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Heparin Protects Against Septic Mortality via ApoE-antagonism

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Abstract

Background—Apolipoprotein E (apoE), a component of plasma lipoproteins, increases septic mortality in a rodent model of sepsis, presumably by enhancing lipid antigen presentation to antigen-presenting cells via the LDL receptor (LDLR). Downstream, this culminates in Natural Killer T (NKT) cell activation and cytokine secretion. To determine whether apoE antagonism would protect against septic mortality in mice, apoE-LDLR binding was antagonized using heparin, which can inhibit apoE's LDLR-binding site.

Methods—C57BL6 mice underwent cecal ligation and puncture (CLP) and heparin infusion. Serum partial thromboplastin time and alanine aminotransferase (ALT) were measured at 24 hours and survival was monitored for 7 days after CLP. LDLR^{+/+} and ^{-/-} fibroblasts were incubated with apoE and heparin to measure apoE internalization. Hepatic NKT cells and cytokine levels were quantified via FACS.

Results—Heparin decreased CLP-induced mortality by 50% versus saline-treated controls, independent of anticoagulation. LDLR^{+/+} fibroblasts displayed decreased uptake of apoE when treated concurrently with heparin for 12 hours. In septic mice, hepatic ALT levels, hepatic NKT cells, and plasma cytokine levels decreased after heparin treatment.

Conclusions—Our study demonstrates that heparin protects against septic mortality independent of its anticoagulant effect. This protective effect is associated with the inhibition of apoE-LDLR binding, diminished NKT proliferation and cytokine production, and hepatic dysfunction. These findings indicate a potential clinical role for apoE-antagonism in the treatment of sepsis.

Keywords

Apolipoprotein E; Sepsis; Cecal Ligation and Puncture; Heparin; Low Density Lipoprotein Receptor; Natural Killer T Cells

Introduction

Apolipoprotein E (apoE), a 34 kDa glycoprotein, is a multifunctional component of plasma lipoproteins that is thought to play important roles in the pathophysiology of cardiovascular and Alzheimer's disease. The extent of apoE's involvement in these disease processes is not completely understood, but it is believed that inflammation contributes significantly to the pathology of both. Recently, a link between apoE and sepsis has been uncovered.

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Earlier studies indicated a protective role for apoE in sepsis. With lipopolysaccharide (LPS) injection in mice, exogenous administration of apoE (iv injection) decreased mortality, while reducing endogenous levels of apoE (apoE knockout mice) increased mortality^{1–3}. On the other hand, when a more clinically relevant model of gram-negative bacterial sepsis, cecal ligation and puncture (CLP), was chosen as the model system, apoE infusion in rats increased septic mortality in a dose dependent manner⁴. In addition, the infusion of apoE in sepsis was also accompanied by an increase in hepatic NKT cell frequency and an increase in T_H1 cytokine production. This finding was consistent with the recent evidence that implicated apoE in the activation of NKT cells by acting as a molecular chaperone for bacterial antigens. The pathogenic antigen is delivered to antigen-presenting cells as part of a triglyceride-rich lipoprotein or bound to apoE. The lipid complex binds to the LDL receptor, is internalized and while trafficked through the endosomal compartments, is processed and loaded onto a CD1d receptor for display on the cell surface where it activates NKT cells (Figure 1)⁵. Predominantly located in the liver, activated NKT cells can secrete large amounts of T_H1 and T_H2 cytokines and appear to serve as a bridge between innate and acquired immunity⁶. Thus, the association between apoE and Gram-negative septic mortality appears to be strong.

We sought to determine whether apoE inhibition would protect against septic mortality in mice. To examine the effect of inhibiting apoE activity on mortality following CLP, we have exploited the apoE-binding property of heparin. ApoE is a ligand for members of the LDL receptor family, heparin sulfate proteoglycans (HSPGs) and heparin^{7–9}, reflecting the physiologic role of apoE binding to HSPGs on hepatocytes in the clearance of circulating lipoproteins by the liver. The dominant heparin binding site on apoE (residues 136–147) colocalizes with the LDL receptor binding site. Since heparin has been shown to competitively inhibit the binding of apoE to members of the LDL receptor family¹⁰, we examined the ability of heparin to protect mice from sepsis-induced mortality following CLP. We also looked at the effect of the heparin derivative octasaccharide which has diminished anticoagulation potency, and at lepirudin which has no known apoE binding affinity, during sepsis in an attempt to further define the role of apoE antagonism in sepsis. The survival data was compared to the level of anticoagulation in an attempt to determine the relative contribution of anticoagulation on survival. We analyzed heparin's ability to alter septic parameters by looking at hepatic NKT cell frequency and cytokine levels. Finally we attempted to provide more direct evidence of an interaction between heparin, apoE and LDLR using LDLR-expressing and knockout mouse embryonic fibroblasts treated with heparin and apoE and then looking at apoE uptake.

