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Activated Protein C Up-Regulates IL-10 and Inhibits Tissue Factor in Blood Monocytes

Lisa J. Toltt,∗‡ Suzanne Beaudin,†‡ Patricia C. Liaw, 2†‡ and the Canadian Critical Care Translational Biology Group

The protective effect of recombinant activated protein C therapy in patients with severe sepsis likely reflects the ability of recombinant activated protein C to modulate multiple pathways implicated in sepsis pathophysiology. In this study, we examined the effects of recombinant activated protein C on the anti-inflammatory cytokine IL-10 and on the procoagulant molecule tissue factor (TF) in LPS-challenged blood monocytes. Treatment of LPS-stimulated monocytes with recombinant activated protein C resulted in an up-regulation of IL-10 protein production and mRNA synthesis. The up-regulation of IL-10 required the serine protease activity of recombinant activated protein C and was dependent on protease-activated receptor-1, but was independent of the endothelial protein C receptor. At the intracellular level, p38 MAPK activation was required for recombinant activated protein C-mediated up-regulation of IL-10. We further observed that incubation of LPS-stimulated monocytes with recombinant activated protein C down-regulated TF activity levels. This anticoagulant effect of recombinant activated protein C was dependent on IL-10 since neutralization of endogenously produced IL-10 abrogated the effect. In patients with severe sepsis, plasma IL-10 levels were markedly higher in those treated with recombinant activated protein C than in those who did not receive recombinant activated protein C. This study reveals novel regulatory functions of recombinant activated protein C, specifically the up-regulation of IL-10 and the inhibition of TF activity in monocytes. Our data further suggest that these activities of recombinant activated protein C are directly linked: the recombinant activated protein C-mediated up-regulation of IL-10 reduces TF in circulating monocytes. The Journal of Immunology, 2008, 181: 2165–2173.

Sepsis is a devastating condition characterized by systemic activation of inflammatory and coagulation pathways in response to microbial infection of the blood or tissues (1). Severe sepsis, defined as sepsis associated with at least one dysfunctional organ, is one of the leading causes of morbidity and mortality in the Western world, with an estimated mortality rate of 30–50% (2).

Activated protein C, a plasma serine protease, is a physiologically important anticoagulant (3, 4). The recombinant form of human activated protein C (recombinant activated protein C; Drotrecogin alpha (activated)) is the first biological agent to improve survival in patients with severe sepsis (5). In 2001, the PROWESS study demonstrated that, compared with placebo, a 4-day infusion of supraphysiological levels of recombinant activated protein C produced a reduction in the relative risk of death of 19.4% and an absolute reduction in the risk of death of 6.1% (p = 0.005) (5). In 2005, the ENHANCE trial provided further evidence of a favorable benefit-risk profile of human recombinant activated protein C in the treatment of severe sepsis, reporting an efficacy and safety outcome similar to that of PROWESS (6).

Why does a 4-day infusion of recombinant activated protein C reduce mortality in septic patients? Over the past 15 years, impressive research advances have provided novel insights into the diverse biological activities of this molecule. Activated protein C is now not only viewed as an anticoagulant but also as a signaling molecule that provides a pivotal link between the pathways of coagulation, inflammation, apoptosis, and vascular permeability (reviewed in Refs. 7, 8). The protective effect of recombinant activated protein C therapy in patients with severe sepsis likely reflects the ability of activated protein C to modulate the complex changes associated with sepsis pathophysiology.

Although activated protein C is well established as a plasma serine protease that degrades blood coagulation cofactors Va and VIIIa, less is known about the ability of activated protein C to modulate cellular functions. We and others have focused on the effects of activated protein C on blood monocytes, cells that play a critical role in initiating, perpetuating, and modulating the immediate host response to invading microorganisms. Activated protein C has been shown to exert direct anti-inflammatory effects on monocytes by inhibiting the production of proinflammatory cytokines. For example, in the THP-1 monocytic cell line and in peripheral blood monocytes, activated protein C down-regulates TNF (9–11), IL-1β, IL-6, and IL-8 (12), presumably by inhibiting NF-κB nuclear translocation (10) and/or down-regulating the transcription of NF-κB subunits (13). In vivo studies of animals challenged with endotoxin have shown that activated protein C...
prevents the production of proinflammatory cytokines (14, 15) and inhibits leukocyte accumulation in injured tissues (16).

