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Protein C anticoagulant activity in relation to anti-inflammatory and anti-apoptotic activities

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TABLE OF CONTENTS

1. ABSTRACT

The anticoagulant protein C system is a dual function cofactor-dependent system. On one hand, it is designed to regulate coagulation, maintain the fluidity of the vasculature and prevent thrombosis. On the other hand, the protein C pathway provides anti-inflammatory and cytoprotective activities. Protein C, a vitamin K-dependent serine protease zymogen that circulates in plasma, is converted by limited proteolysis to activated protein C (APC) by the thrombin-thrombomodulin-endothelial protein C receptor complex on endothelial surfaces. APC and the cofactors of the protein C pathway exert two major distinct types of activities, namely a well-studied anticoagulant activity and a more recently revealed cytoprotective activity due to direct effects on cells. Because of these pleiotropic properties, APC and the protein C pathway components have important roles in the body's host-defense system and provide opportunities for therapeutic treatment of complex and challenging medical disorders, including thrombosis, severe sepsis and stroke.

2. INTRODUCTION

The physiologic importance of the anticoagulant protein C system is most clearly demonstrated by the massive, usually lethal, thrombotic complications occurring in infants with homozygous protein C or protein S

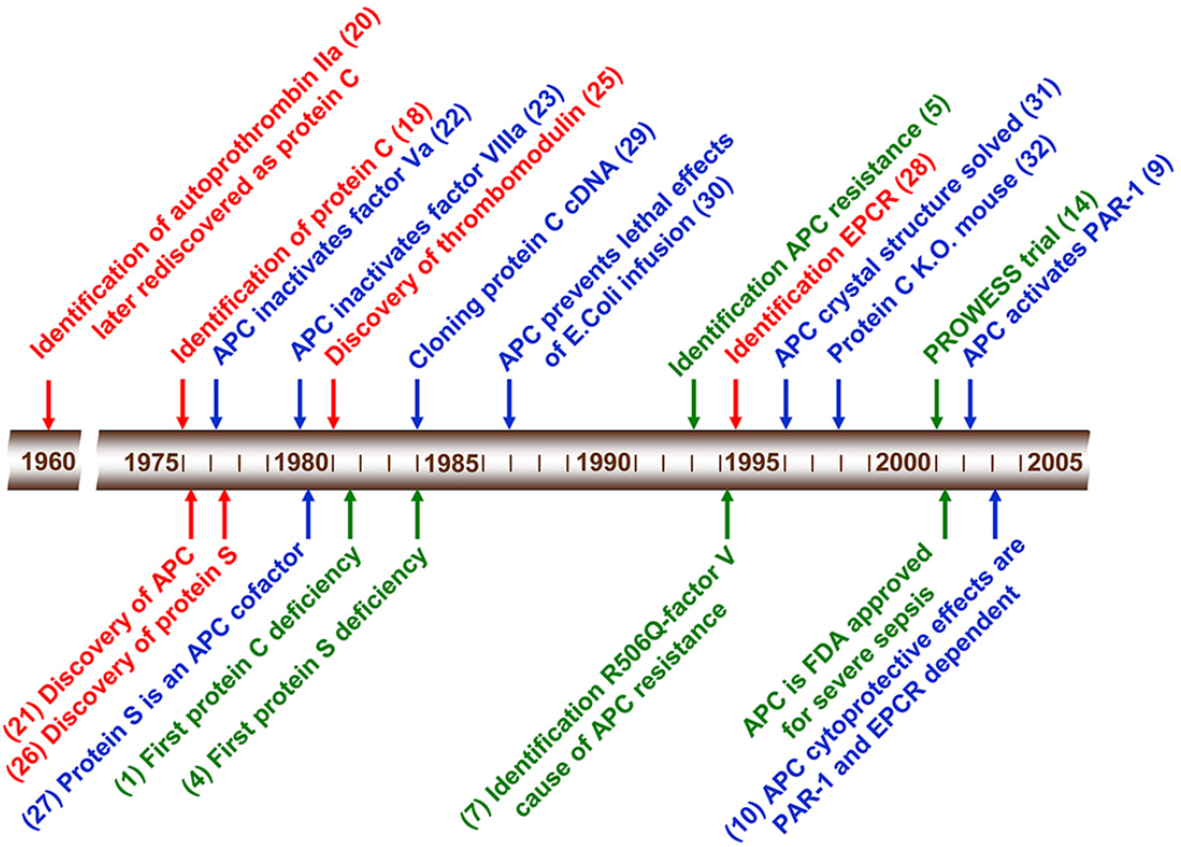

Figure 1. Historic timeline of selected historic events in the elucidation of the protein C pathway. Identifications of new proteins are indicated in red, major mechanistic advances are marked in blue, and discoveries with major clinical implications are in green. References describing the observation are given in parenthesis.

deficiency and the significantly increased risk for venous thrombosis in heterozygous deficient adults (1-4). The most commonly identifiable hereditary risk factor for venous thrombosis among Caucasians involves an APC-cleavage site (Arg506Gln, factor V Leiden) that is the major target for factor Va inactivation by APC (5-7).

In exerting its well-studied anticoagulant activity, APC targets factors Va and VIIIa, major procoagulant cofactors for thrombin generation. To provide direct cytoprotective effects on cells that include anti-inflammatory activity, anti-apoptotic activity, endothelial barrier protection and regulation of gene expression, APC targets two key receptors known as Protease Activated Receptor 1 (PAR-1) and Endothelial Protein C Receptor (EPCR) (8-13). The ability of APC, but not of other anticoagulants such as antithrombin and tissue factor pathway inhibitor, to reduce mortality in severe sepsis patients implicates the less well-defined cytoprotective actions of APC on cells as very important for APC's pharmacologic success (14-16).

Because of its multiple biologic activities, APC has the potential to play a major role in the pharmacologic treatment of complex and challenging medical disorders; for example, recent work suggests that ischemic stroke is a promising target for evaluation of APC therapy (17). This review summarizes current knowledge of the protein C pathway with particular emphasis on the recently elucidated, novel direct cellular effects due to APC and its cofactors, thrombomodulin, EPCR, protein S and factor V.

3. HISTORY OF THE PROTEIN C PATHWAY

Almost 30 years have passed since the identification of a new vitamin K-dependent protein from bovine plasma by Stenflo and colleagues (18,19). Although protein C was originally identified as Autoprothrombin-IIa, it was later rediscovered and named protein C, after its elution position in the third peak ("peak C") on an ion exchange chromatogram (Figure 1) (18,20). Protein C was then shown to be the zymogen of the anticoagulant serine protease, APC, with anticoagulant, anti-inflammatory, anti-apoptotic and cytoprotective activities (8,10,11,21-24). Three decades later, interest in protein C biochemistry and pathophysiology still flourishes due in large part to the growing complexity of the protein C pathway and the clinical highlights marking its history (Figure 1). The identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C, thrombomodulin (TM), provided a rational how a kinetically inefficient enzyme such as thrombin could be a physiologically significant activator of protein C (25). The discovery that protein C deficiency is a hereditary risk factor for venous thrombosis

and neonatal purpura fulminans underscores its physiological relevance (1,3). The identification of plasma protein S as a non-enzymatic cofactor for APC in the inactivation of factors Va and VIIIa and the discovery of congenital deficiencies in protein S linked to thrombosis confirmed and emphasized the clinical importance of the protein C pathway (4,26,27). The description of APC resistance as a risk factor for venous thrombosis and subsequent identification of the underlying defect as a factor V polymorphism (Arg506Gln-factor V, factor V Leiden) was a milestone in hereditary thrombophilia research (5,7). At about the same time, identification of the endothelial protein C receptor (EPCR) by Esmon sparked the efforts to elucidate the molecular basis of the anti-inflammatory effects of APC (28). The mysterious success of APC in reducing mortality in severe sepsis and the concomitant FDA approval of recombinant APC (Xigris^{TM}) for treatment of severe sepsis in adults further stimulated studies of APC multiple activities (14). The observation that APC could induce cell signaling through activation of protease activated receptor-1 (PAR-1) using EPCR as an essential receptor provided a major breakthrough in understanding APC cellular effects (9). To date, PAR-1 and EPCR have been implicated as required for APC's anti-inflammatory, anti-apoptotic and cytoprotective effects both in vitro and in vivo (10,11). In spite of the continual advances in unraveling the protein C humoral and cellular pathways, much remains to be elucidated concerning the molecular mechanisms for APC direct effects on cells and for the evaluation of APC's utility as a pharmacologic agent.

