The association of lipopolysaccharide and inflammatory factors with hepatopulmonary syndrome and their changes after orthotopic liver transplantation

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Background: The level of lipopolysaccharides (LPS) and inflammatory factors were higher in end stage liver disease patient than in normal person for the damage of intestinal mucosal barrier function. Hepatopulmonary syndrome (HPS) was a common pulmonary complication in end stage liver disease. But the association of LPS and inflammatory factors such as toll like receptor 2 (TLR2), TNF-α and ET-1 with the development of HPS was undefined.

Methods: Thirty-one HPS patients were researched (26 patients were performed liver transplantation, five were not). Ten healthy volunteers were recruited as negative control. Blood was collected from the 26 HPS patients before and 3, 7, 14, 21 and 28 days after orthotopic liver transplantation (OLT), and from five HPS patients without OLT and ten healthy volunteers once to detect TLR2 mRNA and iNOS mRNA in peripheral blood monocytes and plasma LPS, TNF-α and ET-1 level. Their levels before and after OLT were compared.

Results: TLR2 mRNA, iNOS mRNA, LPS, TNF-α and ET-1 before OLT in HPS patients were 336,594.1±366,901.1, 63,982.2±74,127.5 copies/ugRNA, 4.3±3.3, 90.1±76.0 and 319.9±124.4 ng/L, respectively. They were 10,338.3±3,814.6, 19,168.5±2,417.4 copies/ugRNA, 0.94±0.69, 2.7±0.1 and 84.2±10.6 ng/L in normal control group. They were significantly higher in HPS patients than those in control group (P<0.05). After OLT, liver function improved to normal. Also TLR2 mRNA, TNF-α and ET-1 decreased in HPS patients after OLT compared with those before OLT. And PaO_2 and PaO_2/FiO_2 improved greatly with intrapulmonary shunt decreased to normal after OLT.

Conclusions: Lipopolysaccharides at the end stage of liver disease with the release of series of inflammatory factors may be associated with the development of HPS.

Keywords: Hepatopulmonary syndrome (HPS); orthotopic liver transplantation (OLT); toll-like receptor (TLR); inducible nitric oxide synthase (iNOS); tissue necrosis factor alpha (TNF-α); mechanism

Introduction

Hepatopulmonary syndrome (HPS) is a relatively common and severe pulmonary vascular complication of advanced liver cirrhosis and/or portal hypertension (PH), occurring in 10-30% of patients with cirrhosis (1). It is characterized by pulmonary microvascular dilatation and remodeling, resulting in impaired oxygenation in the absence of marked intrinsic cardiopulmonary disease (2,3). Medical therapy for severe HPS has generally been ineffective. Orthotopic liver transplantation (OLT) is the only successful treatment and typically results in complete resolution of the gas exchange impairment (4-8). However, the pathogenesis of HPS is unknown. Inflammation might play a role in its pathogenesis and development (9). The objectives of our study were to investigate the role of lipopolysaccharide (LPS), toll-like receptor 2 (TLR2), inducible nitric oxide synthase (iNOS), tissue necrosis factor alpha (TNF-α) and endothelin-1 (ET-1) in HPS and the change in the levels of these factors after OLT.

Materials and methods

Patient selection

From March 2004 to January 2006, 279 patients with end-stage liver disease received OLT in the Liver Transplantation Center of Sun Yat-sen University. During this period, 31 patients with HPS were evaluated in our center and were included in this analysis. Among these 31 HPS patients, 26 underwent OLT and the other five patients did not undergo OLT due to either late timing or expectation of a poor outcome. Written informed consent was obtained from each patient included in the study. The study protocol was approved by the institution's Human Research Committee and the Medical Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University. Ten other healthy volunteers were selected as the healthy control group.

Diagnostic criteria for HPS

HPS was diagnosed by: (I) evidence of chronic liver disease with clinical manifestations of PH (varices, ascites, splenomegaly); (II) abnormal arterial oxygenation (PaO$_2$ ≤70 mmHg) while breathing room air; and (III) "positive" 99m technetium macroaggregated albumin (99mTc-MAA) lung and brain perfusion scans suggesting intrapulmonary vascular dilatation (abnormal pulmonary shunt >7%), or positive contrast echocardiography. Non-HPS patients were those with end-stage liver disease without hypoxemia (PaO$_2$ ≥70 mmHg) (10).