One intriguing feature of monocyte biology is the ability of these cells to produce both pro- and anti-inflammatory cytokines (17, 18). IL-10 is an anti-inflammatory cytokine that is produced mainly by monocytes and macrophages (19). IL-10 inhibits the production of numerous inflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8, as well as IL-10 itself (20, 21), thereby protecting the host from the harmful effects of a prolonged and unbalanced inflammatory response. In murine sepsis models, increases in IL-10 confer protection and are associated with survival (22, 23). In humans, elevated levels of IL-10 early post-insult are protective (24) and may be beneficial to counter-regulate the early inflammatory response (25).

In the current study, we investigated the effects of recombinant activated protein C on IL-10 production in LPS-stimulated monocytes. Since IL-10 has been shown to inhibit the procoagulant molecule tissue factor (TF)\(^1\) on monocytes (26-28), we also explored the possibility that recombinant activated protein C has novel anticoagulant effects via the inhibition of TF procoagulant activity in activated monocytes. Our studies reveal novel regulatory functions of recombinant activated protein C and novel crosstalk between the pathways of inflammation and coagulation.

Materials and Methods

**Materials**

The MACS midiMACS separation kits, LS columns, and anti-CD14 microbeads were all from Miltenyi Biotec. RPMI 1640, Escherichia coli 026:B6 LPS, hirudin, and Ponceau S were from Sigma-Aldrich. Human recombinant tissue factor; PPACK, phe-pro-arg-chloromethylketone; PAR-1, protease-activated receptor-1. ATAP-2, was purified human IgG was from Jackson ImmunoResearch Laboratories. The LPS, hirudin, and Ponceau S were from Sigma-Aldrich. Human recombinant TF, tissue factor; PPACK, phe-pro-arg-chloromethylketone; PAR-1, protease-activated receptor-1.

**Materials and Methods**

**Materials**

The MACS midiMACS separation kits, LS columns, and anti-CD14 microbeads were all from Miltenyi Biotec. RPMI 1640, Escherichia coli 026:B6 LPS, hirudin, and Ponceau S were from Sigma-Aldrich. Human recombinant activated protein C (Drotrecogin alpha (activated); Xigris) was from Eli Lilly. Phe-pro-arg-chloromethylketone (PPACK) was from Calbiochem. Chromopure human IgG was from Jackson ImmunoResearch Laboratories. The anti-human protease-activated receptor-1 (PAR-1) Ab, ATAP-2, was purchased from Santa Cruz Biotechnology. The anti-human endothelial protein C receptor (EPCR) Abs (JRK 1535 and 1495) were provided as gifts from Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). Anti-human phospho-p38 MAPK primary Ab was from Cell Signaling Technology. Anti-human β-actin primary Ab was from BioLegend. Anti-human IL-10 Ab was from R&D Systems and the anti-human TF-FITC Ab was from Affinity Biologicals. Factors VIIa, Xa, and X were all from Hematologic Technologies. The S-2366 and CS-11 chromogenic substrates were from DiaPharma Group and Aniara.

**Isolation of human monocytes from whole blood**

Human peripheral blood monocytes were isolated from the whole blood of healthy volunteers by MACS as previously described (12). The isolation and use of monocytes from healthy donors was voluntary, imposed minimal risk to the donor, and did not require approval of the Research Ethics Board of the Hamilton Health Sciences.

**Cell culture conditions**

Human blood monocytes were cultured in RPMI 1640 media supplemented with 10% FBS and 100 U/ml penicillin-streptomycin and incubated at 37°C with 5% CO\(_2\). Cells used for experimentation were plated at 1 × 10\(^6\) cells/ml in 6-well or 24-well microtiter plates. Monocytes were incubated with LPS (20 ng/ml) in the absence or presence of recombinant activated protein C (5, 30, 60, or 120 nM) or active site blocked recombinant activated protein C (120 nM). For certain conditions, a monoclonal anti-EPCR antiantibody (120 nM) was added to the cell culture before the addition of recombinant activated protein C. To ensure recombinant activated protein C did not contain trace amounts of bovine thrombin, hirudin (1 μg/ml) was added to the cell culture before the addition of recombinant activated protein C or PPACK-recombinant activated protein C.

