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Modulation of Monocyte Function by Activated Protein C, a Natural Anticoagulant

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Activated protein C is the first effective biological therapy for the treatment of severe sepsis. Although activated protein C is well established as a physiological anticoagulant, emerging data suggest that it also exerts anti-inflammatory and antiapoptotic effects. In this study, we investigated the ability of activated protein C to modulate monocyte apoptosis, inflammation, phagocytosis, and adhesion. Using the immortalized human monocyctic cell line THP-1, we demonstrated that activated protein C inhibited campothecin-induced apoptosis in a dose-dependent manner. The antiapoptotic effect of activated protein C requires its serine protease domain and is dependent on the endothelial cell protein C receptor and protease-activated receptor-1. In primary blood monocytes from healthy individuals, activated protein C inhibited spontaneous apoptosis. With respect to inflammation, activated protein C inhibited the production of TNF, IL-1β, IL-6, and IL-8 by LPS-stimulated THP-1 cells. Activated protein C did not influence the phagocytic internalization of Gram-negative and Gram-positive bioparticles by THP-1 cells or by primary blood monocytes. Activated protein C also did not affect the expression of adhesion molecules by LPS-stimulated blood monocytes nor the ability of monocytes to adhere to LPS-stimulated endothelial cells. We hypothesize that the protective effect of activated protein C in sepsis reflects, in part, its ability to prolong monocyte survival in a manner that selectively inhibits inflammatory cytokine production while maintaining phagocytosis and adherence capabilities, thereby promoting antimicrobial properties while limiting tissue damage. The Journal of Immunology, 2006, 177: 2115–2122.

Sepsis is a devastating disorder characterized by uncontrolled activation of inflammation and coagulation in response to microbes and/or microbial toxins (1). Sepsis is the leading cause of death in noncoronary intensive care unit patients and the 13th most common cause of death overall (2). Severe sepsis, defined as sepsis with the associated dysfunction of one or more organs, afflicts ~750,000 people in the United States annually, with an estimated mortality rate of 30–50% (2).

Over the past 20 years, many potential treatments for sepsis have shown early promise yet failed to improve survival in phase III clinical trials. These agents attempted to treat sepsis through attenuation of inflammatory mediators or by the neutralization of endotoxin (3). The current understanding of sepsis pathophysiology is that inflammation, coagulation, and apoptosis act together in the disease process. The recombinant form of human activated protein C, a natural anticoagulant, is the first effective biological agent to have improved the outcome of patients with severe sepsis (4). Compared with placebo, a 4-day infusion of recombinant activated protein C resulted in a reduction in the relative risk of death of 19.4%, corresponding to an absolute reduction of 6.1% (p = 0.005). This was the first human phase III randomized trial to demonstrate a survival benefit in the treatment of severe sepsis.

The protective effect of activated protein C supplementation in patients with severe sepsis likely reflects the ability of activated protein C to modulate multiple pathways implicated in sepsis pathophysiology. Activated protein C, a plasma serine protease, is best known for its ability to inhibit blood clot formation. In conjunction with its cofactor protein S, activated protein C degrades coagulation cofactors Va and VIIa, thereby attenuating the coagulation cascade (5, 6). The importance of the anticoagulant properties of activated protein C is highlighted by the fact that patients with congenital or acquired deficiencies in the activated protein C anticoagulant pathway are prone to venous and arterial thrombosis (7–12).

More recently, activated protein C has been shown to inhibit inflammation (13–18), apoptosis (19–22), and to prevent increases in vascular permeability (23, 24). With respect to its anti-inflammatory properties, activated protein C has been shown to down-regulate the production of TNF by monocytes (13–15) and to suppress expression of leukocyte adhesion molecules in endothelial cells (16), presumably by inhibiting NF-κB nuclear translocation (14) and/or down-regulating the transcription of NF-κB subunits (16). In animals challenged with endotoxin, activated protein C inhibited the production of proinflammatory cytokines (17, 18) and inhibited leukocyte accumulation in injured tissue (25). Likewise, the PROWESS trial revealed that recombinant activated protein C infusion reduced levels of IL-6, a proinflammatory cytokine (4).

