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Old drug, new Trick? The rationale for the treatment of COVID-19 with activated protein C

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ABSTRACT

As the COVID-19 pandemic continues, researchers seek to identify efficacious treatments. Current approaches to COVID-19 therapeutics focus on antiviral agents, convalescent plasma, monoclonal antibodies, immunomodulators and more traditional therapies such as steroids [1-6]. Reversing disturbances in coagulation has also been identified as a priority area for candidate therapies, such as through the Accelerating COVID-19 Therapeutic Interventions and Vaccines 4 adaptive clinical trial (ACTIV-4) which is currently evaluating aspirin, heparins and apixaban [7]. Since there is a clear relationship between mechanisms of coagulation and the immune response, it is possible that reversing disturbances in coagulation may diminish the dysregulated immune response observed in COVID-19. The basis for this hypothesis is described below and is followed by discussion of a proposed candidate therapy - activated protein C. By treating COVID-19 patients using a novel approach, which does not focus on immune-based or antiviral treatments, but instead which addresses both the anti-thrombotic and inflammatory consequences of infection, the hope is that new therapeutic targets can be considered and new candidate therapies, such as activated protein C, may be evaluated.

Background

Anticoagulant mechanisms of activated protein C

Thrombomodulin, which is expressed on the surface of vascular endothelial cells, binds to thrombin. In this bound state, thrombin is inhibited from cleaving both fibrinogen and factor V [8]. Thrombin bound to thrombomodulin is more potently inactivated by both antithrombin and protein C inhibitor [9]. These mechanisms of thrombin inhibition blunt thrombin's roles in platelet activation, the catalysis of fibrinogen to fibrin monomers, and activation of factors V, VIII, and XIII.

The thrombin-thrombomodulin complex not only diminishes coagulation by these direct effects on thrombin, but also through the conversion of the protein C zymogen to its activated form – activated protein C. The binding of thrombin to thrombomodulin increases this rate of conversion at least 20,000-fold through an increase in Vm and decrease in Km, a reaction which is calcium-dependent and largely occurs in the microcirculation as opposed to major vessels [5,10]. The endothelial protein C receptor (EPCR) is a transmembrane protein also expressed on the surface of endothelial cells, and serves to enhance the activation of protein C by the thrombomodulin-thrombin complex, through a decrease in Km and possibly through other mechanisms, and potentiates the activation of protein C [10,11].

Activated protein C, in association with the co-factor protein S, then has anti-coagulant effects by cleaving activated factor V [12,13]. In association with cofactors protein S and inactivated factor V, activated protein C also inactivates factor VIII [14]. The aggregate effects of factor V and VIII inactivation impair the activity of the tenase and prothrombinase complexes, diminishing further thrombin production. By forming a tight complex with plasminogen activator inhibitor 1, activated protein C decreases this inhibitor of tissue plasminogen activator and promotes fibrinolysis [15].

Interrelationship between inflammation and coagulation

Pathways have been described which shed light on a relationship between mechanisms of inflammation and the coagulation cascade - such pathways point in both directions. Cytokines have been demonstrated to have an impact on the above-described coagulation pathways. Interleukin 1 and tumor necrosis factor are known to modify the characteristics of endothelial cells, and specifically synthesize tissue factor and suppress thrombomodulin [16–18]. Further, TNF-induced suppression of the expression and degradation of thrombomodulin in endothelial cells appears to have a lasting effect, persisting for hours

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[19]. TNF also has been demonstrated to reduce EPCR, which may impair the efficacy of the inflammatory effects of both proteins C and activated protein C [20]. Interleukin 6 neutralization in the chimpanzee response to endotoxin was demonstrated to reduce coagulation [21]. Interestingly, administration of an IL-6 neutralizing monoclonal antibody in chimpanzees did not blunt the brisk rises in TNF or IL-8, nor did administration of these same monoclonal antibodies reduce the extent of neutrophilia, neutrophilic degranulation, or reduce fibrinolysis. In contrary, the IL-6 antibody markedly attenuated only coagulation.

