of action and the specificity of the enzyme. These observations were quickly extended when Vehar and Davie6 observed that factor VIII was a substrate for activated protein C and that factor VIIIa was the preferred substrate. Although the broad functions of the system were now clearly established, physiological activators were yet to be described, and the role of this system in the pathogenesis of thrombotic disease and in normal control of hemostasis remained to be investigated.

Properties of the Protein C Pathway

The protein C pathway as currently envisioned is outlined in Figure 1. The pathway is initiated by complex formation between thrombin and thrombomodulin.8 The complex catalytically generates activated protein C and enhances the rate of protein C activation by thrombin at least 1,000-fold.9 In addition, complex formation blocks the procoagulant activity of thrombin, resulting in the loss of thrombin’s activation of fibrinogen,10-15 factor V,10 platelets,11,16 endothelial cells,17 and factor XIII18 and inactivation of protein S.19,20 Thrombin can still be inhibited by its physiological inhibitor, antithrombin III, while bound to thrombomodulin. When this occurs, the thrombin–antithrombin III complex dissociates rapidly, freeing thrombomodulin for continued protein C activation.21 The thrombin–antithrombin III reaction appears to be facilitated by the presence of a chondroitin sulfate, indicated in Figure 1 by a line, with the sulfate on one end of the molecule.13,14 Once protein C is activated, it forms a complex with protein S on endothelium,22 platelets,23-25 and possibly other cells, where it can function optimally as an anticoagulant. The anticoagulant activity involves proteolysis of factor Va, but the proteolytic products remain associated.7 In bovine plasma, an additional factor, referred to as protein S-binding protein, has been described by Walker,26 but its mechanism of action, structure, and existence in other species await further investigation. Of considerable interest to our research program has been the observation by Dahlback and Stenflo27 that C4b-binding protein binds reversibly to protein S. Subsequent work has shown that only free protein S is functional in the anticoagulant pathway, giving rise to the possibility that alterations in the amount of circulating complex might modulate protein S activity. Once activated protein C is formed, it is inhibited in the plasma rather slowly (half-life =15 minutes) by complex formation with either protein C inhibitor or α1-antitrypsin.28,29

This picture of the anticoagulant pathway may be a reasonable depiction of its function in health, but results from many laboratories imply that the system and the endothelial cell surface may be dramatically different in diseases, especially those involving major inflammatory events. A working model of the vasculature after inflammation is shown in Figure 2. Inflammatory cytokines and other agents like interleukin-1,30 tumor necrosis factor (TNF),31-34 and endotoxin35 can lead to the disappearance of thrombomodulin from the surface of endothelium. At least with TNF, this results in rapid inhibition of transcription,34 degradation of message, and subsequent internalization and degradation of thrombomodulin.32,36 At the same time these mediators block thrombomodulin synthesis, they induce the synthesis and expression of tissue factor,30,31,35 a protein that initiates coagulation, and leukocyte adherence molecules (ELAMs) that bind monocytes and neutrophils on activated endothelium.37 Other mediators such as histamine or thrombin mobilize another adherence molecule, GMP-140, from the Weibel Palade bod-
FIGURE 1. The protein C anticoagulant pathway under normal conditions. In this model, injury occurs and prothrombin (Pro) activation results in thrombin (T) formation. Prothrombin activation involves factor Va (Va) and factor Xa (Xa). Thrombin then binds to thrombomodulin (TM) on the lumen of the thrombin—endothelium, illustrated by the heavy line, and the thrombomodulin complex converts protein C (PC) to activated protein C (APC). Thrombin bound to thrombomodulin can be inactivated very rapidly by antithrombin III (ATIII), at which time the thrombin—antithrombin III complex rapidly dissociates. Activated protein C then binds to protein S (S) on cellular surfaces. The activated protein C—protein S complex then converts factor Va to an inactive complex (Vi), illustrated by the slash through the larger part of the two-subunit factor Va molecule. The cleavage product does not appear to dissociate.7 Protein S also circulates in complex with C4bBP, which may in turn bind serum amyloid P (SAP). Activated protein C is inhibited by forming complexes with either the protein C inhibitor (PCI) or α1-antitrypsin (α1AT). See text for a more complete discussion.

