Protective effects of the SEPS1 gene on lipopolysaccharide-induced sepsis

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Abstract. Septic shock and sequential multiple organ failure are the main cause of mortality in patients with sepsis. The induction of inflammation during sepsis is a complex biological cascade, which requires successful therapeutic intervention. Selenoprotein S (SEPS1) is a novel endoplasmic reticulum-resident protein and is important in the production of inflammatory cytokines. The present study attempted to assess the effect of SEPS1 suppression by small interfering RNA (siRNA) on mice with lipopolysaccharide (LPS)-induced sepsis. In total, 30 mice were randomly assigned to three groups: i) H group (LPS-induced sepsis group; n=10): Mice with intraperitoneal injection of LPS (10 mg/kg); ii) K group (scrambled siRNA group; n=10): Mice transfected with scrambled control siRNA 12 h prior to injection with LPS; and iii) L group (SEPS1 siRNA group; n=10): Mice transfected with SEPS1 siRNA 12 h prior to injection with LPS. The effects of siRNA were evaluated by SEPS1 gene and protein expression, biochemical parameters including serum alanine transaminase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), lactic dehydrogenase (LDH), creatine kinase (CK) and myocardial kinase (CK-MB), as well as the cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). The phosphorylation of p38 mitogen-activated protein kinases (p38 MAPK) was also detected by western blot analysis. In the SEPS1 siRNA group, SEPS1 gene and protein expression decreased significantly, while the levels of TNF-α and IL-6 increased compared with the control group. The biochemical parameters of ALT, AST, BUN, LDH, CK and CK-MB were markedly increased in the SEPS1 siRNA group. The phosphorylation of p38 MAPK was also significantly activated. The decrease in SEPS1 gene and protein expression and the production of TNF-α and IL-6 may correlate with the activation of the p38 MAPK pathway. Biochemical factors and pathological results demonstrated that the damage to vital organs was aggravated. In conclusion, these findings suggested that SEPS1 may protect mice against LPS-induced sepsis and organ damage. Therefore, SEPS1 may be a new target to resolve LPS-induced sepsis.

Introduction

Immune system activation is involved in the pathogenesis of numerous common complex diseases, including cardiovascular disease, diabetes and cancer (1). Ongoing inflammatory insults may contribute to the development of such diseases. Inflammatory activation, a result of cellular exposure to stress conditions, is reflected by increases in levels of circulating proinflammatory cytokines (2). Sepsis is an increasingly common cause of morbidity and mortality, particularly in elderly, immunocompromised and critically ill patients (3). The most common cause of sepsis is an exposure to the structural component of a gram-negative bacterial membrane lipopolysaccharide (LPS), with key symptoms including hypotension and vasoplegia, which may lead to multiple organ dysfunction and, ultimately, mortality (4-6). Bacterial LPS in the bloodstream induces the overexpression of various inflammatory mediators, including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and a large quantity of inflammatory mediators produced in the body are hypothesized to contribute to the LPS-induced symptoms of septic shock and mortality (7).

Selenoprotein S1 (SEPS1) has previously been identified as an endoplasmic reticulum stress response protein that is likely to correlate with an inflammatory response (8-10). Genetic variation in the SEPS1 gene was observed to correlate with circulating levels of pro-inflammatory cytokines in human populations and SEPS1 may regulate cytokine production in cultured macrophage cells (11,12). The protective effect of SEPS1 on mice with sepsis is unknown. In order to investigate the role of SEPS1 in mice with sepsis, a LPS-induced sepsis model was used in the present study. SEPS1 small interfering RNA (siRNA) was used to silence the SEPS1 gene. The associated biochemical indicators as well as gene and protein expression were examined.
Materials and methods

Reagents. SEPS1, p38 mitogen-activated protein kinase (p38 MAPK), phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK) and β-actin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Oligonucleotide primers and dNTP mix were purchased from Bio Basic Inc. (Bio Basic, Toronto, Canada). Protein extraction and BCA protein assay kits were purchased from Promega Co. (Madison, WI, USA). Pyrogen-free water was purchased from Shanghai Yihua Clinical Medicine Technologies Company (Shanghai, China). LPS (from *E. coli* strain O55:B5) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals. Inbred BALB/c mice (male, 17-20 g), were purchased from the Institute of Medical Animal Experimental Center, Peking Union Medical College (Beijing, China). The mice were kept at standard laboratory conditions of temperature and humidity with a 12-h light/dark cycle. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and were treated ethically. The study was approved by the Ethics Committee of PLA General Hospital (Beijing, China).

