Elevated serum ApoE levels are associated with bacterial infections in pediatric patients

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Apolipoprotein E; Heparan sulfate proteoglycan (HSPG); LDL receptor-related protein (LRP); Low-density lipoprotein receptor (LDLR); Sepsis; Syndecan-1 (SDC1)

Background/Purpose(s): We aimed to determine the variations in serum apolipoprotein E (ApoE) levels in pediatric patients with a variety of infectious diseases, and to investigate the potential mechanism of elevated ApoE serum levels during infection.

Methods: A total of 279 pediatric patients with a variety of infections and 58 normal controls were enrolled in this study. Serum ApoE levels were detected using an immunoturbidimetric assay. A mouse sepsis model was established to evaluate the expression of ApoE and its receptors by real-time polymerase chain reaction (RT-PCR) and Western blotting.

Results: Serum ApoE was markedly increased in cases with bacterial infections including sepsis, bacterial meningitis, and bacterial pneumonia, compared to healthy controls. No significantly elevated serum ApoE levels were observed in aseptic meningitis patients or mycoplasma pneumonia patients. The mice sepsis models showed a similar pattern of increased serum ApoE levels in the early stage of infections. We found reduced expression of ApoE and its receptors in the liver tissues in these mice models.

Conclusion: Serum ApoE may represent a novel indicator for diagnosis of bacterial infections, especially sepsis, in pediatric patients. The decreased expression of low-density lipoprotein receptor (LDLR), LDL receptor-related protein (LRP), and heparin sulfate proteoglycan (HSPG) syndecan-1 (SDC1) may contribute to reduced ApoE clearance and accumulation in the blood.

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Apoe levels in pediatric bacterial infections

Introduction

Infections are a set of common and frequently-occurring pediatric diseases caused by multiple microorganisms including bacteria, viruses, and mycoplasma.1–4 They pose a serious threat to the health and lives of children because statistics show that most deaths among children aged < 5 years were due to infections; infectious diseases accounted for 64% of deaths in pediatric patients globally.5,6 Therefore, effective control of infectious diseases in children is still an urgent task worldwide.7 However, the diagnostic values of some indicators are limited at the early stage of infections, which may lead to difficulties for the diagnosis of infections in some cases.8,9

Apolipoprotein E (ApoE) is a glycoprotein of 299 amino acids with a molecular mass of ~34 kDa. Circulating ApoE is mainly produced by hepatocytes but can also be produced at much lower levels in the brain, kidneys, and spleen.10,11 As a component of several plasma lipoproteins, ApoE is cleared principally by the hepatocytes by three mechanisms: the low-density lipoprotein receptor (LDLR), LDL receptor-related protein (LRP), and heparin sulfate proteoglycan (HSPG).12–14 Recent evidence highlights the multiple immunomodulatory effects of ApoE, such as the activation of a variety of immune cells or suppression of tumor necrosis factor-alpha (TNF-α) and cytokine production.15,16 It could also interact with other cytokines or pathogens ultimately influencing the inflammatory state and even protection from microbes.17,18

The aims of our study were (1) to detect the variations in ApoE levels in the serum of pediatric patients with a variety of infectious diseases, and (2) to determine the expression of ApoE and its receptors in the liver tissue of a mouse infection model to investigate the potential mechanism of elevated ApoE serum levels during infection.