The active site of recombinant activated protein C was inhibited with the tripeptide inhibitor PPACK. PPACK-recombinant activated protein C was produced by incubation of 4.5 μM recombinant activated protein C in PBS with 20-fold molar excess of PPACK for 2 h on ice. Residual unbound PPACK was removed by dialysis in PBS. The PPACK-recombinant activated protein C was inactive as monitored by the loss of enzymatic activity toward S-2366 chromogenic substrate.

All reagents used were purified from endotoxin contaminants before addition to cell culture using the EndoTrap Blue endotoxin removal system as per manufacturer’s instructions (Cambrex Bio Science Walkersville). The BCA protein assay (Pierce) was performed, according to manufacturer instructions, on EndoTrap-purified reagents. The concentration of protein in each sample was calculated by linear regression analysis of the prepared standard curve.

**Isolation of plasma from patients with severe sepsis**

Blood was collected from patients within 24 h of meeting the definition of severe sepsis. Patients with severe sepsis were identified based on the inclusion criteria described in the PROWESS study (5). Serial samples were obtained daily for the first week and once a week thereafter until study completion. Blood was collected into buffered trisodium citrate tubes as previously described (29). This study was approved by the Research Ethics Board of the Hamilton Health Sciences Corporation.

**Analysis of IL-10 levels in monocyte cultures or in the plasma of patients with severe sepsis**

Quantification of IL-10 levels in monocyte cultures or plasma samples were determined using either the Human IL-10 ELISA Development kit (Biosource) as per manufacturer’s instructions, or the CBA Human Inflammation kit (BD Biosciences) as previously described (12). Monocytes were cultured according to the conditions described above. The supernatants were collected after 4, 12, or 24 h and frozen at −80°C until use.

**Immunoblotting for activation of the p38 MAPK pathway**

Monocytes were treated with recombinant activated protein C (120 nM) alone or in combination with LPS (20 ng/ml) for varying lengths of time. Cells were lysed using a buffer consisting of 1% deoxycholic acid, 1% Triton X-100, in 50 mM Tris-Cl (pH 7.2) containing EDTA (0.25 mM), leupeptin (5 μg/ml), aprotonin (5 μg/ml), pepstatin A (1 μg/ml), PMSF (1 mM), and sodium orthovanadate (0.1 mM). The BCA protein assay was performed on the cell lysates to determine protein concentration. Electrophoresis was performed according to the method of Laemmli (30) using 12% SDS-polyacrylamide gels under reducing conditions. Immunoblotting was performed using a rabbit anti-human phospho-p38 MAPK Ab followed by goat anti-rabbit HRP Ab. The blots were imaged on the Typhoon 9410 Variable Mode Imager (Amersham Biosciences/GE Healthcare). The membranes were stripped of Abs, according to manufacturer’s instructions, and re-probed with an anti-β-actin Ab. Densitometric analyses were performed using Image Quant v5.2 software (Amersham Biosciences).

**Real-time PCR quantitation of IL-10 mRNA levels**

Total RNA was extracted from monocytes for each treatment condition using the RNeasy Mini Mammalian Total RNA kit (Qiagen) as per manufacturer’s instructions. The synthesis of cDNA was performed using the SuperScript III First Strand Synthesis System for RT-PCR with random hexamer primers (Invitrogen) according to the manufacturer’s protocol.

Real-time PCR analysis of IL-10 and β-actin mRNA levels using SYBR GreenER qPCR Supermix (Invitrogen) as per manufacturer’s instructions using the ABI Prism 7300 (Applied Biosystems). Specific primers were used to amplify IL-10 and β-actin mRNA (McMaster University MOBIX Laboratory). The primer sequences are as follows (31, 32):

IL-10 Forward: 5′-AAAGGACGACCAAGCATCAA-3′

IL-10 Reverse: 5′-AGAACAAGACCCAGACATCAA-3′

β-actin Forward: 5′-TGCCATCCTAAAAGCCAC-3′

β-actin Reverse: 5′-TCAACTGGTCTCAGACTGTT-3′

Results are expressed as the fold-increase over the respective β-actin controls.

