Activated protein C and sepsis

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1. ABSTRACT

Protein C is a plasma protease that when activated plays a central role in modulating the function of the vascular endothelium and its interface with the innate immune system. A recombinant form of human activated protein C (APC), drotrecogin alfa (activated), has shown efficacy in a number of preclinical models of thrombosis and ischemia and reduces mortality in patients that have a high risk of dying from severe sepsis. Studies have begun to elucidate the mechanism for the multifunctional role of APC in modulating not only coagulation, but also inflammation and apoptotic processes. From gene profiling to pharmacology studies, drotrecogin alfa (activated) appears to directly modulate endothelial dysfunction by blocking cytokine signaling, functional cell adhesion expression, vascular permeability and preventing the induction of apoptosis. Moreover, APC, via endothelial protein C receptor/protease activated receptor-1 mediated mechanisms, also appears to directly modulate leukocyte migration and adhesion. The ability of APC to suppress pro-inflammatory pathways and enhance cellular survival suggests that APC has a role in the adaptive response at the vessel wall, in which it protects the wall from vascular insult and prolongs endothelial, cellular, and organ survival. The emerging data further suggest that APC effectively modulates the complex changes that occur during multi-system activation and dysfunction in sepsis.

2. INTRODUCTION

Sepsis is an uncontrolled systemic response to inflammation caused by excessive stimulation of the innate immune system by bacteria, fungi, virus or parasites. Sepsis has several grades of severity; the most ill patients develop sepsis associated with acute organ dysfunction (severe sepsis) or hypotension (septic shock) (1-2). Despite advances in treatment and supportive care, the mortality rate is 30-50% and the incidence is rising due to multiple factors including increased numbers of immuno-compromised patients, use of life-sustaining technology and resistance to anti-microbial agents (3-10). Approximately 750,000 people in the United States develop sepsis each year (11). More than half of these patients are older than 65 and the incidence of sepsis is likely to increase during the next two decades as the “baby boom” generation continues to age (12). Numerous attempts have been made to develop therapeutic agents; however, clinical studies targeting several of the inflammatory cytokines that are upregulated in sepsis have been unsuccessful (4, 13-17). Nonetheless, over the last several years, a new understanding of the pathophysiology of sepsis has emerged, which focuses on the tight interplay and coupling of inflammation, microvascular coagulation and endothelial cell dysfunction. In line with this changing view, the use of the recombinant form of the natural anti-thrombotic factor, activated protein C (APC) was examined as a treatment for septic patients. The
administration of recombinant human APC (rhAPC) resulted in a reduction of mortality among treated versus placebo patients in the PROWESS trial (18). In this review, we will overview the biology of human activated protein C and its mechanistic role in the pathogenesis of severe sepsis. In particular, we will focus on its unique modulatory activities that make it an attractive agent for treating severely septic patients that are at a high risk of death and for treating other disorders of endothelial/leukocyte dysfunction.

3. BIOLOGY OF HUMAN PROTEIN C

3.1. Synthesis of protein C

The cDNA for human protein C (hPC) encodes a protein of 461 amino acids (figure 1) (19). The first 42 amino acids consist of a signal peptide (residues -42 to -25), and a pro-peptide (residues -24 to -1) that contains a recognition site for a vitamin K-dependent carboxylase (20). The signal peptide, pro-peptide and an internal dipeptide at Lys156-Arg157 are removed by proteolysis. Approximately 90-95% of the zymogen circulates as a heterodimer consisting of a light chain (residues 1-155), disulfide linked to the heavy chain serine protease domain (residues 158-419). The remaining 5-10% of the secreted mature hPC contains Lys156-Arg157 and circulates as a single chain. Both human plasma derived and recombinant protein C are complex proteins due to several post-translational modifications. In the light chain, the first 9 glutamic acid residues are gamma-carboxylated by the vitamin K-dependent carboxylase and Asp 71 is modified to beta-hydroxyaspartate (21). There are also four Asn-linked glycosylation sites in hPC (Asn 97, Asn 248, Asn 313, Asn 329) (22-23). The complete gamma-
3.2. Activation of protein C

Protein C is part of an integrated pathway that includes thrombin, thrombomodulin, endothelial cell protein C receptor (EPCR) and Protein S (30). Protein C is activated by thrombin-mediated cleavage and the rate of this reaction is increased by 1000-fold when thrombin binds to the cell surface receptor thrombomodulin (31-32). The activation rate of protein C is further increased by approximately 10-fold when EPCR binds protein C and presents it to the thrombin:thrombomodulin complex (33). Platelet factor 4 may also increase APC generation (34). The binding affinity of protein C for EPCR and phospholipid is dependent on the structure of its Gla domain; mutations in this domain reduce the affinity of these interactions (35).