Materials and methods

Participants

A total of 279 pediatric patients (age range 0–6 years, average age 2.9 years) at the Children’s Hospital of Fudan University were enrolled in this study. Neonate patients and those with long courses (> 1 week) were excluded from our study. The cases were subclassified into five groups: 65 cases with sepsis, 47 cases with bacterial meningitis, 67 cases with bacterial pneumonia, 47 cases with aseptic meningitis, and 53 cases with mycoplasma pneumonia (Table 1). An age- and sex-matched population of 58 healthy controls was recruited from among the other patients who had no clinical signs of infection or inflammation. Sepsis was diagnosed in patients showing a positive culture of blood, urea, or other sterile body fluid, except for organisms considered to be contaminants, such as coagulase-negative staphylococcus.19 Bacterial meningitis was diagnosed in those with related clinical symptoms of meningitis, and causative bacteria were identified directly by culturing blood or cerebrospinal fluid (CSF) and indirectly by an antigen test or gram staining of CSF samples.20 CSF pleocytosis [CSF white blood cell (WBC) count > 15 × 10^6/L for infant patients] was analyzed. Aseptic meningitis cases were defined as those presenting with clinical symptoms of meningitis and CSF minimal pleocytosis but showing a negative bacteriological profile in CSF or blood.21 Mycoplasma pneumonia was defined with related symptoms of lung infection where the serum immunoglobulin M (IgM) titer of Mycoplasma pneumoniae (MP-IgM) was greater than 1:160.22 Patients with bacterial pneumonia were enrolled with obvious symptoms of lung infection and X-ray showing related infectious changes and a positive bacterial profile in sputum culture.23

All serum and plasma samples were collected from all enrollees prior to antibiotic treatments or other therapeutic interventions. Infections were subsequently verified, and associated pathogens were identified by routine culture methods using blood, CSF, urine, or abscess samples. Written informed consent was obtained from the participants’ guardians prior to sample collection. The study design and all procedures were approved by the Ethics Committee of the Children’s Hospital of Fudan University.

Laboratory measurements

The blood samples were cultured using a blood culture machine (BacT/ALERT 3D 240; Biomerieux, Lyon, France) and the CSF samples were cultured using blood agar and chocolate agar (Biomerieux, Shanghai, China). The bacteria were identified using the VITEK 60 system (Biomerieux, Lyon, France). The peripheral blood WBC counts were performed using the XS-800i automatic blood analyzer (Sysmex, Kobe, Japan). The CSF protein, glucose, and chloride levels were tested by a one-step method (Wako Pure Chemical Industries, Osaka, Japan), an oxidase assay (KeHua Bioengineering Corporation, Shanghai, China), and

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cases</th>
<th>M/F</th>
<th>Age (y)</th>
<th>ApoE (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>58</td>
<td>31/27</td>
<td>3.8</td>
<td>3.68 ± 1.04</td>
</tr>
<tr>
<td>Sepsis</td>
<td>65</td>
<td>32/33</td>
<td>2.4</td>
<td>6.06 ± 1.98</td>
</tr>
<tr>
<td>Bacterial meningitis</td>
<td>47</td>
<td>27/20</td>
<td>2.6</td>
<td>5.07 ± 1.48</td>
</tr>
<tr>
<td>Bacterial pneumonia</td>
<td>67</td>
<td>41/26</td>
<td>3.4</td>
<td>4.63 ± 1.32</td>
</tr>
<tr>
<td>Aseptic meningitis</td>
<td>47</td>
<td>29/18</td>
<td>3.7</td>
<td>3.62 ± 0.97</td>
</tr>
<tr>
<td>Mycoplasma pneumonia</td>
<td>53</td>
<td>29/24</td>
<td>4.1</td>
<td>3.35 ± 1.02</td>
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<tr>
<td>p &gt; 0.05</td>
<td></td>
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F = female; M = male.
a chloride ion-selective electrode method (HITACHI, Tokyo, Japan), respectively. The *Streptococcus pneumoniae* antisera in the CSF samples were tested using an immunochromatographic membrane assay (Binax, Scarborough, USA). The enterovirus RNA in the CSF samples were detected by using a commercially licensed kit (Da An Gene Co, Guangzhou, China) and carried out by real-time polymerase chain reaction (RT-PCR) assay in our clinical virology laboratory. MP-IgM was detected using a serum MP-IgM detecting kit (HITACHI, Tokyo, Japan).

