Effect of recombinant human activated protein C on the bactericidal activity of human monocytes and modulation of pro-inflammatory cytokines in the presence of antimicrobial agents

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Objectives: To determine the effects of recombinant human activated protein C (rhAPC) on the antimicrobial activity and cytokine production of normal human monocyte-derived macrophages (MDMs) in the presence and absence of Escherichia coli infection, with and without treatment with levofloxacin or ampicillin.

Methods: MDM monolayers were infected with E. coli ATCC 25922 and treated with levofloxacin or ampicillin in the presence or absence of rhAPC. Antimicrobial activity and cytokine (TNF-α, IL-1β, IL-6 and IL-8) concentrations in the supernatants were measured.

Results: When low concentrations of levofloxacin were used, a therapeutic concentration of rhAPC enhanced intracellular antibacterial activity at all time points. With ampicillin, antibacterial activity increased, was unaffected or diminished depending upon the drug concentration and assay time. Without antibiotics, rhAPC had no antibacterial effect. E. coli caused cytokine production to increase many fold. This increase was significantly greater with antibiotics (P < 0.01). Without antibiotics, rhAPC decreased production of TNF-α, IL-1β and IL-6, but not IL-8. At high levofloxacin concentrations, rhAPC was associated with further increases in the concentrations of these cytokines. Cytokine concentrations at 24 h were unaffected by rhAPC in the presence of ampicillin and E. coli.

Conclusions: rhAPC can affect the bactericidal activity and cytokine production of human MDM in the presence of infection and antibiotic therapy. Importantly, factors such as type and concentration of antibiotics, presence of bacteria and timing must be taken into consideration when evaluating cytokine data from septic patients.

Keywords: sepsis, rhAPC, antibiotics

Introduction

The pathogenesis, pathophysiology and therapy of septic patients are complex, and mortality occurs in about one-third.1 Recognition of the importance of the protein C pathway in sepsis, including the activated form of protein C, and its effect on inflammation, clotting and fibrinolysis led to the development of recombinant human activated protein C (rhAPC).2–6 At present, rhAPC is the only pharmacological agent approved by the FDA for the treatment of severe sepsis patients with organ failure and high risk of death, e.g. with APACHE II > 25.5 rhAPC is known to affect the microcirculation, clotting and fibrinolytic processes, as well as cytokine levels.3–6 The possible interactions of rhAPC with antimicrobial agents, Toll-like proteins and other immunity signalling proteins and pathways are not known.

We investigated the effects of rhAPC at currently used therapeutic concentrations8 on the bactericidal activity and pro-inflammatory cytokine production of normal human monocyte-derived macrophages (MDMs) exposed to Escherichia coli in the presence and absence of varying concentrations of levofloxacin or ampicillin known to be active against this microbe.

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Materials and methods

Bacterial strains and preparation of bacterial inocula

*E. coli* ATCC 25922 was used. This strain is susceptible to ampicillin (MIC = 8 mg/L) and levofloxacin (MIC = 0.06 mg/L).

Bacteria were grown in Mueller–Hinton-II broth, harvested by centrifugation, resuspended for opsonization at 2×10⁸ cfu/mL in RPMI 1640 medium (Sigma) containing 20% heat-inactivated, pooled normal human serum and incubated for 30 min at 37°C. Opsonized bacteria were resuspended at 2×10⁷ cfu/mL in RPMI 1640 medium supplemented with 10% fetal bovine serum (RPMI+; Sigma). Viable counts (cfu/mL) were verified by using the standard plate count method.

Results

**rhAPC levels**

The concentration of rhAPC was maintained at therapeutic levels during the 24 h assay (in humans, median = 45 ng/mL, range = 15–115 ng/mL) by the addition of rhAPC (250 ng/mL) at 0 and 4 h. Concentrations were not affected by MDM, *E. coli*, levofloxacin (0.1–5× MIC) or ampicillin (0.1–5× MIC).

**Time–kill assays**

The results of intracellular time–kill assays demonstrating the effects of rhAPC on the antibacterial activity of human MDM against *E. coli* when treated with increasing concentrations of levofloxacin or ampicillin are shown in Figure 1. The antibacterial activity of MDM was either enhanced or unaffected by rhAPC in the presence of levofloxacin (Figure 1a). Significant enhancement of antibacterial activity by rhAPC occurred in the presence of 0.1× MIC of levofloxacin at 2, 4 and 24 h (P < 0.01). At 0.5× MIC of levofloxacin, a concentration with much greater antibacterial activity than 0.1× MIC in our *in vitro* model, enhancement of the antibacterial activity of MDM by rhAPC was demonstrated only at 2 h (P < 0.01). The antibacterial activity of MDM was enhanced, unchanged or decreased by rhAPC in the presence of ampicillin (Figure 1b). A small but significant enhancement of the antibacterial activity of MDM by rhAPC was demonstrated at 5× MIC of ampicillin at 2 h into the assay (P < 0.01). In contrast, antibacterial activity diminished slightly in the presence of rhAPC and 1× and 5× MIC of ampicillin at 4 h (P < 0.01). At 24 h, rhAPC had no effect on the antibacterial activity of MDM in the presence of any concentration of ampicillin studied. Without either levofloxacin or ampicillin, the antibacterial effects of MDM were not affected by rhAPC (Figure 1a and b).