Materials and Methods

Animals

Male C57/Bl6 mice (Charles River, Wilmington, MA) 8 weeks old weighing 20–25 grams were maintained under standard conditions. All procedures were performed in full accordance with the policies of the Institutional Animal Care and Use Committee at UCSF.

Cecal Ligation and Puncture

After general anesthesia via inhaled isofluane was induced, a 1.5 cm midline abdominal incision was made. The cecum was exposed and 40% was ligated with a 4-0 silk suture. The cecum was punctured once using a 25-gauge needle, and a small amount of fecal matter was expressed from the single puncture hole. The cecum was returned to the abdominal cavity. A 500 µl bolus of normal saline, heparin (150 U/kg; APP Pharmaceuticals, Schaumburg, IL), octasaccharide (130 µg/kg; Neoparin, Inc., Alameda, CA), or lepirudin (0.4 mg/kg; Bayer Corporation, Pittsburgh, PA) was given intraperitoneally. The bolus was then followed by an

infusion of saline, heparin, octasaccharide, or lepirudin via a micro-osmotic pump (model 1007D, Durect corporation, Cupertino, CA) placed in the abdominal cavity. The abdominal wall was closed in 2 layers using 4-0 maxon for the fascia, and 4-0 silk for the skin. Survival was monitored in all groups for up to 7 days.

Serum Coagulation and Liver Panel

Samples were obtained via cardiac puncture 24 hours following CLP and pump placement. 100 μ l of mouse plasma was used to obtain activated partial thromboplastin time (PTT) using a fibrometer (Pacific Hemostasis, Cape Town, South Africa). 150 μ l of mouse plasma was used to obtain alanine aminotransferase (ALT) by using L-alanine+oxoglutarate reagent (Diagnostic Chemicals Limited, Oxford, CT) run by a standard chemistry analyzer (Olympus, Center Valley, PA).

Preparation of lymphocytes

Intrahepatic lymphocytes were isolated as described by Watarai et al.¹¹ In brief, mice were perfused via the portal vein with ice cold HBSS. The liver was minced and pressed through a #200-gauge stainless steel mesh. After washing once with RPMI 1640 medium by centrifugation at 800 X g for 5 min, cells were differentially centrifuged over a Percoll gradient (33% Percoll (EMB Biosciences, San Diego, CA) at 800 X g for 30 min. Cells were then resuspended in a red cell lysing buffer (Sigma Aldrich, St. Louis, MO) for 3 min. Cells were washed with RPMI1640 medium (800 X g, 5 min, 4 C) and then resuspended in FACS staining buffer (0.1% BSA, 0.02% Na₂N₃ in PBS) for FACS (Fluorescence Activated Cell Sorting) analysis. Cell viability was determined by trypan blue exclusion.

Flow cytometry analysis

Antibodies used included PB-conjugated rat anti-mouse CD45, APC-conjugated anti-mouse TCR- β , PE-conjugated rat anti-mouse CD4 (BD PharMingen, San Diego, CA). For T-cell counts, 1.0×10^6 cells from the liver were pre-incubated with anti-CD23 to block Fc receptors and decrease nonspecific binding. Samples were then incubated with antibodies for 30 minutes at 4 C in the dark, washed (1200 rpm, 5 min), and resuspended in FACS staining buffer. To determine positive double staining, single chain controls for each surface marker and IgG controls for each color were used. Three-color analysis was performed on a FACS Calibur (BD Biosciences, San Jose, CA) with a 200,000–300,000 event count. Data was analyzed using FlowJo software (TreeStar, Ashland, OR).

Multiplex analysis of serum cytokine concentration

Concentrations of interferon gamma (IFN- γ), interleukin-1 beta (IL-1 β), IL-2, IL-4, IL-10, and tumor necrosis factor alpha (TNF- α) were simultaneously quantified in serum samples using a cytometric bead array flex set (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Dual-color analysis was performed on a FACS Calibur (BD Biosciences, San Jose, CA). Data was analyzed using FCAP Array software (BD Biosciences, San Jose, CA).

