Effects of Recombinant Human Activated Protein C in Human Models of Endotoxin Administration

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Alterations in the generation of activated protein C (APC) as well as in the interactions of APC with the endothelial protein C receptor are present in severe sepsis and acute lung injury. Administration of recombinant human activated protein C (rhAPC) improves the survival of critically ill patients with sepsis, but the mechanisms by which rhAPC produces benefit are not well defined. Human models of systemic and pulmonary endotoxin exposure may provide important insights into the mechanisms of action of rhAPC in critical illness. In volunteers given systemic endotoxin, rhAPC had minimal effects on physiologic parameters, including blood pressure, markers of inflammation, and measures of sepsis-induced coagulopathy. In contrast, in the setting of pulmonary endotoxin exposure, rhAPC decreased neutrophil migration into the airspaces and also diminished neutrophil chemotaxis. Administration of rhAPC did not affect other parameters of neutrophil function, including kinase activation, production of proinflammatory cytokines, or apoptosis. Such results indicate that the effects of rhAPC in inhibiting the infiltration of neutrophils into the lungs and other inflammatory sites may contribute to its beneficial effects in sepsis.

Keywords: activated protein C; endotoxemia; endotoxin; protein C; sepsis; sepsis syndrome

The recognition of activation of the coagulation system in most patients with sepsis, as well as growing appreciation of the procoagulant state in the lungs during acute lung injury, suggested that anticoagulant therapies, particularly those possessing profibrinolytic activities might be effective in the treatment of sepsis (1–4). Several different anticoagulant approaches have been examined in large multicenter trials of sepsis (5–7), but only recombinant human activated protein C (rhAPC) was demonstrated to reduce mortality in this setting. The reasons that rhAPC was effective, whereas other therapies with potent anticoagulant effects, such as tissue factor pathway inhibitor or antithrombin, were ineffective, remain incompletely understood.

Activated protein C (APC) is generated from the cleavage of protein C by thrombin coupled to thrombomodulin (3, 7–9). The anticoagulant effects of APC result primarily from its inhibition of activated factors V and VIII, with associated decreased generation of thrombin (10). APC also has profibrinolytic properties, by decreasing concentrations of plasminogen-activator inhibitor type 1 (PAI-1). In *in vitro* studies, APC can decrease cellular activation, including nuclear translocation of nuclear factor- κ B and production of proinflammatory cytokines (11, 12). Neutrophil adhesion to endothelial cells is inhibited by APC

(13). Recent studies show that neutrophils and monocytes have receptors that interact with protein C, APC, and rhAPC (14). These receptors appear to be similar to the previously characterized endothelial protein C receptor and are involved in modulating neutrophil and monocyte chemotaxis. In particular, incubation of neutrophils or monocytes with protein C, APC, or rhAPC decreases directional migration to chemotactins, including interleukin 8 (IL-8) and formylmethionylleucylphenylalanine. Because activated monocytes and neutrophils, as well as alterations in coagulation and fibrinolytic cascades, all appear to contribute to organ system dysfunction and mortality in sepsis and acute lung injury, there are multiple possible mechanisms through which rhAPC may provide benefit in these clinical settings. However, the relative importance of these actions of rhAPC in patients with sepsis or in relevant *in vivo* models remains incompletely delineated.

There are only limited methodologies that can be used in human volunteers to model physiologic alterations similar to those that are present in critically ill, infected patients. Exposure of volunteers to bacterial products, such as LPS, or proinflammatory cytokines whose release is increased in severe infection, including tumor necrosis factor α (TNF- α) or IL-1, can duplicate some of the relevant findings in sepsis, including hypotension, increased cardiac output, release of proinflammatory mediators, alterations in coagulation and fibrinolytic pathways, and migration of activated neutrophils into relevant tissue sites, such as the lungs.

Reference standard LPS for administration to humans has been prepared from *Escherichia coli* as well as *Salmonella abortus equi* (15–20). The most common protocols have used a single intravenous dose of 1 to 4 ng/kg or an intrapulmonary dose of 4 ng/kg, where the LPS is bronchoscopically introduced into a segment of the lung (21–23). rhAPC has been administered to volunteers who received intravenous or pulmonary endotoxin (24–26), and such studies provide unique insights into the mechanisms through which this molecule may modulate pathophysiologic pathways associated with sepsis.

INTRAVENOUS ADMINISTRATION OF ENDOTOXIN

Systemic symptoms that follow intravenous injection of 2 to 4 ng/kg LPS include fatigue, headache, and nausea (27, 28). Less commonly, there are complaints of rigors, myalgias, drowsiness, mild amnesia, and anorexia. Fever is a consistent finding in human endotoxemia studies, with maximal fever temperatures generally less than 39°C. Hemodynamic findings with endotoxemia include tachycardia and hypotension. In the studies by Martich and colleagues (22) and Suffredini and coworkers (28), mean arterial pressure dropped from an average of 99 mm Hg at baseline to 83 mm Hg 5 h after LPS infusion, and cardiac index was elevated by as much as 50% after endotoxin administration.

