ISOLATION OF BONA FIDE DIFFERENTIALLY EXPRESSED GENES IN THE 18-HOUR SEPSIS LIVER BY SUPPRESSION SUBTRACTIVE HYBRIDIZATION

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ABSTRACT—In late sepsis, it has been established that the liver plays a major role in the initiation of multiorgan failure, which is the most lethal complication in hospitals. The molecular mechanism underlying liver failure that results from sepsis remains elusive. This study was undertaken to identify the bona fide differentially expressed genes in the 18-h septic liver by suppression subtractive hybridization, and the data were corroborated by Northern blot analysis. The differential gene expression profile renders a clue as to the genes involved in septic liver failure. The cecal ligation and puncture (CLP) model of a polymicrobial septic rat was used, with the late sepsis referring to animals sacrificed at 18 h after CLP. We have identified three upregulated genes (TII-kininogen, serine protease inhibitor 2.2 [Sp2.2], and α2 macroglobulin [α2M]) and six down-regulated genes (hydroxysteroid dehydrogenase [3αHSD], EST189895/mouse RNase4, bile acid-CoA-amino acid N-acyltransferase [kan-1/rBAT], IF1, albumin, and α2u-globulins [α2u-G PGCL1]). Among these genes, the 3αHSD and kan-1/rBAT are involved in bile acid metabolism. The IF1 plays a crucial role in any disease that involves ATP hydrolysis by F, F0-ATPase. The α2M, TII-kininogen, and Sp2.2 are protease inhibitors. The functions of the α2u-G PGCL1 and EST189895/mouse RNase4 genes are unknown. The present results suggest that the roles of disturbance of bile acid metabolism/synthesis and the abolishment of ATP production may contribute to liver failure during late sepsis.

KEYWORDS—Septic shock, gene expression profile, ATP production, bile acid metabolism/synthesis, septic liver failure

INTRODUCTION

Sepsis remains a common cause of death in intensive care units despite antibiotic therapy. Even with advances in supportive care, severe sepsis carries a mortality rate of 30% to 50%, and the incidence is expected to increase over the next decade (1). Many approaches to studying sepsis have focused on developing the therapeutic agents targeting various mediators and pathophysiologic stages the progression of the disease. However, the clinical trials in testing the efficacy of antimediator therapeutics are inconclusive (2). The common cause of death in sepsis is multiple organ failure. The liver is thought to be the major organ responsible for the initiation of multiple organ failure during sepsis, as it plays a central role in metabolism and host defense mechanism (3). Liver failure, manifested by hyperbilirubinemia, hypoglycemia, encephalopathy, and coagulopathy, is typically considered to be a complication of late sepsis.

Wichterman et al. (4) reported that rats in early sepsis showed features associated with hyperglycemia and hyperdynamic circulation, whereas those in late sepsis were hypoglycemia and hypodynamic. In our cecal ligation and puncture (CLP) sepsis model, CLP rats show metabolic disturbances by 9 h with hyperglycemic state and followed by a hypoglycemic state at 18 h (5). These results are concordant with previous reports (6, 7). Therefore, we refer to 18-h sepsis induced by CLP as late sepsis. This study was undertaken to investigate the gene expression profile in the late sepsis liver using the 18-h CLP animal as a model. The gene expression profile may give a crucial clue that leads to understanding the molecular mechanisms of septic liver failure. This may lay the foundation for developing an effective therapeutic strategy.

MATERIALS AND METHODS

Animal sepsis model

Twelve male Sprague-Dawley rats (270-320 g) were randomly divided into the 18-h sepsis (late sepsis) and control groups. The late sepsis group was subjected to a CLP operation according to the method of Wichterman et al. (4) with slight modification. Under anesthesia, a laparotomy was performed and the cecum was ligated with a 3-0 silk ligature, punctured twice with an 18-gauge needle, and some internal contents were excreted. The control group received a sham operation (a laparotomy was performed and the cecum was manipulated, but was neither ligated nor punctured). All animals were resuscitated with 4 mL/100 g body weight of normal saline at the completion of surgery and also at 7 h postsurgery. Animals were fasted overnight with free access to water before operations. The mortality rate was approximately 50% and 90% at 18 and 24 h, respectively, after the operation. Liver tissues were collected and were snap-frozen in liquid nitrogen, and animals were sacrificed at 18 h after the operation. The experiments were carried out humanely according to the regulations of the Animal Committee of the Kaohsiung Medical University, Taiwan.

