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

Aldona L. Baltch1,2*, Lawrence H. Bopp1, William J. Ritz1,2, Phyllis B. Michelsen1,2, S. Betty Yan3, Suzane Um3 and Raymond P. Smith1,2

1Infectious Disease Research Laboratory, Stratton VA Medical Center, 113 Holland Avenue, Albany, NY 12208, USA; 2Department of Medicine, Albany Medical College, Albany, NY 12208, USA; 3Lilly Research Laboratories, 355 E. Merrill St, Indianapolis, IN 46225, USA

Received 27 September 2006; returned 11 December 2006; revised 10 January 2007; accepted 25 February 2007

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. 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). 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. rhAPC is known to affect the microcirculation, clotting and fibrinolytic processes, as well as cytokine levels. 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 concentrations 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.

*Corresponding author. Tel: +1-518-626-6416; Fax: +1-518-626-6564; E-mail:aldona.baltch@med.va.gov
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 × 10^6 cfu/mL in RPMI 1640 medium (Sigma) containing 20% heat-inactivated, pooled normal human serum and incubated for 30 min at 37°C. Oposnized bacteria were resuspended at 2 × 10^7 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.

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).

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 Straton 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 × 10^6 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 × 10^7 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-administered 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.

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
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.

Acknowledgements

This study was supported by Eli Lilly Laboratories and in part by the Medical Research Service of the Department of Veterans Affairs.

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.

References

1. Annane D, Bellissant E, Cavaillon JM. Septic shock. Lancet 2005; 365: 63–78.
2. Esmon C. The protein C pathway. Crit Care Med 2000; 28 Suppl: S44–8.
3. Yan SB, Dhainaut JF. Activated protein C versus protein C in severe sepsis. Crit Care Med 2001; 29 July Suppl: S69–74.
4. Dhainaut JF, Yan SB, Margolis BD et al. Drotrecogin alfa (activated) (recombinant human activated protein C) reduces host coagulopathy response in patients with severe sepsis. Thromb Haemost 2003; 90: 642–53.
5. Kallir AC, Coyle SM, Urry JY et al. Effects of drotrecogin alfa (activated) in human endotoxemia. Shock 2004; 21: 222–9.
6. Opal SM, Garber GE, LaRosa SP et al. Systemic host responses in severe sepsis analysed by causative microorganism and treatment effects of drotrecogin alfa (activated). Clin Infect Dis 2003; 37: 50–8.
7. Bernard GR, Vincent JL, Laterre PF et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001; 344: 699–709.
8. Macias WL, Dhainaut JF, Yan SC et al. Pharmacokinetic–pharmacodynamic analysis of drotrecogin alfa (activated) in patients with severe sepsis. Clin Pharmacol Ther 2002; 72: 391–402.
9. Van der Poll T, van Deventer SJ. Cytokines and anticytokines in the pathogenesis of sepsis. Infect Dis Clin North Am 1999; 13: 413–26.
10. Oda S, Hirasawa H, Shiga H et al. Sequential measurement of IL-6 blood levels in patients with systemic inflammatory response syndrome (SIRS)/sepsis. Cytokine 2005; 29: 169–75.