Regulation of Blood Coagulation by the Protein C Anticoagulant Pathway

Novel Insights Into Structure–Function Relationships and Molecular Recognition

Björn Dahlbäck, Bruno O. Villoutreix

Abstract—The protein C system provides important control of blood coagulation by regulating the activities of factor VIIIa (FVIIIa) and factor Va (FVa), cofactors in the activation of factor X and prothrombin, respectively. The system comprises membrane-bound and circulating proteins that assemble into multi-molecular complexes on cell surfaces. Vitamin K–dependent protein C, the key component of the system, circulates in blood as zymogen to an anticoagulant serine protease. It is efficiently activated on the surface of endothelial cells by thrombin bound to the membrane protein thrombomodulin. The endothelial protein C receptor (EPCR) further stimulates the protein C activation. Activated protein C (APC) together with its cofactor protein S inhibits coagulation by degrading FVIIIa and FVa on the surface of negatively charged phospholipid membranes. Efficient FVIIIa degradation by APC requires not only protein S but also intact FV, which like thrombin is a Janus-faced protein with both procoagulant and anticoagulant potential. In addition to its anticoagulant properties, APC has antiinflammatory and antiapoptotic functions, which are exerted when APC binds to EPCR and proteolytically cleaves protease-activated receptor 1 (PAR-1). The protein C system is physiologically important, and genetic defects affecting the system are the most common risk factors of venous thrombosis. The proteins of the protein C system are composed of multiple domains and the 3-dimensional structures of several of the proteins are known. The molecular recognition of the protein C system is progressively being unraveled, giving us new insights into this fascinating and intricate molecular scenario at the atomic level. (Arterioscler Thromb Vasc Biol. 2005;25:1-10.)

Key Words: factor V ■ protein C ■ protein S ■ thrombomodulin

Platelet-dependent primary hemostasis and blood coagulation have evolved as important defense mechanisms against bleeding. The initial occlusion of a vascular lesion is provided by the formation of the platelet plug. This is temporally coordinated with the initiation of the coagulation system by the exposure of tissue factor (TF) to blood and the subsequent binding to TF of circulating factor VIIa (FVIIa). The FVIIa-TF complex efficiently converts factor IX (FIX) and factor X (FX) to active enzymes FIXa and FXa, which together with factor VIIIa (FVIIIa) and factor Va (FVa), respectively, propagate the coagulation process (Figure 1). Factor VIII (FVIII) and factor V (FV) circulate in blood as high-molecular-weight inactive procofactors, which are converted into their active forms, FVIIIa and FVa, early during the coagulation process by thrombin or FXa. FVIIIa and FVa bind to negatively charged phospholipid membranes, eg, on activated platelets, and form complexes with FIXa and FXa, respectively. The FIXa–FVIIIa complex (tenase) activates FX, whereas the FXa–FVa complex (prothrombinase) converts prothrombin to thrombin. In both the tenase and the prothrombinase complexes, the cofactors increase the catalytic efficiency of the respective enzyme by several orders of magnitude. The coagulation process generates large quantities of thrombin, which has multiple procoagulant functions, eg, it activates FV and FVIII and platelets, it converts fibrinogen to a fibrin clot, and it activates FXIII to an active transglutaminase that cross-links fibrin. The reactions of blood coagulation are carefully controlled by several anticoagulant mechanisms, which under normal conditions prevail over the procoagulant forces. Vitamin K–dependent protein C is the key component of an important natural anticoagulant pathway. The protein C system exerts its anticoagulant effect by regulating the activities of FVIIIa and FVa, the cofactors in the tenase and prothrombinase complexes, respectively (Figure 1). The inhibition of FVIIIa and FVa mediated by the protein C system provides a highly efficient and specific regulation of blood coagulation.

The physiological importance of the anticoagulant protein C...
system is most clearly demonstrated by the severe thrombotic disease, purpura fulminans, which affects neonates with homozygous protein C deficiency. In recent years, protein C has been shown not only to be anticoagulant but also to have antiinflammatory and antiapoptotic properties. The unique combination of anticoagulant, antiinflammatory, and antiapoptotic properties of APC has made it an attractive candidate as a therapeutic agent and administration of APC has proven beneficial in the handling of patients with severe sepsis. The protein C system has been intensively investigated and insights into the 3-dimensional (3D) structure of the proteins and the intricate relationships between the structures and their functions have been gained. These new insights are the focus of this review.

