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REVIEW

Protein C anticoagulant system—anti-inflammatory effects

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Abstract Activated protein C (APC) plays active roles in preventing progression of a number of disease processes. These include thrombosis due to its direct anticoagulant activity which is likely augmented by its cytoprotective activity, thereby limiting exposure of procoagulant cellular membrane surfaces on cells. Beyond that, the pathway signals the cells to prevent apoptosis, to dampen inflammation, to increase endothelial barrier function, and to selectively downregulate some genes implicated in disease progression. Most of these functions are manifested to APC binding to endothelial protein C receptor (EPCR) allowing PAR1 activation, but activation of other PARS is also implicated in some cases. In addition to EPCR orchestrating these changes, CD11b is also capable of supporting APC signaling. Selective control of these pathways offers potential in new therapeutic approaches to disease.

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History

The protein C system is best known for its anticoagulant activity seen most clearly in the clinical observation that patients born with a total protein C deficiency exhibit massive neonatal thrombosis that is usually lethal unless treated, reviewed in [1]. Indeed this is also one aspect of the anti-inflammatory functions of the pathway since coagulation, particularly thrombin generation, can trigger a wide variety of pro-inflammatory events including expression of adhesion molecules like P-selectin and activating the Nf-κB pathway [2]. While this is an important aspect of the anti-inflammatory function of the pathway, it does not distinguish the pathway from other anticoagulants. Indeed, heparin has long been noted to have apparent anti-inflammatory functions, in part likely due to its anticoagulant activity.

Some of the first suggestions that this pathway might have additional anti-inflammatory activity came from the treatment of newborns with protein C deficiency. The thrombotic lesions that developed in the newborns were surrounded by an intense red area that retracted rapidly following the administration of protein C, suggesting that protein C was preventing the inflammation in addition to decreasing the thrombosis, reviewed in [1].

These studies were followed by examination of the roles of thrombosis in sepsis. In an early study, Hinshaw and colleagues [3] observed that heparin could prevent the consumptive coagulopathy associated with *Escherichia coli*-induced sepsis in baboons but did not rescue the animals. Later we demonstrated that an active site blocked

form of factor Xa could prevent the disseminated intravascular coagulation (DIC) but again failed to protect against sepsis [4]. Subsequently, Hinshaw and colleagues showed that extracorporeal perfusion without exogenous anticoagulation was protective against endotoxin-induced sepsis [5]. They also observed that an associated anticoagulant was being generated during these studies. Interestingly, the pump could be removed subsequently and the animals were still protected from subsequent bacterial challenge. With the identification of thrombomodulin [6] and demonstration of thrombin-dependent protein C activation in vivo [7], it was possible to test whether the anticoagulant might be activated protein C (APC) generated by thrombin formed by the pump. Indeed, thrombin infusion into dogs challenged with endotoxin was protective [8] despite the fact that the animals would develop DIC without the thrombin infusion. Thrombin infusion decreased both the DIC and inflammation. With the advent of a rapid means for purification of protein C from human plasma [9], it was possible to test the ability of APC to protect baboons from *E. coli*-induced sepsis. When APC was administered with the *E. coli*, the animals survived a normally lethal dose and exhibited reduced coagulation, protection from shock, and decreased inflammation [10]. These older studies highlight that APC could protect against an inflammation-induced disease like sepsis when other comparable anticoagulants could not. In contrast, inhibition of the pathway in the *E. coli* sepsis model, in this case with C4 binding protein, elevated cytokine production in response to *E. coli* challenge [11].

Either reducing protein C levels [12, 13] in mice or blocking protein C activation [10] in baboons increased a sublethal to a lethal challenge with bacteria or endotoxin. In order to perform its full anti-inflammatory functions, the APC must bind to the endothelial protein C receptor (EPCR) [14]. Mice overexpressing EPCR are resistant to endotoxemia [15], whereas those with low-level expression are sensitized [16, 17]. Furthermore, mice with low levels of EPCR have cardiac dysfunction from the challenge [16]. These studies illustrate the important role of the pathway in regulating the host response to acute inflammatory challenges.

How does activated protein C influence inflammation directly?

One of the major mechanisms that augment inflammation is mediated through Nf-κB activation and nuclear translocation from the cytosol [18, 19]. This turns on synthesis of a variety of inflammatory mediators including cytokine production. APC can decrease the synthesis of Nf-κB components [19, 20] and decrease Nf-κB nuclear translocation [18]. Together these activities probably constitute the major mechanisms by which APC downregulates inflammatory cytokine production in inflamed endothelium in culture [21] and in animal models of sepsis [22, 23].