**Measurement of TF surface expression and activity**

Monocyte TF surface Ag levels were measured using a FACSCalibur instrument and CellQuest Pro software (BD Biosciences). In brief, monocytes were removed from culture, washed once, and resuspended in PBS plus 0.1% BSA. To prevent non-specific binding, 200 μg/ml human IgG was added to the monocyte cell suspensions and incubated on ice for 1 h. Membrane monolonal anti-human TF-FITC Ab (2 μg/ml) and an isotype-matched control was added directly to the cell suspensions and incubated for 30 min on ice in the dark. The cells were washed and resuspended in PBS plus 0.1% BSA for flow cytometry.
Monocyte TF activity levels were measured by a chromogenic assay to detect FXa generation by the TF-FVIIa complex (33). In brief, following treatment, the monocytes were washed and resuspended in TBS containing 5 nM FVIIa and 150 nM FX. To initiate the reaction, CaCl2 was added to a final concentration of 5 mM. Following a 30-min incubation at 37°C, monocytes were removed and the supernatants as well as the FXa standards (0–5 nM) were incubated with CS-11 chromogenic substrate at a final concentration of 0.2 mM for 3 min. The reaction was stopped with 50% acetic acid and measured at 405 nm using SpectraMax 340 PC with Softmax Pro software (Molecular Devices).

Statistics
ANOVA was used to compare the results between experimental and control groups. If a significant difference between experimental and control groups was detected, post hoc analyses were performed. For some treatment groups, means were compared by Student t tests. All experiments were performed at least in triplicate and data were considered significant for \( p \leq 0.05 \). Data are shown as mean ± SE.

Results
Effect of recombinant activated protein C on IL-10 protein production and mRNA synthesis in LPS-stimulated human monocytes

Although we and others have reported that recombinant activated protein C down-regulates proinflammatory cytokine production in LPS-stimulated monocytes (9–12), to date there have been no studies examining the effects of recombinant activated protein C on the production of the anti-inflammatory cytokine IL-10 in LPS-stimulated human monocytes. In this study, peripheral blood monocytes were treated with LPS in the absence or presence of recombinant activated protein C, and levels of IL-10 in the conditioned medium were measured. Treatment of LPS-stimulated monocytes with recombinant activated protein C increased IL-10 protein levels in a dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) manner. We observed that after 24 h, recombinant activated protein C treatment resulted in a mean increase in IL-10 protein levels of \( \geq 50\% \) (minimum \( \geq 0\% \), maximum \( \geq 155\% \), relative to LPS only control), compared with monocytes treated with LPS alone (Fig. 1, A and B; Fig. 2). The addition of hirudin (1 \( \mu \)M) did not abrogate the recombinant activated protein C-mediated effect (data not shown), suggesting that this effect of activated protein C was not due to possible trace amounts of thrombin in the recombinant activated protein C.

To ensure that the analyses captured the full nature and magnitude of the response, the following experiments were performed. First, monocytes were treated with various concentrations of LPS (0, 1, 5, 10, 20, 100, 1000 ng/ml LPS) ± recombinant activated protein C. Although we and others have reported that recombinant activated protein C down-regulates proinflammatory cytokine production in LPS-stimulated monocytes (9–12), to date there have been no studies examining the effects of recombinant activated protein C on the production of the anti-inflammatory cytokine IL-10 in LPS-stimulated human monocytes. In this study, peripheral blood monocytes were treated with LPS in the absence or presence of recombinant activated protein C, and levels of IL-10 in the conditioned medium were measured. Treatment of LPS-stimulated monocytes with recombinant activated protein C increased IL-10 protein levels in a dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) manner. We observed that after 24 h, recombinant activated protein C treatment resulted in a mean increase in IL-10 protein levels of \( \geq 50\% \) (minimum \( \geq 0\% \), maximum \( \geq 155\% \), relative to LPS only control), compared with monocytes treated with LPS alone (Fig. 1, A and B; Fig. 2). The addition of hirudin (1 \( \mu \)M) did not abrogate the recombinant activated protein C-mediated effect (data not shown), suggesting that this effect of activated protein C was not due to possible trace amounts of thrombin in the recombinant activated protein C.