Apolipoprotein E

The plasmid encoding the mammalian expression vector apoE3-GFP pCDNA3.1(+) was kindly provided by Yadong Huang (Gladstone Institute, San Francisco, CA). Plasmid was amplified using One Shot Top10 chemically competent E.coli transformation (Invitrogen, Carlsbad, CA), followed by plasmid purification using the Qiagen Plasmid Mega Kit (Qiagen, Inc., Valencia, CA).

HEK293T cells kindly provided by Nigel Bunnett (UCSF, San Francisco, CA) were maintained in DMEM, 10% FBS, and Zeocin (100 µg/ml). Cells were transiently transfected with apoE-GFP plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 48–72 hours following transfection cells were washed with cold 1XPBS and whole cell lysates were prepared using 1% Triton based lysis buffer with protease inhibitor (2 mg/ml) (Roche, Indianapolis, IN). BCA protein assay (Pierce Biotechnology, Rockford, IL) was used to determine lysate total protein content. 500 µg of lysate protein was subjected to immunoprecipitation with 5 µg of GFP polyclonal antibody (ABCAM, Cambridge, MA) using the Classic IP Kit (Pierce Biotechnology, Rockford, IL). Concentration and purity was confirmed with Western blot analysis.

Cell Culture

Wild type mouse embryonic fibroblasts (MEF1) carrying the low density lipoprotein receptor (LDLR) and the equivalent LDLR knockout cells (MEF3) were kindly provided by Joachim Herz, UT Southwestern. Cells were grown as a monolayer in DMEM supplemented with 10% FBS, P/S (100 U/ml penicillin, 0.1 mg/ml streptomycin), and glutamine (4mM) in 95% air/5% CO₂ at 37 C in a humidified incubator.

Cells were grown to 85–90% confluence in 60 mm dishes and treated with varying doses of apoE-GFP and heparin. At 12 and 24 hours after treatment, cells were lysed and subjected to Western blot analysis.

SDS-PAGE and Western Blotting

Cells were washed three times with cold 1XPBS and lysed in 1% Triton X100, 25 mM Tris pH 7.4, 150 mM NaCl, and 2mg/ml protease inhibitor. Lysates were separated by SDS-PAGE (8% acrylamide), transferred to polyvinylidene difluoride membrane (Immobilon FL, Millipore Corp.), blocked in blocking buffer (LI-COR, Lincoln, NE) and incubated with mouse anti-apoE (1:500; 1 hour, room temperature) (Calbiochem, EMD Chemicals, Gibbstown, NJ) and mouse anti-β-actin (1:500; 1 hour, room temperature) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were washed and then incubated with secondary antibodies coupled to IRDye 680 or IRDye 800 (1:10000; 1 hr, room temperature). Immunoreactive proteins were detected with an Odyssey Infrared Imaging System (LI-COR). For the densitometric analysis, the apoE signal and the β-actin signal (used as a loading control) were calculated.

Statistical Analysis

PTT, ALT, and densitometry values were compared using two tailed t-tests. Survival data was analyzed by the chi-square test. A p value of <0.05 was regarded as statistically significant.

Results

Heparin protects against septic mortality independent of its anticoagulation effect

We first investigated whether heparin given as an intraperitoneal bolus and then as a steady infusion via osmotic pump affected septic mortality. Mortality was determined in 3 groups of mice each treated at the time of CLP. The initial dose of heparin was based on pulmonary embolism protocol dosing (150 U/kg bolus, followed by 20 U/kg/hr). The treatment groups consisted of: CLP/saline, CLP/heparin 150 U/kg + 20U/kg/hr, CLP/heparin 150 U/kg + 80 U/kg/hr. The CLP/saline group had a mortality of 71% by 96 hours and out to 7 days (Figure 2). 7 days after CLP, the mice that received heparin 20 U/kg/hr had a 51% reduction in mortality compared to those that received saline with a final survival of 80% (p < 0.05). The high dose heparin (80 U/kg/hr) mice did not demonstrate as profound a survival benefit, but

still had a 16% decrease in mortality versus saline controls ($p = 0.27$). These findings indicate that heparin decreases septic mortality, with the lower dose resulting in improved survival.

Octasaccharide protects against septic mortality

In order to further tease out the contribution that heparin's anti-coagulation effect has on mouse survival in sepsis, we administered heparin octasaccharide in place of unfractionated heparin in our CLP model. Heparin octasaccharide, an oligomeric byproduct of the deaminative cleavage of unfractionated heparin, binds the LDLR-binding region of apoE with comparable affinity to unfractionated heparin^{7,12}. But the anticoagulant effect of the octasaccharide has been reported to be less 13% that of its unfractionated counterpart¹².