Isolation of total RNA and poly(A)+ RNA

Total RNAs were isolated from 50 mg of liver tissue individually from the late sepsis and the sham-operated animals using TRI-REAGENT (Life Technologies, Carlsbad, CA). The total RNAs were used to further isolate mRNAs using an oligo(dT)-coated latex particle mRNA isolation kit (Invitrogen, Carlsbad, CA). The experiments were conducted according to the manufacturers’ instructions.

Suppression subtractive hybridization (SSH)

To isolate the upregulated and down-regulated genes in the 18-h septic livers, SSH was used to carry out forward and reverse subtractions. In the forward subtraction, the 18-h septic liver was used as a tester and the sham-operated liver was used as a driver, whereas in the reverse subtraction, the sham-operated liver was used as...

549
In forward and reverse subtractions, two rounds of subtraction hybridization were carried out in the two adaptor-ligated targets at 68°C, overnight, with the presence of an excess of driver cDNAs. The next day, a nested PCR was performed on the subtracted cDNAs using Advantage cDNA polymerase (Clontech) with the presence of an excess of driver cDNAs. The next day, a nested PCR was performed to reconfirm the SSH results.

** Colony screening**

The nested PCR products were cloned into the pT-Adv cloning vector (Clontech). Ninety-six colonies from each of the forward and the reverse subtracted libraries were randomly picked and were inoculated with Luria Bertani broth containing 50 µg/mL ampicillin/kanamycin (Sigma, St. Louis, MO) and 15% glycerol in a 96-well plate at 37°C overnight. The 96 clones from each subtracted library were dot-lifted onto quadruplicated nylon membranes (Amer sham Pharmacia, Piscataway, NJ) using a 96-well hedg ehog (ABI, Foster City, CA). The membranes were then incubated with 0.1% milk and were then transferred to nylon membranes (Amer sham Pharmacia). The blots were hybridized with probes at 68°C overnight. The next day, the membranes were washed with 2x SSC and 0.05% SDS at room temperature, twice, for 10 min each, and then with 0.1x SSC and 0.01% SDS at 68°C, four times, for 10 min each.

**DNA sequencing and data analysis**

Plasmid DNAs were isolated from positive clones using a Qiafilter Miniprep DNA Purification kit (Quagen, Valencia, CA). The plasmid DNAs were sent for sequencing (Bolsto m Biotechnologies, Taiwan). The sequence data were analyzed by comparing with GenBank/EMBL database using the online computer BLAST program (NCBI).

**Northern blot analysis to reconfirm the SSH results**

The positive clones were further used as probes to reconfirm the bona fide differentially expressed genes (DEG) in the 18-h septic livers. Thirty micrograms of total RNA extracted from sham-operated and 18-h septic livers were subjected to electrophorosis on RNA agarose gels and were then transferred to membranes (Amer sham Pharmacia). The blots were hybridized at 68°C overnight with α-32P [dCTP]-labeled positive cDNAs isolated from the SSH analysis. The blots were then stripped and rehybridized with α-32P [dCTP]-labeled glyceraldehydes 3-phosphate dehydrogenase (GAPDH) for an internal control for the presence of similar amounts of total RNAs in each sample. This experiment was repeated six times. Autoradi ographs were scanned and the relative densities were quantified by Bio-1D V.97 Software program (Vil ber Lourmat, France). The value of relative density is the density of the septic group divided by the density of sham-operated group.

** Antibody production**

The polyclonal antibody of 3αHSD was induced from male New Zealand rabbits by injecting rabbits with a commercial 3αHSD protein (Sigma), according to a previous method (8) with modifications. The kan-1/rBAT antibody was induced using a synthesized peptide (Merck, West Point, PA), Leu-Thr-Arg-Leu-Val-Lys-Arg-Asp-Val-Met-Asn-Arg-Pro-His-Lys, which was designed to correspond to the 88 to 102-amino acid region of kan-1/rBAT. Male New Zealand rabbits (2.5 kg) were immunized with a priming dose 1 mg/mL antigen (i.p.). The antigen was emulsified 1:1 (v/v) with Freund’s complete adjuvant (Sigma) for the priming injection and Freund’s incomplete adjuvant for subsequent boosts. A boost of protein (1 mg) is usually performed on day 14, with subsequent boosts on days 28 and 42.

