ORIGINAL RESEARCH

The significance of Toll-like receptor 4 (TLR4) expression in patients with chronic hepatitis B

Xiu-Qing Wei^{1,2,*}

Yun-Wei Guo^{1,2,*}

Jia-Jun Liu³

Zhuo-Fu Wen¹

Shao-Ji Yang²

Ji-Lu Yao²

¹Department of Gastroenterology, Third Affiliated Hospital of Zhong Shan (Sun Yat-sen) University, 510630 Guangzhou, P.R.China.

²Department of Infectious Disease, Third Affiliated Hospital of Zhong Shan (Sun Yat-sen) University, 510630 Guangzhou, P.R.China.

³Department of Hematology, Third Affiliated Hospital of Zhong Shan (Sun Yat-sen) University, 510630 Guangzhou, P.R.China.

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**co-first authors*

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Abstract

Purpose: To investigate the importance of Toll-like receptor 4 (TLR4) expression on hepatocytes obtained from Chronic Hepatitis B patients as well as on hepatocellular carcinoma HepG2 and HepG2.2.15 cell lines.

Methods: Expression of TLR4 in liver tissues was determined by immunohistochemistry in 75 patients with CHB and in 10 healthy controls. The protein and mRNA levels of TLR4 in hepatocellular carcinoma HepG2 and HepG2.2.15 cells were measured by flow cytometry (FCM) and real-time quantitative PCR (RQ-PCR), and endotoxin triggered TNF- α secretion in HepG2 and HepG2.2.15 cells was evaluated by ELISA.

Results: TLR4 expressed mainly in the cytoplasm and some on cell membrane in hepatocytes. The staining scores of TLR4 expression in the liver tissues of patients with CHB were significantly higher than that of healthy controls. The liver tissues from patients with severe CHB expressed higher level of TLR4 than those from patients with mild CHB. Furthermore, the staining scores of TLR4 expression in the liver tissues of patients with CHB were positively correlated with the grading scores. Our results also showed that the mean fluorescence intensity and TNF- α secretion induced by endotoxin as well as the protein and

mRNA levels of TLR4 in HepG2.2.15 cells were all significantly higher than those in HepG2 cells.

Conclusion: TLR4 was up-regulated in the hepatocytes in patients with CHB. This indicates a potentially important interaction between TLR4 expression and the pathogenesis of CHB.

Mechanisms responsible for chronic hepatitis B remain incompletely understood, although increasing evidence points to immunological rather than direct viral effects playing a central role.^{1,2} However, most studies have focused on adaptive immunity, with little research on the innate immunity. One of the key components of the innate immune response is the family of Toll-like receptors (TLRs), evolutionarily conserved pattern recognition receptors. Activation of TLRs triggered by various motifs common to microorganisms, known as pathogen-associated molecular patterns, can promote the inflammatory response, the anti-infectious response and the maturation of antigen pre-

senting cells.^{3,4} It is also believed that the innate immunity controls the adaptive immune responses.⁵ The Toll-like receptor 4 (TLR4) is one of most important TLRs. Activation of TLR4 can cause inflammation by promoting the secretion of inflammatory cytokines such as TNF- α and interleukin-6 through the MyD88 dependent path way, and anti-virus effects by promoting the secretion of interferon β through the MyD88 independent path way.⁴ TLR4 is expressed in hepatocytes and endotoxin can induce the secretion of TNF- α and interleukin-6, ect by hepatocytes.⁶⁻¹¹ TLR4 is up-regulated in peripheral blood monocytes (PBMCs) and hepatocytes in patients with chronic hepatitis C(CHC) and plays an important role in the pathogenesis of CHC.^{12,13} Systemic endotoxemia often occurs in patients with chronic viral hepatitis¹⁴ and the human 60 kDa heat shock proteins (hHSP 60) are markedly increased in patients with active viral hepatitis with predominant expression in areas of inflammatory infiltrates¹⁵, while endotoxin and hHSP 60 are the major ligands of TLR4, an important member of TLRs.^{16,17} In preliminary studies, we found that TLR4 is up-regulated in the peripheral blood monocytes (PBMCs) in patients with chronic hepatitis B(CHB).¹⁸ However, less detailed data are available about the expression of TLR4 on hepatocytes in patients with CHB. In this study, we analyzed the importance of TLR4 expression in patients with chronic hepatitis B. Our results suggest a potentially important interaction between TLR4 expression and the pathogenesis of CHB.