3.3. Function of protein C

APC plays a central role in vascular function by maintaining vascular patency and modulating the function of the vascular endothelium. Many review articles have been written about the APC anticoagulation pathway and its role in hemostasis and thrombosis (36-43). Recently, a new understanding of the role of APC as an anti-inflammatory and cytoprotective agent in vascular and non-vascular tissues has emerged (44-48). In this section we will overview APC’s anticoagulant/antithrombotic mechanism, but the focus will be on more recent data describing its role as a modulator of endothelial and leukocyte function with properties opposing those of thrombin and pro-inflammatory cytokines (figure 2).

3.3.1. Antithrombotic Mechanism

APC is an important physiologic regulator of coagulation because it maintains hemostasis by controlling the conversion of prothrombin to thrombin. As thrombin is generated, it binds thrombomodulin to form an enzyme complex that modifies the specificity of thrombin for macromolecular substrates and inhibitors (49-50). This interaction reduces the coagulant function of thrombin because it interferes with thrombin’s ability to cleave fibrinogen and factor Va, but increases the rate that protein C is cleaved to its active form (36). APC, along with its cofactor protein S, functions to block further thrombin generation by inactivating the activated forms of factors V and VIII, thus inhibiting the prothrombinase and Factor Xase enzyme complexes, respectively (figure 2). The formation of APC is also tightly controlled by thrombin generation; the activation of protein C ceases carboxylation of the light-chain, beta-hydroxylation of Asp 71 and correct pro-peptide processing are all required for full functional anticoagulant activity (22, 24-29).
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Figure 3. Anti-thrombotic efficacy of APC. A. Antithrombotic activity in a guinea pig arterio-venous shunt model. Adapted from Kurz et al., (263). B. Activity of APC in a primate model of thrombosis. Adapted from Emerick et al., (264). APC decreases thrombus weight/size in a dose dependent fashion.

when thrombin is inhibited. Thrombus formation is determined by the balance between thrombin’s pro-coagulant activities (fibrin generation, platelet activation) and anticoagulant activity (APC generation). Factors that suppress the generation of APC or result in a deficiency of protein C have a significant consequence of shifting this balance towards thrombosis. Most cases of protein C deficiency result from mutations in the protein C gene and these mutations can either result in decreased synthesis of normal protein (type I) or synthesis of dysfunctional protein (type II) (51). Individuals who are homozygous or non-Leiden carriers, suggesting that APC may promote cell survival via anti-apoptosis pathways and these observations have been

3.3.2. Profibrinolytic mechanism

Thrombin converts soluble fibrinogen into a fibrin clot, which is subsequently removed by the fibrinolytic system (80-81). The key components of this system include plasminogen/plasmin, tissue plasminogen activator (t-PA), and plasminogen activator inhibitor (PAI-1). t-PA converts fibrin-bound plasminogen to plasmin, which then digests the fibrin clot resulting in the formation of soluble degradation products. PAI-1 inhibits t-PA and it has been demonstrated that elevated levels of PAI-1 increase the risk for thrombosis, cancer metastasis, vascular complications of diabetes and the development of septic shock (82-83). Thus, PAI-1 plays a critical role in regulating the balance between coagulation and fibrinolysis.

APC appears to play an important role in fibrinolysis because it can inhibit PAI-1 activity (85-87). This is evident in the canine coronary occlusion model and in rabbits infused with endotoxin (88). In the coronary occlusion model Jackson et al., demonstrated reperfusion of occluded vessels following APC administration (89-91). APC may also reduce circulating levels of PAI-1, accelerate t-PA-dependent clot lysis and prevent the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) (92-95), although the physiological importance of this later observation is unclear.

3.3.3. Anti-inflammatory and cytoprotective mechanism

The inflammatory pathway attempts to restore normal hemostasis following injury resulting from a variety of mechanisms including infection. Invading microorganisms can produce toxins such as lipopolysaccharide (LPS) that stimulate endothelial cells, platelets and cells comprising the immune system to release a variety of cytokines such as tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1), IL-2, IL-6, IL-8 and macrophage migration inhibitory factor (MIF). These cytokines recruit neutrophils and monocytes to the site of inflammation and they augment the expression of various adhesion molecules such as intracellular adhesion molecule (ICAM), vascular adhesion molecule (VCAM) and E-selectin, which are required for leukocyte activation and extravasation (96-98). Thrombin is a pro-inflammatory agent because it can also stimulate cells to release cytokines (99-102). APC indirectly modulates the inflammatory pathway because it can inhibit thrombin generation.

