Ulinastatin reduces urinary sepsis-related inflammation by upregulating IL-10 and downregulating TNF-α levels

XIAN CHEN1*, YI WANG1*, HONGMEI LUO2, ZHIGANG LUO1, LISHA LIU1, WUJUN XU1, TAO ZHANG1, NING YANG3, XIANGYANG LONG1, NENG ZHU1, HUANG XIE1 and JUN LIU1

1Department of Urology, The Second Affiliated Hospital of University of South China; 2Department of Histology and Embryology, University of South China, Hengyang, Hunan 421001, P.R. China

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Abstract. The aim of the present study was to determine the efficacy of ulinastatin (UTI) for the treatment of sepsis and to investigate the associated molecular mechanisms. Twenty-four male rabbits were randomly divided into 4 groups, the normal, sham, sepsis model and UTI groups, each containing 6 rabbits. Serum levels of interleukin (IL)-10 and tumor necrosis factor-α (TNF-α) were measured by enzyme-linked immunosorbent assay (ELISA). Liver, kidney and lung tissues were stained with hematoxylin and eosin (H&E) 36 h after sacrifice and morphological changes were observed under an optical microscope. The expression levels of IL-10 and TNF-α proteins in rabbit kidney tissue in each group were determined by immunohistochemical detection and western blot analysis. ELISA results indicated that, compared with the sepsis model, IL-10 levels were significantly higher in the UTI treatment group (31.637±2.770 pg/ml) at 36 h (P=0.000), while serum TNF-α concentration decreased significantly in the UTI treatment group (31.637±2.770 pg/ml; P=0.000). Results of western blot analysis were consistent with the immunohistochemistry, indicating that UTI upregulates IL-10 and downregulates TNF-α levels. In the current study, UTI was demonstrated to effectively treat urinary sepsis and alleviate the inflammatory response in tissues. These effects were mediated by the upregulation of IL-10 and downregulation of TNF-α levels.

Introduction

Urosepsis is a specific form of urinary tract infection, resulting in serious systemic infection by hematogenous spread. Specifically, 20-30% of patients with sepsis develop the condition as a result of a urinary tract infection (1), and urinary tract infection accounts for 5-7% of severe cases of sepsis (2,3). Clinically, acute upper urinary tract obstruction is common and is likely to cause septic shock (4). Therefore, early diagnosis and effective treatment of urinary sepsis is essential for the prevention of mortality.

Tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-10 are cytokines involved in the inflammatory response of sepsis. During the inflammatory response, TNF-α is initially released, regulating IL-6 and IL-8 levels. IL-10 is an important anti-inflammatory and immune inhibitory cytokine secreted by macrophages, which inhibits TNF-α and increases the IL-1 receptor antagonist (5).

Ulinastatin (UTI) is a 143-amino acid, acidic glycoprotein secreted by the liver. UTI inhibits trypsin and is commonly used in the treatment of pancreatitis. In addition, UTI stabilizes lysosomal membranes and inhibits lysosomal enzyme release and myocardial depressant factor production. UTI also inhibits neutrophil activation and transendothelial migration, reduces inflammatory cell infiltration and downregulates inflammatory cytokines and is currently used for the treatment of acute circulatory failure. Early administration of UTI has been demonstrated to inhibit neutrophil protease release and excessive inflammatory responses and reduce the release of oxygen free radicals and consumption of superoxide dismutase (6,7). UTI effectively reduces body temperature, respiratory rate, white blood cell count (WBC) and regulates levels of TNF-α, plasma IL-6, C-reactive protein (CRP) and procalcitonin in patients with sepsis (8).

In our previous study, a non-cytotoxic antitumor reagent was demonstrated to downregulate the expression of Survivin, Bcl-xL and Mta-1 in tumor cells (9) and upregulate expression of Smad-4 (10). In the present study, urinary tract obstruction was performed in rats and the urinary tract was infected with Escherichia coli endotoxin (LPS) to establish a model of sepsis. Successfully established models were used to determine whether UTI induces changes in IL-10 and TNF-α expression. In addition, the efficacy and the associated molecular mechanisms of UTI in the treatment of sepsis were investigated.

Materials and methods

Reagents. LPS (0111:B4) was purchased from Sigma-Aldrich (St. Louis, MO, USA). UTI was purchased from Techpool
Bio-Pharma Co., Ltd. (Guangdong, China). TNF-α and IL-10 kits were purchased from Shanghai Shengke Co. (Shanghai, China). TNF-α and IL-10 antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Rabbit SP-HRP and DAB color kits were purchased from Beijing cwbio tech Co., Ltd. (Beijing, China).