Laboratory alterations induced by intravenous LPS injection include an initial decrease in total leukocyte counts, followed by a later increase. Similar alterations in circulating neutrophil numbers are found. Neutrophil activation is suggested by increased levels of serum neutrophil elastase (29) and by increased expression of neutrophil receptors for the complement opsonin

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C3 (30). Lymphocyte counts drop rapidly within 1 h of LPS administration and then have a more gradual decline until reaching a nadir at 4 h after dosing. Within 1 h after LPS, monocytopenia develops, with return to baseline within 6 h (31).

Administration of LPS intravenously produces increased circulating levels of TNF- α , within 60 to 90 min, followed by IL-1 β , IL-6, and IL-8 at 2 to 3 h, and granulocyte colony-stimulating factor at 5 to 6 h after LPS infusion (32–34). Serum levels of cytokine inhibitors, including soluble 55- and 75-kD TNF receptors (TNFR1 and TNFR2, respectively), as well as the IL-1 receptor antagonist (IL-1ra), also rise after LPS administration (35). Evidence for activation of coagulation cascades is shown by the appearance of elevations of D-dimer levels, thrombin-antithrombin complexes (TATc), and F₁₊₂ fragments in the circulation starting at about 2 h after LPS injection. Factor VIIa (FVIIa) levels decrease steadily after LPS to a 25% reduction at 24 h (36–39). Plasma tissue plasminogen activator (tPA) increases at 2 to 3 h after LPS administration, with elevated circulating PAI-1 levels following those of tPA with a peak at 3 to 4 h (36–39).

rhAPC AND HUMAN ENDOTOXEMIA

Two studies have examined the effects of rhAPC in human endotoxemia. Derhaschnig and colleagues (24) studied 24 healthy male volunteers, of whom 9 received saline and 15 received rhAPC for 8 h at 24 $\mu\text{g/kg/h}$, the standard clinically used dose for severe sepsis. All subjects received a bolus of 2 ng/kg LPS 2 h after the start of rhAPC or placebo.

In the study by Derhasching and colleagues (24), rhAPC infusion produced a steady-state concentration of 60 to 80 ng/ml, an increase in APC levels of 60- to 70-fold above those found at baseline, before rhAPC or LPS administration. In the group receiving placebo, APC levels were significantly increased over baseline after LPS infusion, but this rise was only 2.25-fold over pre-LPS levels. Activated partial thromboplastin time (aPTT) was prolonged by about 20% in the volunteers that received rhAPC.

Administration of rhAPC had no significant effects on LPS-induced changes in coagulation. After LPS, there were no significant differences in markers of thrombin generation (TATc, F₁₊₂), tissue factor (TF) mRNA, D-dimer, tPA, and PAI-1 in subjects treated with rhAPC and placebo. Similarly rhAPC had no significant effects on LPS-induced changes in hemodynamics, including hypotension or tachycardia, circulating cytokine levels, markers of platelet and endothelial cell activation, or leukocyte counts.

A second study in human endotoxemia also found few effects associated with the administration of rhAPC (25). In that experiment, 16 volunteers were randomized in 1:1 fashion to either rhAPC (24 $\mu\text{g/kg/h}$) or 0.1% albumin infusion for an 8-h period, starting 2 h before administration of 2 ng/kg LPS. At a single time point (3 h after LPS administration), mean arterial blood pressure was significantly higher in volunteers receiving rhAPC compared with those receiving placebo (rhAPC 75 mm Hg vs. placebo 65 mm Hg, $p \leq 0.04$). However, no differences in hemodynamics were found at other time points. As in the study by Derhasching and coworkers (24), there were no effects of rhAPC on markers of thrombin generation (F₁₊₂, TATc), D-dimer levels, or circulating concentrations of PAI-1. There were no differences in TNF- α or IL-6 levels between the rhAPC and placebo groups.

