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New Potential Therapeutic Modalities: aPC

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Abstract. **The protein C anticoagulant pathway provides an "on demand" anticoagulant response whenever thrombin is generated. Activated protein C appears to possess both anticoagulant and anti-inflammatory properties. The function of the pathway is impaired in sepsis, including the consumption of protein C and protein S, two plasma components of the pathway. Supplementation with protein C or activated protein C has been shown to inhibit the disseminated intravascular coagulation (DIC) response in experimental animals and patients. Preliminary clinical results suggest that supplementation with components of the pathway may be useful in some patients with sepsis or septic shock.**

Keywords. **protein C, thrombin, disseminated intravascular coagulation, shock, sepsis, thrombomodulin, protein S**

Introduction

The protein C anticoagulant pathway has several components within it that could, based on both experimental results and theoretical considerations, be candidates for the treatment of certain forms of septic shock. The basis for this assertion lies upon three central observations that will be developed further in this manuscript: (1) the protein C pathway can be downregulated or components of the pathway can be consumed by acute inflammatory responses; (2) the pathway plays a major role in regulating blood clotting, especially in the microcirculation; and (3) supplementation with protein C (APC) and some other components of the pathway have been shown to block the coagulation and some of the inflammatory responses that result from bacterial/endotoxin challenge. Thus it follows that supplementation with components of the pathway that could help regulate microvascular coagulation and some of the inflammatory responses might be beneficial in the treatment of at least some forms of sepsis. Before dealing with the biology of this system, it is useful to review briefly the special properties of the system that make it a candidate for therapeutic intervention. For those desiring more information about the clinical or basic aspects of the pathway, there have been a number of reviews written by this author [1–3] or others [4–20].

Biochemical Description of the Protein C Anticoagulant Pathway

The protein C anticoagulant pathway is represented in a highly simplified fashion in Fig. 1. The unique feature of the protein C pathway is its ability to generate an anticoagulant response that is proportional to the thrombotic stimulus [21]. This feature of the pathway is due to the mechanism of protein C activation. Protein C circulates as an inactive precursor. It is converted rapidly to the serine protease, APC, by a complex between thrombin and thrombomodulin (TM). Thus, the anticoagulant response occurs whenever thrombin generation occurs and remains until thrombin generation is controlled. TM is present primarily on the surface of endothelial cells [22,23] and to a lesser extent on several other cell types including monocytes [24,25]. An endothelial cell protein C receptor, EPCR [26], binds both protein C and APC. Binding protein C augments activation by the thrombin-TM complex by increasing the affinity of the complex for protein C [27–29]. Unlike TM, EPCR expression is restricted primarily to endothelium of the larger vessels [22,29]. In addition to accelerating protein C activation, thrombin interaction with TM blocks most of thrombin's procoagulant functions [30] and results in more rapid inhibition of thrombin by antithrombin [15] and by the protein C inhibitor [31]. Thus, thrombin binding to TM not only accelerates protein C activation, but aids in the inhibition of thrombin. Once APC is generated, it binds to protein S and this complex inactivates factors Va and VIIIa. Curiously, factor V, but not factor Va, can serve as a cofactor to enhance the inactivation of factor VIIIa by protein S and APC [32]. A common dimorphin exists in human factor V of Caucasians that results in a substitution of Arg with Gln at one of the APC cleavage sites [9,33,34]. This form of factor V is often called Factor V Leiden for the city in which the mutation was characterized [35]. This mutation causes APC resis-