To ensure that the analyses captured the full nature and magnitude of the response, the following experiments were performed. First, monocytes were treated with various concentrations of LPS (0, 1, 5, 10, 20, 100, 1000 ng/ml LPS) ± recombinant activated protein C.
Activated protein C (120 nM) for 24 h and IL-10 was measured. IL-10 levels increased with increasing concentrations of LPS, and this increase is augmented in the presence of recombinant activated protein C to a similar degree at all tested concentrations of LPS (data not shown). Second, using whole cell lysates from monocytes treated with LPS ± recombinant activated protein C, we measured the levels of IL-10 that may have been taken up by the cells. There were no significant differences in the levels of IL-10 in the lysates of the LPS-treated monocytes vs the monocytes treated with LPS plus recombinant activated protein C. Thus, the uptake of IL-10 does not appear to affect the levels of IL-10 detected in the monocyte culture supernatants.

To gain insight into the mechanism by which recombinant activated protein C up-regulates IL-10, IL-10 mRNA levels were quantified in LPS-stimulated monocytes using real-time PCR. Treatment of LPS-stimulated monocytes with recombinant activated protein C resulted in a 4.65 ± 1.4-fold increase in IL-10 mRNA levels over the LPS control (Fig. 1C). The recombinant activated protein C-mediated increase in IL-10 mRNA paralleled the increases with IL-10 protein production (Fig. 1D). This demonstrates a relationship between the recombinant activated protein C-mediated induction of IL-10 gene expression and the extracellular appearance of IL-10 protein. These results suggest that in activated monocytes, recombinant activated protein C up-regulates the transcription of IL-10 mRNA.

Requirement of serine protease activity for the up-regulation of IL-10 by recombinant activated protein C

Activated protein C is a serine protease derived from the activation of zymogen protein C by the thrombin-thrombomodulin complex (4). To determine whether serine protease activity is essential for the recombinant activated protein C-mediated up-regulation of IL-10, we compared the effect of recombinant activated protein C to that of active site blocked recombinant activated protein C (PPACK-recombinant activated protein C). Monocytes were stimulated with LPS in the presence of either recombinant activated protein C or PPACK-recombinant activated protein C, and levels of IL-10 in the conditioned medium were determined. Unlike recombinant activated protein C, PPACK-recombinant activated protein C did not up-regulate IL-10 protein levels in LPS-stimulated monocytes (Fig. 2), suggesting that the ability of recombinant activated protein C to up-regulate IL-10 levels requires serine protease function.

Requirement of EPCR and PAR-1 for the for the up-regulation of IL-10 by recombinant activated protein C

The cytoprotective effects of activated protein C have been shown to require EPCR and PAR-1 (12, 34–37). To investigate whether EPCR is required for recombinant activated protein C-mediated up-regulation of IL-10, monocytes were treated with recombinant activated protein C and LPS in the presence of two different anti-EPCR mAbs that block activated protein C-EPCR interactions (JRK 1495 or 1535). As shown in Fig. 2, the ability of recombinant activated protein C to up-regulate IL-10 in LPS-stimulated monocytes was not affected by blocking the recombinant activated protein C-EPCR interaction with either Ab. Furthermore, using a range of recombinant activated protein C concentrations (5, 30, 60, 120 nM), blocking recombinant activated protein C-EPCR interaction failed to affect the recombinant activated protein C-mediated up-regulation of IL-10 in monocytes (data not shown).

PAR-1 expression has been demonstrated in human monocytes (38). Since IL-10 production required the serine protease activity of recombinant activated protein C (Fig. 2), we sought to address the question of whether activated protein C-mediated cleavage of PAR-1 is necessary for the ability of recombinant activated protein C to increase IL-10 production. We investigated whether ATAP-2, a mAb that blocks the extracellular activation of PAR-1, could inhibit the up-regulation of IL-10 by recombinant activated protein C in LPS-stimulated monocytes. Monocytes were treated with recombinant activated protein C in the absence or presence of ATAP-2, followed by LPS treatment. Twenty-four hours later, secreted IL-10 levels were determined. As shown in Fig. 2, ATAP-2 completely inhibited the recombinant activated protein C-mediated up-regulation of IL-10 in LPS-treated monocytes, suggesting that the recombinant activated protein C-mediated up-regulation of IL-10 is dependent upon PAR-1 cleavage. Addition of either JRK 1495, JRK 1535, or ATAP-2 Abs alone to monocyte cultures did not increase IL-10 levels in the culture medium (data not shown).