Components of the Protein C Pathway
The protein C pathway comprises multiple proteins involved at different points of the pathway, eg, those affecting the protein C activation, those modulating the proteolytic activity of APC, and those that inhibit the activity of APC (Table 1 and Figure 1). Thus, the activation of protein C is efficiently catalyzed on the endothelial cell surface by thrombin (T) bound to thrombomodulin (TM). TM functions as a cofactor to thrombin, the high affinity binding of thrombin to TM resulting in >1000-fold amplification of the rate of protein C activation. The endothelial protein C receptor (EPCR), which binds protein C, provides a further 20-fold stimulation of the T-TM–mediated activation of protein C in vivo. The anticoagulant activity of APC is enhanced by 2 cofactors, the vitamin K–dependent protein S and the intact form of FV, protein S being sufficient for inactivation of FVa, whereas regulation of FVIIIa in the tenase complex requires the synergistic APC cofactor activities of both protein S and FV (Figure 1). Protein S in human plasma is not only an important component of the protein C pathway but also takes part in the regulation of the complement system as it forms a high-affinity complex with C4b-binding protein (C4BP), a regulator of the classical complement pathway. In human plasma, 30% to 40% of the protein S circulates as free protein, the remaining being bound to C4BP. Only free protein S has the ability to function as a cofactor to APC. Although protease inhibitors such as the protein C inhibitor, α1-antitrypsin, and α2-macroglobulin inhibit APC, the half-life of APC in the circulation is relatively long (~20 minutes).

Activation of Protein C on the Surface of Endothelial Cells
Protein C is composed of a γ-carboxyglutamic acid residue (Gla)-rich domain, 2 epidermal growth factor (EGF)-like domains, a short activation peptide, and the serine protease domain (SP) (Figure 1). The Gla domain of protein C (Figures 1 and 2), SP, serine–threonin-rich domain of TM, which carries a chondroitin sulfate side chain; SP, serine protease domain; Gla, vitamin K–dependent γ-carboxyglutamic acid–rich domain, which binds calcium and negatively charged phospholipid membranes; A, B, and C domains of FV/FVIII attain similar molecular arrangement. During activation of FV and FVIII, the B domains are cleaved off and dissociate from the active molecules.
Characteristics of Components of the Protein C Anticoagulant System

<table>
<thead>
<tr>
<th>Protein (Amino Acids)</th>
<th>Molecular Weight (kDa)</th>
<th>Subunits (Amino Acids)</th>
<th>Domains</th>
<th>Special Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein C (417)</td>
<td>62</td>
<td>Light chain (155), heavy chain (262)</td>
<td>Gla, 2 EGFs AP and SP</td>
<td>Zymogen to an anticoagulant, antinflammatory, and antiapoptotic protease, activated protein C, which cleaves FV/FVa, FVIIIa, and PAR-1</td>
</tr>
<tr>
<td>TM (554)</td>
<td>75</td>
<td>Single chain</td>
<td>Lectin, hydrophobic, 6 EGFs, S/T-rich, transmembrane, cytoplasmic tail</td>
<td>Transmembrane protein in all endothelium; cofactor to thrombin in activation of protein C and TAFI; antithrombotic</td>
</tr>
<tr>
<td>EPCR (221)</td>
<td>50</td>
<td>Single chain</td>
<td>MHC/CD1</td>
<td>Membrane protein; binds Gla domain of protein C, stimulates protein C activation by T-TM and PAR-1 cleavage by APC</td>
</tr>
<tr>
<td>Factor V (2196)</td>
<td>330</td>
<td>Single chain before activation</td>
<td>A1,A2,B,A3,C1,C3</td>
<td>Precursor to procoagulant FVa and to anticoagulant APC cofactor in FVIIa degradation</td>
</tr>
<tr>
<td>Factor VIII (2322)</td>
<td>330</td>
<td>Heavy chain (1313), light chain (684)</td>
<td>A1,A2,B,A3,C1,C3</td>
<td>Precursor to procoagulant FVIIa, cofactor in the tenase complex</td>
</tr>
<tr>
<td>Protein S (635)</td>
<td>75</td>
<td>Single chain</td>
<td>Gla, TSR, 4 EGFs, SHBG (2 LamG domains)</td>
<td>Cofactor to APC; binds C4BP binds to apoptotic cells, stimulates phagocytosis</td>
</tr>
<tr>
<td>C4BP (4078)</td>
<td>570</td>
<td>7 α-chains (549) 1 β-chain (235)</td>
<td>8 CCPs 3 CCPs</td>
<td>α-chains, cofactors to factor I, an enzyme regulating the complement system; β-chain, binds to protein S</td>
</tr>
<tr>
<td>PCI (387)</td>
<td>60</td>
<td>Single chain</td>
<td>Serpin</td>
<td>Inhibitor of APC and of other proteases; stimulated by heparin</td>
</tr>
<tr>
<td>α-1 AT (394)</td>
<td>60</td>
<td>Single chain</td>
<td>Serpin</td>
<td>Inhibitor of APC and of other proteases</td>
</tr>
</tbody>
</table>