99mTc-MAA lung perfusion scan

Every patient with PaO$_2$ ≤70 mmHg underwent a 99mTc-MAA lung and brain perfusion scan to specifically quantify the degree of intrapulmonary vascular dilation (11). Twenty minutes after injection (standing) of 2 mCi of 99mTc-MAA with 90% of particles between 10 and 90 μm (Dupont Pulmolite, Billerica, MA, USA), quantitative brain imaging was performed in the supine position.

Brain uptake or shunt fraction (assuming a constant 13% blood flow to the brain) was obtained by the following calculation: brain uptake (%) = (GMT brain/0.13)/(GMT brain/0.13+ GMT lung). GMT is the geometric mean count of 99mTc-MAA in the brain and lung. An abnormal intrapulmonary shunt was defined as greater than 7%. The severity of liver disease was described by the Child-Turrcote-Pugh (CTP) classification (A, B, or C) and the Model for End-stage Liver Disease (MELD) score. These variables were assessed within one week of the PaO$_2$ measurements.

Blood sample collection

Fasting venous blood samples from all OLT patients with HPS were collected on the morning before transplantation and on postoperative days 3, 7, 14, 21 and 28. Samples were also taken from HPS patients without OLT once, and from healthy volunteers once for controls. Peripheral blood levels of TLR2 messenger ribonucleic acid (mRNA), iNOS mRNA, LPS, TNF-α and ET-1 were measured. Liver function and arterial blood gas (ABG) was examined periodically.

Experimental procedure

The expression levels of TLR2 mRNA and iNOS mRNA were measured by the fluorogenic quantitative polymerase chain reaction (FQ-PCR). The instrument, reagents and technique for real time fluorogenic quantitative reverse transcription-PCR (RT-PCR) were provided by Da An Gene Co., Ltd. of Sun Yat-Sen University. The following procedure was used: (I) peripheral blood total RNA extraction: total RNA was extracted from 500 μL of EDTA-anticoagulated whole blood cells and stored at –80 ℃ with ethanol for preservation; (II) primers and probe:

Table 1 Clinical data for end-stage liver disease patients with HPS before OLT

Clinical data	HPS (n=26)
Age (yr)	48.8±10.9
Sex (male:female)	24:2
Child-Turrcote-Pugh score	11.2±1.7
Child- Turrcote-Pugh classification (cases, %)	
A	2 (7.7)
B	1 (3.9)
C	23 (88.5)
PT (seconds)	32.3±15.2
APTT (seconds)	69.3±39.3
FIB (g/L)	1.6±1.1
ALB (g/L)	35.1±5.7
ALT (U/L)	204.8±314.3
TB (µmol/L)	368.2±294.0
$PaCO_2$ (mmHg)	33.6±4.5
Etiology of cirrhosis (case, %)	
Hepatitis B	23 (88.5)
Hepatitis C	2 (6.5)
Alcoholic cirrhosis	1 (3.2)

OLT, orthotopic liver transplantation; HPS, hepatopulmonary syndrome; PT, prothrombin time; APTT, activated partial thromboplastin time; FIB, fibrinogen; ALB, albumin; ALT, alanine aminotransferase; TB, total bilirubin; $PaCO_2$, partial pressure of carbon dioxide in artery.

the primer sequence of TLR2 was the forward primer 5'-CATTCCCTCAGGGCTCACAG-3' and the reverse primer 5'-TTGTTGGACAGGTCAAGGCTT-3'; the primer sequence of iNOS was the forward primer 5'-AATGGCTGGTACATGGGCAC-3' and the reverse primer 5'-GACGTCA CAGAAGTCCCGGA-3'; (III) reverse transcription: for reverse transcription, 4 µL of extracted RNA were used with the Qiagen RT-PCR Kit (Qiagen, Venlo, Netherlands) in the Perkin Elmer PE 9700 PCR instrument (Perkin Elmer, Waltham, MA). The reaction was conducted using 4 µL of RT-PCR buffer, 0.4 µL (10 pmol/µL) of forward primers, 0.4 µL (10 pmol/µL) of reverse primers, 0.2 µL (25 mmol/L) of dNTP Mix, l µL (10 U/µL) of MMLV, 10 µL of RNase-free water, and 4 µL of total RNA in a final volume of 20 µL. The RT-PCR buffer contains the combination of 50 mmol/L of Tris-HCl (pH 8.0), 50 mmol/L of KCl, 4 mmol/L of $MgCl_2$, and 10 mmol/L of DTT. Reaction conditions were an