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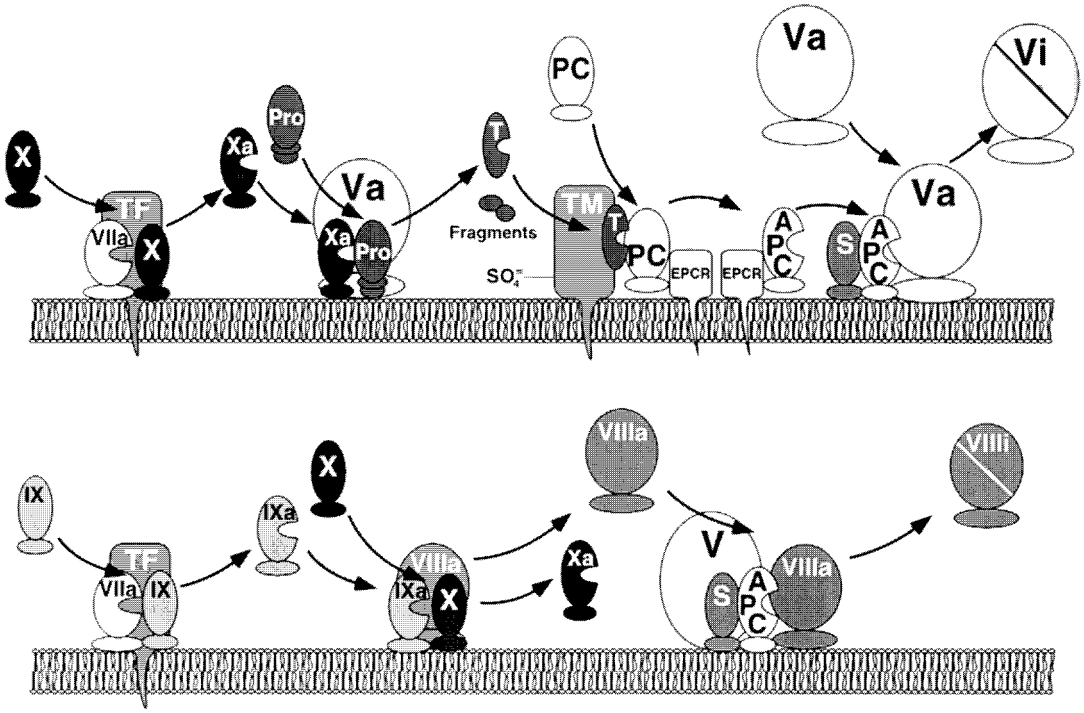

Fig. 1. *The function of membranes and cofactors in blood coagulation. The enzymes associate with cofactors on membrane surfaces. Factor VIIa (VIIa) associates with tissue factor (TF) to activate either factor X (X) or factor IX (IX). Factor IXa (IXa) associates with factor VIIIa (VIIa) to activate factor X. Factor Va (Va) associates with factor Xa (Xa) to activate prothrombin (pro). Thrombin associates with TM to activate protein C, a process which is enhanced on some blood vessels by the presence of the endothelial cell protein C receptor, EPCR. Once activated protein C is formed it dissociates relatively slowly from EPCR to interact with protein S (S) to inactivate factors Va and VIIIa, thereby blocking the coagulation cascade. Factor V (V) serves as a cofactor for the inactivation of factor VIII. See the text for discussions of mechanism. Prothrombin activation probably occurs primarily on platelet surfaces, factor X and IX activation on monocytes and protein C activation on endothelium. (Modified from Esmon, CT: Cell mediated events that control blood coagulation and vascular injury: Annu Rev Cell Biol 1993; 9:1. Copyright © 1993 Annual Review Inc.)*

tance (i.e., a reduced anticoagulant response to APC). In addition, the factor V Leiden does not possess the APC cofactor activity in factor VIII inactivation that is characteristic of normal factor V.

Unlike most serine proteases which are inactivated very rapidly in the circulation with half-lives less than one minute, APC has a relatively long half-life, estimated at approximately 15 minutes [36–40]. Inactivation is mediated by α_1-antitrypsin, protein C inhibitor and α_2-macroglobulin [41–43]. Since α_1-antitrypsin is an acute phase protein and one of the major inhibitors of APC function, the increases in this inhibitor's concentration in response to inflammation would have the effect of dampening the protein C pathway and hence favoring the coagulation response.