Animal transfection. siRNAs for SEPS1 and the scrambled control were synthesized in vitro using a kit from Ambion (Austin, TX, USA; Silencer® Negative Control No. 1 siRNA). Primer sequences are provided in Table I (Beijing AuGCT DNA-SYN Biotechnology Co., Ltd., Beijing, China). The mice were transfected with siRNA oligonucleotides (0.5 μg) formulated by Lipofector 2000 (Beeytime Institute of Biotechnology, Jiangsu, China) via tail vein injection. In total, 30 mice were randomly assigned to three groups: i) H group (LPS-induced sepsis group; n=10): Mice with intraperitoneal injection of LPS (10 mg/kg); ii) K group (scrambled siRNA group; n=10): Mice transfected with scrambled control siRNA 12 h prior to injection with LPS; iii) L group (SEPS1 siRNA group; n=10): Mice transfected with SEPS1 siRNA 12 h prior to injection with LPS. The mice in all groups fasted with free access to water following LPS injection and were continuously monitored over a 24-h period. For each time-point (12 and 24 h), five animals were used.

Assessment of blood biochemical parameters. Following the LPS injections at 12 and 24 h, the blood was collected in heparinized tubes by extirpating the left eyeball of five mice in each group. Serum was isolated by centrifugation at 4,000 x g, 4°C for 15 min and maintained at -70°C until it was required for further analysis. The levels of serum alanine transaminase (ALT), aspartate aminotransferase (AST), serum creatinine (Cr), blood urea nitrogen (BUN), myocardial kinase (CK-MB), creatine kinase (CK) and lactate dehydrogenase (LDH) were assessed using an automatic biochemical analyzer (HITACHI 7170, Hitachi, Ltd., Tokyo, Japan) with commercial kits (LDH-Cytotoxicity Assay Kit II; Biosino Biotechnology Co., Ltd., Beijing, China).

Assessment of TNF-α and IL-6 in the liver homogenate. At each time-point of assessment, the mice were sacrificed once the blood had been obtained. Under strict aseptic conditions, 0.1-0.2 g of liver tissue was removed and placed into a pyrogen-free homogenizer. Three volume equivalents of pyrogen-free saline was added and the liver tissue was homogenized in an ice bath. The liver homogenate supernatant was prepared by centrifugation at 10,000 x g at 4°C for 15 min and maintained at -30°C until it was required for further analysis. The samples were analyzed for TNF-α and IL-6 using an ELISA kit (Endogen Inc., Woburn, MA, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm. The concentrations of TNF-α and IL-6 in the experimental samples were extrapolated from a standard curve.

Western blot analysis. Once the mice had been sacrificed, the liver tissue samples were maintained in liquid nitrogen for western blot analysis. The liver tissue samples were cut up and ground with pre-cooled lysis buffer. Equal quantities of protein were separated by SDS-PAGE (10% gel) and transferred to nitrocellulose (CN) membranes (Hybond Inc., Escondido, CA, USA). The membranes were blocked for 1 h at room temperature with 10% non-fat dry milk and subsequently incubated at 4°C overnight with anti-mouse antibodies (1:300 dilution in blocking buffer). Once the membranes had been washed, they were incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (ZSGB-Bio Co., Beijing, China) for 1 h at room temperature. The blots were visualized using an Enhanced Chemiluminescence kit (Pierce Biotechnology Inc., Rockford, IL, USA), and data were quantified using the Gel Doc EQ system (Bio-Rad, Hercules, CA, USA).

Table I. Primer sequences of SEPS1 and scrambles.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEPS1 antisense</td>
<td>AAGATCTAAATGCCCAAGTTGCGCTGTCCT</td>
</tr>
<tr>
<td>SEPS1 sense</td>
<td>AACAACCTGGGATTTAGATCCCTGTCCT</td>
</tr>
<tr>
<td>Scrambled antisense</td>
<td>AAGTATCTAGGTACACACTACCTGTCCT</td>
</tr>
<tr>
<td>Scrambled sense</td>
<td>AATGAGTGTACCTAGATACCCTGTCCT</td>
</tr>
</tbody>
</table>

RNA extractions and quantitative polymerase chain reaction (qPCR). Total cellular RNA was extracted with TRIzol®...
Results

Confirmation of SEPS1 silencing. In order to examine whether the SEPS1 gene was silenced, the expression of the SEPS1 gene was detected by qPCR and western blot analysis. SEPS1 protein expression in liver tissue decreased 12 and 24 h following transfection with SEPS1 siRNA (Fig. 1). The results of the immunohistochemical analysis also revealed that SEPS1 protein expression in liver and lung tissue decreased in the SEPS1 siRNA group (Figs. 2 and 3). qPCR results demonstrated that SEPS1 gene expression in liver tissue decreased significantly compared with the control group (Fig. 4).