initial incubation at 37 ℃ for one hour, followed by 95 ℃ for 3 min; (IV) positive control cDNA template preparation and quantitative polymerase chain reaction (Q-PCR): for Q-PCR, 5 µL of the RT-PCR cDNA products were used in the Perkin Elmer PE9700 PCR instrument. Reverse and positive control cDNA were in a mixture as follows: 10 µL of 5× SYBR Green 1 buffer, 1 µL (10 pmol/µL) of forward primers, 1 µL (10 pmol/µL) of reverse primers, 0.5 µL (25 mmoL/L) of dNTPs, 1.5 µL (2 U/µL) of Taq DNA Polymerase, 5 µL of cDNA, 31 µL of RNase-free water up to a final volume of 50 µL. Initial reactions at 94 ℃ for three min were followed by 40 cycles of 93 ℃ for 1 min and 55 ℃ for 1 min, and 72 ℃ for 1 min. Final results were automatically analyzed by the computer.

LPS was detected by the EDS-99 LPS detection system (Jinshanchuan Co. Ltd., Beijing) through a dynamic turbidimetric method. TNF-α and ET-1 were detected by Elisa methodology.

Statistical analysis

Patient demographic data are described by the mean and standard deviation for quantitative variables and percentages for binomial variables. SPSS 10.0 software was used for statistical analysis. Measurement data were analyzed by a *t*-test and expressed as mean ± standard deviation. P<0.05 was considered statistically significant.

Results

Patient characteristics

From March 2004 through January 2006, 31 with end-stage liver disease were diagnosed with HPS. Twenty-eight (90%) were male and three (10%) were female, and the mean age was 48 years (range, 28-69 years). Among the 31 HPS patients, 26 (84%) underwent OLT, and five potential OLT candidates (16%) did not undergo transplantation. All patients met minimal listing criteria for liver transplantation (CTP score ≥7 points). The clinical data of 26 OLT recipients including gender ratios, average age, and etiologies of liver cirrhosis, as well as MELD and CTP scores before OLT were listed in *Table 1*.

Levels of inflammatory factors in HPS and healthy control groups

The expression levels of TLR2 mRNA and iNOS mRNA

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Table 2 Levels of TLR2 mRNA, iNOS mRNA, LPS, TNF-α and ET-1 before OLT in HPS and healthy control groups

Inflammatory factors	HPS group	Healthy control group	P value	F value	t value
LPS (ng/L)	4.3±3.3	0.9±0.7	0.042	3.417	2.243
TLR2 mRNA	336,594.1±366,901.1	10,338.3±3,814.6	0.001	4.822	2.151
iNOS mRNA	63,982.2±74,172.5	19,168.5±2,417.4	0.004	4.809	1.462
TNF-α (ng/L)	90.1±76.0	2.7±0.1	0.000	4.798	3.05
ET-1 (ng/L)	319.9±124.4	84.2±10.6	0.000	8.903	4.582

OLT, orthotopic liver transplantation; HPS, hepatopulmonary syndrome; LPS, lipopolysaccharide; TLR2 mRNA, toll-like receptor 2 messenger ribonucleic acid; iNOS mRNA, inducible nitric oxide synthase messenger ribonucleic acid; TNF-α, tissue necrosis factor alpha; ET-1, endothelin-1.

and the plasma levels of LPS, TNF-α and ET-1 before operation are shown for the HPS and healthy control groups in *Table 2*.

After OLT, liver function improved greatly. The decrease of total bilirubin (TB) is shown in *Figure 1*. In all HPS recipients, the levels of TLR2 mRNA (*Figure 2*), TNF-α (*Figure 3*), and ET-1 (*Figure 4*) decreased 28 days after OLT.

Liver function, oxygenation, and intrapulmonary shunt improved after OLT

PaO_2 and PaO_2/FiO_2 in HPS patients improved to normal within 28 days after OLT with the normalization of liver function (*Table 3*). The intrapulmonary shunt measured by 99mTc-MAA decreased to less than 7% when the PaO_2/FiO_2 recovered to normal.