Animal model

Forty C57BL female mice (3–4 weeks old, 10–15 g body weight) were purchased from the Laboratory Animal Research Institute of Shanghai Medical College of Fudan University. All experimental procedures, including the animals’ care and utilization, were approved by the Ethics Committee of the Shanghai Medical College of Fudan University. The mice were housed in a laminar flow environment with a 12-hour light/dark cycle, a stable temperature, and free access to regular diet and water. The sepsis model was generated as follows. Mice were randomly divided into two groups of 20. Sepsis was induced in one group by intraperitoneal injection of 10⁶ colony forming units (CFU)/mL of group B *Salmonella Typhimurium*. The second group was used as the control and was sham-inoculated in the same manner but using physiological saline. The *S. Typhimurium* strain used was isolated from the blood of a pediatric patient with sepsis who was treated in our hospital, and this strain was characterized as resistant to ceftriaxone.

Blood and tissue preparation

After inoculation, mice were euthanized at 0, 1, 3, or 24 hours (n = 5 for each time point). Blood samples were collected from the tail vein, and the serum and plasma were separated and stored separately at −20°C. The whole liver was removed and cut into two sections for subsequent histopathological observation and mRNA or protein analysis. An aliquot of the whole blood sample was cultured overnight on a blood agar plate to verify bacteremia. Sepsis was confirmed by positive blood culture and Lipopolysaccharides (LPS) measurement (described below).

Immunoturbidimetric assay

The concentrations of ApoE in the human serum samples were detected by a latex immunoturbidimetric assay kit (Hitachi 7180 ApoE; Daiichi Pure Chemicals, Tokyo, Japan) and quantitatively measured on an auto-analyzer (Hitachi 7600; Hitachi, Tokyo, Japan) following the manufacturer’s directions. For mouse plasma assays, samples were diluted 1:10 and nonsepsis mouse plasma was used as the standard. LPS was measured in mouse plasma samples by dynamic immunoturbidimetric assay using the Gram-Negative Endotoxin Determination Reagents kit (EKT109; Gold Mountain River Tech, Beijing, China) and an auto-analyzer (MB-80; Goldstream, Beijing, China), according to the manufacturer’s instructions.

Quantitative real-time polymerase chain reaction

Hepatic cells from mice were lysed and total RNA was extracted using the TRizol Max kit (Invitrogen, Carlsbad, USA). The RNA was reverse transcribed to complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Promega, Fitchburg, USA). mRNA expression of ApoE, LDLR, LRP, syndecan-1 (SDC1), and β-actin was determined by quantitative real-time polymerase chain reaction (qPCR) using SYBR Premix Ex Taq (Takara, Dalian, China). The β-actin gene was used as an endogenous control for sample normalization. The following gene-specific primers were designed and synthesized by Sangon Biotech Inc. (Shanghai, China): ApoE, forward 5′-CAGAGCTCCAAAGTACACA-3′, reverse 5′-AGTCGGTGCCTAGATCCTC-3′; LDLR, forward 5′-TGTTCTTGCACTCCTTGATG-3′, reverse 5′-TGTTCTTGCACTCCTTGATG-3′; LRP, forward 5′-CGACACACAAAGAACAGACA-3′, reverse 5′-AGATGTCGTTGCTCCCACT-3′; SDC1, forward 5′-TGCTGA CAAAAGGGATAG-3′, reverse 5′-CCTCCCCATCCTACGTAC-3′; β-actin, forward 5′-GAGACCTTTCAACACCCAGC-3′, reverse 5′-ATGTCACCAGCAACTTCC-3′. The thermal cycling reaction was performed on a 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) using the following conditions: one cycle of 30 seconds at 95°C, 40 cycles of 5 seconds at 95°C, 30 seconds at 60°C. Confirmation of a single gene product was carried out through generation of a dissociation curve following each qPCR cycle. A cycle threshold (CT) value was determined using the iCycler software (Applied Biosystems, Carlsbad, CA, USA), and quantification of gene products, normalized to the expression of the ribosomal β-actin housekeeping gene, was determined using the comparative Ct (2-ΔΔCt) method.

