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Inflammation, sepsis, and coagulation

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ABSTRACT

The molecular links between inflammation and coagulation are unquestioned. Inflammation promotes coagulation by leading to intravascular tissue factor expression, eliciting the expression of leukocyte adhesion molecules on the intravascular cell surfaces, and down regulating the fibrinolytic and protein C anticoagulant pathways. Thrombin, in turn, can promote inflammatory responses. This creates a cycle that logically progresses to vascular injury as occurs in septic shock. Most complex systems are regulated by product inhibition. This inflammation-coagulation cycle seems to follow this same principle with the protein C pathway serving as the regulatory mechanism. The molecular basis by which the protein C pathway functions as an anticoagulant is relatively well established compared to the mechanisms involved in regulating inflammation. As one approach to identifying the mechanisms involved in regulating inflammation, we set out to identify novel receptors that could modulate the specificity of APC in a manner analogous to the mechanisms by which thrombomodulin modulates thrombin specificity. This approach led to the identification of an endothelial cell protein C receptor (EPCR). To understand the mechanism, we obtained a crystal structure of APC (lacking the Gla domain). The crystal structure reveals a deep groove in a location analogous to anion binding exosite 1 of thrombin, the location of interaction for thrombomodulin, platelet thrombin receptor and fibrinogen. Thrombomodulin blocks the activation of platelets and fibrinogen without blocking reactivity with chromogenic substrates or inhibitors. Similarly, in solution, EPCR blocks factor Va inactivation without modulating reactivity with protease inhibitors. Thus, these endothelial cell receptors for the protein C system share many properties in common including the ability to be modulated by inflammatory cytokines. Current studies seek to identify the substrate for the APC-EPCR complex as the next step in elucidating the mechanisms by which the protein C pathway modulates the response to injury and inflammation.

Key words: inflammation, sepsis, coagulation

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The protein C pathway serves as a natural defense mechanism against thrombosis. It differs from other anticoagulants in that it is an *on demand* system that can amplify the anticoagulant response as the coagulant response increases (Figure 1). The mechanisms responsible for this include the fact that thrombomodulin lines the vascular surface, binds to thrombin and this promotes protein C activation. Activated protein C (APC) generation is thus roughly proportional to thrombin concentration.[1] The importance of this system is amply illustrated by the severe thrombotic complications associated with protein C deficiency and the ability to correct the defect by protein C supplementation.[2-4]

Coagulation results not only in thrombosis but also in promotion of inflammation and cell growth. Thrombin thus can promote coagulation, anticoagulation, cell proliferation or inflammation by triggering some of the responses summarized in Figure 2. A major complication and logical inconsistency arises with this picture of thrombin function when one considers that inflammation can also promote coagulation, inhibit anticoagulant responses, and down regulate the fibrinolytic cascade (Figure 3). Left unchecked, this would lead to a self-propagating feedback loop with the predicted consequences illustrated in Figure 4. Since it is clear that inflammation and coagulation do not usually lead to a lethal response, we examined potential mediators that might block the amplification, in particular the possibility that the protein C system might serve this role.

Support for the hypothesis that the protein C pathway could dampen this auto-amplification loop came from early observations that thrombin infusion into dogs could block the subsequent lethal response to challenges with lethal numbers of *E.coli.*[5] With the realization that thrombin infusion *in vivo* could activate protein C,[6] we examined the ability of APC to protect baboons in a similar model. As with thrombin, APC protected the animals from death and organ failure. Inhibition of the pathway exacerbated the response to *E. coli* and enhanced cytokine production.[7,8] Many attempts on our part to obtain APC dependent inhibition of the inflammatory cytokine response *in vitro* met with failure, although others have reported that APC can modulate inflam-