Conversely, proteins involved in the activation and inhibition of coagulation have been demonstrated to affect a diverse set of inflammatory mechanisms. Thrombin appears to be the nucleus for the coagulation cascade's stimulation of inflammation while activated protein C is the main focal point for its anti-inflammatory effects.

In cultured endothelial cells, thrombin promotes neutrophilic adhesion and the production of platelet activating factor which has additional proinflammatory effects [22]. Thrombin signaling, which occurs through the protease-activated receptor-1 (expressed by platelets as well as endothelial cells), has pleiotropic actions on platelets and endothelial cells, some which result in changes in cell shape which may further promote platelet aggregation and vascular permeability [23]. Thrombin additionally stimulates the production of IL-6 and IL-8 in cultured mononuclear and endothelial cells [24], stimulates the secretion of IL-1 and TNF in mononuclear cells [25], and thrombin generation corresponding with fibrin activation are common findings in sepsis [26]. Inhibition of tissue factor with infusion of tissue factor pathway inhibitor protein in baboons exposed to a lethal dose of E. Coli prolonged survival, improved survival rate, and blunted dysregulated coagulation [27]. While administration of this inhibitor was not associated with any changes in TNF levels, IL-6 increases were dramatically blunted, and even decreased, in baboons in the intervention group. A study evaluating the impact of tissue factor pathway inhibitor protein in pigs with peritonitis-induced bacteremia demonstrated attenuation of TNF and IL-8 peak levels [28].

Pathways involving activated protein C counteract these thrombin-mediated effects and downregulate inflammation through several mechanisms. Historically, these effects were suggested when infants with protein C deficiency were found to have a rapid reduction in the thrombosis-associated, intense, local inflammation following the administration of protein C [29]. Since then, sepsis was identified as a model of dysregulated inflammation and coagulation, and a large body of research emerged evaluating the impact that perturbations to the coagulation system could have on inflammatory markers, coagulation proteins, and mortality in animal studies and then in human trials. Blocking protein C activation through use of a monoclonal EPCR antibody demonstrated increased mortality in baboons challenged with sublethal doses of E coli [30]. Baboons receiving the mAb had diverse alterations in coagulation and inflammation, with decreased fibrinogen but increases in fibrin degradation products, soluble thrombin, interleukin 6 and interleukin 8. Further, histopathological findings were notable for thrombosis in the adrenal glands, glomeruli thrombosis with necrosis of the tubular epithelial cells, and leukocyte infiltration in the adrenal glands and liver. Mice with a targeted heterozygous deficiency of protein C had reduced survival in comparison to their wildtype counterparts, and had significantly higher levels of interleukin 1, interleukin 2, interleukin 5, interleukin 6, interleukin 12, and TNF-alpha [31].

The anti-inflammatory properties of activated protein C have additionally been directly studied. Activated protein C has been demonstrated to inhibit the production of TNF by lipopolysaccharide stimulated monocytes in rats [32]. This same study demonstrated that rats treated with activated protein C did not experience vascular lung injury when challenged with lipopolysaccharide. Baboons treated with activated protein C were protected from a lethal dose of E. Coil, and had preserved fibrinogen and normal SGPT levels, in marked contrast to untreated controls [33]. The unique properties of activated protein C were supported by other studies using other anticoagulants, such as heparin and a modified form of factor Xa with its active-site blocked, which did not have the pluripotent protective effects as compared to activated protein C [34]. The diverse anti-inflammatory and protective effects of the activated protein C mechanistically appear to be due to decreased expression and functional activity of the endothelial cell nuclear transcription factor kappa-beta (NFkB, an important intracellular signal for cytokine production), through reduction in cytokine signaling via downregulation of cell surface adhesion molecules (including ICAM-1, E-selectin, and VCAM-1) and through decreased apoptosis via the regulation of antiapoptotic and proapoptotic genes [35]. Thrombomodulin itself has overlapping and complementary anti-inflammatory effects on several of these pathways as well [36].