Materials and methods

Patients

The study was approved by the ethic committee and written consent was obtained from all patients and healthy volunteers.

Patients who had been HBsAg and hepatitis B virus (HBV)-DNA positive for at least six months and had elevated blood alanine aminotransferase (ALT) levels were defined as CHB in this study.¹⁹ Patients with hepatitis delta virus, HIV infection, bacteria in-

fection, concomitant diseases such as autoimmune diseases and gastrointestinal, pulmonary, cardiovascular, renal or neurological disorders or with alcohol abuse were excluded. Seventy-five patients with CHB and 10 healthy controls entered the study. The 10 healthy control liver samples were all of routine biopsies from the donor liver used in liver transplantation surgery and were all excluded of hepatitis viruses infections.

Blood samples

Blood samples were collected one week before the liver biopsy. Clinical routine items such as serum levels of alanine aminotransferase (ALT), serum total bilirubin(TB), serum HBeAg status and serum HBV-DNA levels were tested according to the regular procedures.

Histopathological examination of samples

Percutaneous liver biopsy samples were obtained by using liver biopsy needles after written informed consent was approved. All tissue samples were fixed in 10% buffered formalin solution, embedded in paraffin and stained with haematoxylin-eosin, trichrome and Van Gieson stains for routine histological examination. Each biopsy specimen was evaluated according to the modified grading and staging system described by Scheuer.²⁰ In addition, we classified histological activity of CHB as mild or severe, defining mild CHB as ≤ 2 , and severe CHB as > 2 .²¹

Immunohistochemistry

Standard deparaffinization sections were subjected to microwave oven, immersed in 0.01M citrate buffer pH 6.0 for 20 min and, after washing in PBS, the immunohistochemical procedure was carried out as described as the instruction of the Elivision two step kit (Maxin Biotechnology, China). Polyclonal anti-TLR4 made in rabbit (sc-10741, Santa Cruz Biotechnology, USA) was used in a 1:100 dilution as the primary an-

tibody. DAB-3,3'-diaminobenzidin was used for visualization. PBS instead of the primary antibody served as the negative control. Each section was semiquantitatively evaluated by two independent observers using a Nikon light microscope (Nikon Labophot,, Japan) without the knowledge of either clinical or histological diagnosis. Four distinct items were recorded on separate sheets as follows: 0-no staining; +1-positive staining in <30% of cells per high power field ($\times 250$); +2- positive staining in >30% but in <70% of cells per high power field; and +3-positive staining of >70% of cells per high power field, according to a previously validated scoring system. Finally, data were averaged to median values, giving a numerical score for each liver biopsy specimen, and then used for statistical analysis.^{21,22}

Cell culture of HepG2 and HepG2.2.15

HepG2 and HepG2.2.15 were provided by the laboratory of infectious disease, Third Affiliated Hospital, Sun Yat-Sen University. HepG2 was cultured in six-well plates with DMEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin, in a humidified 5% CO₂ incubator at 37°C. The culture medium for HepG2.2.15 was added 600 μ g/mL G418 to that for HepG2. Cells were passaged twice weekly and routinely examined for mycoplasma contamination. When the monolayers of cells reached about 90% confluency, then cells were harvested by trypsin EDTA treatment.