Effect of recombinant activated protein C on p38 MAPK signaling in monocytes

Cytokine production in monocytes involves the activation of three different MAPK pathways, specifically p38 MAPK, ERK-1/2, and stress-activated protein kinase/JNK (39–42). In monocytes, the induction of IL-10 synthesis requires the activation of p38 MAPK (43). Therefore, we sought to determine whether the recombinant activated protein C-mediated induction of IL-10 in monocytes involves the activation of p38 MAPK. To determine whether recombinant activated protein C activated the p38 MAPK pathway, LPS-stimulated monocytes were incubated with recombinant activated protein C for varying lengths of time, and levels of activated (phosphorylated) p38 were measured in cell lysates by immunoblot analysis. To ensure that equal amounts of cell lysate were loaded onto the polyacrylamide gels, the blots were stained with Ponceau S and immunoblotted for β-actin.

As shown in Fig. 3A, intracellular levels of phosphorylated p38 in LPS-stimulated monocytes were increased when the cells were treated with recombinant activated protein C compared with monocytes challenged with LPS alone (1.6-fold increase after 5 min, 1.2-fold increase after 10 min, 0.8-fold increase after 15 min).
coagulation cofactors Va and VIIIa (3). In this study, we explored the possibility that the anticoagulant activities of activated protein C extend beyond its ability to degrade Va and VIIIa. Specifically, we examined the effects of recombinant activated protein C on monocyte TF expression using flow cytometry. As shown in Fig. 5, recombinant activated protein C inhibited TF Ag expression levels in LPS-stimulated monocytes in a dose-dependent manner.

IL-10 has been shown to inhibit TF mRNA expression in LPS-stimulated monocytes (26) and to inhibit monocyte TF expression in LPS-stimulated whole blood (27). We therefore wanted to determine whether the ability of recombinant activated protein C to down-regulate monocyte TF is mediated by IL-10. LPS-stimulated monocytes were treated with recombinant activated protein C in the presence or absence of a neutralizing anti-human IL-10 mAb. The down-regulation of TF Ag and activity levels in activated monocytes by recombinant activated protein C was abrogated in the presence of the anti-IL-10 Ab. These findings demonstrate that the ability of recombinant activated protein C to inhibit monocyte TF is due to the recombinant activated protein C-mediated up-regulation of IL-10 levels.

**Systemic IL-10 levels in patients with severe sepsis**

To determine whether our in vitro findings correlate with in vivo responses to recombinant activated protein C therapy, we serially measured plasma IL-10 levels in 48 patients with severe sepsis treated either with or without therapeutic recombinant activated protein C. Patients with severe sepsis were identified using the inclusion criteria described by Bernard et al. (5), where some patients received recombinant activated protein C therapy and others did not. Blood samples were obtained daily for the first week and once a week thereafter for the duration of the patients’ stay in the Intensive Care Unit. Baseline characteristics for the patients are shown in Table I. There were significant inter-individual differences in the absolute plasma levels of IL-10 among the patients. However, our analyses of plasma from patients who received recombinant activated protein C therapy showed a trend of increased IL-10 levels compared with patients who did not receive recombinant activated protein C treatment (Fig. 7). In addition, there was a progressive increase in plasma IL-10 in recombinant activated...
protein C-treated patients up until day 7, with a subsequent decline in IL-10 levels after day 7 to below baseline (i.e., day 1) levels observed at day 28.

**Discussion**

During sepsis, an exacerbated and uncontrolled activation of the innate immune system may contribute to tissue damage and, ultimately, death. Unique among cells, monocytes not only secrete a repertoire of pro- and anti-inflammatory cytokines, but are the only blood cells known to synthesize the procoagulant molecule TF (44, 45). In this study, we explored the possibility that recombinant activated protein C dampens the proinflammatory and procoagulant potential of activated monocytes by up-regulating the production of the anti-inflammatory cytokine IL-10 and by inhibiting TF activity.

In these studies, we demonstrate that recombinant activated protein C increases IL-10 production in LPS-stimulated human monocytes by \( \geq 50\% \). In a mouse model of endotoxic shock, increased IL-10 levels of \( \leq 25\% \) dampened the systemic inflammatory response of protein C-treated patients up until day 7, with a subsequent decline in IL-10 levels after day 7 to below baseline (i.e., day 1) levels observed at day 28.

