Research Article

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Role of Toll-like receptor 2 in the immune response against hepadnaviral infection

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Background & Aims: The Toll-like receptor 2 (TLR2) has recently been recognized to play an important role in the pathogenesis of chronic hepatitis B virus (HBV) infection. In the present study, we examined the role of TLR2 in hepadnaviral infection in hepatoma cell lines and the woodchuck model.

Methods: The expression of TLR2 and pro-inflammatory cytokines was quantified by real time RT-PCR. TLR2-associated signaling pathways in hepatocytes were examined by Western blot. HBV replication and gene expression were assessed by Southern blot, Northern blot and specific ELISA, respectively.

Results: TLR2 ligands activated NF-κB, PI3K/Akt, and different arms of MAPK signaling pathways and induced the production of pro-inflammatory cytokines in hepatocytes. TLR2-mediated innate immune responses led to reduction of HBV/woodchuck hepatitis virus (WHV) replication and gene expression in HepG2.2.15 cells and primary woodchuck hepatocytes. Furthermore, the antiviral activity of TLR2 ligands was abolished by pretreatment with U0126 and rapamycin, inhibitors of the MAPK/ERK and PI3K/Akt pathways, respectively. In the woodchuck model, relatively low levels of TLR2 expression were found in peripheral blood mononuclear cells (PBMCs) and in liver tissues from chronic WHV carriers. TLR2 expression in PBMCs was inversely correlated with WHV DNA titers in acute WHV infection and in entecavir-treated chronic WHV carriers.

Conclusions: These data suggest that hepatocytes play an active role in TLR2-mediated antiviral responses during hepadnaviral infection. The mutual inhibition of HBV replication and TLR2 signaling represents an important aspect of HBV infection and should be considered in the new therapeutic concept against chronic HBV infection.

Keywords: Hepatitis B virus; Toll-like receptor 2; Hepatocytes; Woodchuck hepatitis virus; Innate immune response.

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Abbreviations: HBV, hepatitis B virus; CHB, chronic HBV infection; ERK, extracellular signal-regulated kinases; IFN, interferon; IRAKs, interleukin (IL)-1-R–associated kinases; PBMC, peripheral blood mononuclear cells; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary-response protein 88; PHH, primary human hepatocytes; PI3k/Akt, phosphatidylinositol-3 kinase/protein kinase B; PWH, primary woodchuck hepatocytes; TAK1, transforming growth factor (TGF)-β–activated kinase 1; TLR2, Toll-like receptor 2; TNFα, tumor necrosis factor alpha; TRAF6, tumor necrosis factor receptor (TNFR)-associated factor 6; WHV, woodchuck hepatitis virus.

Introduction

Chronic hepatitis B virus (HBV) infection (CHB) is a major public health problem as it is the leading cause of liver cirrhosis and hepatocellular carcinoma worldwide [1]. The outcome of HBV infection is the result of complex interactions between replicating HBV and the immune system [2,3]. While the role of the adaptive immune system in the resolution of HBV infection has been studied extensively [4,5], the contribution of innate immune mechanisms remains to be defined. Notably, recent data provided some evidence that activation of the innate immune system may contribute to controlling HBV infection in hepatocytes [6–13].

Toll-like receptors (TLRs) are evolutionary conserved receptors and play a crucial role in the innate immune response against pathogens by recognizing and responding to pathogen-associated molecular patterns and activation of intracellular signaling pathways [14]. Among the known TLRs, TLR2, in concert with TLR1 or TLR6, recognizes various bacterial components, including peptidoglycans, lipopeptides, and lipoproteins of Gram-positive bacteria, and mycoplasma lipopeptides [15]. TLR2/TLR1 and TLR2/TLR6 heterodimers, in particular, discriminate triacyl lipopeptides and diacyl lipopeptides, respectively [16]. Stimulation of TLR2 in hepatoma cell lines and in primary human hepatocytes results in MyD88-dependent NF-κB activation and production of tumor necrosis factor alpha (TNFα) and interleukin (IL)-8 [17]. Kupffer cells are able to respond to different TLR2 ligands with production of proinflammatory cytokines, upregulation of cell surface molecules relevant for antigen presentation, and promotion of T cell functions [18].

TLR2 shares the MyD88-mediated signaling pathway with TLR4. Unlike TLR4, TLR2 does not activate Toll/IL-1 receptor domain-containing adaptor inducing interferon (IFN)-β/IFN regulatory factor 3 (TRIF/IRF3) pathway to induce IFN production [19]. Previously, we have found that the TLR4 ligand lipopolysaccharide (LPS) was able to activate IFN-independent pathways in hepatocytes and to inhibit woodchuck hepatitis virus (WHV) replication in primary woodchuck hepatocytes (PWHs) [20]. Furthermore, TLR2 activation led to reduction of HBV replication and capsid formation in recombinant HBV baculovirus transduced hepatoma cells [9]. Controversially, recent studies have suggested that HBV is able to interfere with sensors of the innate immune system, in particular by regulating the expression of TLR2. Peripheral blood mononuclear cells (PBMCs) from CHB patients show lower TLR2 expression and impaired pro-inflammatory cytokine production in response to TLR2 ligands [21,22]. Most likely, the interaction of HBeAg with the Toll/IL-1 receptor (TIR) proteins Mal and TRAM leads to disruption of homotypic TIR:TIR interactions that are critical for TLR2-mediated signaling [23].