Table II. Effect of SEPS1 siRNA on the levels of ALT, AST and BUN (n=6).

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/l)</th>
<th>AST (U/l)</th>
<th>BUN (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
<td>12 h</td>
</tr>
<tr>
<td>Sepsis</td>
<td>66.7±15.2</td>
<td>34.4±4.1</td>
<td>19.7±3.2</td>
</tr>
<tr>
<td>Control</td>
<td>71.3±14.6</td>
<td>51.1±12.3</td>
<td>21.2±1.9</td>
</tr>
<tr>
<td>SEPS1 siRNA</td>
<td>77.8±22.1</td>
<td>53.5±7.3</td>
<td>20.0±5.7</td>
</tr>
</tbody>
</table>

‘P<0.05, compared with sepsis group at the same time-point; ‘P<0.01, compared with sepsis group at the same time-point; ‘P<0.05, compared with control group at the same time-point. ALT, serum alanine transaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; SEPS1, selenoprotein S; siRNA, small interfering RNA.

Immunohistochemical detection. Liver and lung tissue samples were deparaffinized three times with xylene for 5 min each, followed by rehydration with a series of absolute, 70% and 50% ethanol for three min each and washed under running tap water for 5 min. Tissue samples were subsequently blocked with 3% hydrogen peroxide for 5 min, placed in distilled water for 5 min, followed by 10 min incubation with a 1:20 dilution of goat serum. Subsequently, tissue samples were incubated with anti-SEPS1 polyclonal serum at 4˚C overnight and washed five times with phosphate-buffered saline and Tween 20 (PBST), for two min each. Tissue samples were then incubated with a 1:1,000 dilution of horseradish peroxidase-conjugated antibody (Sigma-Aldrich) for 30 min and washed again with PBST. Following washing, the samples were developed with 3,3'-diaminobenzidine substrate solution for 3 min and washed again with PBST. Finally, the tissue samples were counterstained with Harris' haematoxylin (Sigma-Aldrich) for 1 min, followed by washing, differentiation with ethanol containing 1% acid alcohol solution, bluing with ammonia in water, another washing step, dehydration with increasing series of alcohols, clearance with xylene and subsequent mounting with dibutyl phthalate xylene (DPX).

Pathological examination. Processed liver and lung tissue samples were deparaffinized three times with xylene for two min each, rehydrated with a series of absolute, 95% and 80% ethanol for two min each, followed by a wash with running tap water for 5 min. Subsequently, the tissue samples were stained with Harris' haematoxylin (Sigma-Aldrich) for 5 min and washed with running tap water. Differentiation with 1% acid alcohol solution was performed for 10 sec, followed by washing and bluing with placing the tissue samples into a solution of ammonia in water for 10 sec. Following a washing step, the samples were counterstained with eosin Y (Sigma-Aldrich) for two min, dehydrated with an increasing series of ethanol for two min each, cleared by three washes with xylene for two min each and finally mounted with DPX.

Statistical analysis. Values are expressed as the mean ± standard deviation from a minimum of three independent experiments. The statistical significance of the results between each treated group was analyzed by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Confirmation of SEPS1 silencing in mice with sepsis increases ALT, AST, BUN, LDH, CK and CK-MB levels. In the present study, important biochemical indicators were detected in order to reflect the effects on the liver, kidney and heart following reagent (Takara, Seoul, Korea) according to the manufacturer's instructions. Reverse transcription was performed at 37˚C for 1 h in a reaction mixture containing 2 mg total RNA, 0.5 mg oligo-dT primer, 10 mM dNTPs, 25 U RNase inhibitor and 200 U M-MLV reverse transcriptase (Promega). qPCR was performed using a SYBR FAST qPCR kit (KAPA, Boston, Massachusetts, USA) with 10 ng cDNA according to the manufacturer's instructions. Cycling conditions were as follows: 95˚C for 1.5 min and 40 subsequent cycles of 95˚C for 3 sec, 56.4˚C for 40 sec and 72˚C for 10 sec. Upon the completion of 40 PCR amplification cycles, melting curve analysis was performed. SEPS1 mRNA levels were normalized to actin, a stable housekeeping gene.
Table III. Effect of SEPS1 siRNA on the levels of CK, LDH and CK-MB (n=5, U/l).