Animal models. Twenty-four rabbits (weight, 1.80-2.20 kg) were purchased from the Department of Animal Experiments, Nanhua University (Hengyang, China). The study was approved by the ethics committee of the University of South China, Hengyang, Hunan, China. Rabbits were randomly divided into 4 groups: the normal, sham, sepsis model and UTI groups. In the normal, sham and UTI groups, rabbits were fed normally. Rabbits were anesthetized with 10% chloral hydrate (3 ml/kg) after being weighed, and were then fixed to the operating table. The abdominal cavity was exposed using a 3-cm longitudinal incision and the left psoas muscle was located. The left ureter was then separated. The abdominal cavity was closed and the intestines were reset. In the sepsis model and sham groups, the left ureter was found and separated using the same surgical approach. The lower end of the ureter was ligated and 1 ml LPS (800 µg/kg) was injected. The ureter was again ligated above the injection point. Following local irrigation, the abdominal cavity was closed. In the UTI group, the surgical approach was identical to that used in the sepsis model group. Following surgery, 1.5x10^7 units UTI were dissolved in 3 ml saline and injected into the marginal ear vein. Following surgery, rabbits from all groups were fed and watered normally.

After 24 h, 4-5 ml blood from the marginal ear vein of surviving rabbits in the sham and sepsis model groups was extracted for subsequent analyses. At 36 h, 2-3 ml blood from the left iliac vein of surviving rabbits was removed for routine blood testing. Following centrifugation (1409 x g) of the venous blood samples, 5-8 ml supernatant was collected and further detection of IL-10 and TNF-α was performed. Rabbits were sacrificed following blood sample extraction. The left kidney, the lungs and the liver were placed in 10% neutral formalin for 24 h and embedded in paraffin. CRP was detected using an ELISA for each well.

Western blot analysis. Total proteins were isolated from tissues, separated on SDS-PAGE gels and transferred to membranes. Membranes were incubated with primary antibodies against IL-10 (1:200), TNF-α (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), β-actin (1:1,000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Next, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (sc-2030; Santa Cruz Biotechnology Inc.), visualized using an enhanced chemiluminescence detection kit (Pierce Biotechnology, Inc., Rockford, IL, USA) and exposed to X-ray film. β-actin was used as a loading control.

Statistical analysis. Data were processed using SPSS 13.0 statistical software. Data are expressed as the mean ± SD. One-way ANOVA was applied for comparison between groups. A two-sample paired t-test analysis method was applied for comparison at 24 and 36 h following surgery. P<0.05 was considered to indicate a statistically significant difference.

Results

Body temperature and respiratory rate of rabbits following surgery. To establish the rabbit model, 24 rabbits were randomly divided into 4 groups, the normal, sham, sepsis model and UTI groups, each containing 6 rabbits. As demonstrated in Table I, at 24 and 36 h postoperatively, body temperature was not found to be significantly different compared with normal rabbits. Respiratory rates between the sham and normal groups at 24 and 36 h following surgery were not identified as significantly different (P=0.731 and P=0.683). The body temperature of sepsis model rabbits began to rise 24 h after establishment of the model. At 36 h, marked changes in body temperature were observed, increasing from 38.6 to 41.0°C (P=0.00), and the respiratory rate increased from 46.5 to 77.5 bpm (P=0.00). Body temperature decreased 36 h after surgery when UTI treatment was administered. When comparing the UTI and model groups, the difference was found to be statistically significant (P=0.010). Respiratory

<table>
<thead>
<tr>
<th>Group</th>
<th>Body temperature (°C)</th>
<th>Respiratory rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>38.6±0.4</td>
<td>46.5±4.3</td>
</tr>
<tr>
<td>Sham</td>
<td>38.6±0.4</td>
<td>45.5±4.2</td>
</tr>
<tr>
<td>Sepsis model</td>
<td>41.0±0.7</td>
<td>77.5±5.1</td>
</tr>
<tr>
<td>UTI</td>
<td>40.0±0.3</td>
<td>72.0±2.8</td>
</tr>
</tbody>
</table>

*P<0.05 vs. normal; †P<0.05 vs. sham; ‡P<0.05 vs. sepsis model. UTI, ulinastatin.

Hematoxylin and eosin (H&E) staining. Tissues were dried in an incubator and dewaxed for 10 min using xylene. Next, samples were dewaxed again with fresh xylene for 5 min. Following H&E staining, samples were observed under a light microscope and images were captured.

Immunohistochemical method. Rabbit kidney tissue specimens were embedded and sliced in a paraffin block to a thickness of 4 mm, according to the manufacturer’s instructions. Tissues were treated with diethylpyrocarbonate (DEPC). The secondary antibody was biotin-labeled goat anti-rabbit secondary antibody solution.

Enzyme-linked immunosorbent assay (ELISA). Blood was coagulated and centrifuged at room temperature for 10-20 min. The supernatant was collected and standards were diluted as the control. Diluted samples (50 µl) were added to blank wells and 50 µl diluted standard was added to standard wells. In the sample wells, 50 µl diluted standard and 10 µl sample was added. The sample was gently mixed and incubated for 30 min at 37°C. OD values at a wavelength of 450 nm were determined by ELISA for each well.