Flow Cytometry for Determination of TLR4 Expression on HepG2 and HepG2.2.15

A total of 1×10^6 single cell suspension were incubated with phycoerythrin-conjugated anti-human TLR4 monoantibody (sc-13593,eBioscience, USA) for 30 min in the dark and washed twice with PBS. Cells incubated with phycoerythrin-conjugated isotype mouse IgG2a were used as negative controls. Analysis was performed on a FACS Calibur flow cy-

tometry (Becton Dickinson, USA). A minimum of 10,000 cells were analyzed in all specimens. The mean fluorescence intensity and the percentage of positive cells were determined using the CellQuest software program (Becton Dickinson,USA) and expressed as the results.

Real-Time RT-PCR for Determination of mRNA level of TLR4 in HepG2 and HepG2.2.15

Oligonucleotide primers were designed and synthesized by Takara (Japan) as follows: 5'-AGGATGATGCCAGGATGATGTC-3'and 5'-TCAGGTCCAGGTTCTTGGTTGAG-3'(198bp) for TLR4, 5'-TGG CAC CCA GCA CAA TGA A-3'and 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'(186bp) for β -actin. The plasmid pBluescript-HBV (constructed by the laboratory of infectious disease, Third affiliated hospital, Sun yat-sen university) coding hepatitis B virus DNA was used to make standard curves. Total RNA was isolated from the cells by Trizol (Invitrogen, USA. One step real-time RT-PCR was performed on ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif. USA), using the SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit (Takara, Japan). The 50 μ L reaction mixture contained: 4 μ L forward primer(0.2 μ M), 4 μ L reverse primer(0.2 μ M) and 4 μ L total RNA or plasmid pBluescript-HBV of different dilutions. The PCR schedule consisted of the following stages: 15 minutes at 42 °C for 1 cycle, 2 minutes at 95 °C for 1 cycle, 5 seconds at 95 °C and then 31 seconds at 60 °C for a total 40 cycles, 15 seconds at 95 °C, 31 seconds at 60 °C and then 15 seconds at 95 °C for 1cycle. The ratio between the mRNA level of TLR4 to β -actin was termed relative TLR4 mRNA level and used for quantitative evaluation.

TABLE 1. Demographic and laboratory variables

	Chronic hepatitis B	Healthy Control
No. of patients	75	10
Sex (male/female)	11 1/2	9
Age (yr)	35±11	36±6
ALT (U/l)	125±76	25±11
Total bilirubin (μmol/l)	63.7±53.5	N/A
Numbers of HBeAg (+) / (-)	2 1/8	N/A
Lg [serum HBV-DNA]	6.19±1.64	N/A
G0/G1/G2/G3/G4 score	0/16/20/18/21	10/0/0/0/0
S0/S1/S2/S3/S4 score	0/14/21/26/14	10/0/0/0/0
TLR4 score 0/1/2/3	2/13/22/38	8/2/0/0

N/A: not applicable

ELISA for Determination of TNF- α Secreted by HepG2 and HepG2.2.15

HepG2 and HepG2.2.15 cells were seeded at a density of 2.5×10^4 cells/cm². All cells were seeded at the same time. Their media were changed every 24 h. Cells were seeded and, on the third change of medium (72 hr after seed), media with or without 1 μg/ml endotoxin from *Salmonella typhimurium* (Sigma, St. Louis, MO) were added.²³ After 24 hr, the medium was collected and centrifuged during 5 min at 30000 rpm, to immediately evaluation of TNF- α . TNF- α was determined by the quantikine human TNF-alpha immunoassay (R&D Systems). The TNF- α secretion ability of HepG2 and HepG2.2.15 cells was presented as the ratio of the TNF- α concentration in the culture supernatant of endotoxin stimulated cells to that of unstimulated cells as control.

Statistical Analysis

Statistical analyses were performed using the Mann-Whitney rank sum test, student-t test and Spearman's rank correlation test as appropriate (SPSS for Windows, version 11.0; SPSS Inc, USA). $P < 0.05$ was considered significant.