In the present study, TLR2-mediated antiviral action and the underlying mechanisms were investigated in the HBV replicating HepG2.2.15 cells. In addition, TLR2 expression and function were examined in the woodchuck model [24]. Our results demonstrated that TLR2-activated intracellular MAPK/ERK and PI3k/Akt pathways in hepatocytes are required for the suppression of HBV replication. Consistently, TLR2 expression in woodchuck PBMCs was downregulated by WHV during acute and chronic WHV infection.

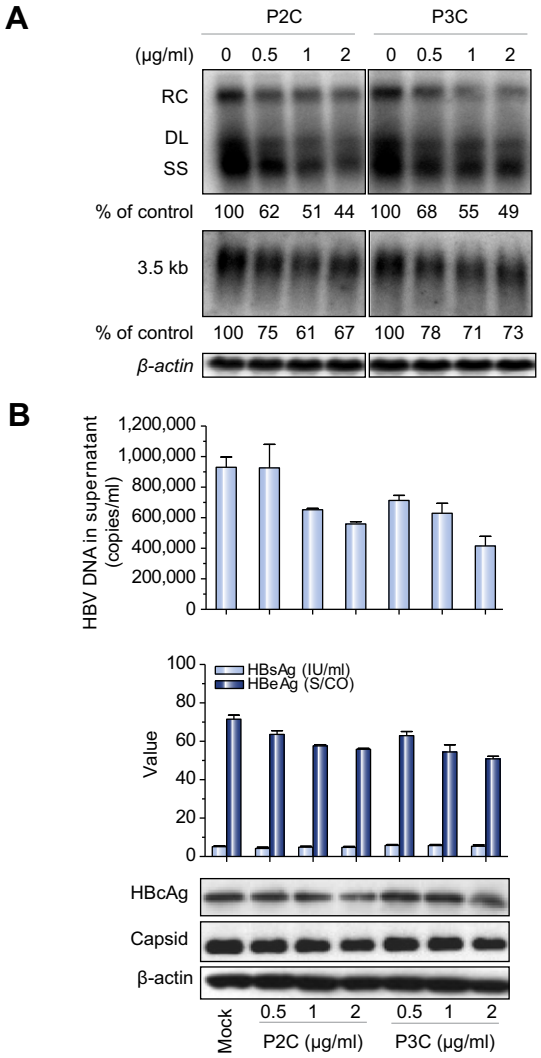

Fig. 1. Suppression of HBV replication and transcription in HepG2.2.15 cells by TLR2 ligands. HepG2.2.15 cells were treated with TLR2 ligands at indicated concentrations for 4 days. (A) HBV replicative intermediates and transcripts were detected by Southern and Northern blot. *β-actin* mRNA was used as loading control. (B) HBV DNA, HBsAg and HBeAg in the culture supernatants were detected by real time PCR and CMIA assay, respectively. Intracellular HBcAg expression and core particles were analyzed by Western blot with β-actin as loading control. RC, relaxed circular; DL, double stranded linear; SS, single stranded.

Materials and methods

Reagents

The synthetic ligands for TLR2/TLR6 Pam2CSK4 (P2C) and TLR2/TLR1 Pam2CSK4 (P3C) were provided by EMC Microcollections (Tübingen, Germany). The ligand for TLR4 (LPS from O26:B6 *Escherichia coli*), NF-κB pathway inhibitor Bay11-7082, MAPK/JNK pathway inhibitor SP600125, and MAPK/p38 pathway inhibitor SB203580 were purchased from Sigma–Aldrich (Steinheim, Germany). The ERK/MAPK pathway inhibitor U0126 was provided by Invivogen (Toulouse, France). The PI3k/Akt pathway inhibitor rapamycin was purchased from LC Laboratories (Woburn, MA). The siRNAs targeting HBV S and X region (Supplementary Table 1) [25], the validated siRNAs targeting TLR signaling adaptor proteins (IRAK1, IRAK4, TAK1, and TRAF6) (Supplementary Table 2) and an unrelated control siRNA (neg.siRNA) were purchased from Qiagen (Hilden, Germany).

Woodchucks

Woodchucks were purchased from North Eastern Wildlife (Ithaca, NY) and kept in the Central Animal Laboratory of University Hospital of Essen. Animal experiments were conducted in accordance with the German Law for the Care and Use of Laboratory Animals and the protocols were reviewed and approved by the District Government of Düsseldorf, Germany. Eight naïve and eight chronically WHV-infected woodchucks were used for the present study.

Preparation of primary human and woodchuck hepatocytes, cell culture and treatment, analysis of HBV and WHV replication and gene expression, real time reverse transcription (RT)-PCR assays of cellular mRNAs, Western blotting analysis, and luciferase reporter assays are included in the Supplementary data.

Statistical analysis

The statistical analysis was carried out with GraphPad (GraphPad Software Inc., San Diego, CA). Analysis of variance with Student's *t*-test was used to determine significant differences in multiple comparisons. $p \leqslant 0.05$ was considered as statistically significant. Representative data from a series of at least three experiments are shown.

Results

Downregulation of HBV replication and gene expression by TLR2 ligands in HepG2.2.15 cells

Firstly, we confirmed *TLR2* mRNA expression in PHHs and three human hepatoma cell lines (HuH7, HepG2, and HepG2.2.15) by real time RT-PCR (Supplementary Fig. 2A). Stimulation with the TLR2 ligands P2C and P3C induced the expression of proinflammatory cytokines in PHHs and the three hepatoma cell lines (Supplementary Fig. 2B and C). We then asked whether TLR2 ligands were able to exert antiviral activities in HepG2.2.15 cells with an established HBV replication. After treatment with 2 μg/ml of P2C