Inflammation, sepsis, and coagulation

CHARLES T. ESMON,°,* KENJI FUKUDOME,° TIM MATHER,° WOLFRAM BODE,° LISA M. REGAN,* DEBORAH J. STEARNS-KUROSAWA,° SHINICHIRO KUROSAWA°

° Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation; † Departments of Pathology, Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, * Howard Hughes Medical Institute, Oklahoma City, Oklahoma, USA; and # Max Planck Institute für Biochemie, Martinsried, Germany

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 and 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.

© 1999, Ferrata Storti Foundation

Key words: inflammation, sepsis, coagulation

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 and 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.

© 1999, Ferrata Storti Foundation

Key words: inflammation, sepsis, coagulation

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. With the realization that thrombin infusion in vivo could activate protein C, 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. 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-
matory cytokine production in vitro as well and that protein C can block selectin-mediated neutrophil binding.

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. 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. Screening of several cell types revealed that APC bound well only to endothelial cells. The binding was moderately high affinity (Kd(app)=30-50 nM), Ca2+ 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 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 VIIa (VIIa) 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 VIIa. For simplicity, the activation of factors VII, V, and VIII are not shown.

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.
Inflammation, sepsis, and coagulation

Inflammation

- Inhibits anticoagulants
- Stimulates coagulants
- Inhibits fibrinolysis

- Thrombomodulin
- a2-AT
- Heparin
- Tissue factor
- Membrane coagulants
- PAI-1

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 a2-AT is a2 antitrypsin.

In addition to homology with the CD1/MHC class I family, EPCR exhibited homology with CCD41 or centrocyclin, a murine protein that had originally been identified as a centrosome associated, cell cycle specific protein. 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 cell 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. 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
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 α1-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.

<table>
<thead>
<tr>
<th>The thrombin-thrombomodulin complex</th>
<th>The PC/APC-EPCR complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Inactivation by antithrombin enhanced or unaffected*</td>
<td>• Inhibition unaffected</td>
</tr>
<tr>
<td>• Chromogenic substrate activity altered very little</td>
<td>• Chromogenic substrate activity altered very little</td>
</tr>
<tr>
<td>• Activation of factor V blocked</td>
<td>• Factor Va inactivation blocked</td>
</tr>
<tr>
<td>• Fibrinogen clotting blocked</td>
<td>• Protein C activation unaffected (soluble)</td>
</tr>
<tr>
<td>• Protein C activation enhanced</td>
<td>• Endothelial cell protein C activation enhanced¹⁹</td>
</tr>
</tbody>
</table>

*Acceleration requires chondroitin sulfate.
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 thrombin is indicated by the solid lines and 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 Ca²⁺ binding site which would be at 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 thrombin is indicated by the solid lines and 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 Ca²⁺ binding site which would be at 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.

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 Ca²⁺ binding site which would be at 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.

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 I 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 is matched only by the complexity of the regulation of the inflammatory response. Understanding these are coupled provides an interesting challenge for future investigation.

Contributions and Acknowledgments

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.

Funding

The research discussed herein was funded by grants awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health (Nos. R37 HL 30340 and P01 HL54804).

Disclosures

Conflict of interest: none
Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 6, 1998; accepted December 28, 1998.

References

259


13. Fukudome K, Esmon CT. Molecule cloning and expression of murine and bovine endothelial cell protein C-activated protein C receptor (EPCR). The struc-