The total numbers of amino acids in the mature proteins is given in parenthesis. The molecular weights are approximate and include post-translational modifications such as carbohydrate side chains. FVIII is synthesized as single chain but processed to 2 chains with heterogeneity of heavy chains. The number of amino acids refers to the longest heavy chain of FVIII.

Abbreviations are explained in the text. References to different proteins and their properties are given in the text; some of the information is derived from protein databases available on the Internet.

All vascular endothelium contain TM, the concentration being particularly high in the capillaries where the ratio between the endothelial cell surface and blood volume reaches its peak. The high concentration of TM in the capillary circulation ensures that thrombin binds to TM (Kd = 0.5 nmol) and activates protein C.7,19,20 The procoagulant properties of thrombin are lost on binding to TM because TM occupies the functionally important exosite I in thrombin and thereby blocks interactions with other thrombin-binding proteins. TM is a type I membrane protein containing several domains: an N-terminal type C lectin domain followed by 6 EGF-like domains, a Ser/Thr-rich region containing a chondroitin sulfate side chain, a transmembrane section, and a short cytoplasmic tail (Figure 1).7,19,20 The EGF domains play a crucial role in the activation of protein C, thrombin binding to EGF5 and EGF6, whereas EGF4 interacts with a positively charged cluster formed by basic residues located in loops 37, 60, 70, and 148 (minor role) in the SP domain of protein C (Figure 2).7,19,21–25 EPCR augments the activation of protein C by binding the Gla domain of protein C, thereby aligning the substrate protein C with the activating T-TM complex.7,14 EPCR is a type I membrane protein and a member of the MHC class 1/CD1 family. Determination of the 3D structure revealed 2 α-helices and an 8-stranded β-sheet creating a phospholipid-binding groove, with the phospholipid binding being important for the ability of EPCR to bind the Gla domain of protein C (Figure 2).26 Thrombin bound to TM is efficiently inhibited by antithrombin (AT) and protein C inhibitor, the chondroitin sulfate side chain stimulating the inhibition.7 Thus, TM has multiple important anticoagulant properties: converting thrombin into an activator of protein C and also accelerating the inhibition of thrombin.

The T-TM complex not only activates protein C but also can activate thrombin-activatable fibrinolysis inhibitor (TAFI), a fibrinolysis inhibitor present in plasma. TAFI circulates as a proenzyme, which after its activation by the T-TM complex functions as a carboxypeptidase B removing C-terminal lysine residues from fibrin.19,27 This results in inhibition of fibrinolysis because these lysines constitute a binding site for plasminogen and the tissue plasminogen activator-mediated activation is stimulated by these lysine residues. Other substrates for TAFI are the pro-inflammatory anaphylatoxins C3a and C5a, which are inhibited by the removal of their C-terminal arginine residues. The structural requirements of the T-TM complex for efficient activation of TAFI differ slightly from those of the protein C activation. Thus, EGF3 of TM is important for TAFI activation, whereas the protein C activation depends on protein C interaction with EGF4. The full physiological significance of the T-TM-mediated activation of TAFI and the resulting inhibition of plasminogen activation and inhibition of anaphylatoxin functions remain to be elucidated.