ies,38 that also binds neutrophils and monocytes. Because platelets contain GMP-140, it is possible that they may adhere to neutrophils attached to the membrane through other adherence molecules. Monocyte adherence may be of particular importance in vivo, as monocytes have been shown to synthesize tissue factor in vivo and the question of tissue factor synthesis by endothelium in vivo remains unresolved.39 With the appearance of ELAMs on the endothelial cell surface, the capacity of the lumen of the endothelium to focally initiate coagulation is ensured through either the controversial endothelial cell–directed synthesis or the well-documented monocyte synthesis. Perhaps equally important, endothelium primed with TNF and subsequently exposed to neutrophils rapidly loses thrombomodulin activity from its surface.40 This synergistic response could potentially link many of the aforementioned processes into a concerted and localized thrombotic response and may explain in part the appearance of circulating thrombomodulin in disease states associated with inflammation.41 In this context, it is especially interesting to note that the in vivo localization of ELAMs is quite restricted to the endothelium of postcapillary venules,45 although in septic shock in baboons, very recent studies have detected ELAMs in capillaries, arterioles, and other areas of the vasculature.46

In addition to changes at the vessel surface, inflammation results in an acute-phase response. Among the many proteins effected is C4bBP.44,45 Because even in normal plasma approximately 60% of protein S is in complex with C4bBP,45,46 changes in the level of this protein can alter the level of free, and therefore of anticoagulantly active, protein S by increasing the amount of complex. This situation may be variably balanced by the fact that serum amyloid P, another weak acute-phase reactant, appears to bind to C4bBP and may alter the affinity for protein S.45-47 Thus, the magnitude of changes seen in specific clinical conditions is variable. Nonetheless, current reports have indicated elevation in C4bBP and/or reduction in free protein S in a variety of clinical situations, including shock,48 the nephrotic syndrome,49 stroke,50,51 after myocardial infarction,52 and deep vein thrombosis.48 These observations have not been universally confirmed, which may relate to differences in treatment, or perhaps more likely, variability in diagnostic methods. Given the complexity of the system as indicated previously, a comprehensive review is beyond the scope of the current article. Reviews have appeared elsewhere.45,53,54 Two features of the protein C anticoagulant pathway will constitute the major focus of the current review: 1) how thrombomodulin functions to accelerate thrombin-catalyzed protein C activation and 2) the impact of inflammation...
FIGURE 2. The protein C pathway after inflammation. In this model, inflammatory mediators lead to the disappearance of thrombomodulin from the endothelial cell surface. Endothelial cell leukocyte adhesion molecules (ELAM) or GMP-140 are synthesized or expressed on endothelial or platelet surfaces. Tissue factor (TF) is expressed on monocytes and binds factor VIIa (VIIa), and this complex converts factor X (X) to factor Xa (Xa), which forms complexes with factor Va (Va) to generate thrombin (T) from prothrombin (Pro). Because little protein C (PC) is formed and the little that forms does not function well because of low protein S (S), factor Va is not inactivated and prothrombin complexes are stabilized. Elevation in circulating C4bBP concentration results in little free protein S. See text for discussion. SAP, serum amyloid P.

on the pathway, with special attention to the potential role of C4bBP in this process.

Molecular Events in Protein C Activation: New Insights Into How the Regulatory Proteins of Protease Cascades May Function

Throughout blood coagulation, complement, and fibrinolysis, a common motif has been established in which the enzyme binds to a surface and/or a specific regulatory protein, and it is this complex that demonstrates the specificity and catalytic competence necessary to effectively activate the appropriate zymogen.55–57 Although a wealth of information about the affinity and localization of the interactions is known, relatively little is known about how the enzyme complexes actually accelerate the reaction or alter enzyme specificity. The dramatic change in thrombin specificity associated with binding to thrombomodulin constitutes a unique advantage in examining enzyme specificity. Perhaps the greatest attribute of the system is that it aids in rephrasing the question of how the regulatory proteins function. It is not that the regulatory proteins (often referred to as cofactors) make the enzymes extremely effective; rather, it is that the enzymes are extremely ineffective by themselves, that is, before complex formation. In the case of the thrombin–thrombomodulin complex, this is clearly pointed out by the fact that the change in protein C activation is compensated for by the loss in fibrinogen clotting. Thus, there must exist either specific structures on the substrate or particular amino acids that interact in an inhibitory fashion and that prevent rapid activation. In this context, a possible and testable mechanism by which the regulatory proteins might function is alteration of the enzyme and/or substrate conformation, thereby overcoming this specific and probably local inhibition. Support for this hypothesis demands that conformational changes within the enzyme or substrate can be documented and that specific inhibitory residues within the substrate and/or enzyme can be identified and altered in a predictable fashion to overcome the inhibition. Ultimately, adequate tests of this hypothesis will combine enzyme and substrate mutagenesis, crystallography, and dynamic methods to analyze enzyme and substrate structure. The studies that follow are a progress report on that overall goal.