Similar to the heparin-treated mice, mortality was monitored for seven days in mice given an initial bolus of 130 $\mu\text{g/kg}$ followed by low and high dose octasaccharide infusion via osmotic pumps (17.5 and 70 $\mu\text{g/kg/hr}$). These doses were based on molar equivalents of the heparin dosing. As in the heparin-treated group, these mice were compared against the mortality of CLP mice treated with saline. Both the low and high dose octasaccharide groups had a mortality of 29% by 4 days and out to 7 days (Figure 3). This was a 51% reduction in mortality compared to the CLP/saline group, which had a mortality of 80% by 7 days ($p < 0.05$). These findings indicate that octasaccharide confers a survival benefit in sepsis that is nearly as pronounced as unfractionated heparin.

Lepirudin does not protect against septic mortality

Finally, we administered lepirudin to septic mice which served as our control to heparin and heparinoid anticoagulant activity. Lepirudin, a hirudin-based anticoagulant that directly inhibits thrombin, has no known apoE-binding affinity¹³. When septic mice were given the standard pulmonary embolism dose of 0.4 mg/kg bolus followed by 0.15 mg/kg/hr. In addition, the high dose group received an infusion of 0.3 mg/kg/hr lepirudin. Mice subjected to CLP did not demonstrate any survival benefit with the administration of lepirudin (Figure 4). In fact, lepirudin+CLP appeared to increase mortality at both doses when compared to CLP+saline mice. Low and high dose lepirudin mice had 90% mortality by 48 hours compared to 59% mortality seen in saline treated mice. By 5 days there were no survivors in the lepirudin treated septic groups, whereas the saline-treated septic mice still had 29% survival ($p = 0.06$). No overt intraperitoneal or intracranial bleeding was noted on autopsy. Lepirudin not only fails to protect septic mice, but it appears to increase mortality.

Heparin and octasaccharide protection is independent of their anticoagulation effect

Much of heparin's anti-inflammatory effects in sepsis are presumed to be from its ability to counteract the process of disseminated intravascular coagulation (DIC)^{14,15}. Heparin activates antithrombin III, thereby decreasing fibrin and thrombin formation which themselves are amplifiers of the inflammatory cascade in addition to direct contributors of microcirculatory thrombosis. Although it has never been formally investigated, octasaccharide—a truncated heparin variant—presumably would act through a similar mechanism. And lepirudin's direct antagonism of thrombin puts it in a position to decrease inflammation as well^{16,17}.

To examine the overall contribution that anticoagulation has on septic mortality, PTT levels were measured at 24 hours after CLP and anticoagulant administration. Septic mice did not demonstrate any difference in PTT compared to control mice. Octasaccharide-treated mice which had a survival benefit with both low and high dose treatment (17.5 and 70 $\mu\text{g/kg/hr}$), failed to produce a significant increase in PTT compared to control mice (Figure 5). Lepirudin treatment (0.15 and 0.3 mg/kg/hr) resulted in a dose-dependent increase in PTT

(CLP vs. high dose $p=0.04$, low vs. high dose $p<0.01$), but this did not prevent septic mortality. While low dose heparin (20U/kg/hr) conferred the optimal survival benefit, high dose heparin (80U/kg/hr) resulted in the greatest degree of anticoagulation compared to CLP-saline and low dose heparin mice (CLP vs. high dose $p=0.02$). For all three anticoagulation agents, the extent of anticoagulation failed to correspond to survival benefit. Thus, it appears that anticoagulation alone is not sufficient to improve septic mortality.

Heparin diminishes liver injury in septic mice

Hepatic dysfunction frequently accompanies sepsis¹⁸. ApoE is thought to play a role in this process via the NKT-mediated mechanism alluded to earlier⁵. ApoE administration in sepsis has been shown to result in NKT proliferation in the liver and to accentuate liver injury⁴. We wanted to determine the effects of an apoE-blocking agent, namely heparin, on liver injury. ALT serum levels were measured 24 hours following CLP and heparin treatment (150U/kg + 20 or 80 U/kg/hr). CLP mice demonstrated an increase in ALT levels when compared to control mice (31.3 ± 12.2 vs. 155.4 ± 21.9 U/L; $p<0.001$). CLP-induced liver inflammation was reduced following the administration of high dose heparin (155.4 ± 21.9 vs. 66.5 ± 11.7 U/L; $p<0.001$). Low dose heparin also appeared to reduce ALT levels when compared to the CLP group, but this difference was not significant (Figure 6). These findings indicate that heparin contributes to increased survival by limiting hepatic damage.

