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 [Spi2.2], 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], IF_1, albumin, and α2u-globulins [α2u-G PGCL1]). Among these genes, the 3αHSD and kan-1/rBAT are involved in bile acid metabolism. The IF_1 plays a crucial role in any disease that involves ATP hydrolysis by F_1F_0-ATPase. The α2M, TII-kininogen, and Spi2.2 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

Table 1. **BLAST research results of the differentially expressed genes in the 8-h sepsis liver by SSH**

Expression pattern	Genes	Base pairs submitted to BLAST	Genbank accession number	Homology
Down-regulated	Rat 3αHSD	651	M64393	100% (498/498)
	Rat kan-1/rBAT	432	NM017300	97% (402/414)
	EST189895/Mouse RNase 4	420	AA800398/NM021472	100% (350/350)/85% (190/219)
	Rat IF$_1$	221	NM012915	100% (198/198)
	Rat α2u-G PGCL1	277	NM147214	100% (250/250)
	Rat albumin	572	NM134326	98% (564/572)
	Rat cytochrome c oxidase subunit II	558	M27315	99% (519/520)
	Rat α-fibrinogen	327	X86561	100% (267/267)
Upregulated	Rat TII-kininogen	300	M14357	100% (234/234)
	Rat α2M	515	NM012488	99% (480/481)
	Rat Spi2.2	226	X13150	100% (190/190)
	Rat urate oxidase	520	X13098	97% (462/476)
	Rat cytochrome P-450	650	X53477	99% (603/608)
	Rat fibrinogen γ-chain	291	X05861	99% (220/222)

a tester and the 18-h septic liver was used as a driver. SSH was carried out using a PCR-select cDNA Subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Briefly, for each subtraction, 2 μg each of poly(A)$^+$ mRNAs from the livers of both groups were used to individually synthesize cDNAs using avian myeloblastosis virus reverse transcriptase with an oligo(dT) primer. The cDNAs were digested with *Rsa* I restriction enzyme. Each tester cDNA was separately ligated with adaptor 1 and 2R (supplied in kit).

In forward and reverse subtractions, two rounds of subtraction hybridization were carried out in the two adaptor-ligated testers 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 three primers contained in the adaptors. The first primer was 5′- CTAATACGACT-CACTATAGGGC-3′ and the nested primer pairs were 5′-TCGAGCGGCCGCCC-GGGCAGGT-3′ and 5′-AGCGTGGTCGCGGCCGAGGT-3′. The first round of PCR cycling parameter was 94°C for 25 sec; 94°C for 10 s, 66°C for 30 s, and 72°C for 1.5 min, for 27 cycles; and 72°C for 8 min and 4°C for 10 min. The second round of PCR was 94°C for 25 s, for 1 cycle; 94°C for 10 s, 68°C for 30 s, and 72°C for 1.5 min, for 10 cycles; and 72°C for 8 min, and 4°C for 10 min. To evaluate the efficiency of cDNA subtraction, a control subtraction experiment (skeletal muscle and *Hae* III-digested Φ×174 cDNAs) supplied with the kit was performed.

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 (Amersham Pharmacia, Piscataway, NJ) using a 96-well hedgehog (ABI, Foster City, CA). The membranes were placed on the top of Luria Bertani agar plates and were incubated at 37°C overnight. The membranes were then denatured (0.5 M NaOH and 1.5 M NaCl), neutralized (0.5 M Tris, pH 7.4, and 1.5 M NaCl), and fixed (0.4 M NaOH). The quadruplicated membranes were hybridized separately with four different α-^{32}P [dCTP]-labeled probes, which were produced from the forward- and reverse-subtracted PCR products without adaptors and nonsubtracted cDNAs from the late septic and the control groups. The membranes were hybridized with probes at 68°C overnight. The next day, the membranes were washed with 2× SSC and 0.05% SDS at room temperature, twice, for 10 min each, and then with 0.1× 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 Minipreps DNA Purification kit (Qiagen, Valencia, CA). The plasmid DNAs were sent for sequencing (Bolssom 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 was subjected to electrophoresis on RNA gels and were then transferred to nylon membranes (Amersham Pharmacia). The blots were hybridized at 68°C overnight with α-^{32}P [dCTP]-labeled positive cDNAs isolated from the SSH analysis. The blots were then stripped and rehybridized with α-^{32}P [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. Autoradiographies were scanned and the relative densities were quantified by Bio-1D V.97 Software program (Vilber 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 blocked with 5% milk for 1 h. The plates were then washed and antiserums diluted from 1:1000 down to 1:16,000 were incubated 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 thereafter the substrate solution (0.4 mg/mL o-dishPhenylenediamine [Sigma] and 0.012% H$_2$O$_2$ in 0.1 M sodium phosphate buffer, pH 6.0) was added to each well. The absorbance at 450 nm was subsequently measured to determine the optimal immunization time points and the dilution factors of the antibodies for Western blot analysis. The 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. Proteins were transferred onto polyvinylidene difluoride (NEN Life Science Products, Boston, MA) 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 then washed and 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 t test.