Because the following arguments are based on structure–function considerations, it is useful to refer to the schematic diagram of protein C, protein S, and thrombomodulin shown in Figure 3. The fundamental question to be addressed is why protein C is a bad substrate for thrombin in the absence of thrombomodulin. Even this problem has two parts, as both calcium and thrombomodulin play major, and somewhat interrelated, roles in determining the rate of protein C activation. Activation by thrombin alone is inhibited in a calcium-concentration–dependent fashion. Surprisingly, with thrombomodulin present, the activation reaction requires calcium (Figure 4). The calcium-concentration dependence of the two processes are indistinguishable, suggesting the possi-
FIGURE 3. Schematic representation of protein C, protein S, and thrombomodulin (TM). The Gla residues of protein C and protein S are indicated by small Y-shaped symbols. Formation of these vitamin K-dependent residues is essential to full activity of protein C and protein S. Gla, γ-carboxyglutamic acid; Th.-sens., thrombin sensitive. (Reproduced from Reference 53 with permission of the American Society for Biochemistry and Molecular Biology, Inc.)

bility that both processes are governed by occupancy of the same Ca\(^{2+}\)-binding site(s). That this site is on protein C is indicated by the observation that proteolysis of protein C by chymotrypsin to remove the first 44 residues containing the vitamin K-dependent γ-carboxyglutamic acid (Gla) residues lowers the calcium dependence of both the inhibition and activation reactions equivalently. Moreover, direct Ca\(^{2+}\)-binding studies and indirect monitors of protein conformation suggest that occupancy of a single Ca\(^{2+}\)-binding site in Gla-domainless protein C results in the structural change that allows recognition by the protein C activation complex but no longer by free thrombin (Figure 5). How and why this occurs remained a mystery until Öhlin and Stenflo\(^{60}\) and Öhlin et al\(^{61}\) demonstrated, in a series of elegant experiments, that the Ca\(^{2+}\)-binding site is in one of the epidermal growth factor (EGF) domains of protein C. Sugo et al\(^{62}\) also demonstrated that occupancy of this site in the EGF domain increases the resistance to reduction of all disulfide bonds in protein C, suggesting a global conformational change. Included in these global changes appears to be a conformational change exactly at the scissile bond involved in protein C activation. This conclusion is based on the similarity of the calcium dependence of the binding of an antibody that recognizes an epitope spanning this site in protein C and the occupancy of the high-affinity Ca\(^{2+}\)-binding site.\(^{59}\) Taken together, the combined results of all of the aforementioned experiments suggest that Ca\(^{2+}\) occupancy of the EGF domain of protein C results in a conformational change that includes the sequence containing the scissile bond and that this conformational change is critical to recognition by the thrombin–thrombomodulin complex. A depiction of the model appears in Figure 6.

Potential insights into the question of inhibitory contacts and the remarkable calcium dependence
began to take shape from a single experiment. We were fortunate to be able to collaborate with Hartmut Ehrlich, Nils Bang, and colleagues, whose experiments resulted in the expression of protein C and mutants thereof. The sequence of human protein C near the scissile Arg-Leu bond is Val-Asp-Pro-Arg-Leu-Ile-Asp. The key experiment postulated that the unusual Asp residue in the P3 site on protein C might be responsible for slow activation, as good thrombin substrates like fibrinogen had neutral (Gly) or hydrophobic (Phe) residues in this site. If the Asp in P3 is responsible for inhibition, then changing the Asp to Gly or Phe by site-directed mutagenesis should improve protein C as a substrate for free thrombin and reduce the fold rate enhancement observed with thrombomodulin. Indeed, this proved to be the case. Surprisingly, the major difference observed was in the presence of calcium, and it was related to a much higher calcium requirement for the inhibition of activation by thrombin alone. Thus, the P3 Asp appears to be both one of the inhibitory residues and one of the residues involved in calcium dependence. An implication of these studies, as yet untested, is that mutation of the activation region perturbs the affinity for Ca\(^{2+}\) binding in the EGF domain of protein C. Subsequent independent experiments by Bang and colleagues and our group have also implicated the P'3 residue in the inhibitory interaction.