With respect to apoptosis, activated protein C has been shown to exert an antiapoptotic effect on vascular endothelial cells (19, 20). The antiapoptotic effect of activated protein C was dependent on the endothelial cell protein C receptor (EPCR),3 as well as protease-activated receptor (PAR)-1 (19, 20). In human brain endothelial cells,

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3 Abbreviations used in this paper: EPCR, endothelial cell protein C receptor; PAR, protease-activated receptor; PPACK, Phe-pro-arg-chloromethylketone; CBA, cytometric bead array.
activated protein C prevented hypoxia-induced apoptosis by inhibiting p53 tumor suppressor protein, by normalizing the proapoptotic Bax:Bcl-2 ratio, and by reducing caspase-3 activation (21). In mouse cortical neurons, activated protein C prevented apoptosis induced by two divergent inducers of apoptosis, N-methyl-D-aspartate and staurosporin, by blocking caspase activation and by inhibiting nuclear translocation of apoptosis-inducing factor (22). The neuroprotective effect of activated protein C required PAR-1 and PAR-3 (22).

Preliminary studies suggest that activated protein C also prolongs monocyte lifespan. Specifically, activated protein C was shown to suppress staurosporine-induced apoptosis of the U937 human leukemia mononuclear cell line (26). This observation is intriguing since it is unclear if prolonged monocyte survival is beneficial or detrimental to the host during sepsis. One perspective is that although monocytes mediate the immediate host response to invading microorganisms, prolonged release of proinflammatory mediators by monocytes may lead to persistence of tissue injury (27). In other words, the monocyte response may be a dual-edged sword: monocytes phagocyte invading pathogens but also produce various inflammatory cytokines, chemokines, and growth factors that can amplify the inflammatory response.

To investigate the functional consequences of an antiapoptotic effect of activated protein C in monocytes, we investigated the ability of activated protein C to modulate monocyte apoptosis, phagocytosis, and inflammation using the immortalized human monocytic cell line THP-1, as well as primary human monocytes. We explored the hypothesis that activated protein C prolongs monocyte lifespan in a manner that maintains phagocytic and adhesiveness capabilities while selectively inhibiting inflammatory cytokine production, thus promoting antimicrobial properties while limiting damage to host tissue.

Materials and Methods

Materials

HUVECs and the immortalized human monocytic cell line THP-1 were purchased from the American Type Culture Collection. RPMI 1640 and DMEM culture media, penicillin-streptomycin, and normal goat serum were purchased from Invitrogen Life Technologies. FBS was purchased from HyClone. BSA and Escherichia coli 026:B6 LPS were purchased from Sigma-Aldrich. Heparin was purchased from American Diagnostica. Human IgG was purchased from Jackson ImmunoResearch Laboratories. Polyclonal anti-human PAR-1 and anti-human CD14 were purchased from Santa Cruz Biotechnology. FITC-conjugated mouse anti-human IgG isotype controls and Human Inflammation Cytometric Bead Array kits were purchased from BD Pharmingen. 293 cells, monoclonal anti-human EPCR Abs (JRK-1513 and JRK-1535), and polyclonal anti-human EPCR Abs (gt254) were provided by Dr. C. Emerson (Oklahoma Medical Research Foundation, Oklahoma City, OK). Recombinant activated protein C was purchased from Eli Lilly. Protein C and Gla-domainless activated protein C were purchased from Hematologic Technologies. Phe-pro-arg-chloromethylketone (PPACK) was purchased from Calbiochem. In Situ Cell Death Detection kits for TUNEL analysis were purchased from Roche Diagnostics. E. coli K-12 and Staphylococcus aureus Wood strain without protein A bioparticles and their respective opsonizing reagents, as well as Calcein-AM and RediPlate 96 EnzChek Caspase-3 Assay kits, were purchased from Molecular Probes. Polyclonal Ab to human procaspase-3 was purchased from Santa Cruz Biotechnology. Annexin V-enhanced GFP was chased from Molecular Probes. Polyclonal Ab to human procaspase-3 was purchased from Cell Signaling Technology. Annexin V-enhanced GFP was purchased from Cell Signaling Technology. Annexin V-enhanced GFP was purchased from BD Biosciences.

Caspase-3 activity assay

Caspase-3 activity assays were performed using the RediPlate 96 EnzChek Caspase-3 Assay kit according to the protocol supplied by the manufacturer (Molecular Probes). THP-1 cells (1 × 10⁶) were treated for 4 h with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C. Annexin V staining was performed by flow cytometry using enhanced GFP-conjugated annexin V protein as recommended by the supplier (BD Biosciences).

Western blot analysis of procaspase-3

THP-1 cells (1 × 10⁶) were treated for 4 h with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C. Electrophoresis of cell lysates was performed according to the method of Laemmli (28) using a 12% SDS-polyacrylamide gel. Immunoblotting was performed using a polyclonal Ab against human procaspase-3.