RESULTS

SSH and colony screening

To validate our SSH assay, we incorporated a subtraction control in SSH in parallel to our experimental groups. The

A 3αHSD

B kan-1/rBAT

C EST189895/ Mouse RNase4

D IF$_1$
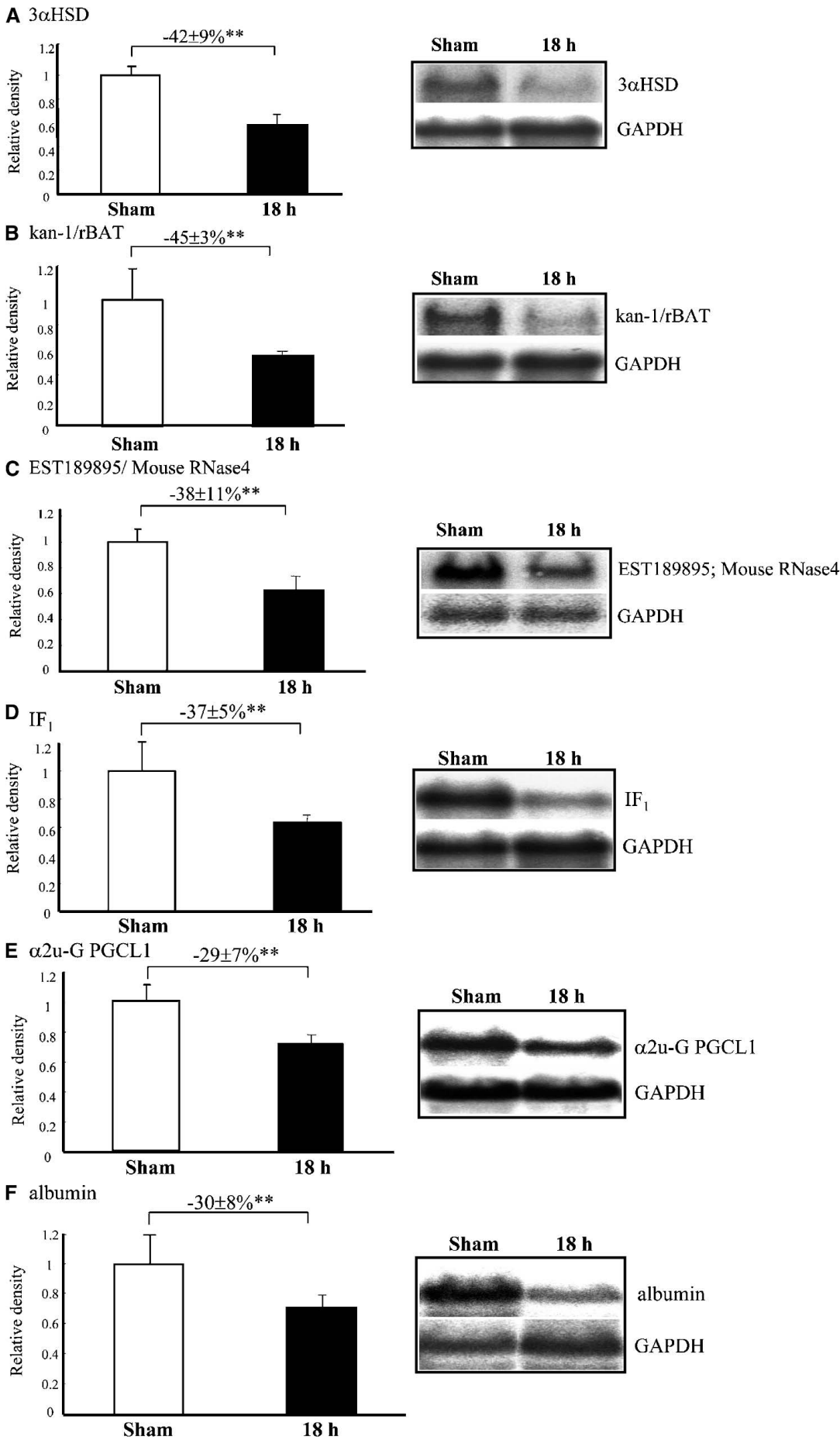

E α2u-G PGCL1

F albumin

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) IF$_1$. (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.

