Decreased Expressions of Toll-Like Receptor 9 and Its Signaling Molecules in Chronic Hepatitis B Virus–Infected Patients

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Objectives.—To determine expression levels of TLR9 messenger RNA along with MYD88, interleukin 1 receptor–associated kinase 1 (IRAK1), tumor necrosis factor receptor–associated factor 6 (TRAF6), and NF-κB in peripheral blood mononuclear cells obtained from chronic hepatitis B virus (CHB)–infected patients.

**Context.**—Toll-like receptors (TLRs) play crucial roles in immune responses, especially innate immunity, against viral infections. Toll-like receptor 9 recognizes intracellular viral double-strand DNA, which leads to the activation of nuclear factor B (NF-κB) through the myeloid differentiation primary response 88 (MYD88) pathway. Defects in the expression of TLR9 and its signaling molecules may cause attenuated immune responses against hepatitis B virus.

**Objective.**—To determine expression levels of TLR9 messenger RNA along with MYD88, interleukin 1 receptor–associated kinase 1 (IRAK1), tumor necrosis factor receptor–associated factor 6 (TRAF6), and NF-κB in the peripheral blood mononuclear cells obtained from chronic hepatitis B virus (CHB)–infected patients.

**Design.**—In this study, 60 CHB patients and 60 healthy controls were recruited and the expression of TLR9 and its downstream signaling molecules was examined by real-time polymerase chain reaction techniques using β-actin as a housekeeping gene.

**Results.**—Our results showed that expression of TLR9, MYD88, IRAK1, TRAF6, and NF-κB in peripheral blood mononuclear cells of CHB patients was significantly decreased in comparison with healthy controls.

**Conclusions.**—According to our results, it appears that CHB patients are unable to appropriately express genes in the TLR9 pathway, which may impede immune responses against hepatitis B virus infection. These results suggest a mechanism that may partially explain the fact that immune responses are disrupted in CHB patients.


Iran has a low endemicity for hepatitis B virus (HBV) infection and Iranian HBV-infected patients appear to carry only the D strain of the HBV genotype. Chronic HBV (CHB)–infected patients demonstrate a clinical form of hepatitis B in which HBV is not completely eradicated from either hepatocytes or sera and show low levels of symptomatic liver disease. Several investigations have proposed that CHB could be one of the main causes of cirrhosis and hepatocarcinoma. The leading mechanisms responsible for CHB pathogenesis and other manifestations of HBV infection have yet to be clarified. Researchers have proposed that immunologic, genetic, and epigenetic parameters of hosts are probably responsible for the differences between individuals with eradication-resistant HBV strains and those who overcome the disease by clearing the virus. Toll-like receptors (TLRs) are the main intracellular/extracellular immune cell receptors that recognize pathogen-associated molecular patterns of the microbes and foreign particles, including viruses, to induce several immune cell functions ranging from migration, nicotinamide adenine dinucleotide phosphate oxidase activation, and phagocytosis to inflammatory cytokine expression. Toll-like receptor 9 induces inflammatory cytokine expression by the recognition of intracellular viral double-strand DNA via the myeloid differentiation primary response 88 (MYD88)–dependent pathway. Assembling of MYD88 (as an adaptor molecule) also leads to the activation of several intracellular signaling molecules, such as interleukin 1 receptor–associated kinase 1 (IRAK1), tumor necrosis factor receptor–associated factor 6 (TRAF6), and nuclear factor κB (NF-κB). Nuclear factor κB is a transcription factor that is reported to bind the regulatory region of several genes, including inflammatory cytokines. Therefore, inadequate expression of these molecules may lead to attenuated immune responses against viral infection. Because the immune system of CHB patients is unable to completely...
remove HBV from hepatocytes, it seems that these patients have deficits in some parameters of their immune systems. Considering the important role of TLR9 and its associated signaling molecules in the recognition and induction of immune responses against viral infection, it may be speculated that these molecules can affect pathogenesis of CHB. Interestingly, an in vivo study has revealed that TLR signaling, via the interactions of TLR3, 4, 5, 7, and 9 with their ligands, inhibits hepatitis B virus replication. Therefore, alteration in the expression levels of these molecules may lead to unimpeded replication and survival of HBV in the infected liver. Thus, the main aim of this study was to evaluate the expression levels of TLR9, MYD88, IRAK1, TRAF6, and NF-κB in the peripheral blood mononuclear cells (PBMCs) of CHB patients.