Degradation of FVa and FVIIIa by APC and the Roles of Protein S and FV

APC is a highly specialized enzyme cleaving only a few peptide bonds in the membrane-bound FV/FVa and FVIIIa.
FV and FVIII are homologous molecules sharing the domain structure A1-A2-B-A3-C1-C2, with the 3 A domains being arranged in a triangular fashion.4,5 The carbohydrate-rich B domains of both FV and FVIII are released as a result of proteolysis mediated by thrombin or FXa and the active cofactors FVa and FVIIIa are composed of the A1-A2-A3-C1-C2 domains. The A domains in FV and FVIII are homologous to A domains in ceruloplasmin, which has allowed the creation of molecular models of the A domains of FVa and FVIIIa.28–33 In addition, experimentally determined 3D structures are available for APC-cleaved bovine FVa lacking the A2 domain and for the C2 domains of both FVa and FVIIIa.34–36 It is noteworthy that the homology-based models are in excellent agreement with the experimentally determined 3D structures (Figure 3). For instance, we proposed a list of residues involved in calcium binding,29 confirmed their role by mutagenesis,37 and noticed that the equivalent residues were coordinating calcium in the x-ray structure of APC-cleaved bovine FVa.36

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The structure of the C1 domain in FVIII has been predicted by homology and a model of phospholipid-bound FVIIIa has been created.30 The C2 domains of both FVIIIa and FVa contain major phospholipid-binding sites, the locations of which have been pin-pointed by mutagenesis studies or crystallography.38–40 Mutagenesis studies also indicated a region of the C1 domain of FVa as important for binding to phospholipids.41 The A2 and A3 domains of FVIIIa and FVa are important for the high affinity binding to FIXa and FXa, respectively, and details of the binding sites on FVa/FVIIIa and FIXa and FXa are beginning to emerge.42–45 It is interesting to note that the orientation of the domains in the light chain differs when comparing the FVIIIa model30 and the x-ray structure of APC-cleaved bovine FV.36 Further investigations are needed to understand better how the 2 molecules could differ in this region.

Three APC cleavage sites have been identified in FVa, at positions Arg306, Arg506, and Arg679.4,5 The Arg306 and Arg506 cleavages have been studied in detail and demonstrated interesting differences with regard to kinetics, requirement of APC cofactors, and remaining activity of the cleaved
FVa. The Arg506 cleavage is kinetically favored over the Arg306 cleavage, is less dependent on the presence of protein S and phospholipid composition, is inhibited by FXa bound to FVa, and only results in partial loss of FXa cofactor activity.\textsuperscript{5,46–49} The Arg306 cleavage, however, is slow, fully dependent on negatively charged phospholipid, not inhibited by FXa bound to FVa, strongly stimulated by protein S, and results in severe loss of FVa activity. Complete loss of FVa activity is caused by dissociation of the A2 fragments after cleavage at Arg306.\textsuperscript{50} The molecular explanation for the described differences in the APC-mediated cleavages at Arg306 and Arg506 is found in the differential involvement of an exosite in the SP domain of APC in the 2 cleavages (Figure 3A).\textsuperscript{51–55} During the cleavage at the Arg506 site, the positively charged cluster in the SP domain of protein C formed by basic residues in loops 60, 37, 70, and 148 (Figure 3B) interacts with a poorly defined negatively charged region in FVa located adjacent to the Arg506 site. As discussed, the same cluster in protein C is important for the activation of protein C by the T-TM complex. Elimination of this positive cluster by mutagenesis affects both the activation of the T-TM complex and the Arg506 cleavage, but has no effect on the cleavage at Arg306. This cluster is also able to bind positively charged heparin, which at high concentrations inhibits the APC-mediated cleavage at Arg506 but not at Arg306.\textsuperscript{54,55}

The regulation of FVIIIa in the tenase complex by APC is more complex than that of FVa and not only protein S but also the nonactivated form of FV serve as cofactors to APC.\textsuperscript{5,56} The explanation for the requirement of 2 APC cofactors may be related to the much lower in vivo concentration of FVIII compared with FV and the complicated task of regulating the highly efficient tenase complex in the presence of a molar surplus of the competing APC substrate FVa.\textsuperscript{5,45,57} In vitro experiments, the FVIIIa in the tenase complex is resistant against APC unless the appropriate APC cofactors are present. The optimal combination of cofactors comprises protein S and the intact FV molecule, with the 2 proteins serving as synergistic APC cofactors.\textsuperscript{58} FVIIIa is cleaved at Arg336 and Arg562 by APC.\textsuperscript{5,55} The molecular events involved in the regulation of the tenase complex by APC-protein S-FV are only partly understood. Presumably, APC and its cofactors interact with each other as well as with the tenase complex on the phospholipid membrane (Figure 1). During the inactivation of FVIIIa, the FV molecule is cleaved by APC at Arg306 and Arg506, with the cleavage at Arg506 being important for the ability of FV to serve as APC cofactor in the reaction.\textsuperscript{5,56,59} In addition, the last portion of the B domain of FV and an intact junction between the B domain and the A3 domain are important for the APC cofactor activity of FV.\textsuperscript{60} The anticoagulant APC cofactor activity of FV is lost on full activation of FV by thrombin when the B–A3 junction is disrupted by the thrombin-mediated cleavage at Arg1545.\textsuperscript{60} Thus, FV is a Janus-faced protein, with the ability to express both procoagulant and anticoagulant functions depending on proteolysis by either procoagulant or anticoagulant enzymes such as thrombin/FXa or APC, respectively.\textsuperscript{5}
The active site in relation to the cleavage sites in FVa and FVIIIa. The 2 LamG-domains, and in particular LamG2, are demonstrated to be important for the synergistic APC cofactor activity of protein S with FV in the degradation of FVIIIa.15,16,62