Activated protein C in the treatment of sepsis

Given the potential for correction of the pro-inflammatory and procoagulant effects of severe sepsis and septic shock, recombinant human activated protein C, or drotrecogin alfa (activated, DrotAA) was studied on patients with septic shock, but ultimately the PROWESS-SHOCK trial demonstrated that DrotAA did not significantly reduce mortality as compared to placebo [37]. This study is notable for a ~ 30% rate of positive blood cultures and ~ 70% rate of infectious organism identification; inclusion criteria are notable for an infection requiring intravenous antimicrobial therapy and persistent septic shock requiring pressors [38]. Other human studies evaluating recombinant activated protein C shed additional light on patient characteristics, which also reinforce the heavy and intended focus on patients with a bacterial etiology of disease. For example, PROWESS, which demonstrated decreased mortality in patients treated with DrotAA, revealed positive blood culture rates again at ~ 30%. RESOLVE, which evaluated DrotAA in pediatric patients, demonstrated that ~ 70% of patients had positive blood cultures and the ADDRESS study, evaluating patients with sepsis with sepsis-induced dysfunction of at least one organ but with lower APACHE II scores, also had positive blood culture rates of ~ 70% [39,40].

Dysregulated inflammation and coagulation in viral infections

Dysregulated coagulation and inflammatory pathways related to consumption of activated protein C have also been studies in viral infections. Early case reports demonstrated evidence of DIC in pediatric patients with influenza A and renal biopsies with fibrin deposition in glomerular capillary walls [41]. A retrospective study evaluating pediatric patients admitted with influenza A infection demonstrated a 24.4% rate of renal involvement, and DIC was present in 63.6% of the patients with renal impairment [42]. Viruses associated with hemorrhagic fevers are complicated by DIC in the most severe cases, and DIC can be found to occur with infections with other viruses including rotavirus, varicella, rubella, rubeola as well as influenza [43]. Humans with H1N1 influenza with increased levels of D-dimer have a higher risk of a respiratory failure and death (higher levels of LDH and a lower absolute lymphocyte count were also associated with a higher risk of respiratory failure and death) [44].

SARS-CoV, which first emerged in 2002 in China and spread to 27 countries by April 2003, with a total of over 8,000 cases, has been associated with elevation in the PTT and D-Dimer [45-47]. There is less robust data available on the SARS-CoV impact on dysregulated mechanisms of coagulation, given the fortunate and relatively rapid regression of human cases of SARS-CoV infection. An observational study of a SARS CoV outbreak in Hong Kong evaluated quantitative RT-PCR in nasopharyngeal aspirates of infected patients and demonstrated a consistent peak at around day 10 following symptom of viral copies/mL, which seemed to correlate with the timing of IgG seroconversion (which started at day 10) [48]. Substantial clinical deterioration frequently occurred following this peak, during a period of decreasing viral

replication and IgG seroconversion; the authors suggested that this observed, latter phase of the illness, which was associated lung injury, could be due to a dysregulated immunologic response. Additional studies were performed to evaluate the molecular mechanism for acute lung injury with SARS-CoV infection; RNA expression patterns, proteonomics, and histology all suggested downregulation of the urokinase and tissue plasminogen activators as well as stimulation of alpha-2-plasmin inhibitor, and these pathways result in a common host response of high levels of fibrin deposition in the lungs [49].

MERS-CoV was first isolated in 2012 in Saudi Arabia and as of December 2019 resulted in 2499 cases in over 27 countries with a case fatality rate of 36% [50,51]. Host factors associated with severe disease include age > 65 years, obesity, diabetes, and other chronic conditions such as heart, kidney and lung disease [52]. A case control study which included 17 patients who tested positive for MERS-CoV demonstrated elevations in aPTT and INR [53]. Thrombocytopenia was also noted in most patients admitted to an ICU with MERS-CoV [54]. Due to the low case count overall, there is even less data available regarding disturbances in coagulation associated with MERS-CoV as compared to SARS-CoV. Tissue samples were lacking, with the exception of two known human cases worldwide, due to the geographic distribution which corresponded with cultural and religious restrictions over autopsies and also as a result of infection control concerns [51]. While a useful animal model for severe MERS-CoV infection has proven challenging, studies using cell lines have demonstrated proinflammatory cytokine induction by MERS-CoV. A human lung epithelial cell line infected with MERS-CoV demonstrated induction of IL-1-beta, IL-6 and IL-8, and these increases were far greater as compared to SARS-CoV.