4. COMPONENTS OF THE PROTEIN C PATHWAY

4.1. Protein C

Protein C was discovered in search for vitamin K-dependent coagulation factors that are not detected by clotting assays (18). The gene for protein C (PROC) has been mapped to chromosome 2 (2p13-14) and is comprised of 9 exons (Table 1). Mature protein C contains 419 amino acids. Approximately 85-90% of the human protein is found in plasma as a two-chain form, linked by a single disulfide bond between Cys141 and Cys277. A significant post-translational modification involves the cleavage of the zymogen between residues 157 and 158 by a furin-like enzyme, followed by the loss of the Lys156-Arg157 dipeptide connecting the light chain (residues 1-155; Mr ~25 kDa) to the heavy chain (residues 158-419; Mr ~ 41 kDa). Other post-translational modifications involve beta-hydroxylation at Asp71, N-linked glycosylation at residues 97, 248, 313, and 329 and gamma-carboxylation at the first 9 Glu residues in the gamma-carboxy glutamic acid (Gla) domain. The 3 subforms of the heavy chain, designated alpha (Mr~ 41 kDa), beta (Mr~ 37 kDa), and gamma (Mr~ 32 kDa) represent tri-, di- and monoglycosylated heavy chain due to partial glycosylation of Asn residues 329 and 248 (33). The characteristic structural elements of protein C include an amino-terminal Gla domain (residues 1 to 37), an aromatic stack (residues 38 to 45), two epidermal growth factor (EGF)-like regions (EGF-1, residues 46 to 92 and EGF-2, residues 93 to 136), an N-terminal activation peptide (residues 158 to 169) on the heavy chain, and the serine protease domain (residues 170 to 419). Thrombin cleavage of the zymogen at Arg169 removes the activation peptide and generates APC, a trypsin-like serine protease with a typical serine protease active site triad (His211, Asp257 and Ser360).

The Gla domain of protein C is essential for binding to negatively charged phospholipids to mediate anticoagulant activity and for binding to EPCR to express cytoprotective activity (34-36). Interactions of protein C with other macromolecules is mediated by functionally important areas on the protease domain surface that are remote from the active site, and these areas are designated "exosites". Mutagenesis studies show that positively charged residues in exosites on the APC protease domain surface are required for rapid inactivation of factor Va (37-42). A distinct partially overlapping exosite is required for interactions of APC with TM, indicating the high degree of specificity of APC protease domain exosites.

4.2. Thrombomodulin

Thrombomodulin (TM, CD141) (43)) was discovered by Esmon and Owen in their search for a physiologic cofactor for thrombin-mediated protein C activation (44). The gene for TM (THBD) has been mapped to chromosome 20 (20p12-cen) and appears to be intron-less (Table 1) (45,46). Based on its cDNA sequence, TM consists of 575 amino acids in the form of a 60.3 kD single chain transmembrane protein (46). Post-translational O-linked and N-linked glycosylation accounts for ~20% of the apparent molecular weight of TM and other modifications include covalently bound chondroitin sulphate moieties (47).

Functionally, six domains on the mature TM can be identified (46). A N-terminal lectin-domain (residues 1 to 226), a repeat of 6 EGF-like domains (EGF-1 (residues 227 to 262), EGF-2 (residues 270 to 305), EGF-3 (residues 311 to 344), EGF-4 (residues 351 to 386), EGF-5 (residues 390 to 421) and EGF-6 (residues 427-462)), a serine and threonine rich region (residues 463 to 496), a transmembrane region (residues 497 to 519), and finally a C-terminal intracellular tail (residues 520 to 557). The serine and threonine rich region following the EGF-like modules is the major site for O-linked glycosylation as well as for glycosaminoglycan attachment at Ser 474 within the chondroitin consensus sequence of Ser-Gly-Ser_{474}-Gly-Glu-Pro. Recently, the lectin-like domain was identified to be required for direct anti-inflammatory activity of TM, although the molecular basis for this remains unclear (48-50). The EGF-like modules mediate protein-protein interactions, as EGF-like modules 5 and 6 mediate thrombin binding, whereas EGF-like module 4 is required for interactions with protein C.

4.3. Endothelial protein C receptor

EPCR (CD201 (43)) was discovered by Fukudome and Esmon in their search for the endothelial cell receptor responsible for binding of APC to the cell surface (28). The gene for EPCR (PROCR) was mapped to chromosome 20 (20q11.2) (Table 1) (51). EPCR is homologous to the CD1/MHC superfamily. MHC class I molecules are type 1 integral membrane proteins and

Table 1. Characteristics of the protein C pathway components

Protein	Gene	Locus	cDNA	Residues	Theoretical mass (kDa)	Observed mass (kDa)	Plasma conc. (nM)	Epsilon (1%; 1 cm)
protein C	PROC	2p13-14	NM_000312	419	47.3	62	65	14.5
thrombomodulin	THBD	20p12-cen	NM_000361	557	60.3	75 (105)[1]	0.05[2]	9.2
EPCR	PROCR	20q11.2	NM_006404	221	25	46	2.5[3]	11.3
protein S	PROS1	3q11.2	NM_000313	635	70.7	77	320[4]	9.5
C4b-binding protein	C4BPA	1q32	NM_000715	549	70	570[5]	260	14.1
	C4BPB	1q32	NM_000716[6]	235	45			
factor V	F5	1q23	NM_000130	2196	249	330	25	8.9
factor VIII	F8	Xq28	NM_000132	2196	265	330	0.7	11.9

Footnotes: [1] Under reducing condition the apparent molecular mass increases to ~105 kDa. [2] Soluble TM is believed to be the product of proteolysis of TM on the cell surface and comprises different molecular forms (Mr 35-65 kDa) that lack the transmembrane sequence. The concentration of soluble TM in normal individuals is ~ 2-5 ng/ml but can increase many-fold under pathological conditions. Increased soluble TM levels are regarded to be a sign of endothelial cell activation. [3] Soluble EPCR is derived from proteolysis of EPCR on the cell surface and lacks the transmembrane domain (Mr ~ 43 kDa). [4] The total protein S concentration (320 nM) includes free protein S (130 nM) and protein S in complex with C4BP (190 nM). [5] C4BP contains two polypeptide chains in varying proportions and circulates in different isoforms, alpha-7-beta-1 being the most common. About 17% (45 nM) of C4BP contains only alpha chains and lacks the beta-chain that binds protein S. The alpha-6-beta-1 and alpha-7 isoforms have a molecular weight of approximately 530 kDa and 500 kDa. [6] Variants 1 (NM_000716), 3 (NM_001017365) and 5 (NM_001017367) encode for the longer isoform 1 but vary in the 5' untranslated region, whereas variants 2 (NM_001017364) and 4 (NM_001017366) encode for isoform 2 with a shorter N-terminus due to an in frame alternate splice site.

consist of three extracellular domains, alpha-1, alpha-2 and alpha-3. The latter domain typically associates non-covalently with beta2-microglobulin. EPCR lacks the alpha-3-domain and thus does not associate with beta2-microglobulin; rather, in EPCR, the transmembrane domain is directly connected to the alpha-2 domain (51). The alpha-1 and alpha-2 domains form a binding groove by each providing an alpha-helix that is located along opposing edges of a planar platform generated by an eight-stranded anti-parallel beta-sheet. The crystal structure of soluble EPCR revealed that a phospholipid was bound in this groove between the two alpha-helices and that the Gla-domain of protein C was bound at one end of the EPCR molecule to one of the alpha-helices (52). Both protein C and APC bind EPCR with similar affinity (K_D~ 30 nM) via the Gla domain (53). Expression of EPCR is readily demonstrated on the surface of endothelial cells, monocytes, CD56+ natural killer cells, neutrophils and eosinophils (28,54).

4.4. Protein S

The vitamin K-dependent plasma glycoprotein, protein S, which was named after its birth city, Seattle, was discovered in 1977, and its function as a non-enzymatic cofactor to APC was subsequently demonstrated (26,27). The relatively large gene for protein S (PROS; PS-alpha) is located on chromosome 3 (3q11.2) and contains 15 exons (Table 1). Chromosome 3 also contains an inactive pseudogene for protein S (PROSP; PS-beta; at location 3p21-cen). The mature polypeptide of protein S has a defined structural organization consisting of 5 distinct domains, including an N-terminal Gla domain (residues 1-37) and aromatic stack (residues 38-45), an unique 29-amino acids sequence known as the thrombin-sensitive region (TSR; residues 46-74), a string of 4 EGF-like domains (EGF-1 (residues 75-115), EGF-2 (residues 116-159), EGF-3 (residues 160-201) and EGF-4 (residues 202-242)), and finally a large 393 amino acid domain on the C-terminus referred to as the sex-hormone binding globulin (SHBG)-like domain (residues 243-635) which is composed of two laminin G-type domains.

Residues involved in interactions of protein S with APC are located in the Gla domain, in the thrombin-sensitive region, and in both EGF-1 and EGF-2 (55-58). The APC-binding interface is thought to contain the polypeptide strand of EGF-1 from Lys97 to Pro106 whereas the Gla domain, the TSR and EGF-2 likely play a supporting structural role rather than directly contributing to macromolecular interactions, although no 3-dimensional structure for the APC-protein S complex has been determined. On the surface of APC, residues that bind protein S are thought to involve hydrophobic stack and EGF-1 residues (59). In the absence of real structures, such interactions are speculative, and more detailed structural data are required to understand interactions between protein S and APC.