**Influence of *E. coli* on cytokine concentrations in the absence of antibiotics and rhAPC**

In the presence of *E. coli* but in the absence of both antibiotics and rhAPC, the concentrations of TNF-α, IL-1β, IL-6 and IL-8 at 0 h were 11, 4, 3 and 277 pg/mL, respectively. TNF-α, IL-1β, IL-6 and IL-8 concentrations were significantly elevated at 4 and 24 h when compared with those at 0 h (P < 0.01, Figure 2). In the absence of *E. coli*, antibiotics and rhAPC, the concentrations of TNF-α, IL-1β, IL-6 and IL-8 at 0 h were 11, 1, 3 and 48 pg/mL, respectively. TNF-α and IL-8 concentrations were significantly elevated at 4 and 24 h when compared with those at 0 h (P < 0.01), whereas concentrations of IL-6 and IL-1β remained stable (Figure 2).

**Influence of antibiotics on cytokine concentrations in the absence of rhAPC**

Results shown in Figure 2 suggest that in the absence of *E. coli*, antibiotics did not usually cause a significant change in the production of cytokines. However, IL-8 did increase at 24 h in the presence of high concentrations of levofloxacin and ampicillin (P < 0.01). In contrast, in the presence of *E. coli* and high concentrations of either antibiotic, significant increases in TNF-α and IL-6 occurred at both 4 and 24 h (P < 0.01), whereas at low

Human monocytes

Monocytes were obtained from the fresh heparinized blood of healthy human donors who had signed an informed consent form approved by the IRB and R&D Committees of the Stratton VA Medical Center (Albany, NY, USA). Mononuclear cells were separated from RBCs and polymorphonuclear leucocytes by centrifugation using Histopaque 1077 (Sigma). Cell viability, determined by using the Trypan Blue assay, was ≥98%.

Infection and treatment of MDM

Mononuclear cells were suspended at 2×10⁶ cells/mL in RPMI+. Aliquots of 500 μL were then added to the wells of 48-well plates and the cells were allowed to adhere overnight at 37°C in air supplemented with 5% CO₂. Following removal of non-adhered cells, MDM monolayers were infected by adding 500 μL aliquots of a bacterial suspension containing 2×10⁸ cfu/mL to the wells. Phagocytosis was for 1 h at 37°C in an atmosphere containing 5% CO₂. Monolayers were washed once with RPMI+ to remove extracellular bacteria, and fresh RPMI+ was added. Antimicrobial agents (0.1–100× MIC) and rhAPC (250 ng/mL) were then added. For time points >4 h, rhAPC was re-applied at 4 h. Supernatants from 0, 2, 4 and 24 h time points were removed, centrifuged at 13 000 g, decanted and stored at −80°C for later determination of cytokine and/or rhAPC concentrations. Monolayers were then lysed with sterile distilled H₂O and the numbers of viable bacteria determined in duplicate by using the standard plate count method. All experiments with MDM were performed in duplicate three times.

Determination of cytokine and rhAPC concentrations

Concentrations of TNF-α, IL-1β, IL-6 and IL-8 were determined in duplicate following the manufacturer’s directions using ELISA plates obtained from R&D Systems, Minneapolis, MN, USA. Concentrations of rhAPC were determined by using both an activated partial thromboplastin time (APTT)-based clotting assay and an enzyme capture assay, as described previously.

Statistical analysis

The analysis of variance methodology with logarithm transformation and post hoc comparisons was used to analyse the data. The level of significance was 0.01.

**rhAPC and antimicrobial agents**

rhAPC was provided by Lilly Research Laboratories (Indianapolis, IN, USA). Levofloxacin and ampicillin were obtained from Sigma Chemical Co. (St Louis, MO, USA).

Concentrations of TNF-α, IL-1β, IL-6 and IL-8 occurred at both 4 and 24 h when compared with those at 0 h (P < 0.01). In contrast, antibacterial activity diminished slightly in the presence of rhAPC and 1× and 5× MIC of ampicillin at 4 h (P < 0.01). At 24 h, rhAPC had no effect on the antibacterial activity of MDM in the presence of any concentration of ampicillin studied. Without either levofloxacin or ampicillin, the antibacterial effects of MDM were not affected by rhAPC (Figure 1a and b).