The identification of inhibitory residues within thrombin that are responsible for the inability to accept P3 and P'3 Asp residues in the substrate site was the next major question. Major hints with respect to the P3 residues came from knowledge that thrombin and trypsin are structurally related but that trypsin accepts acidic residues in the P3 position quite well. Thus, differences in the extended specificity pocket between thrombin and trypsin might account for this difference. Fortunately, B. Le Bonniec joined our group at about this time, and his interests involved precisely the question of which residues in the catalytic center of thrombin are responsible for its remarkable substrate specificity. He chose two candidate residues in thrombin, Glu 192 and Glu 39 in the chymotrypsin numbering system, for modification. Glu 192, three residues from the active Ser, is a Gin in trypsin and is

**Figure 4.** Plot showing influence of calcium ion concentration (\(\mu\text{M}\)) on the activation of Gla-domainless protein C by free thrombin and the thrombin-thrombomodulin (T-TM) complex. Initial rates of activation were determined at each calcium concentration. Conditions were adjusted with excess thrombomodulin so that all thrombin was complexed. Velocities were normalized to optimal conditions for each activator. \(K_{\text{cat}}\) (turnover number for the enzyme in moles per minute per mole of catalytic complex) for free thrombin is approximately 2 mol/min and for the T-TM complex is approximately 250 mol/min. See text for more discussion. Gla, \(\gamma\)-carboxyglutamic acid.

**Figure 5.** Scatterplot showing calcium dependence of protein C activation, fluorescence changes, and Ca\(^{2+}\) binding with Gla-domainless protein C. Fluorescence (\(\Delta F/F_{\text{max}}\), ○) reflects changes in tryptophan environment as a percentage of the maximum change observed at saturating Ca\(^{2+}\). Protein C activation (T-alone, ■; T-Tm, □) reflects the percentage of the maximum initial rate observed under optimal conditions. Calcium binding data (Ca bound, ▲) was obtained from equilibrium dialysis, which indicated a single Ca\(^{2+}\) binding site in Gla-domainless protein C. Gla, \(\gamma\)-carboxyglutamic acid; T, thrombin; Tm, thrombomodulin.
conserved as a Gln in most trypsin-like proteases. Thrombin and protein C, which share primary-sequence specificities, are exceptions. Depending on the particular serine protease, residue 192 can interact with residues P1–P4 of the substrate. The unusual Glu at 192 and the resistance of protein C to proteolysis because of the Asp in the P3 residue made this an extremely good candidate for the key residue responsible for the slow activation of protein C in the absence of thrombomodulin. The experimental results verified that indeed, the isosteric substitution of Glu for Gln at position 192 resulted in a 20-fold increase in the rate of protein C activation in the absence of thrombomodulin. If thrombomodulin functioned by preventing this inhibitory effect, then the thrombomodulin complex with either wild-type or mutant thrombin should activate protein C approximately equivalently. This proved to be the case, with less than a twofold difference in activation rate between the two complexes. As anticipated, the E192Q mutant is not inhibited by peptides with Asp residues in the P3 position. In addition, the mutant also has the interesting property of being more tolerant of the Asp residue in the P'3 position. Thus, residue 192 appears to be a major determinant in the specificity of protein C and accounts in large part for the resistance to accept acidic residues in the P3 position.

A somewhat analogous situation exists with the P'3 Asp residue. In this instance, a Glu at position 39 was substituted by a Lys, and protein C and fibrinogen were examined as substrates. Fibrinogen clotting was inhibited approximately twofold, while protein C activation was enhanced twofold. Examination of the basis of the enhanced protein C activation revealed that mutation selectively blocked the inhibitory influence of the P'3 Asp residue. In the presence of thrombomodulin, there was no difference in the activation rates, again consistent with the concept that thrombomodulin acceleration involves prevention of the inhibitory contacts between Glu 39 and the P'3 Asp.