Besides these indirect effects on inflammation, recent studies have suggested that APC may directly modulate the inflammatory reaction via receptor-mediated effects (reviewed in) (37-38, 41-43, 103). Joyce et al., used broad transcriptional profiling to demonstrate that APC can modulate cell signaling and alter gene expression in two major inflammatory pathways (46-47). Furthermore, APC reduces leukocyte activation by directly suppressing cell surface adhesion molecules such as E-selectin, ICAM, VCAM and CX3C-fractalkine (47) (figure 4). The transcriptional profiling experiment also suggests that APC may promote cell survival via anti-apoptosis pathways and these observations have been
confirmed by cell based assays and animal studies (104-105).

Several studies have established that APC signals through the protease activated receptors (PARs), PAR-1, PAR-2 and PAR-3, but most signaling responses in endothelial cells seem to be mediated by PAR-1 (45-48, 105-106). The PAR receptors are members of the seven transmembrane domain G protein-coupled receptor (GPCR) family, but activation of the PAR receptors is unique compared to other members of the GPCR family. APC or other proteases can cleave at specific sites within the extracellular amino terminus of the PAR receptor, resulting in the exposure of a new amino terminus, which then acts as its own tethered ligand (107). For optimal PAR receptor cleavage, the protease should be juxtaposed to the receptor. This is easily accomplished by thrombin because it can bind to a hirudin like domain on PAR-1 that is located distal to the cleavage site; thus, thrombin can activate PAR-1 on numerous cell types including platelets, endothelium, epithelium, fibroblasts, myocytes, neurons and astrocytes (107-114). Unlike thrombin, APC does not contain a hirudin-binding site and APC must bind to EPCR for it to be juxtaposed to PAR-1 (figure 2). EPCR expression is limited to the endothelial/leukocyte interface of the innate immune system, with expression on endothelial cells, monocytes, natural killer cell, neutrophils and eosinophils, thus the capability of APC to cleave PAR-1 is more limited than that of thrombin (115-117). Nonetheless, APC mediates the suppression of staurosporine-induced apoptosis in many cells that express both PAR-1 and EPCR, including human monocytes, neurons and cultured U937 cells (figure 5) (46). However, APC cannot prevent apoptosis in neutrophils and eosinophils suggesting that different leukocyte populations either require a larger dose of APC to induce or activate proteins of the anti-apoptotic pathway or that PAR-1 signaling may be different in these cell types (117). Nonetheless, APC may still signal through the PAR-1 receptor in neutrophils and eosinophils since it reduces the response of these cells to certain chemotaxis agents (117). APC also reduces the response of human mononuclear phagocytes to phorbol ester, LPS and interferon-gamma and it can uncouple LPS interactions with monocyte CD14, which provides protection against LPS mediated microcirculatory dysfunction (103, 118-122). It should be noted that these results were observed in cell based systems and have not been demonstrated in human studies (123-
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Figure 5. APC inhibits apoptosis. Effect of APC on monocytes treated with staurosporine (SS), which is a potent inducer of apoptosis. The quantity of cells undergoing apoptosis was determined by measuring active caspase-3 by flow cytometry. Results are normalized to the control (no pretreatment with APC). APC suppressed staurosporine-induced apoptosis in a dose dependent fashion, and this effect is mediated by EPCR. Adapted from Joyce et al., (265).

Additional studies have demonstrated that supra-therapeutic concentrations of APC can directly inhibit the generation and release of cytokines and chemokines and this may be related to its ability to inhibit the translocation of nuclear factor kappa B (126-129). APC has been shown to decrease expression of adhesion molecules in vivo, and induce the expression of monocyte chemoattractant protein, which is associated with short-term survival benefits in systemic inflammation because it suppresses IL-12 and TNF-alpha induction (130-132). APC can inhibit the release of macrophage inflammatory protein-1-alpha from THP-1 cells and human monocytes and it can modulate macrophage MIF (127, 133).