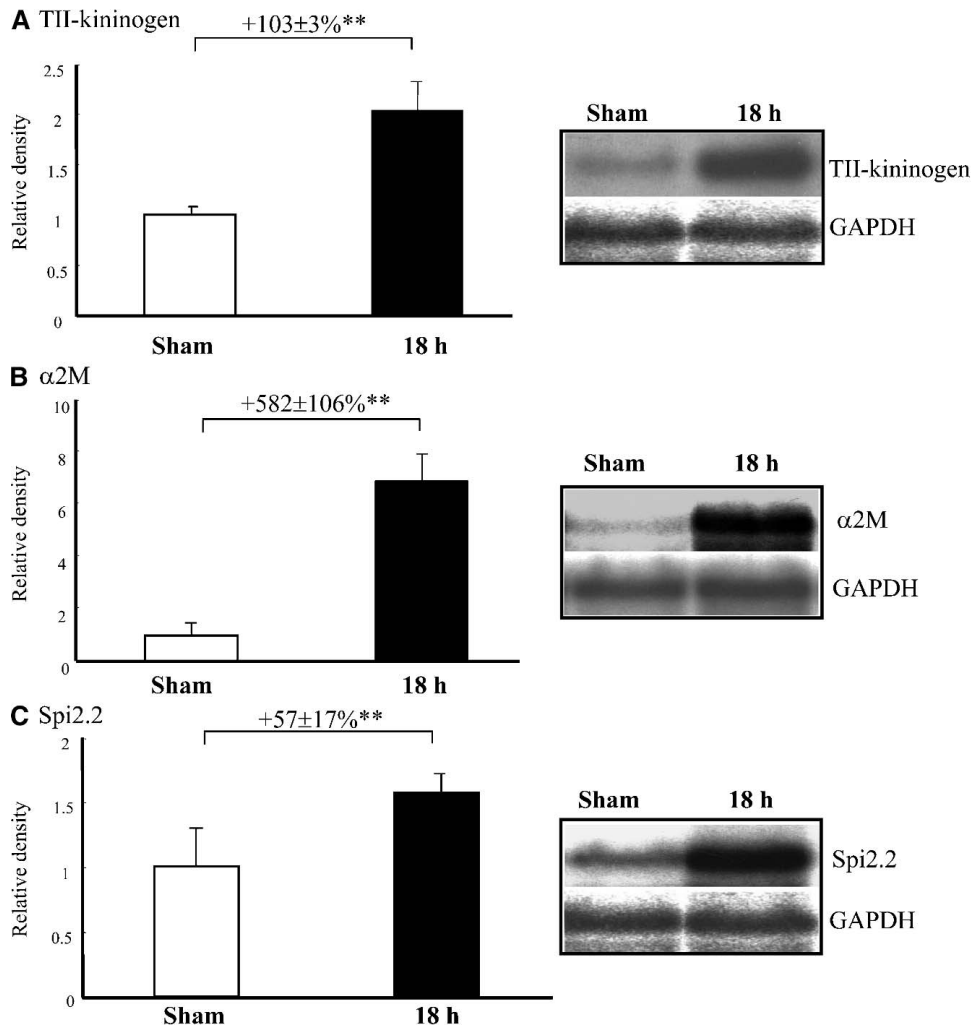**A** TII-kininogen

B α2M

C Spi2.2

Fig. 2. **The comparison of expression levels of the upregulated genes between the sham-operated rats and at 18 h after CLP 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) TII-kininogen. (B) α2M. (C) Spi2.2. GAPDH was used as an internal control. The data are shown as mean ± SE of six samples in each group. **$P < 0.01$. "+", upregulation 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 Φ×174/*Hae* III cDNA (as a tester). The specific fragments from the bacteriophage Φ×174/*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 3αHSD, kan-1/rBAT, EST189895/mouse RNase4, IF_1, α2u-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, α2M,

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).
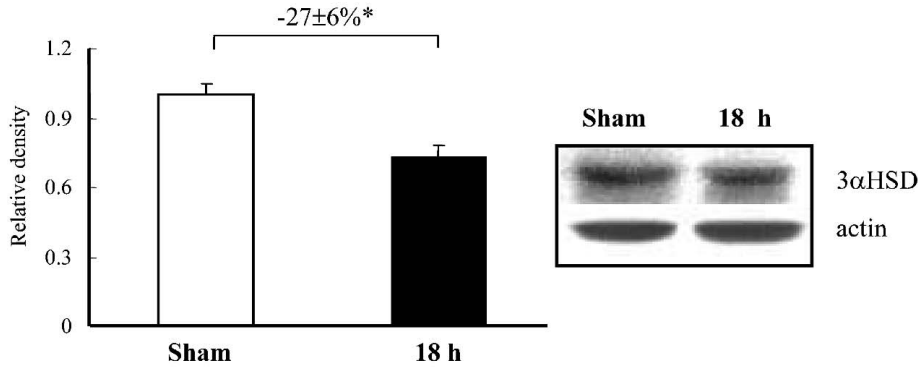
The protein expression level of 3αHSD and kan-1/rBAT by Western blot analysis

The expression levels of 3αHSD and kan-1/rBAT were decreased by 27% and 38%, respectively, in the liver at 18 h after CLP, as compared with the sham-operated livers (Fig. 3).