Mutagenesis of both the substrate and the enzyme was consistent with the central hypothesis formulated above, but this would be viable only if a conformational (structural) change could be documented in the enzyme. Two very fortunate observations allowed a test of the hypothesis that there was structural alteration in the extended specificity pocket related to acceleration of protein C activation. Previous studies demonstrated that an 80-residue domain of thrombomodulin would bind thrombin and block fibrinogen clotting but would not accelerate protein C activation (see Figure 5). A larger fragment, composed of either the terminal three growth factor domains or the terminal six growth factor domains, binds thrombin, blocks fibrinogen clotting, and accelerates protein C activation. Earlier studies with electron paramagnetic resonance spectroscopy had indicated that binding of full-length thrombomodulin to thrombin resulted in multiple conformational changes near the active site Ser. As the thrombomodulin structure-function relations were not known at the time and no probes were available at large distances from the active center, it was not possible to determine which, if any, of the observed changes correlated with the ability to activate protein C. The availability of thrombomodulin fragments and the development of additional probes allowed us to investigate the location of structural changes in thrombin and to correlate these with known structures and functions of the thrombomodulin fragments.

Probes were attached to the active site Ser directly with dansyl fluoride and on the terminal Phe of the covalent inhibitor, d-Phe-Pro-Arg chloromethylketone. Thus, the probes monitored very close to the Arg binding pocket and at a distance approximating four residues from the cleavage site. Comparison of the influence of EGF 5–6 with EGF 1–6, that is, nonfunctional versus functional thrombomodulin fragments with respect to protein C activation, revealed that both altered the environment of the probe attached to the active site Ser, whereas only the larger fragment altered the environment of the probe distant from the active center. Thus, probes located in the vicinity of Glu 192 are sensitive to environmental changes resulting only from binding of active thrombomodulin species. Because both active and inactive thrombomodulin species alter the dansyl environment, the change in the catalytic center could be critical for loss of fibrinogen clotting activity.

Recently, studies were initiated by us and Shaun Coughlin to investigate the influence of peptides on the newly described thrombin receptor sequence on thrombin structure. Proteolysis of this receptor is critical for receptor activation, and it contains an Asp residue in the P3 position, a situation entirely analogous to the protein C activation fragment. In addition, the receptor has a region with remote similarity to the carboxyl terminus of hirudin, the leech thrombin.
bin inhibitor. This domain of hirudin is referred to as hirugen and is known to bind in the anion binding exosite of thrombin.\textsuperscript{75–77} It is interesting that the hirugen-like peptide from the thrombin receptor modifies the dansyl fluorescence in a manner reminiscent of the structural changes in thrombin induced by thrombomodulin, suggesting that this region of the thrombin receptor may alter thrombin conformation to relieve the inhibition of the P3 Asp. Combined with the observation that thrombomodulin prevents thrombin activation of platelets and that hirugen inhibits thrombomodulin acceleration of protein C activation, it is likely that thrombomodulin shares overlapping binding sites with the hirugen-like region of the thrombin receptor.

Specificity is not restricted to local changes within the catalytic center. Binding of thrombin to thrombomodulin on the cell surface results in raising the enzyme nearly 70 Å above the membrane surface.\textsuperscript{78} This complex thus has orientation and properties very similar to other vitamin K–dependent membrane complexes, except that thrombomodulin forms the platform on which thrombin sits, whereas other domains of the same protein serve this function in other vitamin K–dependent proteins.\textsuperscript{79,80}

From this analysis, it is clear that specific residues are responsible for the slow activation of protein C and that conformational changes near these critical contact sites do occur. Furthermore, the topology of the complex is regulated by binding to thrombomodulin. Future advances in understanding the details of this complex process must await crystallization of the complex, hopefully with substrate analogues in place, and subsequent biophysical and structural studies to further test this model. The unique aspects of this complex promise to make it one of the first that is understood at the reaction-mechanism level.