Preparation of PPACK-activated protein C

The active site of activated protein C was inhibited with the tripeptide inhibitor PPACK. PPACK-activated protein C was produced by incubation of 2 ml of 800 nM activated protein C in 0.9% NaCl with 15-fold molar excess of PPACK for 2 h at 4°C. Unbound PPACK was removed by dialysis of the solution in a molecular mass 12- to 14-kDa cut-off dialysis tubing in 0.9% NaCl. The PPACK-activated protein C was inactive as monitored by the loss of enzymatic activity toward S-2366 chromogenic

Isolation of peripheral human monocytes from whole blood

Peripheral human monocytes were isolated from the whole blood of healthy volunteers by MACS. The isolation and use of monocytes from healthy volunteers was voluntary, imposed minimal risk to the donor, and did not require approval of the Research Ethics Board of the Hamilton Health Sciences.

Venous blood from healthy adult volunteers was drawn to a volume of 50 ml into disposable syringes containing 10 U/ml heparin using 18-gauge needles. Whole blood was gently layered over Histopaque 1077 solution, and the mononuclear cells were separated from the polymorphonuclear cells, and the red cells were separated by centrifugation at 1500 rpm for 30 min. The establishment of this density gradient permitted the collection of the white buffy layer (mononuclear cells). Upon collection, the cells were diluted and washed with PBS. Residual RBCs were removed using a lysis buffer of 155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA. After washing and resuspending the cells in PBS, the cells were incubated with anti-CD14 microbeads for 30 min at room temperature. The cells were then washed and resuspended in a MACS buffer of 0.5% BSA and 2 mM EDTA in PBS. This cell suspension was passed through a prepared MACS column. CD14⁺ monocytes were removed from the washed column by plunging MACS buffer through the column outside of the magnetic area. Monocytes were washed using PBS and resuspended in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Cell concentration was determined by physical count of viable cells using a hemocytometer before subjectation to culture conditions. A cell density of 1 × 10⁶ cells/ml was used for monocyte culture experiments.

TUNEL assay

THP-1 cells were treated with camptothecin (5 μM) in the absence or presence of 120 nM of either activated protein C, protein C, or active site-blocked activated protein C (PPACK-activated protein C). Following an 18-h incubation, TUNEL assays of apoptotic cell death were performed with In Situ Cell Death Detection kits as per the protocol of the manufacturer. Briefly, THP-1 cells (1 × 10⁶) were treated with permeabilization buffer (0.1% sodium citrate containing 0.1% Triton X-100) for 2 min at 4°C. Cells were then washed and resuspended in staining reagents, including Tdt and fluorescein-conjugated dUTP, which label DNA nicked during the apoptotic process. Subsequent analysis of PBS-washed cells was performed by flow cytometry. Primary blood monocytes from healthy volunteers were cultured for 48 h in the absence or presence of 30 or 60 nM activated protein C. Apoptosis was studied by TUNEL analysis as described above.

Annexin V staining

The translocation of phosphatidylserine to the outer membrane of the cell is an early marker of apoptosis. THP-1 cells (1 × 10⁶) were treated for 4 h with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C. Annexin V staining was performed by flow cytometry using enhanced GFP-conjugated annexin V protein as recommended by the supplier (BD Biosciences).

Caspase-3 activity assay

Caspase-3 activity assays were performed using the RediPlate 96 EnzChek Caspase-3 Assay kit according to the protocol supplied by the manufacturer (Molecular Probes). THP-1 cells (1 × 10⁶) were treated for 4 h with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C. After isolation of cell lysates, levels of caspase-3 were quantified by the cleavage of the fluorogenic substrate rhodamine 110 bis-(CF-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide).
Results

Effect of activated protein C on camptothecin-induced THP-1 apoptosis

It has been proposed that apoptosis is a significant event mediating the phagocytic and inflammatory behaviors of monocytes during sepsis (27). Thus, the lifespan of monocytes dictates the duration during which microorganisms are ingested and proinflammatory factors are released. In vivo, circulating monocytes constitutively undergo apoptosis (half-life of ~24 h), a process that marks aging monocytes for safe removal by macrophages in the liver and spleen (30, 31).

In this study, we examined the effects of activated protein C on apoptosis of THP-1 cells. THP-1 cells were treated with 5 μM camptothecin, a topoisomerase 1 inhibitor that mediates DNA damage (32), in the presence of increasing concentrations of recombinant activated protein C (0–120 nM). Following an 18-h incubation, apoptosis was studied by detection of DNA fragmentation into nucleosome-length fragments, a characteristic feature of the apoptotic process. Detection of fragmented DNA was assessed by TUNEL analysis. As shown in Fig. 1A, activated protein C exerts a dose-dependent antiapoptotic effect in camptothecin-treated THP-1 cells. TUNEL positivity correlated with exposure of phosphatidylserine, another marker of apoptosis, as measured by annexin V staining (data not shown). To ensure that the antiapoptotic effect was not due to trace amounts of thrombin present in the commercial preparation of recombinant activated protein C, hirudin (1 μM) was added to the activated protein C before its use. The addition of hirudin did not abrogate the antiapoptotic effect of activated protein C (data not shown).