** Enzyme-linked immunosorbent assay (ELISA)**

The titers of the antibodies were measured by ELISA every week after induction. First, each well of ELISA plate (Corning Inc., Corning, NY) was coated with 10 ng/mL 3αHSD and kan-1/rBAT antigen overnight. The plates were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories, Lexington, KY) for 1 h. The plates were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories, Lexington, KY) for 1 h. The plates were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories, Lexington, KY) for 1 h. The plates were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories, Lexington, KY) for 1 h. The plates were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories, Lexington, KY) for 1 h. The plates were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories, Lexington, KY) for 1 h. The plates were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories, Lexington, KY) for 1 h.

Equal proteins (20 µg) were separated by SDS-PAGE using a 12% running gel. The antibodies were transferred onto polyvinylidene difluoride (NEN Life Science Products) transfer membranes by electroblotting for 90 min (120 v). The antibodies were stripped and rehybridized with antibodies immunized for 6 weeks were used for Western blot analysis with dilution factors (1:3000 for 3αHSD and 1:1000 for kan-1/rBAT).

**Western blot analysis**

Equal proteins (20 µg) were separated by SDS-PAGE using a 12% running gel. The antibodies were transferred onto polyvinylidene difluoride (NEN Life Science Products) transfer membranes by electroblotting for 90 min (120 v). The membranes were incubated with the anti-3αHSD and anti-kan-1/rBAT antibodies for 1 h at room temperature. The membranes were washed and then incubated with goat anti-rabbit horseradish peroxidase antibody (Transduction Laboratories) for 1 h. The membranes were washed and then incubated with the enhanced chemiluminescence (NEN Life Science Products) detection solution.

**Statistics**

Data are represented as mean ± SE. The data were analyzed using the one-tailed Student’s t test.
FIG. 1. The comparison of expression levels of the down-regulated genes between the sham-operated rats and at 18 h after CLP by Northern blot analysis. The mRNA levels of the six down-regulated genes were quantified by Northern blot analysis. The autoradiography of the Northern blot analysis is shown in the right panel and the expression levels of the genes presented as the relative density is shown in the left panel. (A) 3αHSD. (B) kan-1/rBAT. (C) EST189895/mouse RNase4. (D) IF1. (E) α2u-G PGCL1. (F) albumin. GAPDH was used as an internal control. The data are shown as mean ± SE of six samples in each group. **P < 0.01. “-”, down-regulation at 18 h after CLP.
subtraction control comprised the skeletal muscle cDNA (as a driver) and the mixture cDNAs between the skeletal muscle cDNA and the bacteriophage \( \Phi x174/Hae \) III cDNA (as a tester). The specific fragments from the bacteriophage \( \Phi x174/Hae \) III cDNA were enriched and highly amplified in the SSH assay (data not shown). It indicated that the SSH assay in this study was successful.

Eight down-regulated genes and six upregulated genes in the liver obtained from 18-h sepsis after CLP were isolated by SSH. Through BLAST research, the 14 cDNA clones have shown more than 97% homology to the partial sequences of the cloned rat genes (Table 1). The disparity in sequences in seven clones (not 100% identical) might be attributed to the sequencing inaccuracy or gene polymorphism.

**Northern blot analysis**

Northern blot analysis was used to quantify the DEG cloned by colony screening in SSH. Of the eight down-regulated genes, the six predicted genes encoding 3\( \alpha \)HSD, kan-1/rBAT, EST189895/mouse RNase4, I\( F \), \( \alpha 2\mu \)-G PGCL1, and albumin in the liver derived from 18 h after CLP were expressed 42%, 45%, 38%, 37%, 29%, and 30% less than those in the sham-operated liver, respectively (Fig. 1). Of the six upregulated genes, the three predicted genes encoding TII-kininogen, \( \alpha 2M \), and Spi2.2 in the liver derived from 18 h after CLP were expressed 103%, 582%, and 57% greater than those in the sham-operated liver, respectively (Fig. 2). The other two down-regulated genes and three upregulated genes were quantified and showed no changes in expression levels by Northern blot analysis (data not shown).