<table>
<thead>
<tr>
<th>Group</th>
<th>CK (U/l)</th>
<th>LDH (U/l)</th>
<th>CK-MB (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
<td>12 h</td>
</tr>
<tr>
<td>Sepsis</td>
<td>2302.9±1378.4</td>
<td>2118.4±923.5</td>
<td>743.6±123.7</td>
</tr>
<tr>
<td>Control</td>
<td>1219.0±358.6</td>
<td>1593.7±439.7</td>
<td>535.2±52.4</td>
</tr>
<tr>
<td>SEPS1 siRNA</td>
<td>2657.6±882.9(^b)</td>
<td>2349.2±127.4(^a)</td>
<td>725.7±186.3(^a)</td>
</tr>
</tbody>
</table>

\(^{a}\)P<0.05, compared with control group at the same time-point. \(^{b}\)P<0.01, compared with control group at the same time-point. CK, creatine kinase; LDH, lactic dehydrogenase; CK-MB, myocardial kinase; SEPS1, selenoprotein S; siRNA, small interfering RNA.
siRNA silencing of the SEPS1 gene. At 12 h following injection with LPS, the concentrations of ALT and BUN exhibited no statistical difference among the three groups (P>0.05); AST and LDH levels exhibited significant differences between the groups except for a non-statistical difference between the H and L groups (P>0.05); the Cr levels showed no statistical difference among the groups except for a significant difference between the K and L groups (P<0.05). At 24 h following injection with LPS, the concentration of ALT was significantly higher in the H group than in the other two groups (P<0.05), and demonstrated no statistical difference between the K and L groups; there were significant differences among the three groups in the concentrations of AST and BUN (P<0.05); there was no significant difference among the three groups in LDH levels (P>0.05); the statistical results of difference in Cr, CK and CK-MB concentrations among the three groups at 24 h were the same as that at 12 h. There was a significant increase in the ALT levels and a significant decrease in the Cr levels at 24 h compared with 12 h among the groups (P<0.05). Furthermore, there was a significant increase in the AST levels and a significant decrease in the BUN levels at 24 h compared with 12 h in the H and K groups (P<0.05); however, the difference in group L was not significant. There was no significant difference in CK, LDH and CK-MB concentrations at 24 h compared with 12 h in the other groups (P>0.05) (Figs. 5, 6 and 7). The results demonstrated that the blood levels of ALT, AST, BUN, CK-MB, CK and LDH all increased in the SEPS1 siRNA group following silencing of the SEPS1 gene (Tables II and III).

SEPS1 silencing in mice with sepsis increases TNF-α and IL-6 levels. To investigate the proinflammatory cytokine levels in mice with sepsis, the effect of SEPS1 siRNA on the circulating levels of primary (TNF-α) and secondary (IL-6) cytokines was examined. At 12 h following LPS injection, IL-6 and TNF-α levels were highest in group K and lowest in group H. However, neither IL-6 nor TNF-α levels exhibited a statistical difference among the three groups (P>0.05). At 24 h, following LPS injection, IL-6 and TNF-α levels were highest in group L and lowest in group K. There was a significant difference in IL-6 levels between all groups except for the difference between groups H and K, whereas no significant difference in TNF-α levels was present between the groups except for a statistically significant difference between groups K and L. There was a significant decrease in the IL-6 and TNF-α levels at 24 h compared with 12 h in the K group (P<0.05). However, there was no significant difference in IL-6 and TNF-α levels at 24 h compared with 12 h in the other groups (P>0.05) (Fig. 8).

SEPS1 silencing in mice with sepsis increases p38 MAPK phosphorylation. MAPKs are involved in signal transduction pathways leading to the regulation of inflammatory mediators. In the present study, the SEPS1 siRNA group enhanced p-p38 protein levels at 12 and 24 h following LPS stimulation (Fig. 9), suggesting that SEPS1 siRNA treatment activated the phosphorylation of p38 MAPK.
**Sepsis in SEPS1-silenced mice causes tissue damage.** Pathological results demonstrated that the liver and lung cells swelled significantly and the inflammatory cells infiltrated the portal area, indicating that cell lesion markedly occurred in the SEPS1 siRNA group compared with the control group, which may correlate with the decrease in SEPS1 gene expression (Figs. 10 and 11).