The interaction between protein S and C4BP is of high affinity in the presence of calcium.15–17 The binding site for C4BP is fully contained in the 2 LamG domains and multiple regions have been suggested to contribute to the binding of C4BP. Molecular models for the LamG domains of protein S have been created based on homology with LamG domains of laminin and Gas6 (the product of growth arrest–specific gene 6), a protein that is structurally closely related to protein S (Figure 4).63,64 C4BP has a unique molecular architecture comprising 7 identical α-chains and a single β-chain forming an octopus-like structure (Figure 4).17 The chains contain multiple complement control protein (CCP) domains arranged in tandem, with the α-chain having 8 CCPs and the β-chain having 3. Each α-chain can bind a C4b (activated complement protein C4) molecule and convert it into a substrate for factor I, a complement regulatory enzyme in blood. Thus, C4BP is an important regulator of the classical complement pathway. The β-chain CCP1 binds protein S, with a hydrophobic patch in CCP1 involving I16, V18, V31, and I33 being particularly important (Figure 4).17,65

Recently it was found that protein S binds to the negatively charged phospholipid surface that is exposed on apoptotic cells and can mediate phagocytosis of the apoptotic cell.66–67 In contrast, binding of the protein S-C4BP complex was found to inhibit the phagocytic process.68 The localization of protein S and protein S-C4BP complexes to apoptotic cell surfaces may be instrumental in local downregulation of coagulation as well as complement. This observation might account for the observed lack of coagulation activation and inflammation in the vicinity of apoptotic cells.

The Protein C System and Venous Thromboembolic Disease

The protein C system is vitally important to keep the blood in a fluid state and the circulatory system open. This is most clearly illustrated by the severe microvascular thrombotic disease that already in the neonatal period affects individuals with complete inherited protein C deficiency. The prevalence of defective protein C alleles in the population is ~1/600 and consequently complete deficiency occurs in ~1/200 000 to 1/300 000 newborn and heterozygous deficiencies in 1/300.69 Heterozygous deficiency is associated with ~5-fold increased risk of venous thrombosis because of the imbalance between procoagulant and anticoagulant pathways. Heterozygous protein S deficiency is affected by similar thrombosis risk as protein C deficiency, and together these defects account for, at most, 5% to 10% of patients with venous thrombosis. The FV Leiden mutation (APC resistance) is the most common gene defect associated with venous thrombosis, and is found in 20% to 40% of patients with thrombosis. The FV Leiden mutation (G1691A) replaces Arg506 with a Gln. Mutant FV has full procoagulant capacity, but the protein C anticoagulant system is affected in 2 ways by the mutation. The first is impaired degradation of mutant FVa by APC because the mutation eliminates 1 of 3 APC cleavage sites in FVa. The second is impaired degradation of FVIIIa because mutant FV cannot be cleaved at Arg506 and is therefore a poor cofactor to APC in the degradation of FVIIIa.5

FV Leiden is the result of a founder effect, with the mutation being ~30 000 years old.72 The mutation is predominantly found in whites and is absent or very rare in Asians, Australian Aboriginals, and black Africans. The population prevalence of FV Leiden varies geographically. With few exceptions, European populations exhibit a north-to-south gradient with highest prevalence (10% to 15%) of FV Leiden in the north and lowest in the south (~2%). In America, where the population is of mixed ethnic background the prevalence is ~5% in the north and somewhat lower in the south.69,71 Heterozygous individuals have ~5-fold increased risk of venous thrombosis, whereas homozygotes have ~50-fold increased risk. The mutation is not a risk factor for arterial thrombosis. The FV Leiden allele appears to have provided a survival advantage during evolution, explaining its high prevalence in certain populations. Thus, women with FV Leiden have reduced bleeding tendency after delivery, which in the history of mankind must have been a major survival benefit.76 In addition, heterozygous FV Leiden is found to be a survival factor in sepsis in humans and mice.73–75