PULMONARY ENDOTOXIN ADMINISTRATION

Because of the important role that endotoxin plays in occupational lung diseases, such as cotton-bract disease or byssinosis, studies have evaluated the effects of inhaled endotoxin on lung function, using either endotoxin itself, killed gram-negative bacteria, or organic dusts containing endotoxin (40–44). Such expo-

sure to inhaled endotoxin results in lung inflammation, with increased numbers of neutrophils and proinflammatory cytokines in bronchoalveolar lavage (BAL) (45, 46). Even with the highest doses of inhaled endotoxin, estimated to be 70 μg or approximately 1 $\mu\text{g/kg}$, the changes in pulmonary function are modest, with a mean 8% decrease in FEV₁ and a mean 17% decrease in diffusing capacity. In that study, fever occurred in 80% of the volunteers and chest tightness in 44% (44). The minimal pyrogenic dose of inhaled endotoxin was estimated to be 0.05 $\mu\text{g/kg}$. In contrast, the minimal pyrogenic dose of intravenous reference endotoxin is considerably less (0.5 ng/kg or 0.0005 $\mu\text{g/kg}$).

Several studies have explored responses when endotoxin is bronchoscopically instilled into a lung segment (20, 26, 47). Under such conditions, there are six- to sevenfold increases in BAL cell counts, with peak numbers of neutrophils, monocytes, and lymphocytes being found approximately 24 h after endotoxin administration. There are only minimal increases induced in BAL alveolar macrophage numbers. BAL protein concentrations significantly rise, as do concentrations of cytokines and chemokines, including TNF- α , IL-1 β , IL-6, IL-8, macrophage inflammatory protein (MIP)-1 α , MIP-1 β , monocyte chemoattractant protein (MCP)-1, epithelial neutrophil activating peptide (ENA)-78, and granulocyte colony-stimulating factor. Levels of cytokine inhibitors, including IL-1ra, TNFR1, and TNFR2, are also elevated in BAL from the endotoxin-exposed lung segment, but not from that instilled with saline. Markers of cell activation, such as L-selectin, angiostatin, myeloperoxidase, gelatinase-B, and lactoferrin, are increased in the lung after endotoxin exposure.

rhAPC AND PULMONARY ENDOTOXIN ADMINISTRATION

Although intravenous endotoxin administration results in increased circulating cytokine levels, activation of coagulation pathways, and hemodynamic alterations, it is unclear how closely such findings duplicate the clinical scenario of sepsis, where infection is generally localized, at least during the initial stages. There are few situations, except perhaps in meningococcemia, where sudden exposure to large amounts of endotoxin are believed to occur. Instillation of endotoxin directly into the lungs induces a localized inflammatory response that resembles that found with bacterial pneumonia, and therefore may be a more appropriate model for use in examining potential mechanisms of action of rhAPC (48).

To investigate the effects of rhAPC on pulmonary inflammatory responses, we performed a double-blinded, placebo-controlled study of rhAPC in the setting of endotoxin-induced pulmonary inflammation (26). In this study, rhAPC was administered at the recommended clinical dose of 24 $\mu\text{g/kg/h}$ for a 16-h period, starting 2 h before instillation of endotoxin. A second bronchoscopy, with BAL, was performed 16 h after administration of LPS, a time point that was 2 h after discontinuation of the rhAPC infusion. Particular attention was focused on examining the effects of rhAPC on neutrophil functions for several reasons. First, in preclinical models of sepsis and ALI, neutrophils have been demonstrated to be important in potentiating ALI. In particular, the severity of ALI is decreased in neutropenic mice exposed to endotoxin or subjected to severe blood loss (49). Second, recent studies have shown that neutrophil migration is inhibited by exposure to rhAPC, through a mechanism involving interaction of rhAPC with the endothelial protein C receptor expressed on neutrophils (14). Such information suggested that rhAPC might inhibit endotoxin-induced accumulation of neutrophils in the airspaces, providing a potential mechanism of action for its beneficial effects in sepsis. Of note, pneumonia was among the sites of infec-

Figure 1. Recombinant human activated protein C (rhAPC) decreases endotoxin-induced neutrophil and total leukocyte accumulation in the airspaces. Total white cells (WBC) and neutrophils present in bronchoalveolar lavage (BAL) obtained 16 h after endobronchial LPS instillation in volunteers administered rhAPC (filled circles; $n = 8$) or placebo (open circles; $n = 7$). Solid bar represents the mean of log values for each group. (A) Total WBC recovered per milliliter of BAL fluid. Administration of rhAPC significantly reduced recovery of all leukocytes ($p = 0.037$ by Student's t test). (B) Total neutrophils recovered per milliliter of BAL fluid. Administration of rhAPC reduced recovery of neutrophils ($p = 0.048$ by Student's t test). Adapted by permission from Reference 26.

tion showing the greatest benefit with rhAPC treatment in the PROWESS (Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis) clinical trial (7, 50).