Activated protein C in the treatment of viral infections

Given a disturbance of coagulation and inflammation in viral infections as well as bacterial infections, the use of recombinant human activated protein C has been studied in settings other than in humans with septic shock. Infection of fourteen rhesus monkeys inoculated with ebolavirus, which is associated with a rapid decline in plasma levels of protein C, demonstrated improved survival in the primates treated with activated protein C. While some monkeys did not appear to exhibit a response to the infusion, a group of responders demonstrated significant, sustained reductions in D-dimer, IL-6, IL-10, monocyte chemotactic protein 1, and TNF-alpha [55]. The relationship of viral-mediated dysregulated inflammatory response, coagulation and thrombus formation has also been studied, as well as the associated mechanisms, and activated protein C has been demonstrated to block several pathways, including endothelial expression of cytokines and adhesion molecules as well as mesangial production of tissue factor in vitro, and thrombosis in vivo [56].

Inflammatory and coagulation disturbances in COVID-19 infections

Coronavirus Disease 2019, which was first recognized in December 2019 and since termed COVID-19, has not only been associated with profound stimulation of both inflammation and coagulation, but the magnitude of coagulopathy, in particular, is noteworthy as is the apparent association of coagulation disturbance with decreased survival. Early case series demonstrated decreases in aPTT and PT in 16% and 30% of patients, and 36% of patients had an increase in D-dimer [57]. A subsequent prospective study in a Wuhan hospital from January 1st through February 3rd 2020 evaluated coagulation tests on admission and during the hospital stay in 183 patients [58]. Using established diagnostic criteria for disseminated intravascular coagulation, 71.4% of the non-survivors had overt DIC while only 0.6% of survivors fulfilled DIC criteria; fibrinogen degradation products and D-dimer were significantly higher on admission in the non-survivors, and later in the course of admission anti-thrombin activity and fibrinogen levels were also significantly lower in non-survivors. Another study of hospitalized patients with COVID-19 similarly demonstrated that D-dimer levels were significantly higher in non-survivors later in the hospitalization as well [59]. Subsequent studies appear to confirm the high incidence of thrombotic complications of COVID-19, and heparin-based thromboembolism prophylaxis is generally recommended for COVID-19 admitted patients [60]. A retrospective study evaluating 449 severe COVID-19 patients demonstrated that elevated D-dimer was associated with higher 28-day mortality and for the group of patients with the highest levels of D-dimer, heparin appeared to reduce mortality by approximately 20% [61]. An observational study of 184 COVID-19 patients admitted to the ICU at one of three Dutch hospitals demonstrated a 31% composite rate of thrombotic complications despite treatment with standard doses of low molecular weight heparin [62]. Another study in Amsterdam similarly demonstrated higher D-dimer and higher rates of thrombosis in patients admitted to an ICU as compared to patients admitted to a general ward, and revealed that rates of venous thromboembolism were high for all patients despite aggressive prophylaxis [63]. A study of 150 ICU patients admitted to a French tertiary hospital demonstrated that 95% of patients had elevations in D-dimer and fibrinogen, factor VIII levels were considerably increased, and of 57 patients who were tested for lupus circulating anticoagulant 50 (87.7%) were positive [64]. This same study, which also matched COVID ARDS patients to non-COVID patients with ARDS, demonstrated a 15 times higher rate of pulmonary embolism and a high rate of circuit clotting among the patients receiving continuous renal replacement therapy. Conversely, the rate of hemorrhagic complications in this study were very low (and when they did occur, appeared to have a traumatic or procedure related etiology). A study evaluating endothelial cell histology in a series of patients with severe COVID-19 infections not only noted thrombotic complications but also identified prominent endotheliitis in several organs, including the intestine, lung, heart, kidney and liver [65]. It is noteworthy that the authors pointed out that viral or immune-mediated factors may cause widespread endothelial dysfunction, which promotes inflammation and a procoagulant state. Evidence of microvascular injury in COVID-19 patients is further provided by a study evaluating markers of endotheliopathy in 68 patients admitted with COVID-19 infection [66]. Not only were robust elevations in D-Dimer again noted but more specific markers of endothelial injury and activation were also observed (including elevations in soluble thrombomodulin and P-selectin, the latter which was also noted to be higher in intensive care unit patients as compared to patients admitted to a general hospital unit). Elevated soluble thrombomodulin concentration were also associated with a lower likelihood of survival