APC signaling

These effects are dependent on APC, EPCR, and protease-activated receptor 1 (PAR-1) [14, 24]. Activation of PAR-1 by the APC–PAR-1 complex leads to different cellular signaling than when thrombin activates PAR-1 despite cleaving the same site on the receptor [25] (Fig. 1). The mechanisms for this change in signaling are currently being elucidated. In one model, protein C binding to EPCR leads to migration of EPCR out of the lipid rafts at which time it interacts with PAR-1 coupled to a different G protein than when it was in the lipid rafts, thus resulting in the altered signaling profile [26–28]. In support of this model, EPCR did appear to migrate from rafts in the presence of protein C [26] and recombinant mutant molecules containing the protein C Gla domain that could elicit signaling similar to that of APC [26].

Inhibition of leukocyte adhesion

Leukocyte adhesion and trafficking APC reduces leukocyte adhesion and activation and protects capillary function in endotoxemia [22, 29–31] in part by reducing chemotaxis [32] and cytokine production [23]. This inhibition of leukocyte attachment could be mediated by decreases in thrombin-dependent mobilization of selectins from Weible Paladi bodies in the endothelium, suppression of ICAM, synthesis, and decreased synthesis of monocyte chemotactic protein-1 [21]. In central venous sinus thrombosis, APC

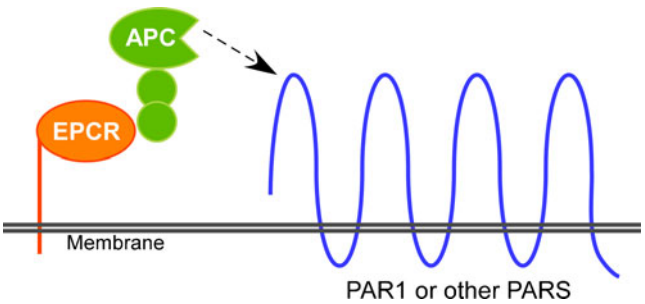

Fig. 1 Cytoprotective signaling by APC. APC binds to EPCR at which time it cleaves PAR1 to generate the active signaling molecule. The APC cleaved PAR1 appears to be linked to a G protein that generates cytoprotective functions—see text for discussion. *APC* activated protein C, *EPCR* endothelial cell protein C receptor, *PAR1* protease-activated receptor-1

decreases inflammatory cell recruitment and protects the microvasculature in this manner [33].

Endothelial barrier function

Endothelial barrier function is compromised in a number of diseases resulting in edema. Thrombin is known to decrease endothelial barrier function, a process that is reversed by APC [26, 34, 35]. APC accomplishes this, at least in part, through the generation of shingosine 1-phosphate receptor transactivation [34, 35]. Improving endothelial barrier function is likely to provide anti-inflammatory effects since it should reduce leukocyte trafficking into the extravascular space. While not directly related to inflammation, one of the features of APC is that it diminishes both endothelial cell and neuronal apoptosis [36–38]. Excessive apoptosis or cellular necrosis leads to release of relatively large amounts of nuclear material in the form of nucleosomes and also the release of mitochondrial contents. Both of these events will trigger inflammation. The histones on nucleosomes induce leukocyte migration into the tissue, platelet activation, and thrombosis [39] and induce cytokine formation, and the mitochondria induce leukocyte activation [40].

Histone neutralization

Extracellular histones are cytotoxic [39], and APC can cleave and neutralize this activity of histones. The importance of the latter observation was apparent in studies that demonstrated that histones were much more toxic in mice where the protein C pathway was blocked and that blocking histone function was protective in endotoxemia [39].

Signaling is required for APC protection in sepsis

Mutants of APC have been developed that retain signaling activity but have very low anticoagulant activity [41]. These mutant forms of APC (5A- aPC and other similar mutants) were effective in preventing mortality in mouse models of sepsis [24, 42].

The original signaling studies were done in endothelium [20]. More recent studies have detected EPCR on leukocytes, particularly CD8+ dendritic cells [43]. Mice with low levels of EPCR (EPCR low) were studied and were less effectively protected from endotoxin-induced sepsis toxicity by 5A aPC than wild-type mice [43]. When bone splenic $CD11c^{hi}$ dendritic cells from wild-type mice were transplanted into EPCR-low mice, they supported protection from endotoxin by 5A- aPC whereas similar cells from EPCR-low mice did not. In vitro, 5A-aPC inhibited the inflammatory response of dendritic cells which appeared to be independent of a requirement for normal levels of EPCR [43]. Thus, protective function seems EPCR dependent but there are cell populations that are responsive to APC in suppressing inflammation that do not appear to require EPCR. A likely receptor for APC on macrophages is CDllb/CD18, also known as Mac-1. APC administration in wild type, but not CD11b null mice, reduced mortality. CD11b was also required for suppression of the endotoxin-induced macrophage inflammatory response [44]. These results indicate that the cellular signaling mechanisms play a dominant role in protection from endotoxemia [45].