APC can induce the formation of microparticles containing EPCR from HUVECs and monocytes and these microparticles may potentially migrate to inflammatory sites and reduce the inflammation/coagulation response (134). Other agents including TNF-alpha also induce the release of microparticles, but these particles do not contain EPCR; therefore, they cannot support activation of protein C and provide the protective responses of the protein C pathway.

The ability of APC to inhibit thrombin generation would reduce the pro-inflammatory response of thrombin. However, the anti-inflammatory effects of APC are not totally dependent on its coagulation effects because the direct inhibition of thrombin by other anticoagulants does not produce the same beneficial effects as APC. Several animal studies with other antiin thrombotics/anticoagulants effectively inhibited disseminated intravascular coagulopathy (DIC), but did not reduce mortality (41, 135-136). Tissue factor pathway inhibitor and antithrombin were evaluated in large phase III sepsis trials (137-141). Both of these proteins corrected the coagulation defect, but did not adequately suppress the inflammatory response. Furthermore, since these proteins attenuated thrombin generation, they directly interfered with the protective effects of the protein C pathway. Antibodies targeting the inflammatory proteins TNF-alpha and IL-1 receptor have also been investigated as a possible treatment for sepsis; however, administration of these proteins did not benefit patients with severe sepsis (142-146). Because thrombin formation and inflammatory stimulation are required for early response to infection, the complete suppression of these factors has proven to be problematic for therapy.

3.4. Differential signaling of APC and thrombin

Although APC and thrombin signal through PAR-1, they often induce different physiological responses. A large-scale gene expression profile experiment using cytokine stimulated endothelial cells demonstrated that some proteins were differentially regulated by APC compared to thrombin; APC downregulated and thrombin upregulated several proapoptotic genes (147).

The opposing cellular effects of thrombin and APC may be explained by dose response and rate of PAR-1 activation. In support of this, Ishii et al., have demonstrated that the rate of PAR hydrolysis determines the magnitude of the resulting cellular signal (148). A low concentration of thrombin (50 pM) protects neurons from apoptosis whereas high concentrations (more than500 pM) cause apoptosis (149). The same kinase and Rho-dependent pathways are activated by both concentrations of thrombin,
but APC increases permeability at supra-therapeutic concentrations (154). Although the differential effects of APC and thrombin may be explained by kinetics, recent data suggests that the initial signaling response measured by calcium flux is distinct between these two mediators (figure 6).

Alternatively, the different signaling pathways induced by APC and thrombin may result from APC activating another receptor. Two recent studies have demonstrated that APC can transactivate the sphingosine-1-phosphate receptor-1 (Edg-1), resulting in decreased endothelial permeability via endothelial cell cytoskeletal rearrangements (figure 2) (151-152). Further studies should be performed to determine if additional proteins are regulated by the transactivation of Edg-1 and if APC can activate additional cell surface receptors.

4. PATHOPHYSIOLOGY OF SEPSIS

The inflammation and coagulation pathways co-evolved and are intimately linked and there is a remarkable degree of integration in the regulation of these pathways, where thrombosis can activate the innate immune system and inflammation can activate the coagulation pathway. When an individual develops an infection, the immune system is rapidly activated so the infection remains localized. A number of cytokines are released to help control and clear the infection and these cytokines affect a number of pathways/processes such as coagulation, oxidation, nitric oxide production, adhesion and apoptosis. In sepsis, the inflammatory reaction is exacerbated because endotoxins or cytokines induce the expression of tissue factor on vascular monocytes and endothelial cells. Tissue factor activates the intrinsic coagulation cascade and causes thrombin generation, which in turn activates the endothelium, platelets and vascular smooth muscle (155). These responses damage the vascular endothelium resulting in leukocyte-endothelial cell adhesion and further tissue factor-dependent activation of coagulation (156-158). Damaged endothelial cells undergo apoptosis and this further contributes to amplifying the coagulation cascade because the disrupted endothelium provides a pro-coagulant surface (159-163). Thrombomodulin and EPCR expression is downregulated, which decreases the anti-coagulant and anti-inflammatory effects of the protein C pathway. Activation of the inflammation and coagulation pathways continue to cycle with little control, leading to enhanced microvascular coagulation and endothelial cell dysfunction. Ultimately, microvascular function is compromised resulting in DIC and microvascular thrombosis, decreased tissue perfusion and hypoxemia and organ dysfunction/failure (164-170). Figure 7 depicts a simplified schematic of the series of events that result from the initial injury through to the release of inflammatory and cytotoxic mediators. Eventually, the infection is cleared or the vascular endothelium is disrupted, which could result in end-organ dysfunction and death. It is rare for the initial infection to be the cause of mortality; rather mortality is the result of the body’s response to the infection.
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5. APC PHARMACOLOGY AND THERAPEUTIC RATIONALE

In this section we will overview the preclinical pharmacology of APC and the rationale for its clinical use. We will also highlight the approved use of drotrecogin alfa (activated), (Xigris®) for the treatment of adult patients with severe sepsis that are at a high risk of death.