Two mechanisms are in place to regulate protein S anticoagulant APC-cofactor function. First, cleavage of protein S in the thrombin-sensitive region (Arg-49, Arg-60 or Arg-70) reduces the affinity of protein S for calcium and phospholipids. Although the thrombin-mediated cleavage has been labeled non-physiological, as it is inhibited by calcium ions, other proteases may be responsible for cleavage of the TSR in protein S *in vivo*. Patients with disseminated intravascular coagulation showed markedly increased levels of cleaved protein S, indicating that cleavage of protein S in the thrombin-sensitive region can occur under certain pathological conditions (60). The second and probably more relevant regulatory mechanism for protein S function is the non-covalent and reversible binding of protein S to the complement factor, C4b binding protein (C4BP), resulting in the loss of protein S APC-cofactor function.

As a component of the complement system, C4BP regulates complement activation by accelerating the proteolytic degradation of activated complement factor, C4b, by factor I. Promotion of C4b degradation by C4BP also inhibits the formation of the C3 convertase (C4b:C2a complex) in the classical pathway of complement activation (61,62). C4BP is an octopus-like shaped

glycoprotein of ~570 kDa and consists of six or seven disulfide-linked, identical alpha-chains (each chain of 549 amino acids; 70 kDa) and one shorter beta-chain (235 amino acids; 45 kDa) (62). C4BP regulates protein S function by binding to protein S via the beta-chain (63). Protein S binds to the C4BP beta-chain in a 1:1 stoichiometric complex with high affinity (K_d ~0.1-0.6 nM) (64). Because the SHBG-like domain of protein S contains surface residues that provide binding sites for C4BP, protein S molecules that are bound to C4BP can still bind to phospholipid membranes and still interact with APC, although interactions of protein S with factor Va are prohibited which effectively shuts down protein S anticoagulant APC-cofactor activity (65-69).

Protein S is predominantly synthesized in the liver but it is also found the endothelium and possibly megakaryocytes. Protein S circulates in plasma at a total concentration of 320 nM, and approximately 60% of the protein S is non-covalently complexed with C4BP such that free protein S is 130 nM in plasma (70). Although C4BP is an acute phase response protein, the beta-chain containing C4BP levels are held stable and thus free protein S levels remain stable during an acute phase response due to differential regulation of expression of C4BP alpha- and beta-chains (71).

4.5. Factor V

Coagulation factor V is a relatively large protein (Mr ~330 kDa) composed of six domains, three A-domains (A1, A2 and A3), one B-domain that is mostly lost during activation and two C domains (C1 and C2), the latter of which are responsible for high affinity binding to phospholipids (Table 1) (72). Activation of factor V involves proteolytic cleavage by thrombin at Arg709, Arg1018 and Arg1545 thereby removing the B-domain to yield activated factor V (factor Va). Factor Va is composed of a heavy chain (A1-A2, residues 1–709) and a light chain (A3-C1-C2, residues 1546–2196) which form a non-covalent heterodimeric complex (73). The three A domains are arranged as a stable heterotrimer in both factor V and factor Va (74,75).

Factor Va is a non-enzymatic cofactor in the prothrombinase complex, the complex that converts prothrombin into thrombin. By binding to both the substrate prothrombin and the enzyme factor Xa on the surface of negatively charged phospholipids, factor Va increases the catalytic efficiency by approximately 10,000-fold (76). Therefore, inactivation of factor Va by APC effectively shuts down thrombin formation.

4.6. Factor VIII

Coagulation factor VIII, similar to factor V, is a relatively large protein (Mr ~330 kDa) composed of six domains, three A-domains (A1, A2 and A3) separated by Asp and Glu rich acidic spacers (*a1*, *a2* and *a3*), a B-domain that is mostly lost during activation and two C domains (C1 and C2) (Table 1). During post-translational processing (77), factor VIII is converted into a two-chain molecule, by endoproteolytic cleavages at Arg1313 and Arg1648 by subtilisin-like enzymes, thereby removing a large part of the B-domain. Factor VIII circulating in plasma is a heterodimer comprised of the heavy chain (A1-*a1*-A2-*a2*-B) non-covalently associated with the light chain (*a3*-A3-C1-C2) in a metal ion-dependent manner (78). In the circulation von Willebrand factor (vWF) forms a tight non-covalent complex (K_D ~0.5 nM) with factor VIII, thereby preventing the incorporation of factor VIII into the tenase complex by shielding both its factor IXa interaction site on the A1 domain as well as its phospholipid binding site on the C1 domain. Upon activation of factor VIII by thrombin or factor Xa activated factor VIII (factor VIIIa) dissociates from vWF and is free to interact with the components of the tenase complex. Thrombin and factor Xa can activate factor VIII by cleaving factor VIII at Arg740, removing the rest of the B-domain, and at Arg372. In addition, a presumed activation peptide in the *a3* acidic spacer is removed by thrombin cleavage at Arg1689, whereas factor Xa removes this activation peptide by cleavage at Arg1721. Thus, factor VIIIa is a heterotrimer whereas factor Va is a heterodimer. Factor VIIIa, expressing full cofactor activity, enhances factor Xa formation by the tenase complex approximately 200,000-fold (79). The importance of factor VIIIa cofactor activity is clearly demonstrated by the bleeding tendency in hemophilia A patients who manifest factor VIII deficiency.

5. THE ANTICOAGULANT PROTEIN C PATHWAY

5.1. Activation of protein C

Physiological proteolytic activation of protein C by thrombin occurs on the surface of the endothelial cells and involves the two membrane receptors TM and EPCR (80). Binding of thrombin to TM on the endothelial surface shields thrombin's procoagulant exosite I and unveils its anticoagulant properties (81). Subsequent activation of protein C by the thrombin-TM complex is facilitated by localization of protein C on the endothelial cell surface by binding to EPCR (Figure 2) (82). The protein C activation pathway appears to be especially sensitive to downregulation by inflammatory responses to endotoxin. Inflammatory mediators such as Interleukin-1 beta and tumor necrosis factor-alpha released by activated leukocytes during inflammation inhibit transcription of TM and EPCR, enhance receptor shedding and promote neutrophil elastase to cleave TM from the endothelial surface, thereby reducing the ability to generate APC on the endothelial cells surface (28,83,84). Impaired activation of protein C is typically observed in patients with inflammation and sepsis (85). Furthermore, protein C levels in patients with severe sepsis often are dramatically decreased, due to a combination of protein C consumption and dysfunctional protein C synthesis in the liver, and low protein C levels correlate with a negative prognosis in septic patients (86).

Recently, because platelet factor 4 (PF4) enhances APC generation, it was suggested to consider infusing PF4 or PF4-related peptides or peptidomimetics as a way of beneficially stimulating "endogenous" APC generation in pathologic disease states such as sepsis (87). PF4 is cationic (pI=7.8) platelet alpha-granule protein that is released from platelets upon activation. In contrast to other cationic proteins, PF4 accelerated APC generation by

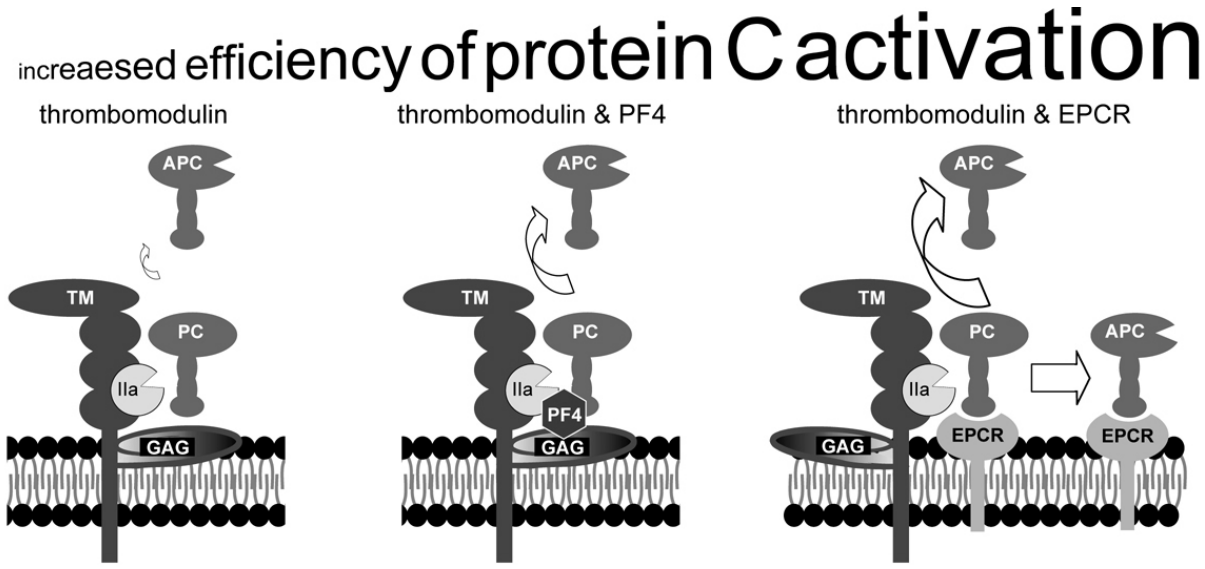

Figure 2. Mechanisms for protein C activation. Protein C (PC) activation occurs on the endothelial cell membrane and requires TM. Binding of thrombin (IIa) to TM shifts thrombin's enzymatic properties from procoagulant to anticoagulant by causing the activation of protein C (left). The efficiency of protein C activation is increased in the presence of additional cofactors that localize protein C also on the endothelial surface. Platelet factor 4 (PF4) is released during platelet activation and forms an electrostatic bridge between the chondroitin sulphate moiety (GAG) of TM and the Gla-domain of protein C. By bringing protein C closer to the thrombin-TM complex, PF4 stimulates protein C activation (middle). Binding of protein C to its endothelial receptor, EPCR, provides for the most efficient activation of protein C (right). Protein C and APC have a similar affinity for EPCR. Dissociation of APC from EPCR allows expression of APC's anticoagulant activity whereas retention of APC bound to EPCR allows APC to activate PAR-1 and express APC's multiple direct cellular activities.