Almost all of the coagulation related activities of the protein C anticoagulant pathway are antithrombotic in nature. Three apparent exceptions exist. TM has recently been shown to augment substantially the activation of a procarboxypeptidase B [44]. This enzyme, sometimes referred to as thrombin activatable fibrinolysis inhibitor (TAFI), inhibits fibrinolysis by removing terminal lysine residues on fibrin. Given, however, that this enzyme removes terminal Arg residues, it is possible, or even likely, that one of its key functions is to inactivate vasoactive substances like C5a which require their terminal Arg to function. If the latter hypothesis is correct, then the TM-thrombin activation of TAFI would be entirely consistent with the anticoagulant/anti-inflammatory functions of the protein C pathway.

TM-thrombin complexes can also stimulate the activation of factor XI somewhat (about 20 fold). This simulation requires the presence of a covalently attached chondroitin sulfate on TM [15]. TM has also been shown to accelerate the inhibition of prourokinase by thrombin [45,46]. This would not only impair fibrinolysis to some extent, but would decrease the ability of the cellular urokinase receptor to activate plasminogen that results in degradation of matrix proteins and thereby decreases cell-cell contacts. *In vivo* studies have demonstrated that the influence of infusion of soluble TM favors a antithrombotic/anti-inflammatory effect very strongly [47–53] indicating

that the dominant function of TM is to suppress these responses.

Congenital and Experimentally Induced Deficiencies of Components of the Protein C Pathway Provide Insights into its Potential Function in Sepsis

Total congenital deficiency of protein C and protein S are associated with neonatal purpura fulminans [54–58]. Visually, these lesions appear similar to the petichiae that form in some cases of severe septic shock. In the case of congenital protein C deficiency [59], these lesions are completely reversed by infusion of protein C indicating that the deficiency is responsible for formation of the lesions and suggesting that acquired protein C deficiency as well as congenital deficiency might predispose to microvascular thrombosis. An example where this seems to be the case is in Warfarin induced skin necrosis. Patients heterozygous for protein C deficiency appear to be at greater risk of skin necrosis [60,61]. As was the case with the homozygous protein C deficient patients, these Warfarin induced skin necrosis patients appear to be treated effectively by protein C supplementation [62,63]. These clinical observations suggest that the acquired deficiency of protein C and the decreased levels of protein S [64–66] observed in many patients with septic shock could contribute to the disseminated intravascular coagulation (DIC) and possibly endothelial cell dysfunction that contributes to the disease morbidity and mortality. This concept is supported further by the observation that a combination of inflammatory cytokines with procoagulant lipids only induces DIC and thrombosis effectively in otherwise healthy animals if the protein C pathway is impaired [67].

A critical role for TM is suggested by gene deletion in the mouse which results in early embryonic lethality [68]. Functional mutations of TM in mice increase thrombotic risk especially when the animals are challenged, for instance with hypoxia [69]. Furthermore, TM mutations have been identified in patients with thrombosis [70], and mutations in the TM gene appear to co-segregate with thrombotic disease in families [71]. Defects in TM have been associated with increased risk of heart attack [71–73]. In experimental settings, inhibition of TM with antibodies increases thrombin induced pulmonary embolism in mice and infusion of soluble TM is protective [74].

At present, there are no published reports of EPCR deficiencies or the influence of blocking EPCR on the pathogenesis of sepsis or thrombosis. Given its important role in regulating protein C activation and function, it is likely that defects in EPCR, whether congenital or acquired, would contribute to a hypercoaguable state.

The Impact of Inflammation on the Protein C Pathway

Several lines of evidence suggest that the protein C anticoagulant pathway function may be down-regulated in disease states. Our current view of the protein C system in normal (Fig. 2A) and inflammatory situations (Fig. 2B) is depicted in these figures. In addition to free protein S depicted in Fig. 1, about half of plasma protein S is complexed with high affinity to C4 binding protein, a regulatory protein of the complement system [75]. Only the free form of protein S has APC cofactor activity [76,77]. Inflammation can reduce free protein S levels as well as reducing TM expression on the endothelium and recruiting leukocytes to the vessel wall where they can inhibit the system by releasing proteases that cleave TM or by releasing inflammatory cytokines that can down-regulate TM and possibly EPCR expression. On the coagulation side, complement activation, particularly generation of complement C5b9 generation, can promote amplification of coagulation by releasing cellular microparticles that provide a procoagulant membrane surface that can amplify the clotting response [78]. Finally, endotoxin and inflammatory cytokines can lead to expression of monocyte tissue factor [79–82]. The vascular damage, increase in procoagulant substances, and decrease in anticoagulant pathway function probably provide insights into the basis for the association between inflammation and thrombosis.