**Influence of Protein C on Inflammation In Vivo: Insights Into Possible Coordinate Regulation of Inflammation by the Protein C Pathway**

A second major component of our research program relates to the impact of inflammatory responses on coagulation. The review of current concepts in the protein C pathway and other inflammatory events is summarized in Figures 1 and 2. From this basic information, we began to postulate that the protein C system might have an impact not only on coagulation but also on inflammation. The basis for this assumption was that in biology in general, products of membrane complexes, except that thrombomodulin forms the platform on which thrombin sits, whereas other domains of the same protein serve this function in other vitamin K–dependent proteins.\textsuperscript{79,80}

From this analysis, it is clear that specific residues are responsible for the slow activation of protein C and that conformational changes near these critical contact sites do occur. Furthermore, the topology of the complex is regulated by binding to thrombomodulin. Future advances in understanding the details of this complex process must await crystallization of the complex, hopefully with substrate analogues in place, and subsequent biophysical and structural studies to further test this model. The unique aspects of this complex promise to make it one of the first that is understood at the reaction-mechanism level.

**Influence of Protein C on Inflammation In Vivo: Insights Into Possible Coordinate Regulation of Inflammation by the Protein C Pathway**

A second major component of our research program relates to the impact of inflammatory responses on coagulation. The review of current concepts in the protein C pathway and other inflammatory events is summarized in Figures 1 and 2. From this basic information, we began to postulate that the protein C system might have an impact not only on coagulation but also on inflammation. The basis for this assumption was that in biology in general, products of membrane complexes, except that thrombomodulin forms the platform on which thrombin sits, whereas other domains of the same protein serve this function in other vitamin K–dependent proteins.\textsuperscript{79,80}

From this analysis, it is clear that specific residues are responsible for the slow activation of protein C and that conformational changes near these critical contact sites do occur. Furthermore, the topology of the complex is regulated by binding to thrombomodulin. Future advances in understanding the details of this complex process must await crystallization of the complex, hopefully with substrate analogues in place, and subsequent biophysical and structural studies to further test this model. The unique aspects of this complex promise to make it one of the first that is understood at the reaction-mechanism level.

**Influence of Protein C on Inflammation In Vivo: Insights Into Possible Coordinate Regulation of Inflammation by the Protein C Pathway**

A second major component of our research program relates to the impact of inflammatory responses on coagulation. The review of current concepts in the protein C pathway and other inflammatory events is summarized in Figures 1 and 2. From this basic information, we began to postulate that the protein C system might have an impact not only on coagulation but also on inflammation. The basis for this assumption was that in biology in general, products of membrane complexes, except that thrombomodulin forms the platform on which thrombin sits, whereas other domains of the same protein serve this function in other vitamin K–dependent proteins.\textsuperscript{79,80}

From this analysis, it is clear that specific residues are responsible for the slow activation of protein C and that conformational changes near these critical contact sites do occur. Furthermore, the topology of the complex is regulated by binding to thrombomodulin. Future advances in understanding the details of this complex process must await crystallization of the complex, hopefully with substrate analogues in place, and subsequent biophysical and structural studies to further test this model. The unique aspects of this complex promise to make it one of the first that is understood at the reaction-mechanism level.

**Influence of Protein C on Inflammation In Vivo: Insights Into Possible Coordinate Regulation of Inflammation by the Protein C Pathway**

A second major component of our research program relates to the impact of inflammatory responses on coagulation. The review of current concepts in the protein C pathway and other inflammatory events is summarized in Figures 1 and 2. From this basic information, we began to postulate that the protein C system might have an impact not only on coagulation but also on inflammation. The basis for this assumption was that in biology in general, products of membrane complexes, except that thrombomodulin forms the platform on which thrombin sits, whereas other domains of the same protein serve this function in other vitamin K–dependent proteins.\textsuperscript{79,80}

From this analysis, it is clear that specific residues are responsible for the slow activation of protein C and that conformational changes near these critical contact sites do occur. Furthermore, the topology of the complex is regulated by binding to thrombomodulin. Future advances in understanding the details of this complex process must await crystallization of the complex, hopefully with substrate analogues in place, and subsequent biophysical and structural studies to further test this model. The unique aspects of this complex promise to make it one of the first that is understood at the reaction-mechanism level.
E. coli elevated the TNF response, which was not exactly the same way but supplemented with protein S did not develop DIC, organ damage, or serum TNF levels were unaltered. Thus, although this inhibitor was very effective, it was anticipated that the inhibitor would be very effective. Infusion of the DEGR-FXa completely prevented the fibrinogen consumption but did not alter organ damage, vascular injury, or the lethal response to the infusion, although death was delayed slightly. TNF levels were unaltered. Thus, although this inhibitor was more effective than activated protein C in preventing DIC, it was not effective in preventing the inflammatory response to the challenge. These results suggest very strongly that activated protein C has influences on the inflammatory response that are not shared by other anticoagulants and that these effects are not manifested solely by preventing thrombin formation.