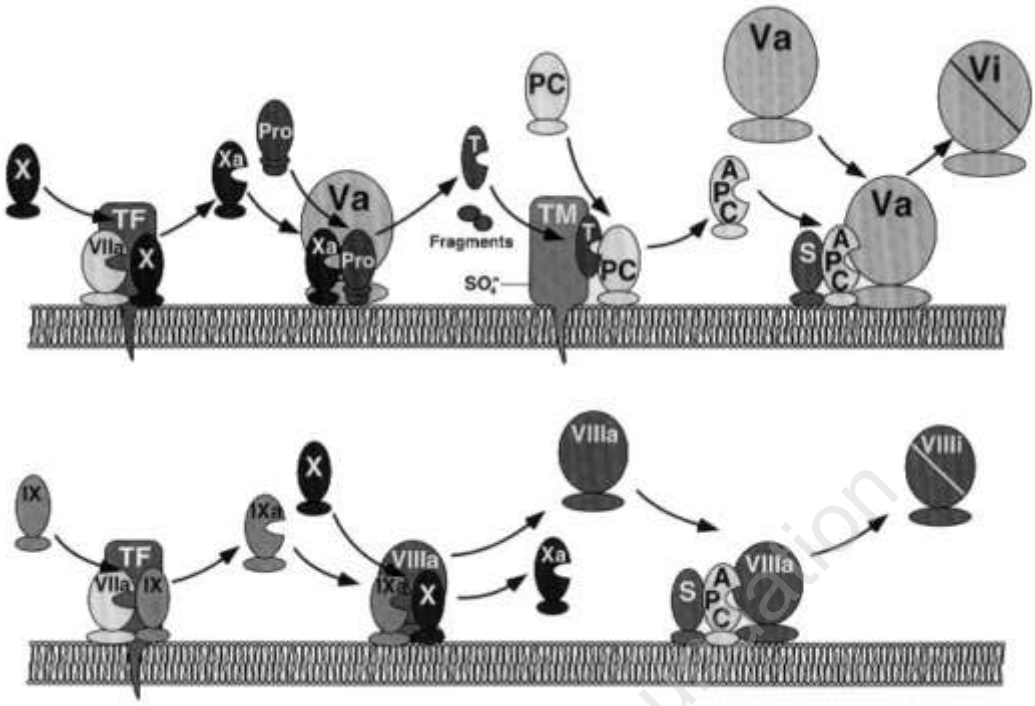

Figure 1 (above). Interaction of the protein C pathway with the extrinsic coagulation system. This is a model in which the coagulation stimulus is tissue factor (TF). TF binds factor VIIa (VIIa) to activate either factor IX (IX) or factor X (X). Factor IXa or Xa then complexes with either factor VIIIa (VIIIa) or factor Va (Va) to activate factor X or prothrombin (Pro), respectively. Thrombin (T) interacts with thrombomodulin (TM) to activate protein C (PC) and the activated protein C (APC) then complexes with protein S (S) to inactivate factor Va or VIIIa. For simplicity, the activation of factors VII, V, and VIII are not shown.

matory cytokine production *in vitro* as well[9] and that protein C can block selectin-mediated neutrophil binding.[10]

Given our inability to observe modulation of these key processes *in vitro*, we explored the possibility that there might be a receptor for APC *in vivo* that could modulate APC function in a manner analogous to thrombomodulin on thrombin.[11] Our hypothesis was that this receptor might bind APC. Therefore, we developed a flow cytometric assay for APC binding to cell surfaces using APC specifically labeled in the active site with fluorescein.[12] Screening of several cell types revealed that APC bound well only to endothelial cells. The binding was moderately high affinity ($K_{d(app)}$=30-50 nM), Ca^{2+} dependent, dependent on the Gla domain and not competed by factor X, properties suggesting a specific receptor. Importantly, we observed that this binding activity was down regulated by TNF-α, further supporting the concept that there was a specific factor, probably a protein, that was involved in the binding interaction. Based on these initial obser-

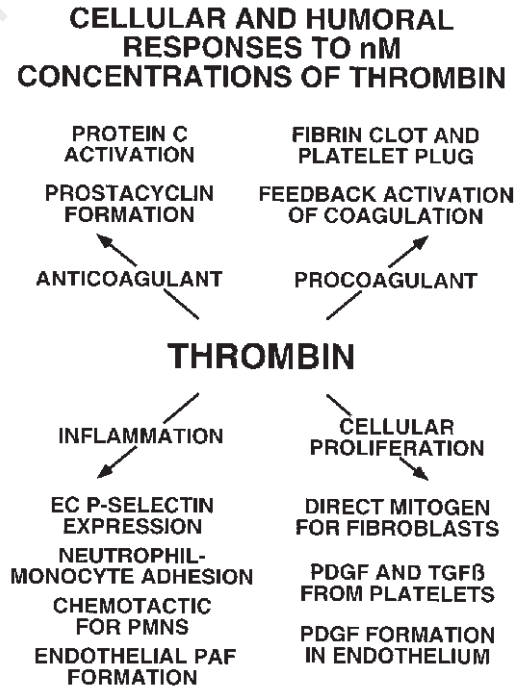

Figure 2. Multiple functions of thrombin. Selected functions of thrombin are illustrated. These include events that modulate coagulation, anticoagulation, inflammation and cell proliferation. EC P-Selectin is endothelial cell P-selectin that is expressed from Weibel Paladi bodies following stimulation with thrombin. *PAF-platelet activating factor, PDGF, platelet derived growth factor; TGFβ, transforming growth factor β.*