Heparin decreases hepatic NKT cell frequencies in sepsis

Next, we wanted to look specifically at the presumed effector cells in the apoE-mediated antigen presentation model: NKT cells. Therefore we investigated whether heparin changed hepatic NKT cell frequency, thereby contributing to the decrease in immune activation and mortality. Lymphocytes from the liver were harvested 24 hours after CLP and saline or heparin treatment (150U/kg + 20 or 80 U/kg/hr). Surface marker staining with the TCR β and CD4 antibodies revealed that NKT cell frequency increased significantly following CLP when compared to control mice (92% increase; $p=0.03$) (Figure 7). Low dose heparin did not significantly reduce NKT cell frequency compared to CLP mice, but high dose heparin reduced the NKT cell frequency by 63% compared to that of CLP mice ($p=0.02$). This finding suggests that heparin might act to limit apoE-mediated lipid antigen presentation to NKT cells.

Decreases in NKT cell populations predominantly correlate with decreases in T_H1 and T_H2 cytokines

Because NKT cells produce a variety of cytokines, we sought to test the systemic effect and contribution of heparin on cytokine secretion. IL-1 β , IL-2, IL-4, IL-10, IFN- γ , and TNF- α cytokine levels were measured in sera from septic mice 24 hours after receiving heparin. Multiplex analysis demonstrated that septic mice had higher levels of the T_H1 cytokines IL-1 β , IFN- γ , TNF- α and the Th2 cytokines IL-4 and IL-10 than did control mice ($p<0.05$) (Figure 8). For all cytokines tested, there appeared to be a trend toward a dose-dependent decrease with the addition of heparin. High dose heparin in septic mice resulted in a dramatic decrease in IL-1 β , IL-4, IL-10, and IFN- γ when compared to non-heparin treated septic mice. Specifically, IL-1 β levels decreased more than seven-fold ($p<0.02$), IL-4 levels decreased more than threefold ($p<0.05$), IL-10 decreased more than two-fold ($p<0.01$), and IFN- γ decreased three-fold ($p<0.02$) over septic mice. These findings indicate that there is a nonspecific downregulation of both T_H1 and T_H2 cytokines with the addition of heparin in septic mice.

Heparin decreases LDLR-mediated uptake of apoE in MEFs

Heparin binds to the LDLR-binding portion of apoE, and presumably can outcompete apoE for LDLR sites. This is the postulated mechanism by which heparin and its LDLR-binding octasaccharide can diminish antigen-presenting cell activation and ultimately septic mortality. To test this competitive binding theory, we treated LDLR-expressing (MEF1) and knockout mouse embryonic fibroblasts (MEF3) with apoE (1.6 $\mu\text{g/mL}$) and heparin concomitantly for 12 and 24 hours. We then harvested the lysates and assayed intracellular apoE protein levels using Western blot analysis.

In LDLR-expressing fibroblasts (MEF1), the addition of exogenous apoE results in an increase in intracellular apoE when compared to control apoE levels ($p < 0.01$). The addition of heparin at all concentrations given significantly decreased intracellular apoE content after 12 hours of exposure, with the most dramatic effect seen at 0.05 $\mu\text{g/mL}$ (Figure 9) ($p < 0.02$). Serving as our negative control, LDLR $^{-/-}$ (MEF3) fibroblasts fail to demonstrate any uptake of apoE with or without the presence of heparin. Thus, heparin appears to decrease the uptake of apoE in fibroblasts, and this process is dependent on the presence of LDLR's.

Discussion

Considerable efforts have been expended to find novel therapeutic strategies to combat sepsis. Unfortunately, most of these strategies failed to improve patient survival when studied in large, multicenter clinical trials^{19,20}. Many of the pathways which were targeted, e.g., IL-1 and TNF- α , are part of an extensive redundant network that cannot be interrupted with an agent that blocks a single pathway. Only activated protein C, with its extensive effects on the coagulation cascade and on downstream inflammatory mediators, has shown any promise in the treatment of sepsis²¹. Our goal was to find a treatment for sepsis utilizing our experience with lipoproteins and their unique role in inflammation.

Apolipoprotein E plays a role in increasing the immune response to polymicrobial sepsis, particularly through increased NKT cell number, proliferation, and downstream responses, which contributes to mortality⁴. ApoE thus provides a potentially valuable upstream target for therapeutic intervention that can exert a more global effect on inflammation. Heparin, an established anticoagulant, fulfills the role of apoE antagonist with its affinity for the LDLR-binding portion of apoE. This study provides evidence that heparin can reduce hepatic NKT proliferation and peripheral cytokine production that occurs in response to sepsis, and thereby contributes to decreased mortality. Our *in vitro* data points to an LDLR-dependent mechanism by which heparin prevents apoE internalization. In addition, heparin variants serve as controls in our efforts to prove that heparin decreases septic mortality independent of its anticoagulant activity.