Influence of *E. coli* on cytokine concentrations in the absence of antibiotics and rhAPC

In the presence of *E. coli* but in the absence of both antibiotics and rhAPC, the concentrations of TNF-α, IL-1β, IL-6 and IL-8 at 0 h were 11, 4, 3 and 277 pg/mL, respectively. TNF-α, IL-1β, IL-6 and IL-8 concentrations were significantly elevated at 4 and 24 h when compared with those at 0 h (P < 0.01, Figure 2). In the absence of *E. coli*, antibiotics and rhAPC, the concentrations of TNF-α, IL-1β, IL-6 and IL-8 at 0 h were 11, 1, 3 and 48 pg/mL, respectively. TNF-α and IL-8 concentrations were significantly elevated at 4 and 24 h when compared with those at 0 h (P < 0.01), whereas concentrations of IL-6 and IL-1β remained stable (Figure 2).

Influence of antibiotics on cytokine concentrations in the absence of rhAPC

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Figure 1. Effect of rhAPC and levofloxacin (a) or ampicillin (b) on the viability of intracellular *E. coli* in human MDM. *Percentage cfu/mL in the presence and absence of rhAPC were significantly different (P < 0.01).“
Figure 2. Effects of rhAPC and of levofloxacin or ampicillin in the presence or absence of intracellular *E. coli* on the concentrations of TNF-α (a and b), IL-1β (c and d), IL-6 (e and f) and IL-8 (g and h) at 4 and 24 h. *Cytokine concentrations in the presence and absence of rhAPC were significantly different (*P* < 0.01).
antibiotic concentrations, decreases ($P < 0.01$) or no change were observed at 4 h. No change was seen in the concentration of IL-8 at 4 h, except at a high ampicillin concentration. However, an increase was observed at 24 h for both concentrations of levofloxacin and for a high concentration of ampicillin ($P < 0.01$). In contrast to TNF-α, IL-6 and IL-8, both antibiotics decreased the IL-1β concentration at both 4 and 24 h.

**Influence of rhAPC on cytokine concentrations**

Figure 2 indicates that in the absence of E. coli, rhAPC was associated with an increase in TNF-α regardless of the presence of antibiotics ($P < 0.01$). No effect was seen on the concentrations of IL-1β or IL-6. IL-8 concentrations varied considerably with the presence or absence of antibiotics and the incubation time. In the presence of E. coli but with no antibiotics, rhAPC did not influence cytokine concentrations at 4 h and decreased TNF-α, IL-1β and IL-6 concentrations at 24 h. Concentrations of TNF-α, IL-1β and IL-6 increased with high levofloxacin concentrations at both 4 and 24 h ($P < 0.01$), whereas ampicillin had little or no effect. rhAPC did not affect the concentrations of IL-8 at either 4 or 24 h in the absence or presence of either antibiotic.

**Discussion**

We are unaware of previous studies such as ours, in which the effects of antimicrobials on intracellular E. coli in MDM in the presence of therapeutic concentrations of rhAPC are described. Although rhAPC alone had no effects on the antibacterial activity of MDM, it facilitated intracellular killing in the presence of low concentrations of levofloxacin. In contrast, rhAPC enhanced killing with higher concentrations of ampicillin at 4 h and decreased this activity at lower concentrations.

Studies of septic patients demonstrate that various cytokines, including IL-6, can be elevated in sepsis. However, the effects of antibiotics on serum cytokine concentrations in septic patients have not been investigated. In our study, the presence of intracellular E. coli in MDM caused significant increases in the levels of all four cytokines tested. Addition of antibiotics to the infected MDM usually caused further increases in TNF-α, IL-6 and IL-8, but not IL-1β. In infected MDM in the absence of antibiotics, rhAPC decreased the concentrations of TNF-α, IL-1β and IL-6, but not IL-8. Further increases in the concentrations of TNF-α, IL-1β and IL-6 occurred in the presence of antibiotics, especially levofloxacin. Administration of rhAPC to uninfected MDM caused minimal increases in TNF-α and no change in IL-1β or IL-6. IL-8 concentration generally decreased in the presence of levofloxacin and increased with ampicillin. Thus, taken together, intracellular E. coli, antibiotics and rhAPC have complex effects on the production of cytokines, and these effects depend on the antibiotic type, its concentration, the presence of rhAPC and time of exposure. These factors must be taken into consideration when evaluating data from septic patients.

In conclusion, our studies show that intracellular E. coli, rhAPC and antibiotics can affect the bactericidal activity of MDM as well as influence the production of pro-inflammatory cytokines by these human cells. Understanding the mechanisms that underlie the complex interactions among MDM, intracellular E. coli, rhAPC and antibiotics requires further study, including the use of an animal model and phagocytic cells obtained from septic patients.

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**Transparency declarations**

S. U. and S. B. Y. are employees and stockholders of Eli Lilly and Company. Xigris (recombinant human activated protein C) is a product of Eli Lilly and Company. Other authors have none to declare.

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