Many of the disease processes associated with thrombotic disease have inflammatory components. Based on *in vitro* data, endotoxin [83] and the inflammatory cytokines (tumor necrosis factor α (TNF) or interleukin-1) can down-regulate TM [84,85] and EPCR [26] expression on endothelial cells in culture. TM levels have been observed to decrease *in vivo* in some instances, such as allograft rejection [86], certain autoimmune diseases like Wegener's granulomatosis [87], and villitis [88]. These may be more complex situations than acute inflammatory injury caused by endotoxin shock. In rat kidney, TM antigen and activity was not altered in kidneys infarcted with thrombi [89]. Based on qualitative immunohistochemical analysis, baboon endothelial cell TM did not appear to be reduced in response to *E. coli* infusion [90]. TM down regulation can be prevented by many factors including interleukin-4 [91] and retinoic acid [92,93] making very incomplete our understanding of the pathophysiologic conditions under which this potentially important phenomenon may occur.

Systemic assays for the presence of TM degradation products would favor the concept that TM is down-regulated by inflammatory processes. Specifically, many inflammatory human diseases, including septic shock, result in large increases in circulating plasma TM levels [87,94–99]. This increase probably results from neutrophil elastase mediated proteolytic cleavage

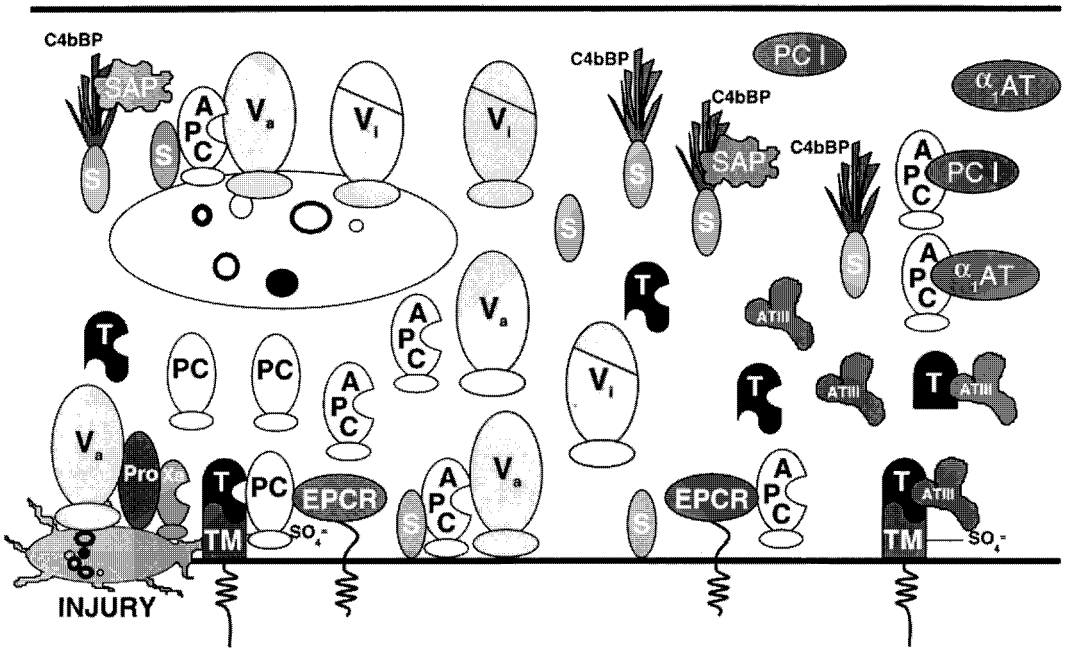

Fig. 2. *Control of coagulation under normal vs inflammatory stituations.* **A:** *The protein C anticoagulant pathway under normal conditions. A wound initiates prothrombin (Pro) activation that results in thrombin (T) formation. Prothrombin activation occurs when factor Va (Va) and factor Xa (Xa) bind on membrane surfaces. Thrombin then binds to thrombomodulin (TM) on the lumen of the endothelium, illustrated by the heavy line, and the thrombin- TM complex converts protein C (PC) to activated protein C (APC). Thrombin bound to TM can be inactivated very rapidly by antithrombin III (ATIII) after which the thrombin-antithrombin III complex rapidly dissociates from TM. Activated protein C (APC) then binds to protein S (S) on cellular surfaces. The activated protein C-protein S complex then converts factor Va to an inactive complex (Vi), illustrated by the slash through the larger part of the two-subunit factor Va molecule. Protein C and activated protein C (APC) interact with an endothelial cell protein C receptor (EPCR). This facilitates protein C activation and will concentrate APC on the endothelial surface. Protein S circulates in complex with C4bBP, which may in turn bind serum amyloid P (SAP). APC is inhibited by forming complexes with either the protein C inhibitor (PCI), a_1-antitrypsin (a_1AT) or a_2-macroglobulin (not shown). See text for a more complete discussion. (Modified from Esmon CT: The protein C anticoagulant pathway. Arterioscl Thromb 1992−(2):135. Copyright © 1992 American Heart Association.)