the soluble thrombin-TM complex *in vitro* up to 25-fold (88). This increase in APC formation is attributable to a 30-fold decrease in the K_M for protein C for the thrombin-TM complex and is dependent on both the Gla-domain of protein C and the chondroitin sulphate glycosaminoglycan (GAG) moiety of TM (89). Presumably, cationic PF4 forms an electrostatic bridge between the anionic Gla domain of protein C and the anionic GAG domain of TM, thereby enhancing the affinity of protein C for the thrombin-TM complex and thereby stimulating the catalytic generation of APC (Figure 2) (87,89). Physiologically relevant concentrations of PF4 enhance thrombin-induced APC generation in cynomologus monkeys *in vivo*, suggesting the potential pharmacologic utility of these phenomena (90). As PF4 has already been successfully used in humans to reverse therapeutic heparinization, the application of PF4 to boost the deteriorating protein C pathway in inflammatory disease and sepsis merits further evaluation (90,91).

5.2. Inactivation of factor Va

APC's potent anticoagulant activity involves inactivation of factors Va and VIIIa (Figure 3) (92,93). Inactivation of factor Va is a complex process involving three APC cleavages of factor Va at Arg306, Arg506, and Arg679. Cleavage at Arg506 is rapid and much faster that cleavage at Arg306, but it only partially inactivates factor Va, whereas cleavage at Arg306 results in the complete inactivation of factor Va (93-95). Cleavage of the connection between the A2 domain and the A1 domain at Arg306 results in the dissociation of the A2 domain fragment, whereas the A1 domain remains associated with the light chain (96). Protein S enhances the APC-mediated cleavage of factor Va at Arg306 by lowering the active site of APC closer to the membrane, presumably thereby placing it in better proximity to Arg306 (Figure 3) (97). In addition, protein S is able to diminish the ability of factor Xa to protect factor Va from inactivation by APC when protein S, factor Xa and factor Va are simultaneously present. This is based presumably on the ability of factor Xa to block the access of APC to factor Va and on the competition of protein S with factor Xa for binding to factor Va.

The importance of factor Va inactivation is observed in patients carrying one of three known mutations in factor V (Arg506Gln, factor V Leiden; Arg306Thr, factor V Cambridge and Arg306Gly, factor V Hong Kong) that render factor Va resistant to APC inactivation, contributing to an increased thrombotic risk. Indeed, for the inactivation of factor Va, the critical events are the cleavages at Arg506 and Arg306 which reduce affinity of factor Va for factor Xa (98). This is in contrast to the mechanism for inactivation of factor VIIIa where dissociation of the A2 domain from the factor VIIIa heterotrimer usually provides the key inactivation step (see below).

5.3. Inactivation of factor VIIIa

Factors Va and VIIIa are generally homologous proteins with similar domain structure and procoagulant

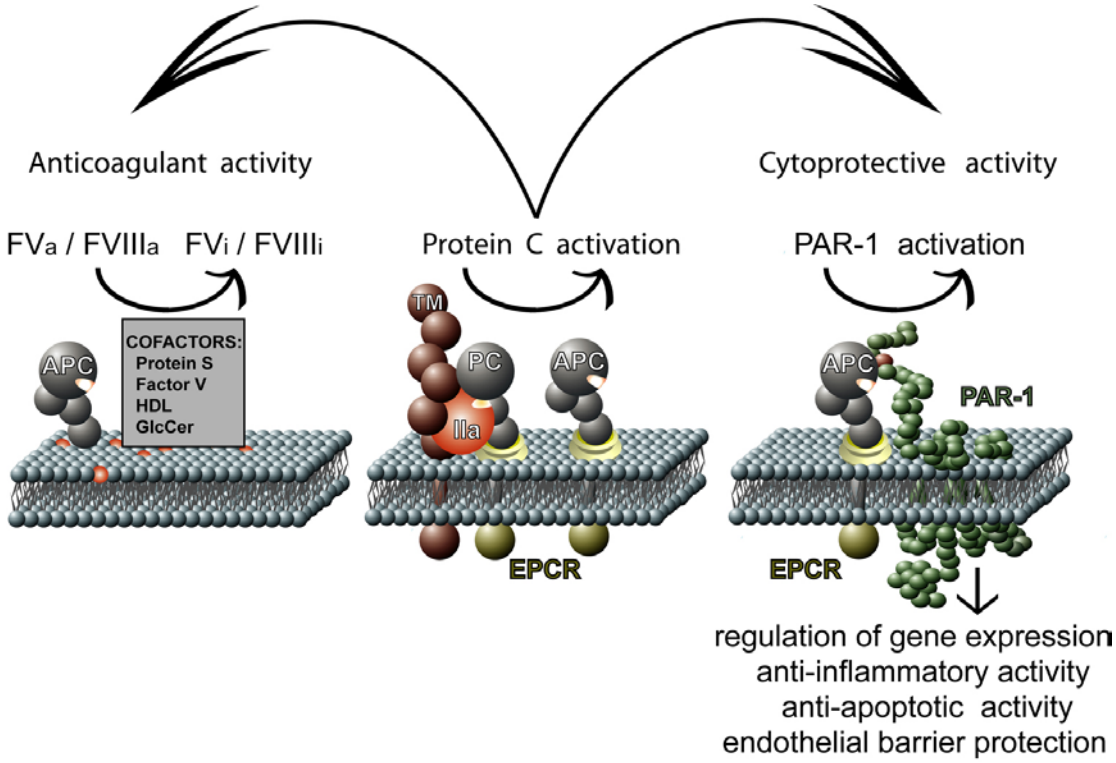

Figure 3. Schematic model of APC's anti-apoptotic and anticoagulant activities. Protein C (PC) is activated by thrombin (IIa) bound to thrombomodulin (TM) located on the endothelial cell surface. EPCR localizes protein C on the endothelial cell membrane and enhances the activation of protein C. Whereas the anticoagulant activity of APC is inhibited when APC is in complex with EPCR, EPCR is a required cofactor for the direct effects of APC on cells, i.e., for APC's alteration of gene expression and anti-inflammatory and anti-apoptotic activities because activation of PAR-1 by APC is EPCR-dependent. When APC dissociates from EPCR, it can express its anticoagulant activity, especially when bound to the surface of activated platelets or endothelial cells, various micro particles, or lipoproteins (eg., HDL). As an anticoagulant, APC cleaves the activated cofactors Va (fVa) and VIIIa (fVIIIa) to yield the inactivated cofactors, fVi and fVIIIi. Inactivation of factors Va and VIIIa by APC is enhanced by a number of different protein and lipid cofactors, e.g. protein S, factor V, HDL, and various lipids (eg., phosphatidylserine, cardiolipin, glucosylceramide, etc.).

function although the former is a heterodimer while the latter is a heterotrimer. This forms the basis for the observation that factor VIIIa, in contrast to factor Va, is quite unstable and has a half life of approximately 2 min due to spontaneous dissociation of the A2-domain. Due to the spontaneous inactivation of factor VIIIa, the physiological relevance of APC-mediated factor VIIIa inactivation has been questioned. On the other hand, factor IXa stabilizes factor VIIIa by linking the A2-domain to the A3-domain, and several arguments support a role for APC in the inactivation of factor VIIIa (see also section 5.4.3. Factor V) (77).

Proteolytic inactivation of factor VIIIa by APC occurs at Arg336 and Arg562, two residues that are homologous to Arg306 and Arg506 in factor Va. Unlike for factor Va inactivation by APC, there is no sequential kinetic order of cleavage in factor VIIIa inactivation as cleavage at either Arg336 or Arg562 results in near complete inactivation of factor VIIIa. Protein S only moderately enhances cleavage at Arg336 (~ 2-fold), whereas it stimulates the cleavage at Arg562 only slightly more (~ 5-fold) (99). Factor V can further enhance the APC-cofactor effect of protein S (Figure 3) (100). The increase in APC activity by factor V is demonstrable only in the presence of protein S (101). Thus, factor V and protein S might act as synergistic cofactors for factor VIIIa inactivation by APC.