Table I. Comparison of body temperature and respiratory rate in 4 groups of rabbits at 36 h postoperatively (mean ± SD, n=6).

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>Body temperature (°C)</th>
<th>Respiratory rate (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>38.6±0.4</td>
<td>46.5±4.3</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>38.6±0.4</td>
<td>45.5±4.2</td>
</tr>
<tr>
<td>Sepsis model</td>
<td>6</td>
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<td>UTI</td>
<td>6</td>
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<td>72.0±2.8</td>
</tr>
</tbody>
</table>

*P<0.05 vs. normal; †P<0.05 vs. sham; ‡P<0.05 vs. sepsis model. UTI, ulinastatin.

Rockford, IL, USA) and exposed to X-ray film. β-actin was used as a loading control.
Peripheral blood cell count and CRP changes. As demonstrated in Table II, at 24 and 36 h postoperatively, when compared with normal group, WBC was slightly elevated in the sham group but was not identified to be significantly different (P=0.099 and P=0.062, respectively; P>0.05). WBC was observed to increase in a time-dependent manner. At 24 and 36 h after modeling, the WBC in the model group was 12.66x10^9 and 15.35x10^9 cells/l, respectively. In the sham group, the WBC 36 h following surgery was identified to be significantly higher than the normal group (P=0.00). WBC decreased in the treatment group compared with the sepsis model group (P=0.008). No significant difference in platelet count was identified between the groups (P=0.686 and P=0.805, respectively). Serum CRP concentration 36 h following surgery was found to be significantly higher in the sham group than the normal group (P=0.001). CRP levels in the model group were observed to be significantly higher compared with the sham and normal groups (P=0.000). CRP levels in the treatment and sepsis model groups were identified to be significantly different, whereby serum CRP concentration was higher in the sepsis model group (P=0.000).

**Table II. Comparison of WBC, PLTs and CRP in 4 groups of rabbits at 36 h postoperatively (mean ± SD, n=6).**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>WBC (10^9 cells/l)</th>
<th>PLT (10^9 cells/l)</th>
<th>CRP (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>6</td>
<td>5.01±1.61</td>
<td>471.83±94.07</td>
<td>1.93±1.26</td>
</tr>
<tr>
<td>Sham</td>
<td>6</td>
<td>7.06±2.68</td>
<td>435.17±63.82</td>
<td>12.27±3.13a</td>
</tr>
<tr>
<td>Sepsis model</td>
<td>6</td>
<td>15.35±1.31ab</td>
<td>451.83±51.29</td>
<td>30.03±7.65ab</td>
</tr>
<tr>
<td>UTI</td>
<td>6</td>
<td>12.29±1.22c</td>
<td>431.50±92.31</td>
<td>19.22±3.19c</td>
</tr>
</tbody>
</table>

*P<0.05 vs. normal; *P<0.05 vs. sham; *P<0.05 vs. sepsis model. WBC, white blood cell count; PLTs, platelets; CRP, C-reactive protein; UTI, ulinastatin.

**Figure 1.** H&E staining of rabbit kidney tissues and immunohistochemical detection of IL-10 and TNF-α in rabbit kidney tissues. (A) H&E staining of rabbit kidney tissues in the normal, sham, sepsis model and UTI groups. (B) Immunohistochemical analysis of levels of IL-10 and TNF-α in rabbit kidney tissues from the 4 groups. Magnification, x400. H&E, hematoxylin and eosin; IL, interleukin; TNF-α, tumor necrosis factor-α; UTI, ulinastatin.
observed to decrease significantly in the UTI treatment group (31.637±2.770 ng/l; P=0.000). These results indicate that UTI treatment increased levels of IL-10, but deceased levels of TNF-α.

H&E staining of rabbit kidney, liver and lung tissues. To examine the effect of UTI treatment on rabbit tissue, H&E staining was performed in all 4 groups. As demonstrated in Fig. 1A, kidney tissues obtained from normal and sham groups exhibited normal morphologies. In the sepsis model group, glomerulus deformities and tubular lumen enlargement were observed. The renal interstitial space was congested with edema and infiltrated with inflammatory cells. There was slight swelling of liver cells and scattered inflammatory cell infiltration in liver biopsies. In lung biopsies, we observed diffuse thickening of the alveolar septa and inflammatory cell infiltration. Pathological deformities were attenuated in the UTI treatment group compared with the sepsis model group. Similar results were also observed in the liver and lung tissues (data not shown).