* **B:** *The protein C pathway after inflammation. Inflammatory mediators lead to the disappearance of thrombomodulin from the endothelial cell surface. Endothelial cell leukocyte adhesion molecules, P-selectin or E-selectin are synthesized or expressed on endothelial or platelet surfaces. Tissue factor (TF) is expressed on monocytes and binds factor VIIa (VIIa), and this complex converts factor X(X) to factor Xa(Xa), which forms complexes with factor Va(Va) to generate thrombin (T) from prothrombin (Pro). Because little activated protein C (APC) is formed and the little that forms does not function well because of low protein S (S), factor Va is not inactivated and prothrombin activation complexes are more stable. Elevation in circulating C4bBP concentration and/or decreased in free protein S results in little free protein S. See text for discussion., SAP, serum amyloid P. (Modified from Esmon CT: The protein C anticoagulant pathway. Arterioscl Thromb 1992; 12(2):135. Copyright © 1992 American Heart Association.)*

of endothelial cell-associated TM [100]. TM activity can be inhibited by eosinophil release products, major basic protein in particular [101], thereby providing an additional mechanism by which inflammation can inhibit the anticoagulant function of the pathway.

Protein S levels decrease in DIC and autoimmune disease. This decrease may be due to changes in synthesis rate, increased binding to C4bBP, or to proteolytic degradation of protein S [66,86,102–113]. In some forms of septic shock, meningococcemia in particular, protein C consumption correlates well with the onset of necrotic skin lesions (purpura fulminans) and a negative clinical outcome [64,114].

Taken together, the clinical manifestations of severe microvascular thrombosis prominent in deficiencies in the protein C pathway, the ability to correct this microvascular thrombosis promptly by replacement therapy and the observation that the pathway is down-regulated by inflammatory mediators, proteolytic inactivation or consumption of components of the pathway in acute inflammatory situations provide a rationale for the use of protein C, protein S and perhaps TM in the treatment of acute systemic inflammatory diseases including sepsis.