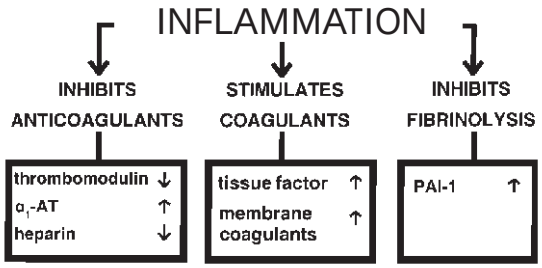

Figure 3. The impact of inflammatory mediators on the regulation of coagulation. Inflammatory mediators such as TNF or endotoxin can mediate the changes indicated. An upward arrow indicates increases in levels and a downward arrow indicates decreases. PAI-1 is plasminogen activator inhibitor 1 and α_1-AT is α_1 antitrypsin.

vations, we prepared an expression library from endothelial cells, and screened transiently transfected 293 T cells for the ability to bind labeled APC. This approach led to the identification of a clone, which when expressed in mammalian cells, resulted in the ability to bind APC. Since this activity appeared to be restricted to the endothelium, we named the protein the endothelial cell protein C receptor or EPCR.

Comparison of the predicted structure of this protein with known proteins revealed that EPCR was homologous to the CD1/MHC class 1 family of molecules, all of which are involved in inflammation.[12-16] This family of proteins normally has three extracellular domains with the one closest to the transmembrane region corresponding to the immunoglobulin-like domain that is involved in β_2-microglobulin binding. EPCR does not contain this immunoglobulin-like domain but has approximately 30% identity with these proteins in the remainder of the molecule. The gene organization of EPCR further supports this evolutionary relationship (Fukudome and Esmon, unpublished). A schematic of EPCR in relation to thrombomodulin, protein C, and protein S is shown in Figure 5.

In addition to homology with the CD1/MHC class 1 family, EPCR exhibited homology with CCD41 or centrocyclin, a murine protein that had originally been identified as a centrosome associated, cell cycle specific protein.[17] To understand the relationship between EPCR and CCD41 better, we cloned the murine form of EPCR and compared this sequence to that of CCD41. Expression of the murine protein in mammalian cells allowed the cells to bind APC. Like its human counterpart, EPCR was down regulated by TNF. Sequence comparison of CCD41 and EPCR suggested that the differences in sequence arose from a few cloning/sequencing errors centered for the most part in regions of the sequence that were difficult to read. We therefore concluded tentatively that murine EPCR and CCD41 were identical. This leads to the following possibility. Since CCD41 is reported to be an intracellular protein, the molecule could elicit protein C binding indirectly by inducing or stabilizing some constitutive cellular protein or facilitating this protein's expression on the cell surface. Two classes of experiments would seem to eliminate this possibility. First, expression of EPCR, truncated at the putative transmembrane domain, leads to the formation of a soluble form of the receptor that can be isolated from conditioned medium.[18] Without truncation of this domain, the receptor remains cell surface associated. The soluble receptor can block APC binding to cells stably transfected with EPCR and the soluble form of EPCR can bind to immobilized protein C. Taken together, these data indicate that EPCR is a transmembrane protein that binds directly to protein C.

The major goal of this work is to identify the function of EPCR and its potential role in the *unexplained* functions of APC. Since the receptor binds both APC

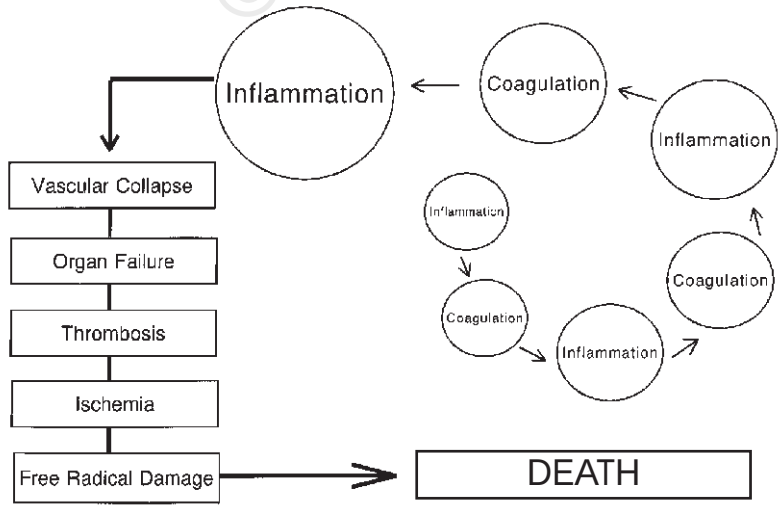

Figure 4. Progression of the inflammation-coagulation autoamplification loop.