Discussion

HPS is a common complication in advanced liver disease patients. In our research, we proved that LPS increased in end-stage liver disease and was especially significant in those with HPS. This means that LPS might play a role in the pathogenesis of HPS. We concluded that the increased LPS might come from the intestine because the intestine is the biggest reservoir for intestinal bacteria and endotoxin, and the intestinal barrier is damaged severely in cirrhosis (12). PH and impaired microvascular circulation of the intestinal mucosa are associated with decreased intestinal movement, poor repair of the damaged barrier, and bacterial overgrowth, resulting in bacterial imbalance and dislocation because of increased permeability of the intestinal barrier. In cirrhosis, the incidence of intestinal bacterial dislocation was 48% (13,14). Bacteria and endotoxin from the intestine

were released into the circulation, resulting in endotoxemia and inflammation with the release of various inflammatory cytokines (14).

In our research, we found that the levels of LPS, TLR2 mRNA, iNOS mRNA, TNF-α and ET-1 were significantly higher in the HPS group before OLT than in the healthy control group (P<0.05). Our results demonstrated that LPS and inflammatory cytokines, such as TNF-α, ET-1 and NO, might play a role in the pathogenesis and pathophysiology of HPS through TLR2. TLR2 (15), part of the transmembrane protein TLR family, was expressed by antigen-presenting cells and cells of the innate immune system, including neutrophils, mastocytes, basophils and eosinophilic granulocytes, as well as those exposed to the external environment, such as epithelial and endothelial cells in the lung and the gastrointestinal tract. TLR2 is essential for the recognition of a variety of pathogen-associated molecular patterns (PAMPs) from gram-positive bacteria, including bacterial lipoproteins, LPS, lipomannans and lipoteichoic acids. These highly conserved, soluble, membrane-bound proteins are collectively called pattern-recognition receptors (PRRs), and it is the PAMP/PRR interaction that triggers the innate immune system (16). LPS is a glycolipid constituent of the outer membrane of gram-negative bacteria, bound to LPS-binding proteins (LBP). With CD14 and MD-2, the LPS-LBP complex combines with TLR4/TLR2 and initiates the TLR2→MyD88→IRAK→TRAF→NIK→IKK→NF-κB signaling cascade in cells, similar to the process followed by TLR4 with a similar downstream effect. Finally, NF-κB is activated and causes the release of IL-12, IFN-γ, TNF-α, IL-1, IL-6, NO, ET-1 (17). ET-1 is released by Kupffer cells in the liver and macrophages in the lungs. Its level increases greatly because of liver dysfunction and collateral
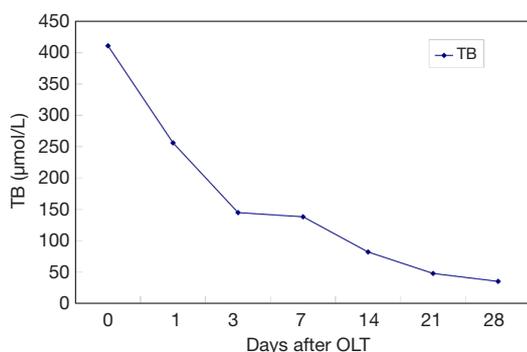
Figure 1 The decrease of TB in HPS patients after OLT. TB, total bilirubin; HPS, hepatopulmonary syndrome; OLT, orthotopic liver transplantation.
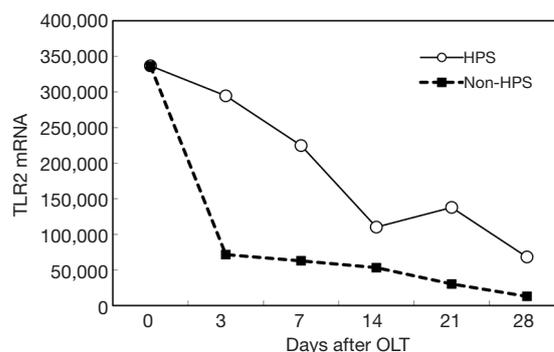