5.4. Anticoagulant cofactors

APC anticoagulant activity is enhanced by multiple cofactors, including protein S (see above), negatively charged phospholipids, neutral glycosphingolipids and high density lipoprotein (HDL) (102-106). Defects in these APC cofactors have been linked to increased risk of venous thrombosis (2,4,105,107). Factor V in the presence of protein S is an APC cofactor for factor VIIIa inactivation (100). Because factor V Leiden lacks this APC-cofactor activity, it was hypothesized that risk for venous thromboembolism in APC resistant subjects who carry factor V Leiden is caused by both defective APC cofactor activity and resistance to APC inactivation with each defective mechanism assumed to contribute equally to increased risk (108-110).

Several protein C pathway cofactors not only enhance the anticoagulant activity of APC but also manifest

their own anticoagulant effects, independent of APC. The anticoagulant activity of TM is most notable its ability to enhance the generation of APC. Nevertheless there are additional mechanisms by which TM contributes to anticoagulant activity that are independent of APC generation. TM has a very high affinity for thrombin (K_D ~ 0.5 nM) and most of free thrombin in the circulation, if not complexed with inhibitors, will be scavenged by TM. Interaction with TM shields the procoagulant exosite I on thrombin, required for interactions with fibrinogen and thrombin-mediated platelet activation by cleavage of PAR-1. In addition, TM enhances inactivation of thrombin by the serpin, protein C inhibitor (PCI) (111).

Protein S also expresses anticoagulant activity independent of APC. Two different molecular mechanisms have been proposed. Protein S can bind to and inhibit factor Xa, factor Va, and factor VIIIa, thereby inhibiting prothrombinase and tenase activity (112,113). Additionally, a more general mechanism of prothrombinase inhibition by protein S was suggested to involve competition for limited amounts of procoagulant phospholipid surfaces (114). An interesting alternative mechanism was recently proposed for protein S direct anticoagulant activity based on the ability of protein S to enhance inhibition of factor Xa by tissue factor pathway inhibitor (TFPI) (Sere and Hacking, Novo Nordisk Haemostasis Award, XXth ISTH Congress, Sydney, Australia).

6. THE CELLULAR PROTEIN C PATHWAY

6.1. Activated protein C

Prior to the relatively recent reports that document the direct effects of APC on cells, it was generally thought that APC's anti-inflammatory activity could well be explained by reason of its ability to down-regulate generation of the proteases of the coagulation pathways. The anticoagulant activity of APC supported a rationale for using APC to counter the emerging coagulopathy associated with the induction of disseminated intravascular coagulation (DIC) caused by sepsis. However, interestingly, some physicians who treated infants homozygous for protein C commented that protein C appeared to be anti-inflammatory (JH Griffin, personal communications). In 1987, APC was found to prevent *Escherichia coli* induced death in baboons (30), and it was suggested that APC indeed provided potent anti-inflammatory activity by down-regulating cytokine generation.

Strong evidence for the direct action of APC on cells came from a transcriptional profiling studies showing that APC directly altered gene expression patterns and modulated cell signaling; the changes involved the major pathways of inflammation and apoptosis and were consistent with favorable alterations of anti-inflammatory and cell survival pathways (8). The next key observation came from Riewald *et al* who showed that APC could induce PAR-1-associated cell signaling and EPCR was identified as essential for these reactions (9). The requirements for PAR-1 and EPCR for APC's *in vitro* action on cells was confirmed and compelling evidence for an *in vivo* requirement for PAR-1 and EPCR to mediate APC's beneficial neuroprotective effects established the physiologic significance of these receptors for APC pharmacologic action (10,11,115). Thus, two cellular receptors for APC likely provide major explanations for the novel direct beneficial and cytoprotective effects of APC on cells that include anti-inflammatory, anti-apoptotic activities, endothelial barrier protection and regulation of gene expression (Figure 3) (8-13).

Alterations of gene expression induced by APC include up-regulation of the anti-apoptotic gene products of Bcl-2, endothelial nitric oxide synthase (eNOS), inhibitor of apoptosis (IAP), and suppression of p53 and Bax mRNA and protein expression (8-10,116). Large-scale gene expression profiling of endothelial cells perturbed by tumor necrosis factor-alpha demonstrated that APC and thrombin can have highly specific, opposite effects. For example, both p53 and thrombospondin-1 expression were inhibited by APC but up regulated by thrombin. These effects of thrombin and APC were dependent on PAR-1 cleavage and binding of APC to EPCR and demonstrated that the same receptor on the same cell type can mediate opposite biological effects (117).

The anti-inflammatory activity of APC includes reduced activation and activity of leukocytes, dampened release of inflammatory cytokines, down regulation of endothelial adhesion molecules and abrogated extravasation of cells at the site of inflammation. Mechanistically, less is known about the anti-inflammatory activity than for the other direct cellular activities. The general consensus is that there is a role for EPCR and possibly also for PAR-1.

The anti-apoptotic activity of APC requires APC's cellular cofactors, EPCR and PAR-1, and a functional APC active site (10,11). The complete molecular mechanism for APC anti-apoptotic activity is unclear. Possibly some of APC's effects on apoptosis can be explained by the altered gene expression profile induced by APC, but other mechanisms may also contribute to this activity. Important changes that are effected by APC include a reduction of activated caspases 3 and 8, an improved Bax/Bcl-2 ratio and a suppression of p53 upregulation that is induced by pro-apoptotic stimuli.

Endothelial barrier protection mediated by APC is a recent novel addition to pleiotropic APC's direct cellular effects. Both APC and thrombin at very low concentrations induced endothelial barrier protection by PAR-1-dependent sphingosine 1-phosphate receptor-1 cross activation, whereas thrombin at higher concentration actually was deleterious and enhanced endothelial permeability (12,13).

The observation that the beneficial cytoprotective effects of APC were mediated through PAR-1 was somewhat surprising as PAR-1 is the archetype thrombin receptor and was considered to mediate predominantly proinflammatory signals (118). This raised the obvious

questions of whether the cytoprotective effects of APC might also be achieved by other PAR-1 agonists or whether the effects are indeed specific to APC, i.e., whether thrombin itself might have activities equivalent to APC; to date, the data on these questions are inconclusive or somewhat contradictory. Differences in amplitude and duration of the signals induced by the PAR-1 agonist peptide and by thrombin were invoked to explain why the mitogenic effects of thrombin are not similarly caused by PAR-1 agonist peptides (119). PAR-1 activation by thrombin occurs with much higher catalytic efficiency than activation by APC, suggesting that differences in PAR-1-dependent signaling amplitude and duration might help partially explain the subtle but important biological differences between the effects of thrombin and APC on cells. Other possible explanations for differences between the messages generated by APC and thrombin include receptor cross activation or modulation of the PAR-1 signal by scaffolding proteins, an emerging concept for prioritizing between the different cell signaling pathways depending on the environmental "state" of the cell. Whatever mechanisms will eventually be clarified, it is clear that much work is needed to decipher APC's signaling and cellular effects.

6.2. The cofactors

Several components of the anticoagulant pathway, in addition to protein C itself, contribute not only to anticoagulant activity but also to cytoprotective, anti-apoptotic and anti-inflammatory functions. Protein C and APC cofactors of interest include protein S, thrombomodulin, EPCR and factor V. These novel anti-inflammatory, anti-apoptotic and cytoprotective activities of cofactors/receptors continue to stimulate new directions for the protein C cellular pathway functional studies.

6.2.1. Protein S

Protein S has long been suspected of acting as a messenger between coagulation and inflammation due to the role of C4BP in regulation of complement activation. The high affinity of protein S for anionic phospholipids prompted the idea that protein S could function as a cellular anchor for C4BP by binding to negatively charged phospholipids on cellular membranes. Maintaining the asymmetric distribution of phosphatidylserine on plasma membranes with this lipid normally in the internal leaflet of the bilayer is essential to maintain patency and fluidity within the vasculature, and activated platelets and cells undergoing apoptosis are significant sources of phosphatidylserine expression. Remarkably, the C4BP-protein S complex binds efficiently to the surface of apoptotic cells, whereas apparently only free protein S binds efficiently to phosphatidylserine on platelet-derived microparticles (120-122). The molecular basis for this difference remains unclear. Noteworthy is the fact that C4BP which is bound to apoptotic bodies by protein S is capable of interacting with its complement partner, C4b. These observations suggest that C4BP can inhibit activation of the complement pathway on the surface of the apoptotic cell (121).