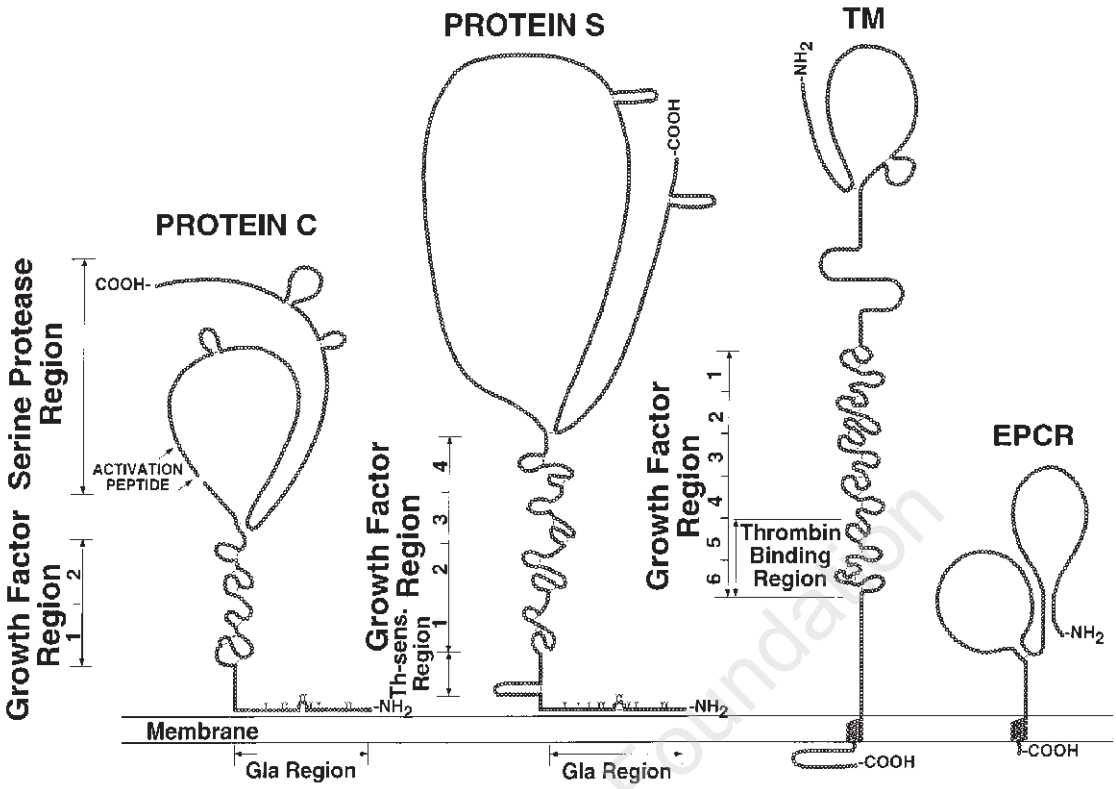

Figure 5. Schematic representation of the proteins of the protein C pathway and their interaction with the membrane surface. Protein C and protein S are vitamin K dependent proteins requiring Ca^{2+} for their interaction with membranes. Thrombomodulin and EPCR are integral membrane proteins. EPCR has an extremely short cytosolic tail (3 residues) making it unlikely to be able to signal directly.

and protein C, it could function by a variety of mechanisms including protein C activation, alteration of APC anticoagulant function, modulation of APC inhibition by plasma proteinase inhibitors or facilitating cleavage of a novel substrate. These possibilities are reminiscent of thrombomodulin, which, when it binds to thrombin, blocks the procoagulant functions of the enzyme, while enhancing protein C activation. Unlike the situation with thrombomodulin, in which the substrate for the receptor enzyme complex was hypothesized as part of the receptor identification, the substrate for the EPCR-APC complex remains unknown. As an initial attempt to compare the properties of these two complexes, we examined the influence of soluble EPCR on APC inactivation of factor Va, protein C activation by the thrombin-thrombomodulin complex, and the influence on APC inactivation by α_1-antitrypsin or protein C inhibitor. We found that EPCR blocked factor Va inactivation, did not influence protein C activation, did not have an impact on APC inactivation, and had little effect on chromogenic substrate activity (Table 1). These types of changes are similar to those exhibited by the thrombin-thrombomodulin complex (Table 1).

Table 1.