**DISCUSSION**

Given the high mortality rate caused by sepsis in intensive care units, some laboratories have endeavored to identify gene expression profiles in multiple organs in sepsis by microarray (9, 10). The major limitations of microarray technology are the relative fidelity of target/probe hybridization, confining in known genes, and the robustness of signal analysis (11). In this study, we used the PCR-based SSH combined with the cDNA library screening technique and corroborated by Northern blot analysis to isolate the *bona fide* DEG in the 18-h septic livers.
The PCR-based SSH is well established as a sensitive method that can isolate rare, abundant, and novel genes. In this study, nine DEG were isolated. Five of the nine genes encoding 3αHSD, EST18985/mouse RNase4, IF1, α2u-G PGCL1, and TII-kininogen have not previously been reported to be linked to sepsis. Of the five genes, the EST18985 clone is a novel rat homolog of the RNase4 gene. We also found two DEG encoding acute-phase reactant α2-macroglobulin and serine protease inhibitor, which are accordant with the results found in a previous study (10). The proteins encoded by the nine isolated genes in this study can be grouped into four in terms of liver functions (Table 2). The real biological functions of the nine genes in 18-h septic liver need further investigation.

In the comparison data among our results and others, surprisingly, it shows how few overlapped DEG there are, despite using a very similar CLP method for inducing sepsis (Table 3). This may be attributed to different species or strains used in the experiments. It is also of interest to know that some overlapped genes show an opposite effect in septic livers in different species. This is not a unique case. Recently, there was a vehement debate about the “stemness” genes of embryonic and adult stem cells identified by analyzing their transcriptional profiling using microarray (12, 13). More than 200 stemness genes each derived from three independent laboratories, however, showed merely one gene commonly identified in the three studies. Although microarray technology is a powerful tool for exploring unearthed secrets in science, the problem of false negatives remains. For example, we have identified seven genes that have not been found in the previous microarray studies (9, 10). Two of the seven genes, 3αHSD and kan-1/rBAT genes, showed that their gene expression levels and protein levels are concomitant in 18 h sepsis. The above indi-

TABLE 2. Possible functions of the isolated differentially expressed genes in the 18-h sepsis liver

<table>
<thead>
<tr>
<th>Genes</th>
<th>Possible functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2M</td>
<td>Restoration of homeostasis</td>
<td>14 and 15</td>
</tr>
<tr>
<td>TII-kininogen</td>
<td>APR; protease inhibitor; cytokine carrier</td>
<td>16 and 17</td>
</tr>
<tr>
<td>Spil2.2</td>
<td>APR; hepatic serine protein inhibitor</td>
<td>18 and 19</td>
</tr>
<tr>
<td>Albumin</td>
<td>Negative APR</td>
<td>20 and 21</td>
</tr>
<tr>
<td>3αHSD</td>
<td>Bile acid metabolism</td>
<td>22 and 23</td>
</tr>
<tr>
<td>Kan-1/rBAT</td>
<td>Synthesis and transportation of bile acid</td>
<td>24 and 25</td>
</tr>
<tr>
<td>IF1</td>
<td>Mitochondria ATPase inhibitor; ATP preservation</td>
<td>26 and 27</td>
</tr>
</tbody>
</table>

APR, Acute-phase reactant.
Table 3. The overlapping data in the DEG in the 6-h, 18-h, or 24-h sepsis livers induced by CLP among our study and the previous studies

<table>
<thead>
<tr>
<th>Clp method</th>
<th>Species</th>
<th>strain</th>
<th>CLP method</th>
<th>Species</th>
<th>strain</th>
<th>Way and line period to detect DEG</th>
<th>Number of detected DEG</th>
<th>ND</th>
<th>ND</th>
<th>ND</th>
<th>ND</th>
<th>ND</th>
<th>ND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microarray</td>
<td>Mouse</td>
<td>C57BL/6J</td>
<td>20-Gauge needle puncture twice and cecal content excretion</td>
<td>6 and 18 h</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>↑↑</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>↑↑</td>
<td>↓</td>
</tr>
<tr>
<td>SSH</td>
<td>Rat</td>
<td>Sprague-Dawley</td>
<td>18-Gauge needle puncture twice and cecal content excretion</td>
<td>18 h</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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</tr>
</tbody>
</table>

Selected cytokine and chemokine at 1, 3, 6, and 18 h of sepsis

- IL-1
- PAI-1/H9251
- MIP-1α/H9251
- TNF-α/H9251
- 2M, plasminogen activator inhibitor; TPA, tissue plasminogen activator.

NOTES:
- ND, Not determined; –, decreased; IL-18, hepatic interleukin-18; TNFα, tumor necrosis factor α; MIP, macrophage inflammatory protein; 2M, α2-macroglobulin; PAI, plasminogen activator inhibitor-1.
- ↑, undetected; ↓, increased.
- The overlaping data in the DEG in the 6-h, 18-h, or 24-h sepsis livers induced by CLP among our study and the previous studies.

REFERENCES