To confirm that TUNEL staining was consistent with activation of the proapoptotic caspase cascade, we assayed for the activation of caspase-3. THP-1 cells were treated with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C. Following a 4-h incubation period, THP-1 cell lysates were assayed for activated caspase-3 activity using the fluorogenic substrate rhodamine 110 bis-(N-CBZ-l-aspartyl-l-glutamyl-l-valyl-l-aspartic acid amide). Activated caspase-3 activity in camptothecin-treated THP-1 cell lysate was greater than that present in the cell lysate of camptothecin- and activated protein C-treated THP-1 cells (Fig. 1B). We also measured levels of procaspase-3 in cell lysates of THP-1 cells using Western blot analysis (Fig. 1C). Normalizing the level of procaspase-3 in untreated THP-1 cells at 100%, treatment of THP-1 cells for 4 h with 5 μM camptothecin decreased procaspase-3 levels to ~50%, indicating that procaspase-3 was activated to caspase-3. In contrast, coincubation of THP-1 cells with 5 μM camptothecin and 120 nM activated protein C decreased procaspase-3 levels to only ~70%. Taken together, these results suggest that activation of caspase-3 in camptothecin-treated THP-1 cells was decreased by activated protein C.

Effect of activated protein C on spontaneous apoptosis of peripheral blood monocytes

To demonstrate that activated protein C also exerts an antiapoptotic effect in primary human blood monocytes, we investigated the effect of activated protein C on spontaneous apoptosis of primary blood monocytes from three healthy volunteers. Primary blood monocytes were cultured for 48 h in the absence or presence of 30 or 60 nM activated protein C. As shown in Fig. 2, activated protein C inhibits spontaneous monocyte apoptosis, although the magnitude of the antiapoptotic effect varied between individual donors.

Statistics

Treatment groups from TUNEL and activated caspase-3 assays were compared by the Student t tests assuming equal variances. Comparison of CBA treatment groups was performed by general linear model ANOVA. Data were considered significant for p < 0.05. Figures denote p < 0.05 with * and p < 0.01 with **.

Cytometric bead array (CBA) analysis of cytokine levels

THP-1 cells were treated in the absence or presence of 120 nM activated protein C for 6 h, followed by a 24-h incubation with 20 ng/ml LPS. Conditioned medium was harvested throughout a 24-h time course, and centrifugation was used to separate cells from the supernatant. Quantification of levels of ILs 1β, 6, 8, 10, and 12p70 in the conditioned medium was performed using CBA Human Inflammation kits. Briefly, unlabeled and 1/100 dilutions of supernatants were incubated with a mixture of six bead populations, each with a unique fluorescence intensity and cytokine-binding specificity. Supplementation with PE-conjugated detection Abs per bead populations, each with a unique fluorescence intensity and cytokine-binding specificity. Supplementation with PE-conjugated detection Abs

Phagocytosis assay

Gram-positive and Gram-negative phagocytosis were assessed with opsonized E. coli and S. aureus Alexa Fluor 488-conjugated bioparticles, respectively. Opsonizing reagents containing rabbit polyclonal Abs specific for S. aureus and E. coli Ags were incubated with bioparticles for 1 h at 37°C before a PBS wash that removes unbound opsonin. THP-1 cells or primary blood monocytes were treated with 120 nM activated protein C for 6 h, followed by incubation with bioparticles at a ratio of 10:1 for 18 h. Samples were collected after 18 h and washed to remove noninternalized bioparticles. Flow cytometry was used to evaluate fluorescence indicative of phagocytosis.

Monocyte adhesion assay

The adherence of primary blood monocytes to HUVECs was studied using a quantitative fluorometric assay as described previously (29). In this method, monocytes are labeled with the fluorescent dye Calcein-AM and incubated with activated endothelial cells. Following coinoculation and gentle washing to remove nonadhering monocytes, the number of adhering monocytes is quantified with a fluorescence microtiter plate reader.