The sublethal model also offered an opportunity to investigate the role of C4bBP in modulating this disease process. As reviewed earlier, C4bBP is elevated in a variety of disease states, many of which are associated with thrombosis. Furthermore, in some patients with abnormal distributions of bound and free protein S, there appeared to be an increased risk of thrombosis. These experiments of nature stop short of determining cause and effect and certainly do not address the important question of whether the deleterious effect of the increased complex formation could be reversed with excess protein S. To resolve these questions, the same sublethal model was employed, but instead of antibodies to protein C, the system was supplemented with C4bBP. This complexed the protein S, and DIC, organ damage, and the major symptoms of shock ensued. Interestingly, the C4bBP in combination with sublethal amounts of E. coli elevated the TNF response, which was undetectable with either agent alone. Animals treated in exactly the same way but supplemented with protein S did not develop DIC, organ damage, or serum TNF levels.

The original work from Cerami's group clearly implicated TNF as a major mediator in septic shock. The previously cited experiments with C4bBP and protein S imply that this mediator may be regulated in part by the coagulation system. To study the possibility that activated protein C worked by altering the TNF response, infusion studies were performed with TNF. Although TNF caused significant responses, it did not induce DIC. To investigate the possibility that the DIC response also required inhibition of the protein C pathway, protein C activation was blocked with the monoclonal antibody to protein C. To our surprise, instead of DIC and shock, the animals developed massive deep vein thrombosis near the site of the catheter.

The thrombosis clearly required the catheter, TNF, and a blockade of protein C activation. In preliminary experiments, it appeared that C4bBP substituted for antibody to protein C and elicited the thrombotic response. This model clearly illustrates that thrombosis and DIC both can be initiated by inflammatory mediators and modulated by the status of the protein C system, but that components of the inflammatory pathway in addition to TNF are necessary to convert the thrombotic response to a DIC response. A summary of these results is presented in Table 1.

**Table 1. Influence of Protein C Pathway Components on the Shock Response**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PMN*</th>
<th>EL*</th>
<th>TNF*</th>
<th>DIC†</th>
<th>DVT‡</th>
<th>SGPT§</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lethal E. coli</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>Activated protein C+lethal E. coli</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>DEGR-Xa+lethal E. coli</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>Sublethal E. coli</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>C4bBP alone</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-protein S</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-protein S+sublethal E. coli</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>C4bBP+sublethal E. coli</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>C4bBP+protein S+sublethal E. coli</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Anti-protein C+sublethal E. coli</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>No</td>
</tr>
<tr>
<td>TNF</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-protein C</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>TNF+anti-protein C</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

PMN, polymorphonuclear leukocytes; EL, neutrophil elastase; TNF, tumor necrosis factor; DIC, disseminated intravascular coagulation; DVT, deep vein thrombosis; SGPT, serum glutamic pyruvic transaminase; DEGR-Xa, active site of factor Xa blocked with dansyl-Glu-Gly-Arg-chloromethylketone; ND, not determined.

*Event occurs within 3 hours of Escherichia coli challenge.
†Event occurs between 3 and 12 hours.
‡Surgically documented thrombus at 48 hours.
§Event occurs after 10 hours.
Possible Involvement of the Protein C Pathway in Protecting Ischemic Tissue