Role of APC in specific disease states

Coronary reperfusion injury One of the events that occur in reperfusion injury is apoptosis. Blocking protein C activation exacerbates reperfusion injury in pig hearts [46]. The ischemia reperfusion also leads to rapid protein C activation in this model [46]. In mouse models of coronary reperfusion, APC reduced coronary apoptosis and decreased inflammation and leukocyte adhesion resulting in improved heart function [47, 48].

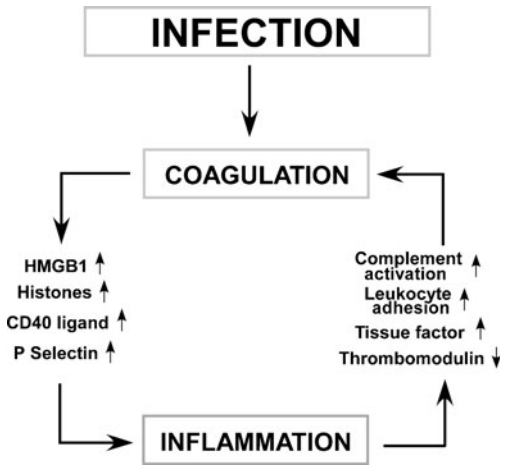

Fig. 2 The links between infection, coagulation, and inflammation. Infection either directly triggers the activation of the intrinsic pathway through activation of factor XII or activates a series of toll-like receptors that can generate cytokines that initiate tissue factor expression. Coagulation leads to platelet activation, releasing CD40 ligand that amplifies inflammation, expression of P-selectin on cell surfaces which aids in leukocyte trafficking, and with ischemia reperfusion injury which leads to the release of HMGB 1 or histones that further trigger inflammation and tissue damage. The resultant amplified inflammatory response leads to additional tissue factor formation, thrombomodulin downregulation, complement activation, and leukocyte activation, further stimulating coagulation. Unchecked, this has the potential for devastating inflammatory and coagulation-mediated injury

Stroke protection APC treatment of ischemic brain endothelium prevents apoptosis in part by blocking P53 function [49]. APC also prevents tissue plasminogen activation-induced Nf-κB-dependent upregulation of matrix metaloproteinase-9 and can prevent neuronal apoptosis by PARs 1 and 3 [37].

Diabetes Diabetes also results in apoptosis of kidney cells. Increasing endogenous APC production decreased apoptosis and improved kidney function in mouse models of type 1 diabetes [50]. As mentioned above, the large degree of apoptosis and necrosis would be anticipated to increase inflammation which is thus indirectly prevented by APC.

Inflammatory bowel disease In inflammatory bowel disease, EPCR and thrombomodulin are downregulated. APC treatment reduced cytokine production, inhibited leukocyte adhesion, diminished weight loss, and reduced the magnitude of the pathological lesions [51].

Tumor adhesion and propagation Endogenous APC decreases the adhesion of tumor cells to the lung and reduces the number of metastatic sites [52, 53]. It does so in part by activating the sphengosine-phosphate-1 system [52] in a PAR-1 dependent reaction.

Trauma Trauma, even sterile trauma, is associated with an increase in inflammation. This may be due to the release of intracellular components that activate the innate immune system, see [54] for a brief review. In mouse models of trauma, APC appears to contribute to the coagulopathy associated with trauma [55]. By use of a selective antibody to murine APC [45], the coagulopathy can be largely prevented by the selective inhibition of endogenous APC's anticoagulant activity with preservation of its cytoprotective functions [55]. APC's cytoprotective/anti-inflammatory functions play a key role in preventing death in this model, in part apparently by preventing excessive thrombosis that might result from tissue necrosis or apoptosis.

Amyotrophic lateral sclerosis A mutant superoxide dismutase gene has been found in amyotrophic lateral sclerosis (ALS) patients, and this gene insertion will elicit ALS-like symptoms in mice [56]. APC mutants with cytoprotective activity can suppress the mutant gene expression and slow the progression of ALS symptoms apparently by crossing the blood–brain barrier and signaling through a PAR1- and PAR3-dependent pathway [56].

Conclusions

APC is a potent modulator of disease processes. Both direct anticoagulant activity and cell signaling are involved. The anti-histone activity of APC has also been implicated. Either the native protein or genetically modified versions of the molecule have potential therapeutic utility. In some clinical conditions like trauma, excess activation of the endogenous protein C seems to contribute to morbidity and mortality. With exogenous APC, mutations of the molecule can selectively alter its function, whereas antibodies can be utilized to modulate the functions of the endogenous APC. As we gain better understanding of the details of the interplay of APC with the complex regulatory systems in vivo, the potential is high to be able to exploit this system for even greater selectivity and clinical benefit. In particular, APC may be able to prevent the autoamplification of inflammation and coagulation depicted in Fig. 2.

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