HUVECs were cultured in 2% gelatin-coated 96-well plates in HUVEC medium (1 ml/99 medium supplemented with 10 μg/ml endothelial cell growth factor containing 20% heat-inactivated FBS, 100 U/ml penicillin-streptomycin, and 12.6 U/ml heparin) at 37°C and 5% CO2. When the cells reached 80–90% confluency, they were treated with LPS (1 μg/ml) for 5 h. Human primary monocytes from healthy volunteers were resuspended in RPMI 1640 medium at a concentration of 5 × 106 cells/ml. The cells were incubated for 30 min at 37°C with Calcein-AM to a final concentration of 5 μM. Following a wash in HBSS buffer (0.5% human serum albumin, 3 mM CaCl2, and 0.6 mM MgCl2), the cells were incubated in the absence or presence of 120 nM recombinant activated protein C for 4 h. Calcein-AM-labeled monocytes (5 × 106 cells in 100 μl) treated with or without activated protein C were added to unstimulated or LPS-stimulated HUVECs. The monocytes were incubated for 45 min at 37°C, and following two gentle washes in HBSS buffer, the fluorescence intensity of the adhering monocytes was determined with a fluorescent plate reader (Gemini XS; Molecular Devices) using excitation wavelength of 494 nm and an emission wavelength of 538 nm. The fluorescence intensity of the monocytes was compared with a standard curve of labeled cells alone.

Statistics

Treatment groups from TUNEL and activated caspase-3 assays were compared by the Student’s t tests assuming equal variances. Comparison of CBA treatment groups was performed by general linear model ANOVA. Data were considered significant for p < 0.05. Figures denote p < 0.05 with * and p < 0.01 with **.
Requirement of serine protease activity for the antiapoptotic effect of activated protein C

Activated protein C is the serine protease derived from activation of zymogen protein C by the thrombin-thrombomodulin complex. To determine whether serine protease activity is essential for the antiapoptotic effect of activated protein C, we compared the antiapoptotic efficacy of activated protein C to protein C as well as to activated protein C with its active site blocked by the tripeptide inhibitor PPACK (PPACK-activated protein C). THP-1 cells were treated for 18 h with camptothecin (5 μM) in the absence or presence of either activated protein C, protein C, or PPACK-activated protein C. Apoptosis was studied by TUNEL analysis. Neither protein C nor PPACK-activated protein C exerted an antiapoptotic effect in camptothecin-treated THP-1 cells (Fig. 3). In addition, treatment of THP-1 cells with the serine protease thrombin (120 nM) did not effect the proapoptotic effect of camptothecin (Fig. 3). These results suggest that the ability of activated protein C to inhibit camptothecin-induced THP-1 apoptosis is specific to activated protein C and requires serine protease function.
protein C (120 nM) in the absence or presence of 0.4 μM clonal anti-CD14 Ab, or 0.4 μM were treated with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C, protein C, PPACK-activated protein C, or thrombin. Following an 18-h incubation, apoptosis was studied by TUNEL analysis. The bars represent the mean, whereas the lines above the bars reflect the SE of three separate experiments. **, p < 0.01.

**FIGURE 3.** Effect of activated protein C (APC), protein C (PC), PPACK-activated protein C, and thrombin on camptothecin (CAM)-induced apoptosis of THP-1 cells. THP-1 cells were treated with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C, protein C, PPACK-activated protein C, or thrombin. Following an 18-h incubation, apoptosis was studied by TUNEL analysis. The bars represent the mean, whereas the lines above the bars reflect the SE of two separate experiments.

**FIGURE 4.** Effect of PAR-1 in activated protein C (APC)-mediated inhibition of camptothecin (CAM)-induced THP-1 apoptosis. THP-1 cells were treated with camptothecin (5 μM) and activated protein C (120 nM) in the absence or presence of 0.4 μg/ml polyclonal anti-CD14 Ab, or 0.4 μg/ml polyclonal anti-PAR-1 Ab. Following an 18-h incubation, apoptosis was assessed using the TUNEL method. The bars represent the mean, whereas the lines above the bars reflect the SE of two separate experiments. **, p < 0.01.

Requirement of EPCR and PAR-1 for the antiapoptotic effect of activated protein C

How does activated protein C, a plasma serine protease, modulate cellular functions such as apoptosis? To date, the only known cellular receptor for activated protein C is the EPCR, a 46-kDa single-chain transmembrane glycoprotein homologous to the MHC class I/CD1 family of molecules (33). EPCR was identified originally on vascular endothelial cells and has since been detected in neutrophils (34), monocytes (34), and in the monocytic cell line THP-1 (35). Recent studies in endothelial cells suggest that the antiapoptotic effect of activated protein C is mediated by EPCR and PAR-1 and EPCR (19–21). PAR-1 is a member of the G protein-coupled receptors that convert extracellular proteolytic cleavage events into intracellular signals (36). Current thinking is that EPCR binds to activated protein C and serves as a coreceptor for activated protein C-mediated proteolytic activation of PAR-1.