Unfractionated heparin clearly increased survival of mice subjected to cecal ligation and puncture compared to saline. 20 U/kg/hr, equivalent to the dose given for the pulmonary embolism protocol, conferred the most survival benefit. To date, animal studies looking at heparin effects during sepsis have been mixed. These studies utilize various methods to induce sepsis, including intravascular *e.coli* infusion, intraperitoneal meningococcal or fecal injection, and biliary fecal injection^{14,22-24}. Our chosen sepsis model, cecal ligation and puncture, has been proven to be both highly reproducible and clinically relevant²⁵. Clinical studies looking at heparin effects in septic patients have produced mixed results. A retrospective Canadian study demonstrated a reduction in 28 day mortality from 69% to 56% in patients given at least one day of heparin therapy for various indications compared to those not given heparin.²⁶ In contrast the first randomized single center trial looking at heparin administration following signs of sepsis (the HETRASE Study) showed no significant change in 28 day mortality (16% vs 14%) when compared to the control group²⁷.

The different outcomes of these two clinical studies could be attributed to the different patient populations in terms of disease severity in addition to the dosage of heparin given. But the inability to demonstrate consistent survival benefit still put into question the assumption that heparin primarily acts in sepsis to counteract the DIC response, specifically to deplete the potent pro-inflammatory generators thrombin and fibrin.

Interestingly, our study showed that anticoagulation was not necessary for the observed survival improvement. When heparin was administered at high doses, 80 U/kg/hr, the survival benefit was minimal even though this group was effectively anticoagulated 24 hours after surgery. In addition, octasaccharide, a heparin variant which has only a fraction of the anticoagulation potency of heparin but similar apoE-binding ability,^{7,12} demonstrated a survival benefit in sepsis without obtaining significant anticoagulation. These results are in line with a recent prospective randomized clinical study out of China demonstrating a 28-day survival benefit in septic patients given heparin when compared to the control group (15.4% vs. 32.4%), but no difference in PTT.²⁸ Similarly, the subgroup analysis of the antithrombin III Kybersept trial showed that in moderate risk septic patients, high dose ATIII therapy decreased the 28-day mortality versus controls (44.4% vs. 35.7%) without a significant change in PTT, PT, or fibrinogen.^{29,30} These findings support previous clinical and animal study findings that allude to the fact that the anticoagulation, anti-DIC effects of heparin are insufficient for septic protection^{27,31,32}. The subgroup analysis of the antithrombin III Kybersept trial demonstrates that moderate risk patients We believe that a significant component of the survival benefit seen with heparin can be attributed to its apoE-binding properties. This theory was further corroborated by our finding that lepirudin, which decreases fibrin and thrombin production but does not bind apoE, effectively anticoagulated septic mice at high doses but failed to demonstrate any improvement in survival and in fact increased overall mortality.³³ Perhaps the lack of apoE antagonism is what makes lepirudin an ineffective anti-sepsis agent.

Our hypothesis is based on the aforementioned mechanism whereby apoE facilitates lipid antigen presentation in an LDLR-mediated proinflammatory cascade (figure 1), and heparin binding of apoE can interfere with this process. In order to define the specific interaction between heparin and apoE, we attempted to monitor what happens to endogenous apoE with the addition of heparin. We found that in the presence of a fixed concentration of apoE, heparin decreases LDLR-mediated uptake of apoE in fibroblasts. This interaction supports the mechanism proposed by van den Elzen et al. and our *in vivo* findings that heparin can diminish apoE-mediated lipid antigen presentation and ultimately downregulate the LDLR pathway that leads to inflammation and death.⁵ The concept of heparin interacting with apoE and the LDLR is not a new one. In the liver, apoE mediates lipoprotein remnant binding to the heparan sulfate proteoglycan (HSPG) – LDLR-related protein pathway, facilitating lipoprotein uptake.³⁴ ApoE contains a C-terminal domain which contains the major lipid-binding (and presumably lipid antigen-binding) site, and a N-terminal domain which contains the LDLR-binding region. This N-terminal domain also contains a high-affinity heparin-binding site overlapping with the receptor region.^{7,9} Conceivably, heparin could bind the N-terminal domain of apoE and interfere with its ability to bind the LDLR either by directly blocking the site or by inducing a conformational change that prevents it from binding the receptor. The C-terminal domain of apoE also contains a lower affinity heparin-binding site.⁹ Heparin could similarly decrease the lipid antigen-binding potential of the apoE C-terminal domain, and thereby reduce lipid antigen presentation and inflammation. Regardless of the specific pathway, decreasing apoE-LDLR or apoE-lipid antigen could both decrease downstream CD1d-restricted NKT cell activity and cytokine release.