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.
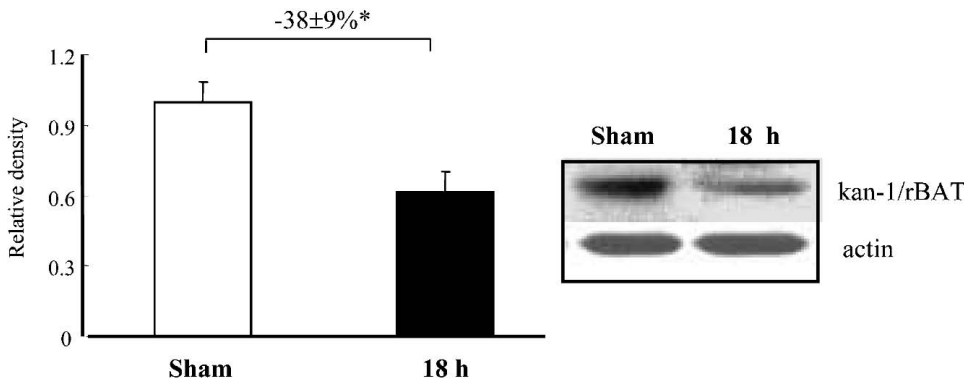
A   3αHSD

B   kan-1/rBAT

Fig. 3. **Protein expression levels of 3αHSD and kan-1/rBAT in the liver derived from sham-operated rats and at 18 h after CLP by Western blot analysis.** The autoradiography of the Western blot analysis is shown in the right panel and the expression levels of the protein presented as the relative density is shown in the left panel. (A) 3αHSD. (B) kan-1/rBAT. The molecular weight of 3αHSD and kan-1/rBAT is 34 and 46 KD, respectively. Actin was used as an internal control. The data are shown as mean ± SE of three samples in each group. $*P < 0.05$. "-", down-regulation at 18 h after CLP.

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, EST189895/mouse RNase4, IF$_1$, α2u-G PGCL1, and TII-kininogen have not previously been reported to be linked to sepsis. Of the five genes, the EST189895 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**

Genes	Possible functions	References
	Restoration of homeostasis	
α2M	APR; protease inhibitor; cytokine carrier	14 and 15
TII-kininogen	APR; cystein protease inhibitor	16 and 17
Spi2.2	APR; hepatic serine protein inhibitor	18 and 19
Albumin	Negative APR	20 and 21
	Bile acid metabolism	
3αHSD	Synthesis and transportation of bile acid	22 and 23
Kan-1/rBAT	Synthesis of bile acid	24 and 25
	ATP production	
IF1	Mitochondria ATPase inhibitor; ATP preservation	26 and 27

APR, Acute-phase reactant.

Table 3. **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**

Studies	Species strain	CLP method	Way and time period to detect DEG	Number of detected DEG	Overlapped DEG						
					IL-1β 6 and 18 h	TNFα 6 h	MIP-1α 6 h	α2M 18 h	Spi2.2 18 h	PAI 24 h	TPA 24 h
Salkowski et al. (28)	Mouse (C57BL/6J)	20-Gauge needle puncture twice and cecal content excretion	Selected cytokine and chemokine at 1, 3, 6, and 18 h of sepsis	18	↑	↑	↑	ND	ND	ND	ND
Chinnaiyan et al. (10)	Rat (Long-Evans)	21-Gauge needle puncture once	Microarray at 6, 12, 18, and 24 h of sepsis	~120	↑	→	→	↑	↑	→	→
Cobb et al. (9)	Mouse (C57BL/6J)	25-Gauge needle puncture once	Microarray at 24 h of sepsis	13	ND	ND	ND	ND	ND	↑	ND
Our study	Rat (Sprague-Dawley)	18-Gauge needle puncture twice and cecal content excretion	SSH at 18 h of sepsis	9	—	ND	ND	↑	↑	ND	→

ND, Not determined; —, undetected; ↑, increased; ↓, decreased; IL-1β, hepatic interleukin-1β; TNFα, tumor necrosis factor α; MIP, macrophage inflammatory protein; α2M, α-2-macroglobulin; PAI, plasminogen activator inhibitor; TPA, tissue plasminogen activator.

cates that there is no all-encompassing technique—neither microarray nor SSH are false-negative proof.

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