Reperfusion injury and damage to ischemic tissue seem to involve many of the same mechanisms apparent in the shock process. This led to the hypothesis that protein C might be activated rapidly in ischemic tissue, as it is in shock, and thus might prevent local injury. To examine this possibility, the left anterior descending coronary artery was ligated for approximately 2 minutes in pigs and dogs and then released. The blood from this ischemic area and from the systemic circulation was analyzed for protein C-dependent anticoagulant activity. Significant anticoagulant activity (approximate doubling of the clotting time) was observed only from the ischemic region. As anticipated, this short-term ligation had no major deleterious effects on heart function. In contrast, when protein C activation was blocked as described in the shock model, 30% of the animals fibrillated compared with none of 20 receiving either no treatment or activated protein C. The animals that did survive the initial reperfusion exhibited myocardial stunning, as reflected by progressive loss of left ventricular function. These studies suggest that protein C plays a major role in preventing ischemic injury. Its potential in therapy is suggested by these studies but not truly documented, as the control animals did not exhibit severe enough myocardial dysfunction to measure statistically significant improvement. The relation between the inhibition of protein C with an antibody and clinical situations is clearly subject to speculation. It is, nonetheless, relevant to point out that patients with unstable angina have been reported to have elevated levels of circulating TF and that postmyocardial infarction patients have been observed with low levels of free protein S. If one makes the reasonable assumption that these situations exist in at least some patients at risk of myocardial infarction, and, when they exist, that they impair the protein C system in these patients, then it follows that activated protein C with or without protein S might help salvage ischemic tissue. A direct test of this hypothesis will require additional animal and clinical study.

In summary, the protein C system offers a myriad of opportunities to investigate both structure–function correlates and the cellular basis of the regulation of coagulation. The near future promises to add dramatically to this rapidly growing area.

Acknowledgments

I would like to express my sincere appreciation to Arthur Johnson, Fletcher Taylor, Thomas Snow, and Philip Comp for the opportunity to collaborate with them and their research groups for the last 12 years. I would also like to thank Naomi Esmon for helpful advice and frequent criticism over this period, not to mention her invaluable help in establishing techniques and performing these studies. The results described above were made possible by the creative input of many excellent former and current students and fellows in my laboratory, especially Deborah Stearns, Shinichiro Kurosawa, Jennifer Galvin, Le-Wen Liu, Bernard Le Bonniec, Ray Rezaie, and Tim Mather. Tireless efforts on the part of Jean Husten, Jia Ye, and Ben Isaacs made the energy-transfer and fluorescence studies possible. I would also like to thank Kevin Moore, David Stern, and Peter Nawroth, with whom we did the initial cytokine studies. The dedicated work of Gary Ferrell, Susan Worsham, Jeff Bex, Clendon Brown, Barb Carpenter, Glenn Pipher, Teresa Burnett, Pam Hagan, and Katherine Pih to the successful completion of these studies is gratefully acknowledged. Finally, I would like to express my thanks to Nils Bang, Betty Yan, Brian Grinnell, and Hartmut Ehrlich for several of the early collaborations on structure–function correlates in protein C and for some of the initial preclinical pharmacology. Many others have contributed equivalently to other aspects of our research program. Any successes that I have had have been totally the result of being able to sustain these long-term collaborations. The recent work on the molecular biology of the system is entirely attributable to the support of the Howard Hughes Medical Institute and the Oklahoma Medical Research Foundation for allowing me to establish these techniques in my laboratory.

References

1. Mammen EF, Thomas WR, Seegers WH: Activation of purified prothrombin to autoprothrombin I or autoprothrombin II (platelet cofactor II) or autoprothrombin II-A. Thromb Diath Haemorrh 1960;5:218–250
Arteriosclerosis and Thrombosis Vol 12, No 2 February 1992


45. Rosenberg RD: Regulation of the hemostatic mechanism, in Stamatsopoulos G, Nienhaus AW, Leder P, Majerus PW


58. Johnson AE, Esmon NL, Laue TM, Esmon CT: Structural changes required for activation of protein C are induced by Ca2+ binding to a high affinity site that does not contain gamma-carboxyglutamic acid-containing region. *J Biol Chem* 1984;259:5703–5710


80. Armstrong SA, Hustedt EJ, Esmon CT, Johnson AE: Membrane-bound prothrombin: The distance of the active site domain above the phospholipid surface (abstract). *Blood* 1987;70:384a


**Key Words**: protein C • protein S • thrombomodulin • thrombosis • inflammation • disseminated intravascular coagulation • endothelium • monocytes
The protein C anticoagulant pathway.

C T Esmon

doi: 10.1161/01.ATV.12.2.135

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/12/2/135.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/