MATERIALS AND METHODS

Subjects

Peripheral blood samples were collected from 60 CHB patients and 60 healthy controls from Rafsanjan and Yazd (southeastern and central region of Iran) in 5.5-mL tubes with and without anticoagulant. Chronic hepatitis B virus patients with HBV and hepatitis C virus coinfection; patients with detectable human immunodeficiency virus antibody; and patients with infections caused by other viruses, such as cytomegalovirus, hepatitis A, C, D, and E viruses, and Epstein-Barr virus, were excluded from the study. The diagnosis of CHB was performed based on the “Guide of Prevention and Treatment in Viral Hepatitis” by an expert internal medicine specialist; in brief, the CHB patients had been hepatitis B surface antigen (HBsAg)—positive for more than 6 months and had normal serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, direct bilirubin, and total bilirubin (Table 1). All of the patients were recruited to the study based on assessment of their previous clinical and experimental records; control patients were selected with the same age, sex, and socioeconomic status (Table 1). Evaluation of socioeconomic condition was based on the levels of education and monthly income of CHB patients and controls. Patients meeting the following criteria were excluded from the study: age younger than 18 years or older than 55 years; pregnant or breastfeeding; having features suggestive of other coexistent liver diseases, including alcoholic liver disease, autoimmune liver diseases, previous liver transplantation, cirrhosis, or Wilson disease; having psychiatric conditions; showing evidence of hepatocellular carcinoma; or taking antiviral drugs or interferon before the biopsy. Patients under therapy with immunosuppressive drugs were also excluded from the study. The samples collected without anticoagulant were centrifuged at 3500 rpm for 4 minutes, and the sera were separated within 8 hours after collection. The plasma samples were stored at −20°C for a maximum of 2 months or at −70°C when longer storage periods were required for analysis. The samples collected with anticoagulant were immediately used for isolation of PBMCs and RNA extraction. Isolation of PBMCs was performed from 20 mL of fresh whole blood collected in plastic tubes containing EDTA as a anticoagulant and diluted (1:2) with phosphate-buffered saline (0.1 M sodium phosphate, 0.14 M NaCl, pH 7.4). The prepared samples were added to a Ficoll-Paque solution (Cinnaclon, Tehran, Iran) and then separated by centrifugation at low speed (2000 rpm) at 25°C for 30 minutes. The enriched PBMC layer was moved to a Falcon tube and washed.

Table 1. Laboratory and Demographic Information of Chronic Hepatitis B Virus (CHB)—Infected Patients and Healthy Controls

<table>
<thead>
<tr>
<th>Factor</th>
<th>Healthy Controls</th>
<th>CHB Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, y</td>
<td>38.41 ± 7</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>Sex, No. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>31 (48.3)</td>
<td>28 (46.6)</td>
</tr>
<tr>
<td>Female</td>
<td>29 (51.7)</td>
<td>32 (53.4)</td>
</tr>
<tr>
<td>Hepatitis B surface antigen—positive, No. (%)</td>
<td>0</td>
<td>60 (100)</td>
</tr>
<tr>
<td>Hepatitis B e antigen—positive</td>
<td>0</td>
<td>5 (8.3)</td>
</tr>
<tr>
<td>Liver function tests, mean ± SD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferase, U/L</td>
<td>28 ± 9</td>
<td>27 ± 12</td>
</tr>
<tr>
<td>Aspartate aminotransferase, U/L</td>
<td>29 ± 5</td>
<td>28 ± 11</td>
</tr>
<tr>
<td>Alkaline phosphatase, U/L</td>
<td>240 ± 20</td>
<td>270 ± 40</td>
</tr>
<tr>
<td>Direct bilirubin, mg/dL</td>
<td>0.1 ± 0.08</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Total bilirubin, mg/dL</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.2</td>
</tr>
</tbody>
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SI conversion factors: To convert bilirubin to micromoles per liter, multiply by 17.104.

Table 2. Primer Sequences of Evaluated Genes

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequences</th>
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<tbody>
<tr>
<td>Toll-like receptor 9</td>
<td>F: 5’-CTCCCCTGTAGGCTGTCC-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-CTCTCACCAGGAGAGACAG-3’</td>
</tr>
<tr>
<td>Myeloid differentiation primary response 88</td>
<td>F: 5’-TGGGACTCTGCTGGCTG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-ACATTCCTTGCTGGCACAGT-3’</td>
</tr>
<tr>
<td>Interleukin-1 receptor associated kinase 1</td>
<td>F: 5’-CAGACTCTTGTGGATGTG-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-AGCTGACCGCTGCAATTT-3’</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor—associated factor 6</td>
<td>F: 5’-AAAGAATTGGGAACTTGGGAGT-3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’-GGTGAGATCTGCTGGCTG-3’</td>
</tr>
<tr>
<td>Nuclear factor κB</td>
<td>F: 5’-GCACCACCGGCAATGAG-3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>R: 5’-CCGATCCGACAGGAGCTT-3’</td>
</tr>
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</table>
twice with phosphate-buffered saline. This study was approved by the ethical committee of the Rafsanjan University of Medical Sciences, and written informed consent was obtained from all participants prior to sample collection.