5.1. Role Of protein C pathway in sepsis

The vascular endothelium secretes numerous proteins that maintain homeostatic balance in the vessel (91, 171). During infection or inflammation, this balance is altered as the endothelium becomes activated, resulting in the expression of pro-inflammatory cytokines, chemokines and cell surface adhesion molecules that are required for leukocyte adhesion and migration (reviewed in (44, 172-174)). This adaptive response plays a critical role in host defense, protecting the endothelium from toxins, shear stress, oxidative stress, hypoxia and various cytokines. However, this balance is lost during severe inflammatory insults, as occurs in sepsis. Endothelial activation becomes unregulated, leading to increased pro-inflammatory activity, procoagulant activity and cell death. The protein C pathway helps maintain normal homeostasis and limits inflammatory responses and endothelial cell apoptosis. During an inflammatory response, many of the natural anticoagulants including protein C are consumed due to increased activation of the coagulation pathway. Furthermore, activation of protein C is decreased because expression of thrombomodulin and EPCR is downregulated by inflammatory cytokines (175). Compounds secreted by leukocytes oxidize Met388 on thrombomodulin, thereby further reducing its activity (176). Also, neutrophil elastase and a metalloproteinase solubilizes thrombomodulin and EPCR, respectively (92, 177-178). Soluble thrombomodulin can still activate protein C, but it is less active than its cellular form since it is no longer juxtaposed to EPCR and it lacks the high affinity thrombin-binding site (179-180). Soluble EPCR inhibits the anticoagulant function of APC by blocking phospholipid interactions (181).

In addition to these pro-coagulant changes, anti-fibrinolytic changes also occur in response to endothelial damage (182-184). Following an inflammatory insult, the fibrinolytic process is inhibited by a mechanism that appears to be independent of the coagulation changes but involves increased levels of the fibrinolysis inhibitors PAI-1 and TAFI (166, 182, 184-186). The decreased activity of the protein C pathway and suppression of the fibrinolysis pathway impairs normal hemostasis.

A number of studies have demonstrated that reduced protein C levels are correlated with mortality in both sepsis and septic shock (187-197). Decreased protein C levels often develop before the onset of the clinical parameters that are used to define severe sepsis or septic shock and therefore could be considered a prognostic indicator (198-202). Protein C deficiency during polymicrobial sepsis in a cecal ligation and puncture model has been shown to exacerbate the inflammatory and hypotensive response in mice (203). These results may be attributed to increased plasma cytokine levels and renal and organ damage in the protein C deficient mice compared to their wild-type littermates. Reduced levels of protein C have also been associated with multi-organ dysfunction in bone marrow transplant patients and may be the cause of multi-organ failure in septic patients (204-205). End-organ dysfunction may be caused by microthrombi formation and proteins/pharmaceuticals that can block thrombus formation may reduce organ damage (182, 206).

Given the essential roles of the protein C pathway in coagulation, fibrinolysis and inflammation, several proteins in this pathway have been evaluated as a treatment for severe sepsis. Plasma-derived protein C has been used as an adjunct to the conventional therapy used in coagulopathy and purpura fulminans in meningococcal sepsis and gram-positive sepsis (39, 121, 136, 207-214). Due to the interest generated from these early case studies, a phase 2 placebo-controlled trial was initiated to assess the activation process of protein C and to determine its dosing regimen in children with purpura fulminans and meningococcal septic shock (215, 216). The outcome of this trial demonstrated that protein C concentrate led to a dose dependent, transient increase of plasma APC resulting in improved hemostasis. No significant improvement in survival was observed in this small phase 2 study.

While the above reports have suggested that protein C can be useful, the endothelium of septic patients often expresses decreased quantities of EPCR and thrombomodulin and this reduces the quantity of protein C that can become activated. Work in model systems demonstrated that generation of APC reduced the pathogenesis of sepsis. Preliminary studies demonstrated that APC could inhibit DIC, decrease markers of inflammation, decrease the coagulation response, reduce the drop in blood pressure and reduce mortality in baboons that were administered lethal doses of E. coli (135, 217). Furthermore, the coagulation and inflammatory responses to E. coli were heightened if protein C activation was prevented.