A single point mutation (G20210A) in the 3′ untranslated region of the prothrombin gene is the second most common genetic risk factor for thrombosis found in 6% to 8% of patients with thrombosis and in ~2% of healthy individuals. The prothrombin function is unaffected by the mutation but the levels of prothrombin in plasma are slightly increased, which may be the basis for the increased risk. Heterozygous deficiency of AT is another rare cause of venous thrombosis, found in 1% to 2% of thrombosis patients and in 1/2000 of the general population.76

Antiinflammatory and Antiapoptotic Effects of the Protein C Pathway

Several of the components of the protein C system exert other biological effects than those strictly referred to as being anticoagulant. For example, protein C and APC have been found to directly inhibit the adhesion of neutrophils to the endothelial cell surface and the transmigration of neutrophils.76 The lectin domain of TM has direct antiinflammatory properties, downregulating NF-κB and the MAP kinase pathway.20,77 Studies of mice having a selective deficiency of the TM-lectin domain demonstrated that this domain decreases leukocyte adhesion and extravasation.77 As mentioned, protein S and the protein S–C4BP complex have antiinflammatory properties.15–17 The antiinflammatory effects of free protein S are related to the binding of protein S to negatively charged phospholipid exposed on the surface of apoptotic cells and the associated protein S-mediated stimulation of the phagocytic process. C4BP is a potent regulator of the complement system and it is thought that localization of the protein S–C4BP complex to negatively charged phospholipid membranes, eg, on apoptotic cells, yields local antiinflammatory effects.
The antiapoptotic effects were dependent on the presence of PAR-1. PAR-1 spans the membrane 7 times and the cytoplasmatic portion of PAR-1 interacts with G proteins. The APC-mediated cleavage of PAR-1 creates a novel N-terminus in PAR-1, which folds back and activates the PAR-1. This results in the activation of the intracellular G proteins generating the antiinflammatory and antiapoptotic responses of APC (see text for references).

APC also has direct antiinflammatory and antiapoptotic properties in vivo and in vitro on many cell types. Many of these effects depend on the simultaneous presence of EPCR and protease activated receptor 1 (PAR-1) in the membrane and the APC-mediated proteolytic cleavage of PAR-1 (Figure 5). PAR-1 is a 7-transmembrane domain, G protein-coupled receptor that is primarily cleaved by thrombin. The novel N-terminus of the receptor exposed after proteolysis activates the receptor, thus triggering intracellular signaling events. Most of the antiapoptotic effects of APC have been demonstrated using primary endothelial cells or endothelial-like cell lines. In cultured human umbilical vein endothelial cells, APC was found to affect gene expression by blocking downstream NF-kB-regulated genes. After treatment of human umbilical vein endothelial cells with APC, the gene expression profile switched toward an antiinflammatory and antiapoptotic direction, eg, the apoptosis-associated genes were suppressed, whereas genes known to downregulate proinflammatory signaling pathways and antiapoptotic mRNA transcripts were upregulated. In the EAhy926 cell line, human APC was shown to inhibit staurosporine-induced apoptosis. The antiapoptotic effects were dependent on the presence of PAR-1 and EPCR and required several hours of pre-incubation of the cells with APC before the addition of staurosporine. These antiapoptotic effects were independent of the basic cluster of the serine protease domain of APC, because 37-loop and 70-loop mutated APC variants expressed full antiapoptotic activity, even though they had compromised anticoagulant activity. It remains to be elucidated whether such APC variants have therapeutic value in the treatment of sepsis or stroke.