Similar to dysregulated coagulation, SARS-CoV-2 has been reported to be associated with a robust proinflammatory state and even a cytokine storm phenomenon similar to what is observed in macrophage activation syndrome or secondary hemophagocytic lymphohistiocytosis. An early, retrospective study of COVID-19 patients in Wuhan identified that IL-6, ferritin and CRP were elevated, and these elevations were considerably higher in those patients who died (median CRP > 100 mg/L, ferritin 1297 ng/mL and IL-6 > 10 pg/mL) [67]. Another study from Wuhan of 41 hospitalized patients demonstrated that ICU patients exhibited higher plasma concentrations of IL-2, macrophage chemotactic protein 1, TNF-alpha, and other cytokines [68]. It is interesting that IL-6 has been demonstrated to induce C-reactive protein, which itself has been demonstrated to be impressively elevated in COVID-19, given that C-reactive protein stimulates the production of tissue factor [17].

Hypothesis: Activated protein C as a novel therapeutic for COVID-19 infections

Treatments for hyperinflammation associated with COVID-19 have been evaluated, and have included steroids, monoclonal antibodies as well as more targeted therapies, and other therapies have been proposed or are currently under investigation, such as intravenous

immunoglobulin, cytokine inhibitors and JAK inhibitors [5,6,69]. Studies have evaluated the impact of tocilizumab (a monoclonal antibody against the IL-6 receptor), anakinra (an interleukin-1 inhibitor), adalimumab (an anti-TNF-alpha antibody) and other inhibitors of pro-inflammatory cytokines [4,70,71]. The impact of these targeted immune therapies on COVID-19 outcomes may be blunted by accompanying disturbances in coagulation which sustain the the dysregulated immune response. Such mechanisms may explain why survival was not improved in hospitalized patients with COVID-19 treated with tocilizumab [4]. Similarly, as was noted above in studies in SARS-CoV, antiviral treatment directed towards COVID-19, such as remdesivir, may not be expected to significantly impact the most severe effects of the illness once potent cytokine release and activation of coagulation are triggered, particularly when these sequelae of COVID-19 occur during the phase of illness when viral replication is already on the descent. The largest trial to date evaluating remdesivir on hospitalized patients with COVID-19 demonstrated no effect on mortality [70]. COVID-19 pathways of injury may be hypothesized that focus on dysregulated coagulation, potent activation of tissue factor and subsequently activation of thrombin, and downregulation of innate mechanisms of anti-coagulation. If these mechanisms are responsible for a potent shift in balance of natural pro-coagulant and anti-coagulant pathways, the consequences of a vigorous procoagulant response would be expected to cause cytokine elevations. Therefore, it is possible that approaching the observed disturbance in coagulation, rather than directly inhibiting mechanisms of inflammation or the virus itself, may be a more efficacious route to positively impact the course of illness, particularly among the sickest patients affected by COVID-19.

This concept is supported by predictors for disease worsening identified in a trial designed to derive and validate a predictive score for disease worsening in inpatients with COVID-19 [71]. Compared with patients admitted to a conventional unit, patients admitted to the intensive care unit had lower levels of antithrombin activity and protein C activity as well as higher D-Dimer and fibrinogen levels. Equally notable is that the multivariate analysis identified elevations in D-Dimer and decreased activity of protein C and antithrombin as significant predictors of worsening disease.