APC Resistance and Factor V Leiden

A potential complication in application of this system to the treatment of sepsis is that there is a common

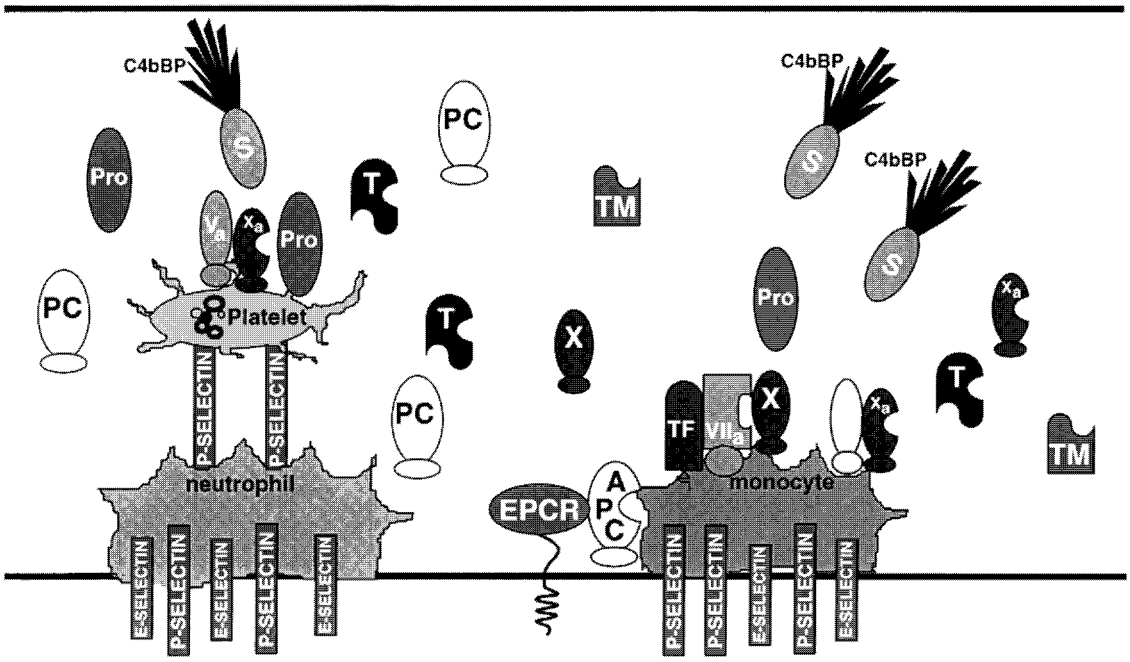

Fig. 2 continued.

factor V variant (Factor V Leiden) that is resistant to proteolytic inactivation by APC [4–10]. This variant results in a complication commonly referred to as APC resistance and is caused by a substitution of Gln at residue 506 in factor V. This corresponds to the first cleavage site in factor Va [115–117]. Applications of the protein C system to the treatment of sepsis may be complicated by this variant that is common in Caucasians (≈5%) [118]. The variant is rare in Black and Oriental populations. Patients with the variant could theoretically be less responsive to APC therapy. This is particularly true since inactivation of factor V Leiden is much more dependent on protein S than normal factor V [117].

Thrombomodulin as an Agent in the Treatment of Sepsis

The ability of TM to block fibrinogen clotting and cell activation, activate protein C, and promote thrombin inhibition suggests that TM could be useful as a therapeutic agent in sepsis and DIC. The most likely candidates are forms truncated above the membrane spanning region [30] which are very soluble proteins. TM exists in two forms, with and without a covalently attached chondroitin sulfate. Soluble TM performs all of the above functions if it contains the chondroitin sulfate moiety, but is less effective in blocking fibrinogen and platelet activation and in promoting thrombin inhibition by plasma proteinase inhibitors when it lacks this moiety.

In addition to its multiple anticoagulant effects, the potential utility of TM as a soluble antithrombotic is suggested by the observation that TM activity is likely reduced in many inflammatory diseases associated with DIC/sepsis (see above), and hence, replacement therapy would target the acquired deficiency state potentially offering optimal response with minimal risk. The author is unaware of published data with TM in humans at this time, but several reports from animal model studies of DIC/sepsis have been published. For instance, in rats soluble TM can block tissue factor- [49] or endotoxin- [48] induced DIC and can block the pulmonary vascular injury that results from endotoxin exposure [50]. These properties appear to be selective for the protein C pathway since the potent and specific antithrombotic agent, active site- blocked factor Xa [119] blocks the coagulation response without protecting from lung injury [50]. APC can also block lung injury in this model [120], suggesting that the TM effect is the result of increased APC formation.