Hypotension is the hallmark feature of septic shock. APC prevents the rapid occurrence of hypotension in rats and healthy human subjects that are administered an intravenous dose of endotoxin (125, 218). The precise mechanism by which hypotension is alleviated is unknown, but intravenous administration of APC prevents the increase in plasma nitric oxide byproducts and iNOS mRNA expression in lung (218). LPS-induced hypotension is inhibited when APC is administered 30 minutes post-LPS administration; however, these LPS-induced effects were not prevented by active site blocked APC (DIP-APC), suggesting that the reversal of hypotension requires the protease activity of APC (218). Decreasing endothelium permeability may also normalize blood pressure.
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Figure 8. Effect of drotrecogin alfa (activated) on 28-day all cause mortality in severe sepsis. Adapted from Bernard et al (18).

Overall the mechanism of action of APC and its physiological role in sepsis made it an attractive agent for therapy. Because APC has a direct anti-thrombotic activity, it may prevent microvascular thrombosis, vascular congestion and resulting organ failure. The anti-inflammatory/cytotoxic activity may reduce the signaling of cytokines and further reduce activation of the coagulation/inflammation cycle. Moreover APC can also suppress the expression of ICAM, VCAM, and E-selectin and this may be important because of the critical role of cell adhesion in the early inflammatory response and sepsis (219-221).

5.2. Clinical development of drotrecogin alfa (Activated) for severe sepsis

Sepsis is a complex disorder that involves a number of proteins in the coagulation and inflammation pathways. Many of these proteins have been the targets for therapeutic intervention, but the majority of these proteins demonstrated little or no success in clinical trials (4, 13-17). However, early phase studies using recombinant human activated protein C (drotrecogin alfa [activated]) suggested a potential trend of a treatment benefit (223-224). This prompted the initiation of the Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) trial, a phase III, international placebo-controlled, blinded, randomized, 28-day-all-cause mortality study, which showed reduced mortality among drotrecogin alfa (activated) treated patients (figure 8) (18). Details of the clinical development of drotrecogin alfa (activated), which led to its approval by global regulatory agencies has recently been reviewed (222, 225). The challenges of expressing fully processed and active recombinant human APC was also recently reviewed in detail (222). Drotrecogin alpha (activated) is currently available for the treatment of severe sepsis in over 50 countries. Details of the labeled indication for commercial drotrecogin alfa (activated) (Xigris™) and for the cloning, production, and formulation can be found at the following web sites: http://www.emea.eu.int/humandocs/Humans/EPAR/xigris/xigris.htm

5.3. Pharmacodynamics of drotrecogin alfa (activated)

Pharmacodynamic analyses of 15 biomarkers of hemostasis, inflammation and endothelial injury were performed on the data collected from patients enrolled in the PROWESS trial (123). Administration of drotrecogin alfa (activated) resulted in decreased levels of thrombin generation markers and an increased prothrombin time (PT) and activated partial thromboplastin time (APTT); however, the increased PT and APTT were limited to the infusion period. Since drotrecogin alfa (activated) has a relatively short circulatory half-life, blood levels of drotrecogin alfa (activated) are often below the level of detection within 2 hours following discontinuation of the infusion (226).

Several studies have suggested that APC may play a role in PAI-1 regulation; however, the profibrinolytic effect of drotrecogin alpha (activated) was not significant in the PROWESS patients as there was only a statistical trend in lower PAI-1 levels (18). A reduction in pro-inflammatory markers following drotrecogin alfa (activated) administration was less well demonstrated in patients from the PROWESS trial compared to in vitro studies and early in vivo non-clinical studies. A dose-dependent and statistically significant reduction in IL-6 levels was observed in the Phase II study; however, in the Phase III study, the reduction in IL-6 levels was statistically significant on the first day and after the fourth day following drotrecogin alfa (activated) administration depending on the type of statistical analysis (227). Several other cytokines including TNF-alpha, IL-beta, IL-8 and IL-10 were analyzed, but there were no statistical differences between patients in the drotrecogin alfa (activated) group and placebo group (123). Subsequent studies have been performed to determine if drotrecogin alfa (activated) can alter the cytokine profile. Healthy volunteers received drotrecogin alfa (activated) or placebo, followed by a single low dose of endotoxin two hours later (124, 228). Analyses demonstrated that drotrecogin alfa (activated) did not directly affect the cytokine profile suggesting that drotrecogin alfa (activated) has minimal to no effect on the early response cytokines that characterize the acute response to infection. Moreover, studies demonstrating a direct impact of drotrecogin alfa (activated) on endothelial/leukocyte function are limited. Although many studies have demonstrated that drotrecogin alfa (activated) exerts beneficial effects for severe sepsis patients, the exact mechanism by which this drug functions is uncertain but most likely involves the antithrombotic, profibrinolytic, anti-inflammatory and cytoprotective properties of APC.