**Detection of Serologic HBV Markers**

All of the samples were screened for HBsAg and hepatitis B e antigen by enzyme-linked immunosorbent assay (Behring, Marburg, Germany) according to the manufacturer's guidelines.

**HBV DNA Extraction and Real-Time Polymerase Chain Reaction Conditions**

Viral DNA was purified from 200 μL of plasma from HBsAg-positive patients using a commercial kit (Cinnaclon) according to the manufacturer's guidelines. Hepatitis B virus DNA amplification was also performed using a commercial kit from the Primer Design Company (London, United Kingdom) following the manufacturer's instructions.

**RNA Extraction, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted from PBMCs using the RNX extraction kit from Cinnaclon. The extracted RNA quality was determined by electrophoresis on an ethidium bromide–pretreated agarose gel as well as by measuring absorption at 260/280 nm by spectrophotometer. The RNA was converted to complementary DNA using a complementary DNA synthesis kit (Parstous, Tehran, Iran) with both oligo(dT) and random hexamer primers. The reverse transcription step was performed using the following steps: 70°C for 10 minutes (without reverse transcription enzymes), −20°C for 1 minute (cooling), addition of reverse transcription enzymes, 42°C for 60 minutes, and a final step at 95°C for 10 minutes to inactivate the reverse transcription enzymes. Real-time polymerase chain reaction was performed using a SYBR Green Master Mix (Parstous), combined with 200 ng of template complementary DNA with the appropriate primers (Table 2) in a Bio-Rad CFX96 system (Bio-Rad Company, Foster City, California) using the following program: 1 cycle of 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Primers were synthesized by Cinnaclon. Real-time polymerase chain reactions were carried out in triplicate and the β-actin housekeeping gene was used for normalization of amplification signals of target genes. The relative amounts of polymerase chain reaction product were determined using the 2^−ΔΔCt formula. The dissociation stages, melting curves, and quantitative analyses of the data were performed using CFX Manager software version 1.1.308.111 (Bio-Rad). Polymerase chain reaction products were also analyzed by electrophoresis on a 1% gel agarose containing 0.5 mg/mL ethidium bromide.

**Figure 1.** Messenger RNA levels of toll-like receptor 9 (TLR9) (A), myeloid differentiation primary response 88 (MYD88) (B), interleukin-1 receptor–associated kinase 1 (IRAK1) (C), tumor necrosis factor receptor–associated factor 6 (TRAF6) (D), and nuclear factor B (NF-kB) (E) in peripheral blood mononuclear cells of chronic hepatitis B virus (CHB)–infected patients and healthy controls. Results are shown as mean ± standard error. *Significant difference between CHB patients and healthy controls.
The parametric statistical analyses were performed by a t test using SPSS software version 18 (Chicago, Illinois). When a P value was less than .05 it was considered significant.

RESULTS

Detection of HBsAg and Hepatitis B e Antigen

Our results demonstrated that all of the patients were HBsAg positive, and 5 of them were also hepatitis B e antigen positive (Table 1).

Quantification of HBV DNA Copy Numbers

Our results showed that all of patients had detectable HBV DNA. Thirty-seven, 12, and 11 of the patients carried less than 20 000, between 20 000 and 400 000, and greater than 1 000 000 HBV copies/mL, respectively.

Expression Levels of Target Genes

Our results showed that expression levels of TLR9, MYD88, IRAK1, TRAF6, and NF-κB in the PBMCs of CHB patients were 9.52-, 2.99-, 30.67-, 3.92-, and 1.5-fold decreased, respectively, in comparison with healthy controls (Figure 1). Statistical analysis revealed that the differences between CHB and control groups regarding expression of TLR9 (P = .04), MYD88 (P < .001), IRAK1 (P = .007), and TRAF (P < .001) were significant (Figure 1), whereas the decreased expression level of NF-κB in CHB patients was not significant (P = .17). The results also demonstrated that expression levels of TLR9 (P = .53), MYD88 (P = .20), IRAK1 (P = .39), and NF-κB (P = .52) were not significantly different among CHB patients with different viral loads, whereas the messenger RNA (mRNA) levels of TRAF were significantly decreased (P = .003) in CHB patients with HBV DNA viral loads less than 20 000 copies/mL when compared with patients suffering from higher viral loads (Figure 2).