NKT cells, which are predominantly expressed in the liver, appear to act as a bridge between the innate and acquired immune systems. Hepatic NKT cell frequency decreased significantly in septic mice treated with heparin when compared to septic mice given saline. ApoE has been shown to increase CD1d-restricted NKT cell activity in sepsis⁴. We speculated that the addition of the presumed apoE-antagonist, heparin, could decrease NKT proliferation. Not only did heparin decrease NKT cell frequency, but it also decreased ALT levels which we interpreted as an index of apoE-induced NKT cell hepatotoxicity. Upon stimulation, NKT cells can promptly secrete large amounts of Th1 and Th2 cytokines. Previous research suggests that both pro-inflammatory cytokines such as TNF- α , IL-1 β , and IFN- γ , as well as anti-inflammatory cytokines such as IL-4 and IL-10, have relevant roles in sepsis³⁵. ApoE activation of NKT cells appears to produce a mixed cytokine response, with Th1 cytokines predominating (IL-10, TNF- α , IL-1 β , and IFN- γ)⁴. All these cytokines were reduced in septic mice treated with heparin when compared to saline treated mice. IL-4, which is the main Th2 cytokine produced by NKT cells, was also reduced with heparin administration. Both the NKT cell and cytokine decreases appear to be dose-specific, with only high dose heparin resulting in a significant reduction. Interestingly, although both low and high dose heparin decreased hepatic NKT cell frequency and cytokine production, only high dose resulted in a significant reduction when compared to untreated septic mice. This is in contrast to our survival data, in which the low dose heparin demonstrated optimal survival benefit compared to high dose heparin. One possible explanation is that heparin is acting through an additional pathway that does not involve NKT cells. Another possibility is that unlike high dose heparin, low dose heparin achieves the optimal balance of minimizing the detrimental hyperinflammatory response without being overly immunosuppressive.

We acknowledge that heparin is a diverse molecule that has the potential to exert an array of anti-inflammatory effects. From inhibition of leukocyte adhesion³⁶, to selectin and PECAM binding³⁷⁻³⁹, heparin has been revealed to be much more than just an anticoagulant. Although how these functions play out in *in vivo* has not been completely elucidated. These alternate functions could very well exist in parallel to the mechanism that we feel makes a significant contribution to septic mortality. Our focus is to highlight the significant role that apoE-mediated immunomodulation plays in septic death and how heparin acts to counteract these detrimental effects. In order to define heparin's specific role in the apoE-mediated inflammatory response, we will have to repeat our *in vivo* experiments in apoE, LDLR, and NKT (*J α -18*) knockout animals. These future experiments will allow us to isolate the integral nature of each component of the pathway. Why heparin to date has not consistently shown survival benefit in sepsis is still to be determined. It is possible that the timing of the treatment is critical to significantly blunting the systemic inflammatory response before the cascade propagates out of control. In our animal study, we have the luxury of initiating treatment at the time of the insult. Further studies are necessary to address the therapeutic window for heparin in sepsis.

In conclusion, our study provides evidence that heparin can decrease inflammation and mortality in a murine model of sepsis independent of its traditional anti-thrombotic effects. Research has highlighted a growing role for agents of lipid metabolism, specifically apoE, as playing a significant role in foreign lipid antigen processing and in the immune response. Heparin binds with high affinity to apoE, and this unique interaction leads to downregulation of the LDLR-mediated hyperinflammatory cascade. Ultimately, the goal will be to find an agent that can minimize the pro-inflammatory effects of endogenous apoE without the potential side effect profile of heparin. Potential candidates include heparin variants such as octasaccharide, apoE-mimetic peptides, which can occupy LDLR sites without facilitating lipid antigen presentation, apoE-antibodies, and soluble apoE-receptors. Our research highlights the importance of apoE in regulating infection and immunity, and the importance of cultivating this role for future sepsis therapy.

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Figure 1.