**FIGURE 6.** Effects of recombinant activated protein C and IL-10 neutralization on TF Ag and procoagulant activity in LPS-stimulated human monocytes. Monocytes were treated with recombinant activated protein C (120 nM), followed by the addition of a neutralizing anti-IL-10 mAb and LPS stimulation. Following incubation, levels of TF Ag (A) and procoagulant activity (B) were measured. Data in A are expressed as mean fluorescence intensity (MFI) ± SE relative to the LPS control and in B as FXa generation relative to the LPS control. * denotes \( p \leq 0.05 \) (\( n = 6 \) for A, \( n = 4 \) for B).

**FIGURE 7.** Plasma IL-10 levels in patients with severe sepsis. IL-10 levels were measured in the plasma from patients with severe sepsis treated with recombinant activated protein C (○, \( n = 7 \)) or without recombinant activated protein C (●, \( n = 41 \)). Recombinant activated protein C was administered to the patients for 4 days starting from day 2. IL-10 levels are expressed as the absolute levels for each day shown and are expressed as mean ± SE.

**Table I.** Baseline characteristics of 48 patients with severe sepsis

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<tr>
<th>Characteristic</th>
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<td><strong>MODS score</strong></td>
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<td>Mean ± SD (minimum, maximum)</td>
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<td>12 ± 2.9 (8, 15)</td>
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<td><strong>Primary site of infection, no. (% of total)</strong></td>
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<td>Lung</td>
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<td>Abdomen</td>
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response and limited end-organ injury (46). We also observed that recombinant activated protein C increased IL-10 production in LPS-stimulated monocytes at the mRNA and protein levels, and required recombinant activated protein C serine protease activity as well as PAR-1 cleavage. The requirement of PAR-1 for this anti-inflammatory effect of recombinant activated protein C is consistent with previous studies demonstrating that recombinant activated protein C inhibits endothelial and monocyte apoptosis via PAR-1 (12, 34, 35, 47, 48). Interestingly, the recombinant activated protein C-mediated up-regulation of IL-10 in monocytes was not significantly affected by blocking recombinant activated protein C-EPCR interaction. Thus, in our monocyte model, the up-regulation of IL-10 by recombinant activated protein C appears to be independent of EPCR. Consistent with this finding, studies by Zheng et al. (49) demonstrated that deficiency of EPCR in non-hematopoietic cells (i.e., vascular endothelial cells) exaggerated the host responses to LPS, whereas deficiency of EPCR in hematopoietic leukocytes played a much less important role (49). Furthermore, recent reports demonstrate that recombinant activated protein C can induce a cell signaling response via ERK activation through a PAR-1/S1P1-dependent, but EPCR-independent, mechanism (50). Other studies have shown that blocking the binding of activated protein C to EPCR did not affect the inhibition of TNF-α production by activated protein C in LPS-stimulated human monocytes (11).

It is also possible that there may be an alternative activated protein C-binding receptor on the surface of monocytes (51). Using Ca²⁺-free conditions (which prevents activated protein C-EPCR interactions) (52) or mAbs that block activated protein C-EPCR interactions (53), we observed that recombinant activated protein C retained the ability to bind to the monocyte cell surface (unpublished observations). In contrast, the binding of recombinant activated protein C to endothelial cell surfaces is blocked by anti-EPCR Abs as well as by the removal of calcium in the binding buffer (unpublished observations). Taken together, these studies suggest that EPCR is not required for the anti-inflammatory effects of activated protein C in human monocytes.

The precise mechanism(s) by which activated protein C signals in monocytes is(are) not fully elucidated; however, previous studies have shown that activated protein C induces MAPK activation in endothelial cells via PAR-1 activation (34, 50). Although not fully understood, IL-10 production involves the p38 MAPK pathway for the activation of the Sp1 transcription factor (43). Consistent with this, our data suggest that p38 MAPK activation is important for the up-regulation of IL-10 by recombinant activated protein C in monocytes. We demonstrate that recombinant activated protein C promotes p38 MAPK phosphorylation in monocytes, an effect shown by recombinant activated protein C alone and by recombinant activated protein C in combination with LPS. Furthermore, our observations show that a cleavage-blocking Ab against PAR-1 inhibits monocyte p38 MAPK activation. Because IL-10 function and signaling are important for controlling inflammatory responses, this effect of recombinant activated protein C may offer a controlled mechanism for promoting an anti-inflammatory response via the up-regulation of IL-10.