APC was also found to alter cytosolic calcium flux in endothelium from human brain or umbilical veins in an EPCR-dependent and PAR-1–dependent manner. Moreover, APC inhibited apoptosis in hypoxic human brain endothelium through transcriptionally dependent inhibition of the tumor suppressor p53, normalization of the Bax/Bcl-2 ratio, and reduction of caspase 3 activation. These effects of APC were dependent on EPCR and PAR-1. Moreover, APC has been found to be neuroprotective, both in a stroke model in mice and in cultured cortical neurons, with the effects being both EPCR-dependent and PAR-1–dependent. It remains to be determined whether the beneficial effects in vivo are caused by direct cytoprotective effects on the neurons or mediated through improved blood flow, antiinflammatory effects, or decreased apoptosis of endothelial cells. Interestingly, in the same mouse model of stroke, the administration of protein S was likewise found to provide neuroprotection, the mechanism of action for which is unknown.

There are many unresolved questions related to the APC-mediated effects on PAR-1 in vivo, eg, it is difficult to understand the relationships between the APC-mediated and thrombin-mediated cleavages of PAR-1 and their physiological roles. The recent demonstration of thrombin being several orders of magnitude ($10^5$) more potent than APC in cleaving PAR-1 will stimulate research aiming at elucidation of the physiological significance of the APC-mediated PAR-1 cleavage in vivo.

APC can also express antiinflammatory properties that are unrelated to EPCR binding and PAR-1 cleavage. Thus, APC inhibits the interferon-γ-induced, PMA-induced, and endotoxin-induced pathways of monocyte activation, resulting in decreased production of IL-1 and tumor necrosis factor-α, decreased cell surface exposure of TF, and selective prevention of downregulation of certain membrane receptors (CD11b, CD14, and CD18). Another potentially interesting observation is the APC-mediated stabilization of monocyte chemoattractant protein-1 mRNA, a chemokine that is controlled by the activation of NF-kB.

The Protein C System and Severe Sepsis

During sepsis, blood coagulation is activated by TF expressed on the endothelium and monocytes/macrophages in response to cytokines (eg, tumor necrosis factor, IL-1, and IL-6) that are produced after the septic challenge. Disseminated intravascular coagulation (DIC), microvascular thrombosis, circulatory collapse, organ failure, and shock may develop in severe cases. Protein C is consumed during development of severe septic shock and the decrease in the plasma level of protein C may contribute further to the development of microvascular thrombosis and DIC. Moreover, the cytokine response in sepsis results in decreased expression levels of TM and EPCR on endothelium and thus decreased activation of protein C. However, the protein C system can counteract the deleterious effects associated with sepsis. Thus, the DIC response that develops from infusion of a sublethal dose of Escherichia coli in baboons was found to be aggravated by blockage of the protein C activation or by direct inhibition of APC activity. Furthermore, inhibition of EPCR binding of protein C or APC was also found to exacerbate the septic response to E. coli infusion, the effects being related to inhibition of protein C activation and to inhibition of EPCR/PAR-1–dependent APC effects. Inhibition of protein S either by monoclonal antibodies or by infusion of C4BP, which binds free protein S, gave similar aggravating effects of sublethal E. coli infusion, as did inhibition of protein C. In contrast, the administration of APC in the baboon sepsis model resulted in prevention of shock and DIC, which
was suggested not only to depend on the anticoagulant activity of APC but in addition on antiinflammatory effects. These results suggested that APC administration might be beneficial for the treatment of sepsis in humans. In a large study (1690 patients) of severe sepsis (PROWESS), the administration of recombinant APC (drotrecogin α-Xigris) was found to result in a 19.4% reduction in the relative risk of death and an absolute reduction of 6.1% (30.8% mortality at 28 days in the placebo group versus 24.7% with APC). From these data, it can be concluded that the protein C system plays an important role in the defense against sepsis, the anticoagulant, antiinflammatory, and antiapoptotic properties of APC presumably all contributing to the beneficial effects.

**Conclusion and Perspectives**

The elucidation of the molecular mechanisms of blood coagulation and the intricate protein C system has provided insights into a fascinating molecular world and also knowledge of great clinical relevance. Significant insights have been gained into the structure-function relationships of large macromolecular complexes important for the activation of protein C, the regulation of tenase and prothrombinase complexes, and the cell surface interactions with EPCR/PAR-1 resulting in antiinflammatory and antiapoptotic effects. However, many unanswered questions remain and some may be particularly challenging, eg, the molecular interactions of the synergistic APC cofactor activity of FV and protein S in the regulation of FVIIIa in the tenase complex and the elucidation of the cell surface and intracellular events associated with the antiinflammatory and antiapoptotic functions of the protein C system. The coming years will no doubt bring further exciting novel insights into these mechanisms.

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