One of the characteristics of apoptosis that makes it distinct from necrosis is that the death of the cell does not elicit a local inflammatory response. This requires rapid clearance of the apoptotic cells which is facilitated by blebbing of the apoptotic cell into small phagocytosis-ready apoptotic bodies and the expression of phosphatiylserine on the outer membrane which stimulates phagocytosis. Binding of early components of the classical complement pathway, especially C1q, is essential for clearance of apoptotic cells. C1q binds specifically to the surface of blebs of apoptotic cells and stimulates the uptake of apoptotic cells by macrophages; accordingly, deficiency in C1q result in increased circulation apoptotic bodies consistent with a defect in clearance of apoptotic cells (123-125). However, binding of C1q and other early classical complement components also brings about the risk for generation of inflammatory mediators such as C3a and C5a when not properly controlled. Therefore, there is a high need for the presence of a strong complement inhibitor on the surface of apoptotic cells, consistent with an hypothesized physiologic role for C4BP anchored by protein S on the surface of apoptotic cells (61).

In addition to localization of C4BP on the apoptotic cell surface, protein S is also implicated directly in stimulating phagocytosis of apoptotic cells by macrophages. Although the molecular mechanism is unknown, involvement of the family of receptor tyrosine kinases (see below) was suggested (122,126). Although primarily based on extrapolation and on circumstantial evidence, this novel role of protein S in the clearance of apoptotic cells might have considerable implications for the clinical manifestations such as those in systemic lupus erythematosis (SLE). SLE is characterized by the presence of auto antibodies against cell components associated with apoptotic cells, which are normally not exposed for prolonged time to the immune system. Deficiencies in early components of the classical complement pathway affecting clearance of apoptotic bodies constitute significant genetic risk factors for SLE (127). With this in mind, it is remarkable that anti-protein S autoantibodies and acquired protein S deficiency have been reported in SLE patients (128).

Protein S has also been implicated in mediating cell signaling based on the homology of protein S with growth arrest specific gene 6 (Gas6) which has substantial homology to protein S. Protein S and Gas6 might share a common receptor belonging to the Tyro3/Axl family of receptor tyrosine kinases (129,130). Protein S is a potent mitogen in various cell types and inhibits apoptosis *in vitro* (131,132). Furthermore, independent of APC, protein S exhibits both *in vitro* and *in vivo* neuronal protective effects. For example, infusion of protein S in a murine model of ischemic stroke decreases motor neurologic deficit and infarction volume in the brain (133).

6.2.2. Thrombomodulin

Both receptor cofactors of protein C activation on the endothelial cell membrane, TM and EPCR, were implicated to mediate direct cellular functions. Targeted gene deletion of TM showed that the embryonic lethality caused by TM deficiency developed before the development of a functional cardiovascular system and was

independent of TM's ability to promote APC generation because knock-in of the gene for a TM variant (TM^{Pro}; Glu404Pro) that does not support protein C activation actually rescued mice and caused normal survival (134,135). This implies that TM has a physiologic role independent of its anticoagulant activity. Participation in favorable cell signaling reactions by TM is one such role. Although the cytoplasmic tail of TM contains a Tyr, a Ser and two Thr residues that are potential substrates for kinases, deletion of the TM cytoplasmic tail did not affect embryonic survival (134,136). Therefore, it seems that most of the direct anti-inflammatory effects of TM are mediated by the extracellular portion of the protein, especially the N-terminal lectin-like domain. Consistent with this idea, trangenic mice lacking the lectin-like domain have a reduced survival upon challenge with endotoxin and increased infarcts after myocardial ischemia-reperfusion injury (48). Furthermore, the TM lectin-like domain dampens the proinflammatory effects of high-mobility group-B1 protein (HMGB1), phosporylation of mitogen-activated protein kinases and expression of leukocyte adhesion molecules (48,49). Remarkably, deletion of the lectin-like domain did not affect protein C activation and provides more evidence that the anti-inflammatory functions of TM are indeed independent of APC.

TM can indirectly exert anti-inflammatory effects by stimulating the activation of thrombin activatable fibrinolysis inhibitor (TAFI). Activated TAFI (TAFIa) is a basic carboxypeptidase that removes C-terminal Lys and Arg residues from proteins and polypeptides and is also a regulator of fibrinolysis with the potential to play an important role in processes such as blood pressure regulation, inflammation and wound healing (137). The list of TAFIa reactions includes conversion of bradykinin into inactive des-Arg^9-bradykinin and inactivation of the anaphylatoxins C3a and C5a by the inactivating removal of their C-terminal Arg, thereby reducing their proinflammatory effects. TAFI may also be important for the anti-inflammatory effects of recombinant soluble TM. Injection of recombinant human soluble TM rescues rats from massive and lethal thrombotic reactions in an *in vivo* glomerulonephritis model (138). The soluble TM decreases infiltration of leucocytes and increases TAFIa levels, whereas administration of a TAFIa inhibitor increases the infiltration of leucocytes and neutrophils, suggesting that anti-inflammatory activity of thrombomodulin functions at least in part via TAFI activation (138). Thus, in summary, TM can provide anti-inflammatory activity either directly, via cellular mechanisms that require the lectin-like domain, or indirectly via the activation of protein C or of TAFI.

6.2.3. Endothelial protein C receptor

Structural homology of EPCR and the MHC class 1/CD1 family of proteins, most of which are involved in inflammatory processes, suggests that the function of EPCR exceeds its ability to localize protein C and APC on the endothelial membrane (52). EPCR promotes *in vitro* and *in vivo* neuroprotective and cytoprotective effects of APC by acting as an essential cofactor in the activation of PAR-1 by APC, but does EPCR also possess direct cellular effects independent of its cofactor function? EPCR is considered incapable of intracellular signaling by itself due to its extremely short intracellular C-terminal tail (Arg-Arg-Cys-COOH). Nevertheless, nuclear translocation of EPCR with co-translocation of APC, but not of protein C, has been suggested (139). The murine centrosomal protein, CCD41, that is involved in cell-cycle regulation is identical to murine EPCR though it lacks the first N-terminal 31 amino acids. Alteration of gene expression profiles induced by transfection of K293 cells with human EPCR might indicate that the conclusion that EPCR is devoid of intracellular signaling is premature and warrants more thorough investigation (139,140). Mice that are genetically engineered to be null for EPCR exhibit embryonic lethality, showing there is a physiologically essential role for EPCR (141).

Another role for EPCR might involve the regulation of adhesion and migration of leukocytes. The beta-2 integrin, CD11b/CD18 (Mac-1), on monocytes is the ligand for ICAM and plays a pivotal role in the adhesion and migration of leukocytes (142,143). Soluble EPCR binds CD11b/CD18 and an antibody that blocks beta-2 integrin-mediated adhesion also inhibited soluble EPCR binding to CD11b/CD18. This suggests that the binding of soluble EPCR to CD11b/CD18 might interfere with leukocyte adhesion (144). Proteinase-3 (PR3), a serine protease with elastase-like properties stored in granules of neutrophils, binds soluble EPCR, implying that PR3 might mediate binding of soluble EPCR to CD11b/CD18 (144). While functional roles for the EPCR-PR3-CD11b/CD18 complex remain elusive, it is very interesting that the protein C/APC binding site on EPCR remains available in the soluble EPCR-PR3 complex, suggesting a possible role of this complex in mediating APC cellular signals and/or activation of protein C on leukocytes.

6.2.4. Factor V

Factor V was portrayed as a Janus-faced protein due to its two paradoxical functions as a procoagulant cofactor in the prothrombinase complex and anticoagulant cofactor to APC in the inactivation of factor VIIIa (109). Curiously it appears that factor V Leiden heterozygotes had a 2.8 times better odds of survival than non-Leiden patients in the PROWESS trial of APC treatment for severe sepsis (145). Mouse model studies of LPS-induced endotoxemia corroborate these findings in that the heterozygote factor V Leiden mice had approximately 2-fold increased survival compared to wild-type or homozygous factor V Leiden mice (145). Whether or not this survival advantage is linked to thrombin generation needs further investigation. In the meantime, it is tempting to speculate that the factor V Leiden polymorphism is a balanced polymorphism and is maintained in the gene pool due to a survival advantage of heterozygosity in inflammation-related diseases such as severe sepsis similar to the survival advantage in malaria bequeathed by the sickle cell hemoglobin polymorphism.