Current thinking is that the severity of sepsis is determined by the balance of pro- and anti-inflammatory cytokines (17, 22). In a murine model of sepsis, increased levels of IL-10 were associated with less severe sepsis (22), and administration of IL-10 was highly effective at protecting mice from lethal endotoxemia (23). In humans, increased plasma IL-10 levels are presumed to limit the inflammatory response induced by invading microorganisms (54). IL-10 is a potent anti-inflammatory cytokine that inhibits an exaggerated response of proinflammatory cytokine release, and, thus, it is likely that regulated levels of IL-10 play an important role in preventing the development of various pathologic events seen in sepsis. The anti-inflammatory effect of recombinant activated protein C, specifically the inhibition of proinflammatory cytokine production and up-regulation of IL-10, as well as its anti-coagulant effect, through the degradation of FVα and FVIIIα and inhibition of TF, could contribute to the therapeutic efficacy of recombinant activated protein C in patients with severe sepsis.

In this study, we measured IL-10 levels in the plasma of patients with severe sepsis and found that patients receiving recombinant activated protein C had higher IL-10 compared with patients that did not receive recombinant activated protein C therapy. Specifically, in the patients treated with recombinant activated protein C, IL-10 levels were found to progressively increase until day 7, but, beyond that, plasma IL-10 levels declined back to baseline levels. Studies have demonstrated that elevated levels of IL-10 early post-insult confer protection following trauma, endotoxemia, and sepsis (23, 24). In accordance with these findings, studies have also shown that reducing IL-10 at a later time point following the diagnosis of sepsis exerts beneficial effects (55). A recent study using a rat endotoxemia model reported higher IL-10 levels in rats treated with recombinant activated protein C compared with untreated animals; however, this increase was not statistically significant (56). The increase in IL-10 synthesis and production by recombinant activated protein C in activated monocytes may offer protection and a mechanism of regulating an unbalanced immune response.

In vivo, TF is the primary physiologic initiator of blood coagulation (57–59). TF is a transmembrane glycoprotein constitutively expressed on cells that are not normally in contact with blood (e.g., fibroblasts and smooth muscle cells) (60). Upon vascular injury, plasma coagulation factor VIIa contacts extravascular TF, thereby initiating coagulation. Importantly, monocytes are the only circulating cells in which TF expression is subject to inducible regulation and the only intravascular cells in which TF expression has been detected in vivo (60). Thus, activated monocytes can contribute to the dissemination of local as well as systemic procoagulant potentials. Another intriguing finding in this work is that recombinant activated protein C inhibits TF Ag and procoagulant activity in LPS-activated blood monocytes. In vitro studies have demonstrated that the procoagulant potential of various cells is mediated by the concentration of cell surface TF (61–63). Thus, the decrease in monocyte cell surface TF Ag and procoagulant activity we report here is likely physiologically important. Using the commercially available human monoblastic promyeloid leukemia cell line U937, Shu et al. (64) demonstrated that activated protein C inhibited TF expression in phorbol ester-stimulated cells in an EPCR-dependent manner (64). We add to this by suggesting that, in human monocytes, the recombinant activated protein C-mediated down-regulation in TF expression and procoagulant activity is dependent upon the recombinant activated protein C-mediated up-regulation of IL-10. Furthermore, our findings demonstrate that the ability of recombinant activated protein C to up-regulate IL-10 is EPCR-independent, suggesting that recombinant activated protein C-mediated effects on blood monocytes and U937 cells occur via distinct mechanisms.

In summary, these studies reveal novel regulatory functions of recombinant activated protein C, specifically the up-regulation of IL-10 via an EPCR-independent, but PAR-1-dependent, mechanism, as well as the inhibition of TF expression and procoagulant activity in LPS-stimulated monocytes. We also demonstrate that these activities of recombinant activated protein C are directly linked: the recombinant activated protein C-mediated up-regulation of IL-10 inhibits monocyte TF activity. These data suggest
that part of the protective effect of recombinant activated protein C therapy may reflect the ability of recombinant activated protein C to dampen the proinflammatory and procoagulant potential of activated monocytes.

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Disclosures

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