PAR-1 is expressed in human monocytes (37). To address the question of whether PAR-1 is required for the antiapoptotic effect of activated protein C, we investigated whether anti-PAR-1 polyclonal Abs can inhibit camptothecin-induced THP-1 apoptosis. THP-1 cells were treated with camptothecin (5 μM) and activated protein C (120 nM) in the absence or presence of 0.4 μg/ml polyclonal anti-PAR-1 Ab, 0.4 μg/ml polyclonal anti-CD14 Ab, or 0.4 μg/ml bulk human IgG. Following an 18-h incubation, apoptosis was assessed by TUNEL analysis. As shown in Fig. 4, activated protein C exerted an antiapoptotic effect on camptothecin-treated THP-1 cells. Neither bulk human IgG nor polyclonal anti-CD14 Abs interfered with the antiapoptotic effect of activated protein C. In contrast, polyclonal anti-PAR-1 Abs inhibited the activated protein C-mediated down-regulation of apoptosis in camptothecin-treated THP-1 cells. These findings suggest that the antiapoptotic effect of activated protein C is dependent on PAR-1.

We next determined if the ability of activated protein C to prolong THP-1 survival is also dependent on EPCR. The EPCR-binding domain on activated protein C has been localized to the N-terminal Gla domain of the enzyme (38). To determine whether the antiapoptotic effect of activated protein C is dependent on EPCR, we compared the effects of activated protein C and Gla-domainless activated protein C on camptothecin-induced apoptosis. THP-1 cells were treated with camptothecin (5 μM) and 120 nM of either activated protein C or Gla-domainless activated protein C for 18 h. As shown in Fig. 5, activated protein C exerted an antiapoptotic effect on THP-1 survival in the absence or presence of activated protein C (120 nM). Phagocytic capacity was determined by evaluating the relative internalization of fluorescently conjugated E. coli bioparticles. The bars represent the mean, whereas the lines above the bars reflect the SE of two separate experiments. **, p < 0.01.

**FIGURE 5.** Effect of EPCR in activated protein C (APC)-mediated inhibition of camptothecin (CAM)-induced THP-1 apoptosis. THP-1 cells were treated with camptothecin (5 μM) in the absence or presence of 120 nM activated protein C. The effect of EPCR in activated protein C-mediated inhibition of camptothecin-induced THP-1 apoptosis was examined in two ways. First, THP-1 cells were treated with camptothecin in the presence of 120 nM Gla-domainless activated protein C (GD-APC). Second, THP-1 cells were treated with camptothecin (5 μM) in the presence of activated protein C (120 nM) and polyclonal anti-EPCR Ab (0.4 g/ml). Following an 18-h incubation, apoptosis was assessed using the TUNEL method. The bars represent the mean, whereas the lines above the bars reflect the SE of three separate experiments.

**FIGURE 6.** Effect of activated protein C (APC) on THP-1 phagocytosis. The phagocytic capacities of THP-1 cells were examined in the absence or presence of activated protein C (120 nM). Phagocytic capacity was determined by evaluating the relative internalization of fluorescently conjugated E. coli bioparticles. The bars represent the mean, whereas the lines above the bars reflect the SE of two separate experiments.
Effect on camptothecin-treated THP-1 cells, whereas Gla-domain-less activated protein C did not have an effect. Furthermore, the addition of anti-EPCR polyclonal Abs before activated protein C treatment abolished the antiapoptotic effect of activated protein C. These results suggest that the antiapoptotic effect of activated protein C is dependent on EPCR.

Effect of activated protein C on THP-1 and primary monocyte phagocytosis

Host responses to sepsis include the phagocytosis and clearance of microbial infections. Historically, most cases of sepsis resulted from Gram-negative pathogens, although recent clinical trials indicate that Gram-negative and Gram-positive infections occur with similar frequencies (4, 39, 40). To determine whether activated protein C alters monocyte phagocytic capacities, we investigated THP-1 phagocytosis in response to activated protein C. THP-1 cells were treated with 120 nM activated protein C for 6 h, followed by incubation with fluorescently conjugated *E. coli* and *S. aureus* bioparticles for 18 h. Flow cytometry was used to evaluate fluorescence indicative of phagocytosis. As shown in Fig. 6, activated protein C did not influence the internalization of either Gram-negative or Gram-positive bioparticles, suggesting that activated protein C does not compromise the phagocytic capacity of monocytes. Similar observations were obtained using primary blood monocytes (data not shown).