Western blot

Total hepatic cell protein levels were assayed using the BCA Protein Assay kit (Beyotime, Shanghai, China). Lysates (50 µg total protein) were mixed with 5× sample buffer, heated to 100°C for 5 minutes, and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were transferred onto a 0.45-µm polyvinylidene fluoride (PVDF) membrane by using the Mini-Protean 3 electrophoresis system (Bio-Rad, Hercules, CA, USA). The membrane was probed with primary rabbit anti-mouse antibodies against ApoE (1:1000; Abcam, Cambridge, USA), LDLR (1:5000; Epitomics, Burlingame, USA), LRP (1:5000; Epitomics, Burlingame, USA), SDC1 (1:1000; Abcam, Cambridge, USA), and β-actin (1:3000; Santa Cruz Biotechnologies, Santa Cruz, USA). Immunoreactive bands were detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000; Santa Cruz Biotechnologies, Santa Cruz, USA) and then visualized by chemiluminescence. Protein bands were quantified with Quantity One software (Bio-Rad, Hercules, USA) and normalized to corresponding β-actin bands.

Statistical analysis

Statistics were analyzed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as arithmetic
mean ± standard deviation (SD). Differences among the groups were analyzed with the independent-samples t test. One-way analysis of variance (ANOVA) was used for multiple mean comparisons. Two-sided p values of less than 0.05 were considered statistically significant.

**Results**

**Serum ApoE levels were increased in patients with bacterial infections**

We screened the ApoE levels in serum samples from a total of 279 pediatric patients with various infections and 58 matched normal controls. Their clinical characteristics are shown in Table 1. There were no significant differences in age and sex among the five groups.

Serum ApoE was markedly increased in bacterial sepsis patients (6.06 ± 1.98 mg/dL), bacterial meningitis patients (5.07 ± 1.48 mg/dL), and bacterial pneumonia patients (4.63 ± 1.32 mg/dL) compared with healthy controls (3.68 ± 1.04 mg/dL, p < 0.01). Furthermore, bacterial sepsis patients showed higher serum ApoE levels than both bacterial meningitis and bacterial pneumonia patients, but no significant difference was observed between bacterial meningitis and bacterial pneumonia patients.

There were no significantly elevated serum ApoE levels in aseptic meningitis patients (3.62 ± 0.97 mg/dL) or mycoplasma pneumonia patients (3.35 ± 1.02 mg/dL) compared to healthy controls. We also calculated the differences in serum ApoE between patients with bacterial infections and those with nonbacterial infections. As expected, patients with sepsis, bacterial meningitis, and bacterial pneumonia exhibited much higher ApoE levels than those with aseptic meningitis and mycoplasma pneumonia.

**Bacterial infection increased circulating LPS in mice**

In mice, positive whole blood cultures were found at 1 hour, 3 hours, and 24 hours after S. Typhimurium inoculation. The

<table>
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<th>Table 2</th>
<th>Plasma lipopolysaccharide (LPS) levels in mice (pg/mL)</th>
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<tr>
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<td>0 h</td>
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<tr>
<td>Control</td>
<td>0.10 ± 0.00</td>
</tr>
<tr>
<td>Sepsis</td>
<td>0.10 ± 0.00</td>
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</tbody>
</table>

**p < 0.01 vs. control.

Figure 1. Liver pathology during the progression of sepsis. Liver sections stained with hematoxylin and eosin from post-inoculation time points (A) 0 hour, (B) 1 hour, (C) 3 hours, and (D) 24 hours. Magnification 10×.
LPS concentrations in plasma of the mice were found to be significantly increased at 3 hours and 24 hours after bacterial inoculation (p < 0.01) (Table 2).

Bacterial-induced inflammatory cell infiltration in hepatic tissue

Slight infiltration was observed at 1 hour after bacterial inoculation (Fig. 1B). By 3 hours postinoculation, the number of inflammatory cells around the center veins in the hepatic lobules were significantly increased (Fig. 1C). By 24 hours postinoculation, the liver tissue was full of inflammatory cells, both in the hepatic lobules and portal areas (Fig. 1D).