The thrombin-thrombomodulin complex	*The PC/APC-EPCR complex*
' Inactivation by antithrombin enhanced or unaffected*	' Inhibition unaffected
' Chromogenic substrate activity altered very little	' Chromogenic substrate activity altered very little
' Activation of factor V blocked	' Factor Va inactivation blocked
' Fibrinogen clotting blocked	' Protein C activation unaffected (soluble)
' Protein C activation enhanced	' Endothelial cell protein C activation enhanced[19]

**Acceleration requires chondroitin sulfate.*

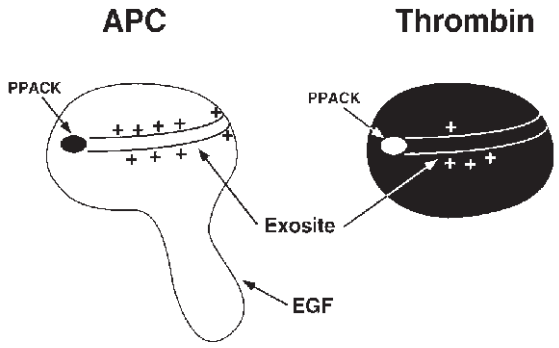

Figure 6. Schematic comparisons of thrombin with APC. In these models, the location of the active site inhibitor, PPACK, is indicated. The groove corresponding to anion binding exosite 1 in thrombin is indicated by the solid lines. The putative corresponding domain is indicated by the solid lines in APC. The basic residues are more plentiful in APC than in thrombin but those on the lower rim are somewhat more distant from the groove. In both cases the groove is oriented toward the Ca^{2+} binding site which would be to the far right in this picture. These schematics are rotated up and to the left of the *standard* orientation. This allows the EGF domains to face downward. The Gla domain was not present in the APC molecule that was crystallized. The two EGF domains extend from the protease domain with a slight bend.

The properties exhibited by the two complexes are similar, but considerable work has been done on the mechanisms by which thrombomodulin modulates thrombin function and the binding sites on thrombin. In the case of thrombin, thrombomodulin binds in the anion binding exosite 1 in a site that overlaps the sites of fibrinogen and platelet thrombin receptor interaction (probably to identical residues).[20] This probably accounts for the ability to block these two procoagulant reactions, reviewed in ref. #11. Occupancy of this site in thrombin is also known to modulate thrombin specificity. Therefore, this site represents a candidate site for allosteric regulation of serine proteases that share this structure. In addition, this long groove terminates near the Ca^{2+} binding region in many serine proteases (but absent from thrombin).[21] Binding Ca^{2+} to serine proteases is known to alter their activity.[22] Given the similarities between these two enzyme-receptor properties and the fact that thrombomodulin utilizes anion binding exosite 1 in thrombin, we wished to determine whether APC might have a similar site. Fortunately, the crystal structure of APC has been recently determined.[23] Like thrombin, APC has an extended groove that extends to the carboxyl (P') side of the substrate binding site. This groove has basic residues on one lip of the groove, hydrophobic residues in its base, and aromatic residues on the opposite face. Like anion binding exosite 1 in thrombin, the groove extends toward the Ca^{2+} binding site. No direct evidence for EPCR binding to this groove is yet available, but factor Va and EPCR appear to cross compete for binding to APC since the concentration of EPCR required to inhibit factor Va inactivation is much higher than that predicted based on competition binding analysis in the absence of factor Va. Our current working model is that factor Va and EPCR share overlapping binding sites on APC and that one of these binding sites is the exosite in APC. If it is correct that EPCR binds to this exosite in APC, then it is likely that occupancy of this site will modulate the residues near the catalytic center to alter the substrate specificity of APC.

Future directions currently being pursued to identify the mechanisms by which APC modulates the inflammatory response include inhibition of EPCR function *in vivo*, identification of the preferred substrates for EPCR-APC complexes, and the identification of additional receptors that may be able to bind to components of the protein C pathway and modulate their function. Most members of the CD1/MHC class 1 family of proteins form heterodimers that are important for their function. We have yet to identify an EPCR binding protein, but immunoprecipitation data suggest that this protein may interact with other cell surface proteins that may further modulate function or allow cellular signalling. It is important to realize that the protein C pathway is one of the last to be described and the complexity of its regulation[24] is matched only by the complexity of the regulation of the inflammatory response. Understanding how these are coupled provides an interesting challenge for future investigation.

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CTE is an investigator at the Howard Hughes Medical Institute and was primarily responsible for the conception of this article and writing of the manuscript. The remaining authors participated in the research discussed herein.

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Disclosures

Conflict of interest: none

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