Proposed model of apolipoprotein E (apoE)-mediated lipid antigen presentation and NKT cell activation. Pathogenic microbial lipids (e.g. lipopolysaccharide, LPS) are delivered to antigen-presenting cells as part of a triglyceride-rich lipoprotein (i.e. chylomicrons and very low density lipoproteins) or bound to apoE. The lipid complex binds to the LDL receptor (LDLR), is internalized and while trafficked through the endosomal compartments, is processed and loaded onto CD1d for display on the cell surface where it activates CD1d-restricted NKT cells (adapted from van den Elzen et al., Nature 2005)

Figure 2.

Administration of heparin increases survival of mice (C57BL6) following CLP. After CLP, an intraperitoneal heparin (150 U/kg) or vehicle bolus (saline) was followed by an infusion of low or high dose heparin (20 U/kg/hr and 80U/kg/hr) or vehicle via an intraperitoneally placed osmotic pump. Survival was monitored for 7 days. Differences between survival at day 7 were determined by chi-square test. Results are expressed as percentage of survival $p < 0.005$ [CLP/saline (n=21) vs. CLP/low heparin (n=20)]; $p = 0.3$ [CLP/saline vs. CLP/high heparin (n=20)]; $p < 0.05$ [CLP/low heparin vs. CLP/high heparin].

Figure 3.

Administration of heparin fragment, octasaccharide, increases survival of mice (C57BL6) following CLP. After CLP, an intraperitoneal octasaccharide (130 µg/kg) or vehicle bolus (saline) was followed by an infusion of low or high dose octasaccharide (17.5 µg/kg/hr and 70 µg/kg/hr) or vehicle via an intraperitoneally placed osmotic pump. Survival was monitored for 7 days. Results are expressed as percentage of survival. $p < 0.05$ [CLP/saline (n=10) vs. CLP/low octasaccharide (n=14)]; $p < 0.05$ [CLP/saline vs. CLP/high octasaccharide (n=14)].

Figure 4.

Administration of lepirudin does not increase survival of mice (C57BL6) following CLP. After CLP, an intraperitoneal lepirudin (0.4 mg/kg) or vehicle bolus (saline) was followed by an infusion of low or high dose lepirudin (0.15 mg/kg/hr and 0.3 mg/kg/hr) or vehicle via an intraperitoneally placed osmotic pump. Survival was monitored for 7 days. Results are expressed as percentage of survival. $p = 0.06$ [CLP/saline (n=17) vs. CLP/low lepirudin (n=10)]; $p = 0.06$ [CLP/saline vs. CLP/high lepirudin (n=10)].

Figure 5. Survival following sepsis does not depend on anticoagulation. Murine plasma was drawn 24 hours following CLP and saline/heparin/octasaccharide/lepirudin treatment. PTT analysis was then performed. Results are expressed in seconds. Data represent 3 independent experiments. Error bars indicate standard deviations. * $p < 0.05$.

Figure 6.

Heparin reduces liver injury in mice subjected to CLP. Serum concentrations of ALT were measured 24 hours after mice were subjected to CLP and treated with saline/heparin/octasaccharide/lepirudin. Data represent 3 independent experiments. Error bars indicate standard deviations. * $p < 0.05$.

Figure 7.

Heparin decreases hepatic NKT cell frequency in mice subjected to CLP. Lymphocytes were isolated from the livers of mice treated with saline or heparin. (A) Numbers in flow cytometry panels indicate the percentage of gated lymphocytes (B). TCR β : β T cell receptor; PE: lymphocyte cell-associated marker (CD4). Data represent three to four independent experiments and are reported as a fraction of control. Error bars indicate standard deviations. * $p < 0.05$.

Figure 8.

Heparin reduces cytokine levels in mice subjected to CLP. Cytokine concentrations 24 hours after CLP/heparin were determined by cytometric bead array multiplex of mouse sera. (A) IL-1 β , (B) TNF- α , (C) IFN- γ , (D) IL-10, (E) IL-4, (F) IL-2. Data represent 3 to four independent experiments. Error bars indicate standard deviations. * p < 0.05.

Figure 9.

Heparin reduces apoE internalization in LDLR-expressing mouse embryonic fibroblasts (MEF1). MEF1's were cultured for 12 hours with: no apoE-GFP, no heparin; 1.6 $\mu\text{g/mL}$ apoE-GFP, no heparin; and 1.6 $\mu\text{g/mL}$ apoE-GFP with various doses of heparin (0.01 $\mu\text{g/mL}$, 0.05 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, and 5.0 $\mu\text{g/mL}$ heparin). Cells were then washed, lysed, and immunoblot with antibody against apoE was performed to assess intracellular concentration. Data represent 3 independent experiments. Error bars indicate standard deviations. * $p < 0.05$.