Immunohistochemical detection of IL-10 and TNF-α in kidney tissues of each group. To detect whether UTI induces alterations in levels of IL-10 and TNF-α, immunohistochemical analysis of rabbit kidney tissues was performed. Images are presented in Fig. 1B. Levels were calculated based on immunohistochemistry detection. As demonstrated in Table IV, differences in the expression of IL-10 and TNF-α between the normal and sham groups were not significant. IL-10 protein levels in the UTI treatment group were higher than in the sepsis model group. However, levels of TNF-α in the UTI treatment group were lower than those in the sepsis model group.

Western blot analysis of IL-10 and TNF-α in kidney tissue of each group. To further determine whether UTI induces alterations in the levels of IL-10 and TNF-α, total proteins were extracted from rabbit kidney tissues in every group and western blot analysis was performed. As demonstrated in Fig. 2, IL-10 and TNF-α protein levels were quantified by western blot analysis. **P<0.01, vs. the control group; ##P<0.01 vs. the sepsis group.
levels in the normal and sham groups were similar. In the sepsis group, IL-10 levels were increased. However, IL-10 levels in the UTI treatment group were found to be significantly increased compared with the sepsis group.

As revealed in Fig. 3, TNF-α levels in the normal and sham groups were similar. In the sepsis group, TNF-α levels were increased compared with the control group. However, TNF-α levels in the UTI treatment group were significantly decreased compared with the sepsis group. Results of western blot analysis were consistent with the immunohistochemistry results, indicating that UTI upregulates IL-10 levels and down-regulates TNF-α levels.

Discussion

Animal models provide a basis for pathogenesis studies of the mechanisms of sepsis and are essential for the development, prevention and control of this condition. Models of sepsis simulate the etiology, pathogenesis, development and clinical characteristics of sepsis to provide insight into methods for its prevention and treatment. Several sepsis models have been established and are each associated with specific characteristics (5). In the present study, a model of urinary tract infection and acute upper urinary tract obstruction was established by injection of LPS and ligation of the ureter to induce acute obstruction and a model of urinary sepsis.

In this study, no significant differences in rabbit rectal temperatures, respiratory rates, peripheral blood leukocytes and serum TNF-α and IL-10 levels were identified in the sham and normal groups at each time-point. These observations indicate that the surgery itself did not affect organ function. In the sepsis model group, body temperature began to rise at 24 h, respiratory rate accelerated in a time-dependent manner, TNF-α and IL-10 levels were observed to be significantly increased, rectal temperature increased from 38.6 to 41.0°C and peripheral blood leukocytes (15.35×10^9/l) and CRP (31.03±8.06 mg/l) increased significantly compared with the control group. In addition, liver, kidney and lung morphologies were altered, indicating that the urinary sepsis model was established successfully.

Inflammatory cytokines, including TNF-α, IL-6 and IL-10, were previously revealed to cause sepsis-related multiple organ dysfunction syndrome (MODS) (11-14). TNF-α is largely generated by activated monocytes/macrophages and endothelial cells, which induce systemic inflammatory response syndrome/MODS. IL-6 is a major cytokine that is activated by monocytes, macrophages and endothelial cells in the acute response phase and is induced by IL-1β and TNF-α. IL-6 is a hepatocyte-stimulating factor that induces liver cells to produce acute CRP and enhances the destructive inflammatory response of the host. Continually increasing IL-6 levels indicate poor prognosis (15). IL-10 is an important cytokine produced by T cells, macrophages and monocytes. It inhibits mRNA transcription for a variety of immune active cytokines and the activation of monocytes and macrophages to secrete other cytokines, including TNF-α and IL-1, which regulates damage by the excessive inflammatory response triggered by proinflammatory cytokines (16). IL-10 is a protective and anti-inflammatory cytokine in sepsis and MODS that reduces the systemic inflammatory response and organ damage (17). CRP is an extremely sensitive and nonspecific indicator of sepsis diagnosis, which is an active protein in the acute phase of an infectious disease and is significantly elevated in inflammation and tissue damage. Increasing levels of CRP are closely associated with the severity of tissue damage.

Molor-Erdene et al (18) demonstrated that UTI inhibited TNF-α induced by LPS and significantly reduced TNF-α expression in a dose-dependent manner in rat lung tissue. UTI reduces TNF-α and IL-6 and improves IL-10 levels to prevent further MODS (19). In the current study, compared with model rabbits, TNF-α levels were decreased and IL-10 levels were increased in the control group, as demonstrated by western blot analysis and immunohistochemistry. CRP was decreased significantly in the treatment group, consistent with previous studies (8,19). These observations indicate that UTI may represent an important agent for the prevention and treatment of urinary sepsis by inhibiting synthesis and release of TNF-α and upregulating IL-10. UTI was found to balance the internal environmental and reduce tissue damage.

The current study indicates that UTI may represent a novel therapy for the prevention and treatment of urinary sepsis and suggests that the mechanism via which UTI exerts its effects may involve the upregulation of IL-10 and downregulation of TNF-α levels.

References


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