Results

There were no differences in age or sex distribution between the patient and control groups. Demographic

parameters and laboratory findings (ALT, serum total bilirubin, HBeAg status and lg[serum HBV-DNA]) of the patients are shown in Table 1.

Few TLR4-positive hepatocytes could be traced in the liver tissues of normal controls whereas, they existed discreetly or diffusely in the liver tissues of the patients with CHB. Positive staining of TLR4 was located mainly in the cytoplasm with some on the cell membrane of hepatocytes. Other cells in the liver were not stained (Figure 1). TLR4 expression scores in CHB liver tissue were higher than that of healthy controls ($P < 0.001$, table 2). Staining scores of TLR4 expression in the severe CHB group were much higher than in the mild CHB group ($P < 0.001$, Table 2). There were positive correlations between staining scores of TLR4 expression and the grading scores in CHB ($\gamma = 0.579$, $P < 0.001$). There were no correlations between the staining scores of TLR4 expression and the levels of serum ALT, lg[serum HBV-DNA] and the staging scores. No difference was found between the HBeAg positive group and the HBeAg negative group for expression of TLR4 in hepatocytes. The level of

FIGURE 1. 1 Toll-like receptor 4 (TLR4) expression. A, B: Healthy control, no staining for TLR4 expression (x100, x400). C, D: Liver biopsy from a patient with chronic hepatitis B (x200), TLR4 expressed on hepatocytes, positive staining mainly located in the cytoplasm but not the nuclei (x100, x400).

TABLE 2. TLR4 Scores

	No. of patients	Score of Expression of TLR4
Normal Control	10	0.20±0.42
CHB	75	2.17±0.88 *
Mild CHB	36	1.75±0.87*
Severe CHB	39	2.56±0.68 *,#

Notes: CHB, chronic hepatitis B; *, $P < 0.05$ vs normal control; #, $P < 0.05$ vs mild CHB

serum ALT did not correlate with the histopathological grading or staging score.

The mean fluorescence intensity of TLR4, percentage of TLR4 positive cells and relative TLR4 mRNA level in HepG2.2.15 were all higher than those in HepG2 (Table 3). After stimulation with endotoxin for 24 hr, secretion of TNF- α by HepG2.2.15 increased much more than that by HepG2 (3.3 ± 0.2 vs 2.6 ± 0.3 , $n=18$, $P < 0.001$).

Discussion

The expression of both mRNA and protein levels of TLR4 were much higher in HepG2.2.15 cells than in HepG2 cells and that secretion of TNF- α by HepG2.2.15 stimulated by endotoxin increased much more than by HepG2. Also, levels of TLR4 in CHB patients were higher than in healthy controls, and TLR4 expression was much more remarkable in the severe than in the mild CHB group. There was a positive correlation between the staining scores of TLR4 expression and the grading scores in CHB. However, there was no correlation between the staining scores of TLR4 expression and the levels of serum ALT and or between scores of TLR4 and levels Ig[serum HBV-DNA].

TLR4, barely detected in the normal hepatocytes, can be up-regulated by NS5A protein of hepatitis c virus and beta-glucan of *Aspergillus fumigatus*, etc.^{24,25} Our results agree with those of Mozer-Lisewska¹³, but differ from those reports that HBV virus has no effect on the expression of TLR4 on hepatocytes²⁶ We found that HBV virus could up-regulate the expression of TLR4 in hepatocytes.

These results may be explained as follows:

TABLE 3. Expression of TLR4 in HepG2 and HepG2.2.15

	n	MFI	TLR4 positive cells(%)	Relative TLR4 mRNA
HepG2	12	1.03±0.34	0.39±0.12	0.13±0.03
HepG2.2.15	12	10.72±2.79*	16.34±6.97*	0.60±0.09*

Notes: MFI, Mean fluorescence intensity ; *, $P < 0.05$ vs HepG2

1. TLR4 exists not only on the cell membrane but also in the cytoplasm.^{27,28} The level of TLR4 on the cell membrane can be altered by exocytosis and endocytosis. Visvanathan studied only the expression of TLR4 on cell membrane of hepatocytes in patients with CHB, while we studied the total expression of TLR4 in both cell membrane and cytoplasm.