TM with or without the chondroitin sulfate was shown to be effective in the tissue factor mediated DIC model [49]. The TM containing chondroitin sulfate was more effective on a mass basis, but was also cleared from the circulation faster, $T_{1/2}$ = 20 min vs 1 h. TM appeared to have less effect on bleeding time than heparin, a feature that is likely to be beneficial in sepsis/DIC cases. When expressed as the concentration required to double the bleeding time vs the concentra-

tion required to block the decrease in platelet count 50%, TM with and without chondroitin sulfate were respectively about 3 and 2 fold better than standard heparin.

Protein C as an Agent in the Treatment of DIC/Sepsis

The initial clinical information about the use of protein C in the treatment of thrombotic disease in humans came from the treatment of congenital deficiencies which are often manifested by microvascular thrombosis of the skin capillaries (purpura fulminans) [55,59,121,122]. Replacement therapy with protein C has been shown to prevent further progression of these lesions which subsequently healed rapidly [123,124] but these lesions are not prevented by heparinization [56].

DIC and septic shock are additional situations in which microvascular thrombosis can occur. This is particularly common in meningococcemia [64,125]. Protein C consumption in humans with meningococcemia correlates better with the formation of the purpura like lesions and death than other markers examined [64,114]. Of course, this correlation between protein C levels and disease progression or outcome does not prove that the decrease in protein C levels is causally related to the clinical progression of the disease. The earliest observations that suggested a causal relationship came for the observation that thrombin infusion into dogs that were subsequently challenged with lethal concentration of *E. coli* resulted in survival and prevention of DIC [126]. Thrombin infusion under these conditions leads to systemic protein C activation *in vivo* [36]. Therefore, to test whether the APC so generated could protect from sepsis, lethal *E. coli* concentrations were injected in baboons with or without APC. The animals infused with APC were protected from death, DIC and organ dysfunction [127]. Blocking the protein C pathway made these primates hyper-responsive to sublethal concentrations of *E. coli* [127,128]. Blocking the pathway increased the DIC response to the sublethal *E. coli* as expected, but it also increased the circulating TNF α levels compared to control animals given the same dose of *E. coli.* Restoration of the protein C system prevented the DIC, organ damage, and elaboration of elevated cytokine levels. Taken together, these results suggest that protein C is a major regulator of microvascular thrombosis and that the system modulates the inflammatory response by as yet unknown mechanisms. The links presented above between protein C deficiency and microvascular thrombosis provided a rationale for the use of protein C in the prevention of some complications of septic shock. Treatment with protein C of severely ill patients with relatively advanced septic shock, usually due to meningococcemia, have been reported [1,129,130]. In general, protein C infusion has been associated with normalization of circulating protein C levels and reversal of organ dysfunction including a rapid regain of consciousness and kidney function [129,130] and reviewed in [1]. In addition to meningococcemia, one patient with a group A β-hemolytic streptococcal infection and varicella developed septic shock (DIC) had undetectable protein C levels probably due to consumption, and purpura [131]. His condition improved rapidly following protein C supplementation. Most recently, the results of phase 2 studies of severe septic patients were reported using APC [132,133]. At the highest dosages used (24 or 30 μg/kg/hr), a 40% reduction in 30 day mortality was observed (21% vs 35% and a trend toward decreased time on the ventilator, in the ICU and in the hospital). The low numbers of patients studied (131 total, 41 control, 51 low dose and 39 high dose) resulted in a P value for significance of only 0.21. Consistent with reported anti-inflammatory events, circulating IL-6 levels in these patients were decreased compared to placebo ($P = 0.05$) [132]. Thus, although the rationale and preliminary anecdotal clinical results appear promising, a larger clinical trial will be needed to verify the validity of this approach.