6. ADDITIONAL PHARMACOLOGY OF APC

As indicated above, APC (both plasma-derived and recombinant) has been shown to be effective in a wide variety of venous and arterial thrombosis models and in models of sepsis. In addition to these animal studies, recent
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preclinical data have shown the effect of APC in models of inflammatory/ischemic insult, stroke and lung injury.

6.1. Ischemic injury

Prospective epidemiologic studies demonstrated that increased plasma protein C levels might be a protective factor for ischemic stroke and low APC levels were often observed in patients that suffered from an ischemic stroke following an infection (229-230). The potential role of APC in stroke has recently been studied in murine models of focal cerebral ischemia (105, 231). APC provided remarkable anti-inflammatory and neuroprotective effects in vivo and increased survival at 24 hours. Furthermore, APC reduced neutrophil extravasation and decreased ICAM expression on cerebral blood vessels, which resulted in a reduction in the transmigration of circulating leukocytes across the blood-brain barrier. APC also reduces fibrin deposition, microvascular obstructions, tissue levels of CD11b and decreases the number of apoptotic cells by reducing the pro-apoptotic protein Bax and hypoxia-induced increases in p53 mRNA and by increasing the anti-apoptotic protein Bcl-2 (231-232). Both PAR-1 and PAR-3 are required for protective factor for ischemic stroke and low APC levels that increased plasma protein C levels might be a potential role of APC in stroke has recently been studied in murine models of focal cerebral ischemia (105, 231). Current, the only approved therapy for acute ischemic stroke is t-PA, but improved therapies are necessary since t-PA must be administered shortly following a stroke to be efficacious. Furthermore, t-PA may cause damage to neurons and about one third of arteries re-occlude following treatment. Of interest, APC has been shown to protect neurons from t-PA induced damage by inhibiting the activation of caspase 8 (233).

APC reduces intestinal ischemia/reperfusion injury in rats (234). Administration of APC reduces IL-6 plasma levels and decreases thrombin generation and fibrin degradation deposits following superior mesenteric artery occlusion. APC can also reduce ischemia/reperfusion-induced renal injury in rats (235). Renal levels of inflammatory cytokines including IL-8 and TNF-alpha were decreased following APC administration. Because leukocytopenia also reduced these cytokines, the effect of APC was attributed to the inhibition of leukocyte activation rather than inhibiting coagulation. Decreased leukocyte activation may also be the mechanism by which APC attenuates endotoxin-induced pulmonary vascular injury in a rat model (236). These in vivo results are consistent with in vitro data, which demonstrate that APC, in conjunction with EPCR, suppresses the expression of cell adhesion molecules on endothelial cell surfaces (46-47). APC can also reduce spinal cord injury induced by trauma or ischemia (237-238). APC has been shown to induce vascularization, which may be beneficial in ischemic conditions. It induced the formation of new blood vessels in an in vivo rat cornea model and the increase in endothelial cell proliferation may result from activation of the mitogen-activated protein kinase pathway (239).

6.2. Lung injury

Low protein C levels are associated with worse clinical outcomes in patients with acute lung injury and acute respiratory distress syndrome (240). APC also inhibits the inflammatory responses in asthma and reduces lung injury induced by acid aspiration and smoke inhalation (241-242). In addition, APC has been shown to inhibit endotoxin-induced pulmonary vascular injury in rats by inhibiting neutrophil activation via inhibition of TNF-alpha production (243). In humans, APC reduced endobronchial, endotoxin-induced pulmonary inflammation via inhibition of neutrophil chemotaxis (244). APC appears to protect the lung from damage by inhibiting the expression of cytokines reducing the quantity of leukocytes and neutrophils that accumulate in airspaces and decreasing PAI-1 activity (241, 244-246).