2. Visvanathan reported that the change of expression of TLR4 on the cell membrane of HepG2 was caused by HBV by acute transduction. This should be a model of acute HBV infection, while HepG2.2.15 is a model of persistent stable chronic HBV infection of HepG2. The differences may indicate that HBV has different effects on the expression of TLR4 on hepatocytes during the course of chronic and acute infection. We found that there was no correlation between the staining scores of TLR4 expression and the levels of serum ALT. This agreed with the observation that there was no correlation between histopathological grading scores and levels of serum ALT. In fact, the levels of serum ALT may often not reflect the severity of CHB.²⁹

HepG2.2.15 is a cell line of HepG2 with stable chronic HBV infection. We concluded that up-regulation of TLR4 expression in HepG2.2.15 cells might be caused by HBV itself. Systemic endotoxemia often occurs in patients with chronic viral hepatitis¹⁶ and human 60 kDa heat shock proteins (hHSP 60) are increased in patients with active viral hepatitis with predominant expression in areas of inflammatory infiltrates.¹⁷ Both endotoxin and hHSP 60 are major ligands of TLR4.^{14,15} Elevated expression of TLR4 increases sensitivity to the ligands and leads to enhanced production of pro-inflammation cytokines.^{24,25}

FIGURE 2. Typical TLR4 in HepG2 (A) and HepG2.2.15 (B). Cells were stained with directly conjugated antibodies to TLR4. Diagrams represent 10,000 gated cells.

We found that secretion of TNF- α by HepG2.2.15 stimulated by endotoxin increased more than that by HepG2. Thus, hepatocytes could produce high levels of TNF- α in chronic hepatitis. Also, TNF- α plays an important role in the pathogenesis of CHB.^{30,31} In this study, there was a positive correlation between the staining scores of TLR4 expression and the grading scores in CHB. Taken together, the elevated TLR4 expression in hepatocytes may play a role in the pathogenesis of CHB. In animal models, activation of TLR4 caused inhibition of replication of HBV.^{32,33} However, there was no correlation between the scores of TLR4 and the levels of Ig[serum HBV-DNA] in this study. There may be no role of elevated expression of TLR4 in hepatocytes for inhibition of replication of HBV in CHB. Activation of TLR4 can cause liver injury in several pathological processes such as hemorrhagic shock, hepatic ischemia/reperfusion injury, alcohol-induced liver injury and hepatic failure.³³⁻³⁷ Inhibition of TLR4 with a TLR4 antagonist or recombinant soluble forms of extracellular TLR4 domain and MD-2 can attenuate myocardial ischemia-reperfusion injury, dampen lipopolysaccharide-induced pulmonary inflammation in mice

and have anti-inflammatory effects in murine models of inflammatory bowel disease.³⁸⁻⁴⁰ Our finding of elevated expression of TLR4 in hepatocytes may have a role in the pathogenesis of CHB while little effect on the inhibition of replication of HBV, TLR4 antagonist may be helpful in the treatment of CHB.

In conclusion, we have demonstrated for the first time that TLR4 expression may play an important role in the pathogenesis of CHB. This indicates that TLR4 may be a useful therapeutic target for the treatment of CHB.

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Correspondence to:

Dr. Zhuo-Fu Wen
 Department of Gastroenterology,
 Third Affiliated Hospital of Zhong Shan (Sun Yat-sen)
 University, 510630 Guangzhou, P.R.China
 Email: wenzfu@163.com or
 weixiuqing1975@yahoo.com.cn