**Discussion**

SEPS1 is a newly identified member of the selenoprotein family that contains enzymes, including thioredoxin reductase and glutathione peroxidase (13). The human gene SEPS1 is located on chromosome 15q26.3, consists of six exons and encodes a 189-amino acid protein. This region of chromosome 15 was previously suggested to contain quantitative trait loci that influence inflammatory disorders, including insulin-dependent diabetes mellitus, Alzheimer's disease and celiac disease (14-17). It has been observed that genetic variation in the SEPS1 gene correlated strongly with circulating levels of pro-inflammatory cytokines in human populations and that SEPS1 may regulate cytokine production in cultured macrophage cells (11,18). It has also been reported that cytokines have a direct impact on SEPS1 levels (19). Thus, there appears to be a regulatory loop whereby cytokines stimulate the expression of SEPS1, which in turn suppresses cytokine production. In order to clarify the importance of SEPS1 in multiple organ dysfunction in mice with sepsis, siRNA was used to inhibit SEPS1 gene expression and observe the impact on organ function and prognosis.

RNA interference (RNAi) is an RNA-dependent gene silencing process controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in the cell cytoplasm, where they interact with the catalytic RISC component, argonaute. RNAi is widely used in the study of gene function and biological genetic improvement. Synthetic double-stranded (ds) siRNA in mammalian cells exerted a target gene-silencing effect. siRNA is favored as a gene therapy drug due to its specific efficiency in addition to its enormous potential. Although it is difficult to introduce long dsRNA strands into mammalian cells due to the interferon response, the use of siRNA mimics has been more successful (20). In the present study, siRNAs for SEPS1 and a scrambled control were synthesized. In order to examine whether the SEPS1 gene was silenced, SEPS1 gene and protein expression were assessed.
results demonstrated that SEPS1 protein expression in liver tissue decreased 12 and 24 h following injection with SEPS1 siRNA. SEPS1 gene expression in liver tissue also decreased significantly. These results demonstrated that the SEPS1 gene was silenced.

Sepsis is defined as a systemic inflammatory response to a microbial infection that results from excessive stimulation of the host immune system by pathogen components, producing various proinflammatory cytokines. The overproduction of these cytokines then causes systemic inflammation that may lead to the lethal multiple organ damage (21). In the present study, the influence of important organ function and prognosis in mice with sepsis was investigated following silencing of the SEPS1 gene with SEPS1 siRNA. Several biochemical parameters were selected in order to reflect the important organ condition. The results demonstrated that the levels of ALT, AST, BUN, CK-MB, CK and LDH all increased in the SEPS1 siRNA group following SEPS1 siRNA silencing of the SEPS1 gene, indicating that damage to the liver, kidney and heart was aggravated. Thus, SEPS1 has an important role in protecting the liver, kidney and heart from damage due to sepsis.

In order to investigate the protective role of SEPS1 in mice with sepsis, the effect of SEPS1 siRNA on circulating levels of primary (TNF-α) and secondary (IL-6) proinflammatory cytokines was assessed as an index of inflammation (22-24). The TNF-α and IL-6 levels in the SEPS1 siRNA group significantly increased compared with the control group. SEPS1 siRNA increased the levels of TNF-α in liver tissue and may have subsequently aggravated liver dysfunction.

The MAPK signaling pathways consist of a series of kinases that are sequentially activated and consequently phosphorylate
the downstream kinases and transduce extracellular stimuli into intracellular responses. The MAPK family includes extracellular signal-regulated kinases, c-Jun N-terminal kinases and p38 MAPK. One of the major functions of MAPK is the activation of transcription factors, several of which bind to the promoters of pro-inflammatory cytokines (25,26). Furthermore, the MAPKs have been previously implicated in the signaling pathways relevant to LPS-induced inflammation. LPS activates all three MAPK type kinases in mouse macrophages (27). p38 is activated by LPS stimulation and has been theorized to have an important function in the control of IL-6 and TNF-α gene expression. Numerous downstream targets of the p38 MAPK pathway are transcription factors which regulate transcription of proinflammatory mediators. In the present study, the SEPS1 siRNA group enhanced the phospho-p38 expression at 12 and 24 h following LPS stimulation, suggesting that SEPS1 siRNA treatment increased the phosphorylation of p38 MAPK. From this it may be inferred that an increase in the levels of proinflammatory cytokines is due to the activation of the p38 MAPK pathway.

The pathological results demonstrated that liver and lung cell lesions markedly occurred in the SEPS1 siRNA group as compared with the control group, which may correlate with the decrease in SEPS1 gene expression. Therefore, it may be inferred that the SEPS1 gene exhibited a protective effect on the livers and lungs of mice with sepsis.

These data suggest that SEPS1 may have an important role in regulating processes associated with sepsis, particularly in protecting important organs from damage and decreasing the production of inflammatory cytokines. Increasing SEPS1 gene expression may be a new way to reduce sepsis, liver and other organ damage.

Acknowledgements

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