When considering therapeutics that impact the activated protein C pathway, options include not only wild-type activated protein C, but also 3K3A-APC and soluble human recombinant thrombomodulin. Several variant forms of activated protein C have been evaluated in preclinical injury models, and 3K3A-APC, a recombinant human 3K3A-APC, has been evaluated in phase 1 and phase 2 human trials in patients with acute ischemic stroke [72,73]. The 3K3A variant, however, has $<$ 10% anticoagulant activity as compared to activated protein 3. While the enhanced anticoagulant effect of activated protein C may result in more non-serious and serious bleeding events, human studies of activated protein C did not consistently demonstrate a significantly increased incidence of serious bleeding in patients treated with activated protein C [37]. It is also noteworthy that the above-described high rates of small and large vessel thrombosis in patients with COVID-19 could make activated protein C the optimal formulation for the treatment of severely ill COVID-19 patients. Activated protein C, therefore, may not only benefit COVID-19 patients through immunologic pathways which diminish endothelial injury and consequent tissue injury, but may also provide additional benefits specifically through antithrombotic properties. The fact that therapeutic anticoagulation is being evaluated in non-hospitalized and hospitalized patients with COVID-19 also lends merit to studying wild-type activated protein C, given its anticoagulant effects. While ART-123, a soluble human recombinant thrombomodulin, has been evaluated in patients with sepsis associated with coagulopathy and could be considered for treatment in patients with COVID-19, this therapy depends upon sufficient concentrations of thrombin and protein C. Given evidence that protein C activity is decreased in patients with severe COVID-19 infections, the impact of ART-123 could be dampened in this population of patients, again making wild-type activated protein C a more attractive candidate therapy.

While it would be ideal to more rigorously study the characteristics of some of the sickest COVID-19 patients, specifically through the collection of additional data, such as protein C, soluble thrombin, tissue factor, soluble thrombomodulin, protein C inhibitor, alpha-1 antitrypsin, and their relationship to outcomes as well as pathological data, given the current crisis and the need for effective therapeutics, it is appropriate to trial investigational, candidate therapies which could significantly alter the course of the illness without the usual, more complete, supporting data. Evaluating the effect of activated protein C on patients with COVID-19 is, therefore, appropriate. Studying activated protein C both as a solitary therapy for patients with COVID-19 and in combination with dexamethasone, which has been demonstrated to reduce mortality in patients with severe COVID-19 infections, may be instructive given potentially additive or synergistic therapeutic effects.

Given that the sickest patients, such as those in intensive care on mechanical ventilation, presumably have experienced the most profound dysregulation of coagulation and inflammation, a subpopulation of these patients would be the most appropriate target population to trial this candidate therapeutic. Selection of severely ill patients not only would identify those with vigorous pathophysiologic mechanisms that can lead to tissue injury but also would represent a group of patients which may stand to benefit the most. Patients on mechanical ventilation were demonstrated to benefit most with administration of dexamethasone in the RECOVERY trial, presumably through the impact of the steroid on the host immune response to infection, which supports the selection of patients on mechanical ventilation as appropriate initial candidates for treatment with activated protein C as well.1

In addition to severity of illness, it is equally important to consider the duration of illness. Patients with particularly prolonged illness, for example, may have experienced the consequences of dysregulated coagulation and inflammation, such that considerable tissue injury has taken hold, such as through fibrin deposition in the lungs. Therefore, it will be important to identify a population of patients that are both severely ill but not too far along the continuum of the illness such that treatment can still have a therapeutic impact. In the RECOVERY trial, patients in the invasive mechanical ventilation group had a symptom onset median of 13 days at the time of randomization, with the interquartile range 8–18 days. Using the 75th %ile, 18 days or less from symptom onset, as an initial eligibility criteria for the initiation of treatment with activated protein C would be reasonable. In addition to optimizing patient selection based on severity and duration of illness, direct markers of dysregulated coagulation and a vigorous host immune response should be used for eligibility. D-Dimer and CRP, which are readily available and frequently monitored in patients with COVID-19 infection, could easily be applied to identify this subset of patients. Based on the observed relationship of elevations in D-Dimer and CRP to mortality in patients with COVID-19 infection, eligibility for treatment with activated protein C could be based on a D-dimer$>$10-times the upper limit of normal and CRP $>$ 100 mg/L [57,62].

Conclusion

In summary, the potent coagulation disturbance associated with severe COVID-19 infections, combined with evidence of a frequently associated hyperimmune state, when considered in the context of the well-established link between the inflammatory and coagulation pathways, may position a therapeutic with mechanistic roots in reversing the coagulation dysfunction as an effective in the treatment of COVID-19.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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