COMMENT

Results of the current study showed that the expression levels of TLR9, MYD88, IRAK1, and TRAF6 in the PBMCs from CHB patients were significantly decreased in comparison with healthy controls. Based on these results, it may be concluded that CHB patients may be unable to express suitable levels of these signaling molecules to induce expression of several required inflammatory factors, including proinflammatory cytokines, that are required for clearance of the HBV infection. Therefore, the TLR9
pathway may be compromised in CHB patients and this may be the mechanism that can explain how infection is sustained in CHB patients. Our results also revealed that mRNA levels of NF-κB were not different between the CHB and healthy control groups. Nuclear factor κB is a common transcription factor that is involved in several pathways, including TLRs, cytokine/cytokine receptor interaction, T cell receptor (TCR) and B cell receptor (BCR) signaling pathways, and so on; hence, it may be concluded that its normal expression is related to normal signaling in other pathways than TLRs. Interestingly, Isogawa et al., using injection of ligands specific for TLR9, demonstrated that the TLR9-ligand (microbial double-strand DNA) interaction leads to inhibition of HBV replication in an animal model. These results would support a model in which the down-regulation of TLR9 and its downstream molecules may be implicated in the defective immune responses seen in CHB patients. The investigation by Xu et al. also demonstrated that TLR9 mRNA levels were decreased in CHB and related hepatocellular carcinoma. Zhou et al. also reported that expression levels of TLR9 were significantly decreased in the HB patients. In parallel with our results, Xie and colleagues also showed that expression levels of TLR9 mRNA and protein levels were decreased in plasmacytoid dendritic cells of CHB patients when compared with healthy controls. All of these studies measured only TLR9 mRNA levels, whereas our study evaluated some of the downstream signaling molecules and revealed that these molecules also were decreased. Therefore, based on these results we conclude that the TLR9 pathway is an important intracellular viral double-strand DNA sensor, and that expression of the signaling molecules may be defective in CHB patients. Interestingly, Vincent et al. reported that HBV can block interferon-α production by blocking the MYD88-IRAK4 axis. The authors concluded that HBV used several escape mechanisms to down-regulate and inactivate TLR9 in both plasmacytoid dendritic cells and B lymphocytes. Hirsch et al. also demonstrated that HBV can lead to decreased expression of TLR9 and interferon-α in plasmacytoid dendritic cells via interaction with the regulatory receptors of plasmacytoid dendritic cells. Using synthetic ligands of TLR9, they concluded that HBV can block plasmacytoid dendritic cell stimulation. Our results revealed that the expression of TLR9 and its molecular signaling were decreased in the PBMCs of Iranian CHB patients; hence, it seems that HBV may use the same mechanisms to suppress PBMCs to reduce expression levels of TLR9 and its downstream signaling molecules.

Our results demonstrated that the expression levels of TLR9, MYD88, IRAK1, and NF-κB were not affected by different levels of HBV DNA, whereas TRAF6 was decreased in the patients with lower HBV DNA copy numbers. It seems that immune cells are induced to express TRAF6 in response to HBV replication in our cohort of patients. In contrast with our results, Zhou et al. showed that TLR9 expression levels were lower in CHB patients with high HBV DNA copy numbers when compared with patients with low HBV DNA copy numbers. Xu et al. also reported that HBsAg can suppress TLR9 transcription in plasmacytoid dendritic cells. It is likely that the populations studied by Zhou et al. and Xu et al. were genetically and ethnically different from the Iranian patients evaluated here; hence, it seems that more studies on Iranian patients are needed to confirm our results.

In addition, the sample sizes of our patients with different HBV DNA viral loads were not the same, and the majority of them carried fewer than 20,000 HBV DNA copies/mL; therefore, it would be important to evaluate more patients with medium and high virus copy numbers in future studies. This would improve the power of our study in relation to HBV replication and expression of TLRs and their molecular signaling in the in vivo condition.

Finally, based on our results, it seems that future therapies could focus on early detection and treatments that could upregulate the expression of TLR9 and its signaling molecules as a way of reactivating the immune system and facilitating the clearance of HBV from CHB patients.

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References


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