**FIGURE 6.** Effect of activated protein C (APC) on monocyte phagocytosis. The adherence of primary blood monocytes to HUVECs was studied using a quantitative fluorometric assay as described in Materials and Methods. HUVECs were incubated in the absence or presence of 1 µg/ml LPS for 5 h. Calcein-AM-labeled monocytes were treated in the absence or presence of 120 nM activated protein C for 4 h. Following coincubation of the cells for 45 min at 37°C, nonadherent monocytes were removed by gentle washing, and the number of adherent monocytes was quantified in a fluorescent plate reader by comparison to a standard curve of fluorescent cells alone (n = 3).

**Effect of activated protein C on THP-1 and primary monocyte phagocytosis**

Host responses to sepsis include the phagocytosis and clearance of microbial infections. Historically, most cases of sepsis resulted from Gram-negative pathogens, although recent clinical trials indicate that Gram-negative and Gram-positive infections occur with similar frequencies (4, 39, 40). To determine whether activated protein C alters monocyte phagocytic capacities, we investigated THP-1 phagocytosis in response to activated protein C. THP-1 cells were treated with 120 nM activated protein C for 6 h, followed by incubation with fluorescently conjugated *E. coli* and *S. aureus* bioparticles for 18 h. Flow cytometry was used to evaluate fluorescence indicative of phagocytosis. As shown in Fig. 6, activated protein C did not influence the internalization of either Gram-negative or Gram-positive bioparticles, suggesting that activated protein C does not compromise the phagocytic capacity of monocytes. Similar observations were obtained using primary blood monocytes (data not shown).

**Effect of activated protein C on cytokine production from LPS-stimulated THP-1 cells**

In addition to revealing a survival benefit for activated protein C, the PROWESS trial revealed that the serum of patients with severe sepsis contained elevated levels of cytokines. To investigate whether activated protein C affects cytokine production, we examined cytokine expression by LPS-stimulated THP-1 cells. THP-1 cells were treated with 120 nM activated protein C or no activated protein C for 6 h, followed by stimulation with 20 ng/ml LPS for 24 h. Levels of TNF (A), IL-1β (B), IL-6 (C), and IL-8 (D) from the conditioned medium were determined using the CBA. Absolute cytokine concentrations were extrapolated by nonlinear regression from standard curves with recombinant cytokines.
stimulation with 20 presence of 120 nM of activated protein C for 6 h, followed by as well as TNF-5 pg/ml (data not shown).

Although ILs 10 and 12p70 were assayed with the CBA, no treatment group expressed either cytokine at concentrations exceeding ment group expressed either cytokine at concentrations exceeding 5 pg/ml (data not shown).

Discussion
Circulating monocytes play a major role in the immediate host response to invading microorganisms. The primary functions of monocytes include the phagocytosis of invading pathogens and the synthesis and secretion of proinflammatory cytokines, chemokines, and growth factors (42). In vivo, circulating monocytes constitutively undergo apoptosis with a half-life of ~24 h (30, 31). Although monocytes protect the host from infection, prolonged monocyte survival may potentially lead to the persistence of inflammatory tissue injury via the release of proinflammatory mediators. In this study, we determined the functional consequences of an activated protein C-dependent antiapoptotic response of monocytes. Using the THP-1-immortalized human monocytic cell line, as well as primary blood monocytes, we investigated the ability of activated protein C to modulate monocyte apoptosis, phagocytosis, adhesion, and inflammation.

Our studies indicate that activated protein C exerts an antiapoptotic effect on camptothecin-treated THP-1 cells in a dose-dependent manner (Fig. 1). The antiapoptotic effect of activated protein C is specific and requires the serine protease domain of the enzyme (Fig. 3). Our results also indicate that both PAR-1 and EPCR are necessary for the antiapoptotic effect of activated protein C on THP-1 cells (Figs. 4 and 5). The requirement of both PAR-1 and EPCR for the antiapoptotic effect of activated protein C on THP-1 cells is consistent with previous reports, demonstrating that activated protein C-mediated up-regulation of antiapoptotic genes in endothelial cells is mediated by PAR-1 and EPCR (19–21). In primary blood monocytes from healthy volunteers, activated protein C inhibits spontaneous apoptosis (Fig. 2).