6.3. Other disorders

By reducing proinflammatory cytokine release, APC preserves the function of islet cells following transplantation in diabetic mice (247). The APC treated mice exhibited better glucose control, higher glucose disposal rates and higher arginine-stimulated acute insulin release. The beneficial effects of APC observed in these mice suggest that APC may enhance the therapeutic efficacy of pancreatic islet cell transplantation in diabetic patients.

APC reduces stress-induced gastric mucosal injury via attenuation of activated-neutrophil-induced endothelial cell injury (248). In an animal model of endotoxemia, APC has been shown to improve capillary perfusion and provide protection from LPS-mediated microcirculatory dysfunction (122). APC has shown efficacy in some patients with cancer and placental abruption and it demonstrated favorable results in a phase III DIC study (249-252). In addition, APC has also been used in the treatment of veno-occlusive disease of the liver after bone marrow transplantation and as an adjunctive therapy to thrombolytics in patients with acute myocardial infarction (70, 253-256). Low levels of APC are often observed in patients that do not survive severe necrotizing pancreatitis and decreased thrombomodulin and EPCR expression has been observed in diabetic rats and in atherosclerotic lesions and vasculitities in humans (257-259).

7. FUTURE STUDIES

As indicated above, the protein C pathway appears to play an important regulatory role in many disorders characterized by endothelial dysfunction and activation. While recombinant drotrecogin alpha (activated) is currently used to treat severely septic patients that are at a high risk of death, the preclinical studies outlined above suggest that further clinical work may explore the utility of this protein for the treatment of ischemic tissue damage in disorders such as stroke, cardiogenic shock, transplantation, acute lung injury and acute renal failure. Drotrecogin alfa (activated) decreases the mortality rate in severe sepsis, but not all patients respond to therapy. Basic studies are progressing to determine protein markers that can be used to predict which patients will have a poor outcome and to optimize the drotrecogin alfa (activated) treatment. Analyses of plasma samples collected from 78 septic
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patients demonstrated that antithrombin and protein C were the most reliable markers that were associated with organ dysfunction and data from a rat model of sepsis suggested that in addition to low protein C, increased levels of macrophage inflammatory protein-2 and the cytokine KC precede poor outcome (260-261). Moreover, a retrospective evaluation of the placebo arm from the PROWESS clinical trial indicated that severe protein C deficiency was associated with early death resulting from refractory shock and multiple organ failure in sepsis (18, 225). Also, a bi-allelic polymorphism located in the promoter of the TNF-alpha gene, which influences its plasma levels, is associated with outcome in patients with severe sepsis (262). These and other future prospective studies hold promise to improve the identification and treatment of severe sepsis patients.

8. SUMMARY AND PERSPECTIVE

The antithrombotic role of APC is well established but recent data suggests that APC can modulate the fibrinolytic and inflammatory pathways, which become deregulated during sepsis. APC has an indirect effect on inflammation because it limits thrombin generation, but it also has a direct effect on inflammation. Many of these effects can be attributed to the signaling effects of APC/EPCR, which suppresses proinflammatory signaling, inhibits the expression of cytokines and adhesion molecules, decreases adhesion, activation and extravasation of leukocytes and modulates the apoptotic pathway. During sepsis, the excessive activation of the inflammation and coagulation pathways damages the endothelium and decreases the functions of the protein C pathway, which can ultimately result in vascular dysfunction, end organ failure and death. Drotrecogin alfa (activated) or rhAPC improves organ function and decreases mortality in patients with severe sepsis. The exact mechanism by which drotrecogin alfa (activated) improves survival is currently uncertain but is most likely attributed to its ability to modulate multiple pathways. Ongoing studies have been designed to elucidate the mechanism of APC function and to optimize its use as a therapy for severe sepsis patients. Because of its ability to modulate multiple pathways, drotrecogin alfa (activated) may be useful in treating other acute critical coagulation/inflammation disorders.

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Abbreviations: APC: activated protein C; EPCR: endothelial cell protein C receptor; PAR: protease activated receptor; rhAPC: recombinant human activated protein C; hPC: human protein C; t-PA: tissue plasminogen activator; TAFI: thrombin activatable fibrinolysis inhibitor; LPS: lipopolysaccharide; TNF-alpha: tumor necrosis factor-alpha; MIF: migration inhibitory factor; ICAM: intracellular adhesion molecule; VCAM: vascular adhesion molecule; IL: interleukin; DIC: disseminated intravascular coagulopathy; Edg-1: sphingosine 1-phosphate receptor-1

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