Figure 2 The decrease of TLR2 mRNA expression in HPS patients after OLT. TLR, toll like receptor; HPS, hepatopulmonary syndrome; OLT, orthotopic liver transplantation.
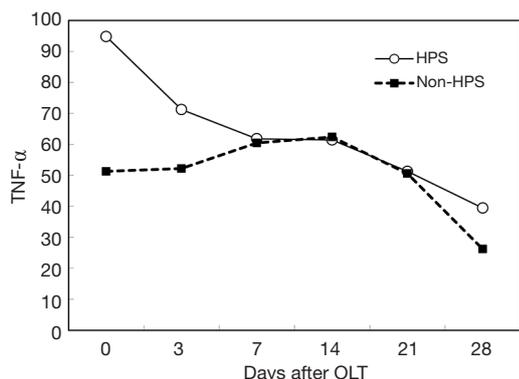

Figure 3 The decrease of plasma TNF-α level in HPS patients after OLT. TNF-α, tissue necrosis factor alpha; HPS, hepatopulmonary syndrome; OLT, orthotopic liver transplantation.
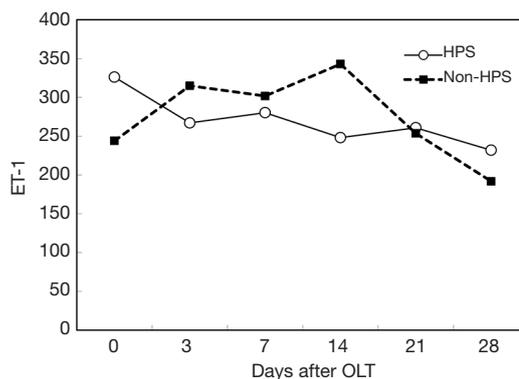

Figure 4 The decrease of plasma ET-1 level in HPS patients after OLT. ET-1, endothelin-1; HPS, hepatopulmonary syndrome; OLT, orthotopic liver transplantation.

Table 3 The levels of PaO_2, PaO_2/FiO_2 and TB in HPS patients after OLT

Variables	Pre-operation	Days after OLT					
	0	1	3	7	14	21	28
PaO_2	64.47	90.16	82.74	80.89	76.67	78	81.27
PaO_2/FiO_2	287.73	210.49	210.19	222.31	281.78	341.35	382.28
TB	410.94	256	144.72	138.07	81.86	47.63	34.96

OLT, orthotopic liver transplantation; PaO_2, partial pressure of oxygen; PaO_2/FiO_2, the ratio of partial pressure of oxygen to fraction of inspire oxygen; TB, total bilirubin; HPS, hepatopulmonary syndrome.

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circulation, and through the ETB receptor, it activates eNOS (18,19), causing accumulation of macrophages in the pulmonary vasculature and overexpression of iNOS and HO-1. The overproduction of NO and carbon monoxide (CO) results in widespread pulmonary vasodilatation at the precapillary and capillary level that leads to a right-to-left shunt, pulmonary V/Q unbalance and hypoxemia.

On the other hand, with the improvement of liver function in HPS patients, TLR2 mRNA, TNF-α and ET-1 decrease, and then, hypoxemia is relieved gradually. In our study, the decrease of these inflammatory factors did not reach statistical significance at 28 days after OLT; however, longer observation might be needed. LPS and iNOS mRNA decreased early after OLT, but they increased again later. HPS patients are extremely prone to the complication of pneumonia. We hypothesized that a later increase in LPS and iNOS mRNA might be associated with subsequent infection in some HPS patients.

Our research demonstrated that LPS might upregulated TLR2 mRNA expression and activation of the downstream inflammatory factor production in cirrhosis before OLT. The overproduction of inflammatory factors might play an important role in the pathogenesis and pathophysiology of HPS. We supposed that LPS came from the intestine because of a damaged intestinal barrier in cirrhosis. After OLT, the liver and intestinal function recovered, and the intestinal barrier was repaired; thus, the levels of inflammatory factors decreased as the hypoxemia of HPS was relieved gradually and completely.

There were limitations in our research in that we could not prove that the origin of LPS was from the intestine. Nor did we detect the expression level of TLR4, which was predominantly activated by LPS. Further research is needed to clarify the function of immune cells and how they work through LPS and TLR activation in HPS.

Conclusions

Above all, LPS and inflammatory factors increased at the end stage of liver disease before OLT, but decreased after OLT with the recovery of liver function. Oxygenation of lungs also got better and the HPS was cured. Our results showed LPS with the release of series of inflammatory factors may be associated with the pathogenesis and development of HPS.

Acknowledgements

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