7. CLINICAL, PRECLINICAL AND EXPERIMENTAL APPLICATIONS OF APC

7.1. APC therapy for severe sepsis

The landmark Protein C evaluation in Severe Sepsis (PROWESS) clinical trial introduced a new era in

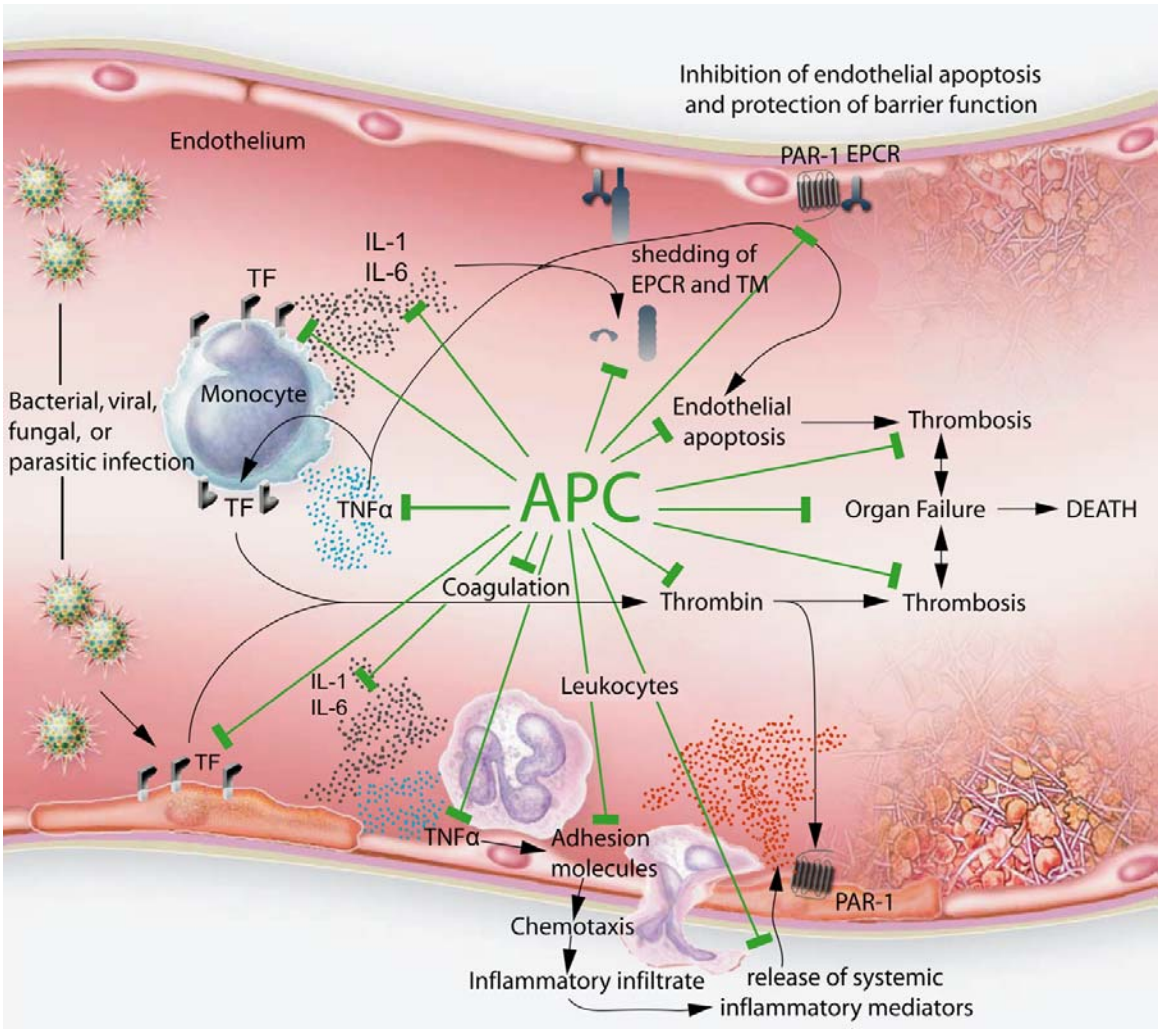

Figure 4. Beneficial multiple effects of APC. The broad spectrum of anti-inflammatory, anticoagulant and cytoprotective activities of APC is depicted. APC was successful for reducing mortality in the PROWESS clinical trail for severe sepsis while other anticoagulant or anti-inflammatory agents were not, presumably due to its multiple beneficial activities. Based on numerous reports, it is hypothesized that APC breaks the vicious spiral of inflammation and thrombin generation by simultaneously inhibiting both prothrombotic processes and pro-inflammatory processes. In addition, APC preserves and promotes endothelial cell viability and tissue integrity such that the blood vessel environment within which pathologic reactions occur is favorably affected so that when inflammation abates then the tissue has been preserved. Abbreviations used: TF, tissue factor; IL-1, interleukin 1; IL-6, interleukin 6; TNF-alpha, tumor necrosis factor alpha; PAR-1, protease activated receptor 1; EPCR, endothelial protein C receptor; TM, thrombomodulin. This figure is modified with permission from a scheme of Bernard *et. al.* (14).

protein C and activated protein C studies (14). This positive study demonstrated that treatment with recombinant APC caused a significant reduction of 28-day all-cause mortality in patients with severe sepsis. In this randomized, double-blind, placebo controlled multi center trial, APC (Drotrecogin alpha-activated) reduced the risk of death by 6.1% (relative risk reduction 19.4% [95% confident interval, 6.6% to 30.5%]) (14). These results were especially remarkable since two other potent anticoagulant proteins, tissue factor pathway inhibitor (TFPI) and antithrombin III (ATIII), failed to significantly reduce mortality in similar phase III severe sepsis clinical trials (OPTIMIST and KyberSept) (15,16). The failure of these potent anticoagulant agents implied that APC anticoagulant activity alone cannot explain its success in severe sepsis and suggested that APC therapeutically utilizes less well defined activities of APC such as its direct effects on cells that promotes anti-inflammatory and anti-apoptotic activities. Indeed, recent *in vitro* and *in vivo* data now support important roles for APC's direct anti-inflammatory and cytoprotective activities (see Figure 2). Clinical studies and animal model systems for treating a variety of thrombotic disorders with APC have shown promising results (10,146-148). We speculate that the mysterious success of APC in reducing death in severe sepsis patients is based on the duality of APC's humoral anticoagulant activity and direct cellular activities, and further speculate that APC's cytoprotective activities derive from a varied palette of beneficial actions (see Figure 4).

7.2. APC for ischemic stroke

Not only is APC treatment beneficial in severe sepsis, there is an emerging rationale for development of clinical trails of APC in ischemic stroke (17). The prospective epidemiologic Atherosclerosis Risk in Communities (ARIC) study reported that plasma protein C appears to be protective for ischemic stroke (149). Moreover, acquired APC resistance may be a marker or risk factor for ischemic stroke (150). In murine models of focal cerebral ischemia, APC provided remarkable anti-inflammatory and neuroprotective effects *in vivo* and increased survival (147,151-153). Interestingly, in mice both PAR-1 and PAR-3 were implicated in mediating the neuroprotecive effects of APC (147).

Currently, tissue-type plasminogen activator (tPA) is the only approved therapy for the treatment of acute ischemic stroke in the USA (154). Treatment with tPA effectively induces the fibrinolytic system to dissolve the thrombus that causes ischemic occlusion, but it also presents significant problems because tPA increases risk of symptomatic brain hemorrhage, has a brief 3-hour time window of efficacy, and is capable of directly causing damage to neurons (155-157). Clearly, improved therapies for ischemic/thrombotic stroke are needed (158). A potential breakthrough for ischemic stroke treatment is suggested by the recent *in vivo* results of combined APC and tPA therapy in a mouse N-methyl-D-aspartate (NMDA) excitotoxic injury and in mouse and rat ischemic stroke models (153,159). The cytoprotective activity of APC effectively counteracted tPA's neurotoxic effects on neurons, prevented neuronal apoptosis exacerbated by tPA and improved overall neurological deficits (159). Remarkably, APC could ameliorate the deleterious effects induced by both the intrinsic apoptotic pathway that involved hypoxia-induced caspase 9 activation and the extrinsic apoptotic pathway that involved tPA-induced caspase 8 activation (159). Thus, the combination of tPA's fibrinolytic activities with APC's cytoprotective and neuroprotective activities might provide major advancements for future stroke treatment.

7.3. APC for transplantation

Another potential application for APC therapy is suggested by a recent study showing beneficial effects of APC on pancreatic islet transplantation. Pancreatic islet transplantation has been validated as treatment for type I diabetes. However, clinical and preclinical studies indicate that significant loss of functional islet mass occurs in the peritransplant period due to inflammation and thrombosis. Administration of APC at the time of transplant improved the overall outcome in a mouse diabetes model (148). When islets were transplanted into diabetic mice, APC reduced loss of functional islet mass, improved glucose control, increased insulin release and reduced islet apoptosis, suggesting that APC therapy might enhance the therapeutic efficacy of pancreatic islet transplantation in diabetic patients.