Serum ApoE levels were increased at the early stage of sepsis in mice

Compared with the nonseptic control group, the plasma ApoE levels in the septic mice were increased at 1 hour postinoculation (1.32 ± 0.28 vs. 1.06 ± 0.27 mg/dL, p < 0.05), at 3 hours (1.62 ± 0.44 vs. 1.04 ± 0.24 mg/dL, p < 0.01) and at 24 hours (2.20 ± 0.34 vs. 1.12 ± 0.29 mg/dL, p < 0.01) (Fig. 2B).

Bacterial infection downregulated ApoE and its receptor expression in hepatic cells

As shown in Fig. 3A, the expression of ApoE mRNA in the mice hepatic cells did not change significantly from control levels at 1 hour after inoculation but was decreased by 0.29-fold at 3 hours and by 0.26-fold at 24 hours (p < 0.01). The mRNA expressions of LDLR, LRP, and SDC1 were all found to be significantly reduced as sepsis progressed. The LDLR gene expression was the most robustly decreased at 1 hour, 3 hours, and 24 hours postinoculation (by 0.38-, 0.40-, and 0.41-fold, respectively) (Fig. 3B, p < 0.01). SDC1 was only significantly decreased at 1 hour after inoculation (0.52-fold, p < 0.05, Fig. 3C). LRP mRNA expression were decreased by 0.42-fold at 1 hour, then by 0.50-fold at 24 hours (Fig. 3D, p < 0.01).

The protein results from Western blotting were highly correlative to those for mRNA from qPCR, indicating that the protein expression of these receptors was also dynamic and distinctive with sepsis progression. All the bands had been digitalized and calculated. The expression of ApoE was decreased 3 hours and 24 hours after inoculation (p < 0.05) and LDLR expression decreased at 1 hour, 3 hours, and 24 hours after inoculation (p < 0.05). SDC1 was only decreased at 1-hour time point postinoculation (p < 0.05). LRP expression was decreased significantly at 1 hour and 24 hours (p < 0.01) (Fig. 4).

Discussion

In this study, we found that serum ApoE increased in pediatric patients with bacterial infections (sepsis, bacterial meningitis, and bacterial pneumonia) compared to normal controls. By contrast, no obviously elevated serum ApoE levels were seen in patients with nonbacterial infections (aseptic meningitis and mycoplasma pneumonia). These interesting results demonstrate that compared with nonbacterial infections, bacterial infections, especially sepsis, could lead to higher ApoE levels in serum samples from pediatric patients. The results of our clinical study were similar to those reported by the previous studies showing the increased serum ApoE levels in adult patients with sepsis.24,25 Furthermore, in patients with sepsis, this high variation could develop for
A long duration of time, which might benefit the sensitivity of the diagnosis.

The diagnosis of infections is a common problem in pediatric practice.26,27 The clinical manifestations of bacterial and nonbacterial infections are nearly the same, therefore routine laboratory tests including C reactive protein (CRP) and procalcitonin (PCT) remain a challenge in differentiating between children at risk for a severe bacterial infection and those with a localized bacterial or viral infection.28,29 Here, we reported serum ApoE levels elevated in pediatric patients with bacterial infections compared with no equivalent ApoE levels in those with nonbacterial infections. It may help us to discriminate the types of microbe at the early stage of infection and aid us in