Several mechanisms, in addition to inhibition of thrombin formation, have been identified that might contribute to useful effects of protein C in the treatment of sepsis. APC has been reported to inhibit tumor necrosis factor elaboration by monocytes *in vitro* [134], to prevent interferon gamma mediated Ca^{2+} transients and cellular proliferation [135], and to bind to the monocyte cell surface apparently through an as yet uncharacterized cell surface receptor. In addition, protein C has also been reported to inhibit leukocyte adhesion to selectins [136]. EPCR on the endothelium is structurally related to the major histocompatibility (MHC) class 1 molecules, a class of molecules involved in inflammation. EPCR is regulated by inflammatory cytokines suggesting the possible, but unproven, role of this receptor in control of inflammatory processes [26]. It remains unclear what the relative contribution of the anti-inflammatory activities vs anticoagulant activities of this system are to the host response against sepsis.

Protein S as an Agent in the Treatment of DIC/Sepsis

Protein S has not been studied extensively as a therapeutic agent. Protein S deficiency has been described in patients with Warfarin induced skin necrosis [137] and patients with homozygous protein S deficiency may develop purpura fulminans [58] indicating that the protein plays a critical role in preventing microvascular thrombosis. Free and total protein S levels are often low is septic shock or following thrombosis [66,110,138]. Therefore, it is reasonable to infer that

protein S supplementation might be helpful in patients with acquired deficiencies of protein S.

In one study of sepsis, a baboon model of *E. coli* induced septic shock was employed. Protein S cofactor activity for APC was inhibited by infusion of excess human C4BP to complex the free protein S. This exacerbated the response to sublethal levels of E. coli resulting in death. In this model, when protein S levels are normal, infusion of 10% of the lethal concentration of *E. coli* results only in an acute phase response. However, when free protein S is reduced by infusion of C4bBP or an antibody to protein S, the same dose of bacteria leads to death, organ failure, and either DIC or microvascular thrombosis [128]. Protein S supplementation protects the latter group of animals from death and DIC. While protein S levels have been observed to decrease in septic shock, the preliminary clinical experience with protein C infusion in septic shock patients suggests that it is effective without simultaneous protein S supplementation. Whether protein S supplementation would improve the efficacy of protein C or whether it may be required in some patients for protein C to be effective may be clarified during the clinical trials in progress.

Conclusion

The protein C system has all of the hallmarks of being at an interface between inflammation and coagulation apparently serving a negative regulatory process in both systems. Especially because this key regulatory system often becomes impaired in severe systemic inflammatory situations like sepsis, it follows that a return of the system to normal status might be beneficial. This concept appears to be borne out in experimental sepsis models which have shown protective effects by supplementation of components of the system and deleterious effects by inhibition of the system. The preliminary clinical results appear promising with several clinical reports of rapid improvement in gravely ill patients following protein C or APC infusion. Whether this potential clinical benefit will be confirmed in larger trials remains to be determined.

With respect to protein C therapy, there are a number of important issues that remain unresolved. For instance, it is unclear whether there are patients who would benefit more from protein C than APC infusion. In those patients with ongoing coagulopathies, protein C is rapidly activated *in vivo*. The potential advantages of protein C over APC is that the highest levels of APC would be generated at the sites of greatest thrombin generation and in the microcirculation where it might be most efficacious. In addition, protein C has the theoretical advantage of being self regulated. Once thrombin formation is halted, protein C activation ceases and the anticoagulant response slowly disappears unless another wave of thrombin generation begins. These features might help prevent excess anticoagulation and provide a safety margin not present with APC. From the available data, protein C may have been more effective clinically than APC, but it is important to note that protein C has been used most often with meningococcemia patients whereas APC has been used in "severe sepsis" patients in general. If one assumes that APC has protective anti-inflammatory activities as suggested from the *in vitro* studies, APC might prove effective in patients even without overt DIC. In these patients, it is unlikely that protein C supplementation would be beneficial since little of the added protein C would be activated. Hopefully the clinical trials now in progress will provide answers to these unresolved questions. Ultimately, it will be important to know which, if any, therapeutic agents work effectively in combination with components of the protein C anticoagulant pathway.

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