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Endogenous protein C inhibits activation of coagulation and transiently lowers bacterial outgrowth in murine *Escherichia coli* peritonitis

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5.1 Introduction

Sepsis is the leading cause of death in critically ill patients. Peritonitis is the second most common cause of sepsis with *Escherichia (E.) coli* being one of the major pathogens involved. Severe bacterial infection results in systemic activation of coagulation accompanied by downregulation of anticoagulant mechanisms and fibrinolysis. In murine peritonitis coagulation is activated intraperitoneally, resulting in intra-abdominal fibrin formation, which on the one hand limits bacterial spreading but on the other hand hampers bacterial killing. Indeed, administration of the coagulation inhibitor activated protein C (APC) not only decreased coagulation activation, but also improved bacterial clearance and survival in a murine model of peritonitis. The endogenous PC system is important for the regulation of coagulation and inflammation, as illustrated by studies in mice with very low PC levels due to genetic deficiencies, which display severe procoagulant and inflammatory responses to lipopolysaccharide. We here investigate the role of the endogenous PC system in local and systemic activation of coagulation and fibrinolysis, bacterial outgrowth and inflammation during murine *E. coli* peritonitis.
5.2 Methods

Ten weeks old female C57BL/6 mice were used. All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center. Peritonitis was induced by intraperitoneal administration of $10^4$ CFU of E. coli O18:K1. Thirty minutes prior to induction of peritonitis mice received an intraperitoneal injection with 200 μl isotonic saline containing 200 μg of either MPC1609, a rat monoclonal antibody (mAb) directed against murine PC (anti-PC), or MCO1716, a cross-matched mAb targeted against the mouse keyhole limpet hemocyanin protein (control antibody). Mice were sacrificed after 6, 14 or 20 hours. Samples were harvested and processed as described. PC plasma activity levels were measured by an amidolytic assay. Thrombin-antithrombin complexes (TATc; Behringwerke AG, Marburg, Germany) and plasminogen activator inhibitor-1 (PAI-1) were measured by ELISA. Plasminogen activator activity (PAA) was determined by an amidolytic assay. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, interferon (IFN)-γ and IL-10 were measured by cytometric bead array multiplex assay (BD Biosciences, San Jose, CA).

5.3 Results and Discussion

Coagulation and fibrinolysis

Anti-PC treatment strongly reduced PC levels in peritoneal lavage fluid (PLF) and plasma (Figure 5.1A and B). TATc levels in PLF were significantly higher in anti-PC treated animals as compared to mice treated with control antibody at 14 hours ($p < 0.05$) and 20 hours ($p < 0.001$) after infection (Figure 5.1C). Plasma TATc levels were higher in anti-PC treated animals already at 6 hours after infection and remained higher during the course of peritonitis ($p < 0.01$ versus mice treated with control antibody at all time points) (Figure 5.1D). These findings are in line with previous studies in which anti-PC enhanced plasma TATc levels after intravenous administration of lipopolysaccharide in mice and triggered fibrinogen consumption after intravenous infusion of E. coli in baboons. Notably, however, heterozygous PC deficient mice showed unaltered systemic activation of coagulation during polymicrobial periton-
Results and Discussion

Inflammatory response

Anti-PC treatment did not impact on cell counts or differentials or on cytokine concentrations in PLF at any time point after infection (data not shown). Whereas no differences in cytokine levels were detected at 6 hours after infection, at 14 hours plasma levels of IL-6, MCP-1 and IL-10 were substantially higher in anti-PC treated animals (p < 0.01 versus mice treated with control antibody; shown for IL-6 and MCP-1 in Figure 5.1I and J); after 20 hours, these differences had subsided. There were no differences in TNF-α and IFN-γ levels between groups at any time point (not shown). IL12p70 levels were below detection at all time points.

Bacterial outgrowth

Of interest, at 14 hours post-infection anti-PC treatment enhanced bacterial loads approximately ten-fold in PLF (Figure 5.1K), blood (Figure 5.1L), liver and lung (not shown) (p < 0.05 versus mice treated with control antibody in all compartments tested); after 20 hours of infection, bacterial loads were very high in all mice and differences between groups had subsided. The inhibiting effect of endogenous PC
Figure 5.1: Treatment with anti protein C antibodies enhances activation of coagulation, increases PAI-1 levels, inhibits systemic fibrinolytic activity, enhances systemic cytokine production and enhances bacterial outgrowth in murine Escherichia coli peritonitis. Peritoneal lavage fluid (PLF) and plasma levels of A. B. protein C (PC) activity, C. D. thrombin-antithrombin complexes (TATc), E. F. plasminogen activator inhibitor (PAI)-1 and G. H. plasminogen activator activity (PAA), plasma levels of I. IL-6 and J. MCP-1, and bacterial outgrowth in K. PLF and L. blood 6, 14 and 20 hours after induction of Escherichia coli peritonitis in control antibody (white) and anti protein C (grey) treated mice. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8 mice per group at each time point). *, ** and *** indicate statistical significance as compared to control antibody (p < 0.05, p < 0.01 and p < 0.001 respectively, Mann-Whitney U test). N.D. = not determined.
Figure 5.1: Treatment with anti protein C antibodies enhances activation of coagulation, increases PAI-1 levels, inhibits systemic fibrinolytic activity, enhances systemic cytokine production and enhances bacterial outgrowth in murine Escherichia coli peritonitis (continued).

Peritoneal lavage fluid (PLF) and plasma levels of A. B. protein C (PC) activity C. D. thrombin-antithrombin complexes (TATc), E. F. plasminogen activator inhibitor (PAI)-1 and G. H. plasminogen activator activity (PAA), plasma levels of I. IL-6 and J. MCP-1, and bacterial outgrowth in K. PLF and L. blood 6, 14 and 20 hours after induction of Escherichia coli peritonitis in control antibody (white) and anti protein C (grey) treated mice. Data are expressed as box-and-whisker diagrams depicting the smallest observation, lower quartile, median, upper quartile and largest observation (8 mice per group at each time point). *, ** and *** indicate statistical significance as compared to control antibody (p < 0.05, p < 0.01 and p < 0.001 respectively, Mann-Whitney U test). N.D. = not determined.
on bacterial growth and dissemination is remarkable considering that (A)PC is not known to impact on antibacterial mechanisms per se and considering that anti-PC did not influence the inflammatory response to *E. coli* in a way that might have impaired antibacterial defense. It is conceivable that lower bacterial loads in PLF of mice not treated with anti-PC results from less activation of coagulation, which could deprive bacteria from an intraperitoneal niche provided by fibrin, as was suggested earlier\(^3\). This is in correspondence with studies from our group that linked less activation of coagulation or enhanced fibrinolysis to lower bacterial counts in murine *E. coli* peritonitis\(^6,7\). Differences in blood, liver and lung bacterial outgrowth in our study are probably secondary to different outgrowth at the primary site of infection.

### 5.4 Conclusions

We show that endogenous PC decreases local and systemic activation of coagulation and enhances systemic fibrinolytic activity in murine *E. coli* peritonitis, thereby limiting the procoagulant trigger elicited by this rapidly disseminating infection. Moreover, we demonstrate that endogenous PC transiently lowers bacterial outgrowth, possibly by preventing bacteria to use fibrin clots to escape from bacterial killing. These data reveal endogenous PC as an important regulator of an adequate antibacterial host response during abdominal sepsis.

### References