Figure 3. Acute sepsis affected the mRNA expression of apolipoprotein E (ApoE), low-density lipoprotein receptor (LDLR), LDL receptor-related protein (LRP), and syndecan-1 (SDC1) at different times. After injection with *Salmonella* Typhimurium, mice were sacrificed at 0 hour, 1 hour, 3 hours, and 24 hours. Hepatic expression of (A) ApoE, (B) LDLR, (C) SDC1, and (D) LRP mRNA was detected by quantitative real-time polymerase chain reaction (qPCR). Expressions were normalized to β-actin, and all the data are shown as "-fold" of β-actin. * p < 0.05, ** p < 0.01.
Serum ApoE predominantly originates from the liver tissue. Thus, to investigate the potential mechanism of elevated serum ApoE levels, we established a sepsis mice model by lumbar injection of group B S. Typhimurium. Group B S. Typhimurium is a pathogen of humans and mice, showing a high predisposition for mice infection. Within 24 hours after injection, the sepsis mice group showed an acute inflammatory response; group B S. Typhimurium could be detected from the blood of the sepsis mice group at three time points (1 hour, 3 hours, 24 hours), whereas plasma LPS levels were significantly higher than the corresponding control group, and the live tissue was infiltrated by inflammatory cells. Given that serum ApoE levels were elevated in the experimental group at the early stage of infection, this result was highly consistent with the increased serum ApoE levels in pediatric patients with sepsis.

We determined the expression of ApoE in liver tissue, which is considered the major organ involved in the metabolism of ApoE. Instead of elevated ApoE expression, however, a reduced appearance of ApoE mRNA and protein was found in liver tissue at 3 hours and 24 hours after injection in sepsis mice. The data showed that the high plasma ApoE levels were not due to increased synthesis. The mechanism for such unconformity remains elusive. Recent evidence indicated that cytokines such as interferon-gamma (IFN-γ) and interferon-beta-1b (IFN-β-1b) could exert inhibitory effects on ApoE production in macrophages. In consideration of the high levels of these cytokines during infection, the hepatocytes may suffer from the similar inhibitory effects, resulting in declined ApoE synthesis. Some researchers have also hypothesized that decreased ApoE production in the liver could conserve biosynthetic resources to serve functions against infection.

In conclusion, we evaluated serum ApoE levels in 279 pediatric patients with a variety of infections. Our data demonstrated elevated ApoE levels in patients with bacterial infections but no equivalent ApoE levels in those with nonbacterial infections. Using a sepsis mice model, we found that ApoE increased in the early stage of infections in serum samples but reduced in live tissues. The decreased expression of LDLR, LRP, and SDC1 might decrease the hepatic uptake of important lipids, making them available within peripheral sites against infection and inflammation. Thus, this unbalanced production and clearance of ApoE finally leads to a high serum ApoE level, which would be expected to increase apoE concentrations in lymph and interstitial fluids, where apoE could exert its immunomodulatory effects during infection.

In conclusion, we evaluated serum ApoE levels in 279 pediatric patients with a variety of infections. Our data demonstrated elevated ApoE levels in patients with bacterial infections but no equivalent ApoE levels in those with nonbacterial infections. Using a sepsis mice model, we found that ApoE increased in the early stage of infections in serum samples but reduced in live tissues. The decreased expression of LDLR, LRP, and SDC1 might contribute to reduced ApoE clearance and accumulation in the blood. Serum ApoE may represent a novel indicator for diagnosis of bacterial infections, especially sepsis, in pediatric patients.

Conflicts of interest

The authors declare that they have no conflicts of interest, financial or otherwise, related to the publication of this study.

Acknowledgments

We would like to thank the numerous researchers in the Microbiology Department who participated in collecting blood samples from the pediatric patients, those in the Pathology Department who carried out the pathological sectioning of mice tissues, and those in the Department of Laboratory Animals (Shanghai Medical College of Fudan

![Figure 4. Acute infection of Salmonella Typhimurium decreased protein expression of apolipoprotein E (ApoE) and its three receptors in the liver. Mice were sacrificed at 0 hour, 1 hour, 3 hours, and 24 hours after inoculation. Hepatic ApoE and its receptors [low-density lipoprotein receptor (LDLR), LDL receptor-related protein (LRP), and syndecan-1 (SDC1)] were measured by Western blotting using equal amounts (50 µg) of liver total protein from (A) control and (B) sepsis mice. Semi-quantitative analysis of the bands was performed as described in the Materials and methods. The experiments were repeated once and yielded similar results.](image-url)