Although thrombin is the prototypical activator of PAR-1, we demonstrated that thrombin failed to modulate camptothecin-induced THP-1 apoptosis (Fig. 3). This suggests that signaling by EPCR-bound activated protein C through PAR-1 is distinct from signaling by thrombin through PAR-1. One possibility is that thrombin and EPCR-bound activated protein C cleave at distinct sites within the PAR-1 N terminus, triggering unique cytosolic G protein coupling events. Although thrombin and activated protein C have been shown to cleave a synthetic PAR-1 N-terminal peptide at the same site (between Arg(10) and Ser(15)) (43), it is unknown if EPCR-bound activated protein C also cleaves at this site. Since the binding of EPCR to activated protein C has been shown to alter the active site of activated protein C (44), the role of EPCR may be to bind activated protein C in the vicinity of PAR-1, as well as to alter the macromolecular substrate specificity of activated protein C. Another possibility is that the palmitoylation site on the intracellular C terminus of EPCR (45) modulates the coupling of G proteins to the PAR-1 C terminus. A third possibility is that EPCR-bound activated protein C may activate additional receptors.

Because monocytes mediate sepsis pathology by their expression of proinflammatory cytokines, the prolongation of monocyte survival is believed to be detrimental to host tissue. Thus, we explored the possibility that activated protein C prolongs monocyte survival in a manner that selectively inhibits inflammatory cytokine production while maintaining functional responses associated with removal of microbes. Assays of innate immune function revealed that activated protein C had no significant effect on THP-1 phagocytosis of Gram-negative and Gram-positive pathogens (Fig. 6). Similar observations were obtained using primary blood monocytes. Our results are consistent with subgroup analysis of the PROWESS trial, which revealed that improvement in survival associated with recombinant activated protein C therapy was similar for patients with Gram-positive and Gram-negative bacterial sepsis (46). Our results are also consistent with studies by Sturn et al. (34) that examined the effect of activated protein C on neutrophil phagocytosis. The authors reported that activated protein C inhibited neutrophil chemotaxis but did not affect neutrophil respiratory bursts nor phagocytic activity. Thus, in vitro results suggest that the phagocytic responses of monocytes and neutrophils are maintained in the presence of activated protein C.

The generation of an efficient innate immune response is also dependent on the ability of monocytes to migrate from the blood into infected tissues. A key step in monocyte migration is adhesion to the endothelium. Thus, we examined the effects of activated protein C on monocyte adhesion to activated endothelial cells (Fig. 7) and on the expression of monocyte adhesion molecules. Our results suggest that activated protein C does not compromise the ability of monocytes to adhere to activated endothelial cells and does not affect the expression of monocyte adhesion molecules. Recently, EPCR has been shown to be expressed by human lymphocytes (47). Interestingly, pretreatment of lymphocytes with protein C or activated protein C reduced their migration toward IL-8, RANTES, MCP-1, and substance P. These studies suggest that modulation of lymphocyte function may be among the protective effects of activated protein C.

We next measured levels of ILs 6, 8, 1β, 10, and 12p70, as well as TNF in the conditioned medium of LPS-stimulated THP-1 cells. To our knowledge, this is the broadest analysis of cytokine secretion by activated protein C-treated monocytic cell lines to date. Treatment of LPS-stimulated THP-1 cells with 120 nM activated protein C resulted in a reduction in the levels of ILs 6, 8, and 1 by 53, 35, and 19%, respectively (Fig. 8). Interestingly, an increase in plasma levels of IL-6 has been shown to correlate with disease severity in septic patients (48). Although ILs 10 and 12p70 were assayed with the CBA, no treatment group expressed either cytokine at concentrations exceeding 10 pg/ml (data not shown). We also observed a minor but significant 8% down-regulation of TNF-1 TNF expression by 120 nM activated protein C. Such a small effect may appear inconsistent with previously published results that revealed inhibitions exceeding 50% (14). However, activated protein C concentrations in these previous studies exceeded 3.6 μM, representing a probable cause for this discrepancy.

In summary, our study demonstrates that activated protein C exerts an antiapoptotic effect in THP-1 cells and in primary blood monocytes. We have also shown that activated protein C inhibits THP-1 expression of ILs 1β, 6, and 8, as well as TNF-α, but does not compromise the phagocytic capacity of THP-1 cells or primary
blood monocytes. Activated protein C also did not affect the ability of primary monocytes to adhere to LPS-stimulated endothelial cells. We hypothesis that the protective effect of activated protein C treatment in sepsis reflects, in part, the ability of activated protein C to prolong monocyte lifespan in a manner that maintains phagocytosis and adherence capabilities while selectively inhibiting inflammatory cytokine production, thereby promoting anti-inflammatory properties while limiting damage to host tissue.

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Disclosures

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