8. IMPROVING APC THERAPEUTIC PROPERTIES

8.1. Risk of bleeding

Inherent to APC therapy is a significant concern for APC-induced bleeding due to its anticoagulant activity. An increased risk for serious bleeding was a side effect of APC in the PROWESS trial, especially during the 4-day infusion period (14,160). Although APC reduced mortality in severe sepsis, at least some of APC's life saving effects might have been counteracted by an increase in hemorrhage as cause of death (2.5% (6/236) vs. 0.7% (2/273), APC vs. placebo; P=0.1) (160). APC has multiple activities beside its anticoagulant action that is due to inactivation of factors Va and VIIIa, and these other activities might have contributed to the therapeutic efficacy of APC in the PROWESS trial. To construct potentially safer yet effective APC variants, we decided to prepare APC variants with a reduced risk of bleeding due to reduced anticoagulant activity but with normal direct effects on cells (161). Multiple observations implied that this might be possible. *In vivo* data suggested there might be an important distinction between the anticoagulant and cell protective activities of APC (10,147). APC neuroprotective effects in a murine ischemic stroke model were observed at low APC doses that had no observable effect on fibrin deposition or on restoration of blood flow, indicating that APC's neuroprotective effects, at least in part, were independent of APC's anticoagulant activity (10). Furthermore, binding of APC to EPCR is required for APC cytoprotective activity, but occupation of the phospholipid-binding Gla domain of APC by soluble EPCR inhibits APC anticoagulant activity and presumably APC bound to endothelial EPCR is also devoid of anticoagulant activity (162,163). In addition, two other potent anticoagulant proteins, tissue factor pathway inhibitor (TFPI) and antithrombin III (ATIII) failed to significantly reduce mortality in similar phase III clinical trials for severe sepsis. This indicates that the anticoagulant activity of APC alone cannot explain its success in the clinical trial.

To construct potentially safer APC variants, we assumed that: 1) bleeding was caused by APC's anticoagulant activity; 2) the beneficial therapeutic effect of APC is significantly due to APC's direct effects on cells; 3) the anti-apoptotic activity of APC is representative for APC's cytoprotective effects *in vivo*; 4) APC's cytoprotective effects require APC-mediated PAR-1 activation; 5) cleavage at Arg^{41} in the N-terminal tail of PAR-1 by APC has different exosite requirements than cleavage at Arg^{506} in factor Va and 6) the factor Va exosite and the PAR-1 exosite in APC are at least partially non-overlapping. Therefore, we attempted to alter factor Va exosites in APC without affecting exosites that might recognize PAR-1. The interaction of APC with the Arg506 cleavage site in factor Va that is critical for APC's normal anticoagulant activity has been extensively characterized and data show that a factor Va binding site on the positively charged surface of the protease domain includes loop 37 (protein C residues 190-193, equivalent to chymotrypsin (CHT) residues 36-39), the Ca^{++}-binding loop (residues 225-235, CHT residues 70-80) and the autolysis loop (residues 301-316, CHT residues 142-153) (Figure 5A) (31,37,39-42,164). To provide proof of principle for our concept, two APC variants were selected with Ala mutations in two critical APC surface loops and involved Ala replacements of Arg229 and 230 and of Lys191, 192 and 193. When tested in assays of

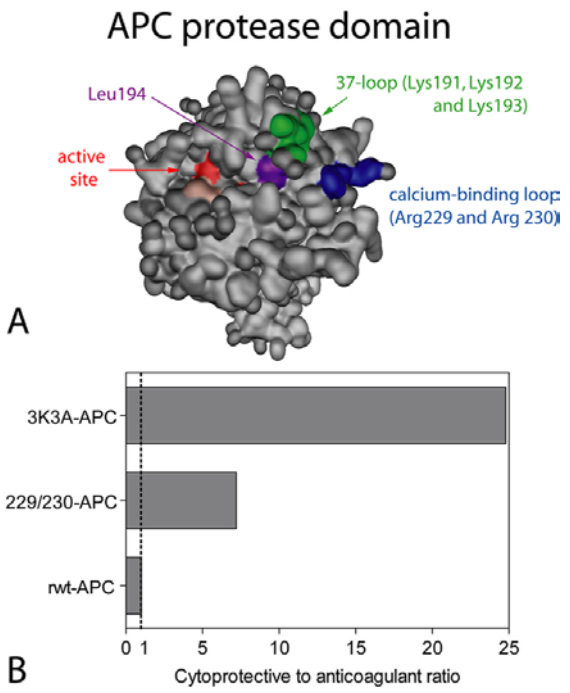

Figure 5. APC various biologic activities by exosite specificity. Interactions of APC with its macromolecular substrates are dictated by exosite interactions, and APC protease domain surface loops directly provide important exosites that specifically recognize various APC substrates. A. The surface contour of the serine protease domain of APC is shown with the active site residues His57, Asp102 and Ser195 in red, the 37-loop residues Lys191, Lys192 and Lys193 in green and Leu194 in purple, and the calcium-binding loop residues Arg229 and Arg230 in blue. Exosite specificity is illustrated by the fact that both the 37-loop residues (green) and the calcium-binding loop residues (blue) are required for normal cleavage of factor Va at Arg506 and thus for normal anticoagulant activity while they are not involved in proteolytic activation of PAR-1 that generates anti-apoptotic, cytoprotective activity. Whereas residue Leu194 in the 37-loop is not required for normal anticoagulant activity, it seems essential for normal interactions of APC with the serpins, protein C inhibitor and alpha$_1$-antitrypsin. The serine protease domain structure of APC is taken from Protein Data Bank entry 1AUT (31). B. APC variants with altered substrate specificity related to anticoagulant and anti-apoptotic activities. To design APC variants with reduced risk of bleeding due to reduced anticoagulant activity but with full cytoprotective activity, a molecular engineering approach of APC surface loops required for anticoagulant activity was used (161). This figure illustrates the increased ratio of cytoprotective (anti-apoptotic) to anticoagulant activities (7 to 25 fold) for two of such APC variants, 3K3A-APC (residues Lys191, 192 and 193 mutated to Ala) and 229/230-APC (residues Arg229 and 230 mutated to Ala) compared to the reference ratio of 1.0 for recombinant wild-type APC (rwt-APC). This figure is based on the values for anticoagulant and cytoprotective activities reported by Mosnier *et. al.* (161).

staurosporine-induced endothelial cell apoptosis, these two APC variants retained normal anti-apoptotic activity that requires PAR-1 and EPCR and exhibited a normal ability to cleave a PAR-1 N-terminal peptide at Arg41 (161). When the anticoagulant and anti-apoptotic activities of these APC variants were normalized to wild-type APC, the two APC variants exhibited 7-times and 25-times greater anti-apoptotic activity relative to anticoagulant activity compared to wild-type APC (Figure 5B). Thus, genetic engineering strategies aimed at reducing APC's anticoagulant activity while preserving the EPCR-dependent ability of APC to signal cells via PAR-1 activation are feasible. Preclinical and clinical studies will be needed to establish whether the *a priori* assumptions listed above are valid and whether the therapeutic use of such APC variants will reduce serious bleeding events associated with clinical use of wild-type APC while providing the beneficial effects of APC acting directly on cells. Safer APC variants not only might reduce bleeding complications associated with the current APC regimen but also might allow increased dosage and/or prolonged infusion periods for APC therapy, and such variants with altered dose regimens might achieve an additional reduction in mortality in severe sepsis patients.

8.2. Increasing APC half-life

Another approach for potentially improving APC therapeutic efficiency is to extend its half-life in the circulation as has been successfully used for improving tPA variants. Inactivation of the serine proteases involved in blood coagulation principally involves plasma serine protease inhibitors (SERPIN's) via a suicide-inhibition mechanism. The major inhibitors of APC in plasma are protein C inhibitor (PCI) and alpha$_1$-antitrypsin, and their neutralization of APC significantly helps to determine the half life of APC in humans that is approximately 23 min (60,165,166). Several residues in the 37-loop of protein C have been implicated in the reaction with inhibitors (167). Although mutation of Leu194 (CHT residue 40) resulted in an 8-fold increased half-life *in vitro*, only marginal benefits for prolonging half-life were found when APC variants with Leu194 mutations were characterized in various animal models. Although theoretically promising, this approach for improving APC therapy has yet to be successful.

9. CONCLUSION

At 30 years since the purification of protein C and after many years of elucidating the anticoagulant actions of APC, much remains to be characterized about the protein C pathway and about the potential therapeutic applications of APC. The recently discovered direct activities of APC on cells provide windows for novel insights into the biology, pharmacology and pathology of the protein C pathway. Future studies of this pathway and its components will be focused on molecular mechanisms and therapeutic opportunities. Insights into important natural defense mechanisms, into the relationships between inflammation and thrombosis, and into improved APC therapeutic variants are likely to accrue from further studies of each of the components of the protein C cellular pathway.

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Abbreviations: APC, activated protein C; C4BP, C4b-binding protein; CHT, chymotrypsin; eNOS, endothelial nitric oxide synthase; GAG, glycosaminoglycan moiety; GAS6, growth arrest specific gene 6; Gla, gamma-carboxy glutamic acid domain; IAP, inhibitor of apoptosis; IL-1, interleukin 1; IL-6, interleukin 6; EPCR, endothelial protein C receptor; NMDA, N-methyl-D-aspartate; PAR-1, protease activated receptor 1; PCI, protein C inhibitor; PF4, platelet factor 4; PR3, proteinase 3; serpin, serine protease inhibitor; SLE, systemic lupus erythematosis; TAFI, thrombin activatable fibrinolysis inhibitor; TF, tissue factor; TFPI, tissue factor pathway inhibtor; TM, thrombomodulin; TNF-alpha, tumor necrosis factor alpha; tPA, tissue-type plasminogen activator; TSR, thrombin sensitive region; vWF, von Willebrand factor

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