Administration of rhAPC significantly reduced the total number of cells as well as neutrophils in the BAL samples from the endotoxin-exposed lung segment (Figure 1). Neutrophils recovered from BAL, but not from the peripheral circulation, of volunteers receiving rhAPC demonstrated decreased chemotaxis *ex vivo* (Figure 2). As noted above, previous studies (14) had shown that incubation of neutrophils with rhAPC decreased chemotaxis. We duplicated this finding, using IL-8 as a chemotactin. Of note, in the previously reported experiments (14), chemotaxis was examined at only a single time point, 30 min after initiation of the chemotaxis gradient. In our experiments, we found that the inhibition of chemotaxis produced by exposure to rhAPC was even greater at later time points.

There were no alterations in the concentrations of BAL cytokines, chemokines, or cytokine inhibitors in volunteers receiving rhAPC. In particular, administration of rhAPC did not produce changes in BAL levels of IL-6, IL-8, TNF- α , MCP-1, IL-1ra, TNFR1, or TNFR2. No differences were detected in gene expression using comprehensive human gene arrays, kinase activation, cytokine release, cell survival, or apoptosis of neutrophils recovered in the presence or absence of rhAPC.

This study in human volunteers demonstrates that rhAPC reduces both endotoxin-induced accumulation of leukocytes in the airspaces and neutrophil chemotaxis (26). These rhAPC-induced effects on neutrophil function may represent a mechanism by which rhAPC improves survival in patients with sepsis. Of note, the neutrophils that were recovered from the airspaces in rhAPC-treated volunteers, aside from their decreased chemotaxis, did not differ from those in the placebo group. These findings suggest that beneficial effects of rhAPC in severe infection may result from inhibiting neutrophil migration into tissue sites, such as the lungs and other organs, and thereby reducing injurious effects due to excessive accumulation of activated neutrophils. Because the suppression of neutrophil chemotaxis by

Figure 2. rhAPC decreases neutrophil migration. (A) Effect of rhAPC on neutrophil chemotaxis *in vitro*. Peripheral blood neutrophils isolated from healthy volunteers were exposed to rhAPC (solid diamonds: 10^{-4} g/ml; asterisks: 10^{-6} g/ml; solid triangles: 10^{-8} g/ml; solid circles: 10^{-10} g/ml) or left untreated (open squares) for 20 min. The mean chemotaxis index is shown ($n = 5$) for migration using an interleukin-8 (1 nM) gradient over a 2-h period. Pretreatment of the neutrophils with rhAPC resulted in a significant reduction in migration for all concentrations tested when compared with untreated cells (10^{-4} g/ml, $p < 0.0001$; 10^{-6} g/ml, $p < 0.0001$; 10^{-8} g/ml, $p < 0.0001$; 10^{-10} g/ml, $p = 0.01$, by two-way analysis of variance [ANOVA]). (B) Administration of rhAPC results in significant decreases in neutrophil chemotaxis *ex vivo*. Neutrophils were isolated from BAL obtained 16 h after endobronchial LPS instillation in volunteers treated with rhAPC (solid circles) or placebo (open circles). Mean index $\pm$ SEM ($n = 3$ for rhAPC-treated group, $n = 4$ for untreated group; $p = 0.012$ by two-way ANOVA). This research was originally published in Reference 26.

rhAPC appears to be independent of effects on other neutrophil functions or inflammatory responses, rhAPC may act as a very selective intervention that reduces excessive neutrophil recruitment into vulnerable organ sites without producing immunosuppression that might reduce resistance to the infectious insult.

IMPLICATIONS AND FUTURE DIRECTIONS

The *in vitro* and *in vivo* studies discussed in this article suggest that the beneficial actions of rhAPC in decreasing the severity of multisystem organ dysfunction and reducing mortality in patients with sepsis may not result from its effects on coagulation pathways, but rather may occur through decreasing the accumulation of activated neutrophils in the lungs and other organs. If this is indeed the case, then modifications of rhAPC that lack anticoagulant properties, but still affect neutrophil function, may be possible to develop. Such formulations would decrease the risk of bleeding, which is the major side effect associated with the

use of this drug, without limiting efficacy. Of note, recent data indicate that the neuroprotective effects of APC are distinct from its anticoagulant effects, and rather, relate to antiapoptotic and antiinflammatory properties (51). In addition, if the major actions of rhAPC are on neutrophil chemotaxis, this may have clinical implications for its use. Because activated neutrophils rapidly traffic to the lungs in experimental models of sepsis-induced acute lung injury (49, 52, 53), early use of rhAPC in appropriate patients (i.e. those with significant risk of mortality as defined by an APACHE (Acute Physiology and Chronic Health Evaluation) II score of at least 25, or more than two sepsis-induced organ dysfunctions) (54) would be expected to have greater benefit than delayed institution of this therapy. Similarly, if the primary effects of rhAPC are on neutrophils, there should be diminished benefit with this agent in neutropenic patients with sepsis as compared with patients with normal or elevated neutrophil counts. Analysis of patient populations treated with rhAPC should be able to address these hypotheses.

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