Research Article

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Early inhibition of hepatocyte innate responses by hepatitis B virus

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Background & Aims: The outcome of hepatitis B virus (HBV) infection may be influenced by early interactions between the virus and hepatocyte innate immune responses. To date, the study of such interactions during the very early step of infection has not been adequately investigated.

Methods: We used the HepaRG cell line, as well as primary human hepatocytes to analyze, within 24 h of exposure to HBV, either delivered by a physiologic route or baculovirus vector (Bac-HBV), the early modulation of the expression of selected antiviral/pro-inflammatory cytokines and interferon stimulated genes. Experiments were also performed in the presence or absence of innate receptor agonists to investigate early HBV-induced blockade of innate responses.

Results: We show that hepatocytes themselves could detect HBV, and express innate genes when exposed to either HBV virions or Bac-HBV. Whereas Bac-HBV triggered a strong antiviral cytokine secretion followed by the clearance of replicative intermediates, a physiologic HBV exposure led to an abortive response. The early inhibition of innate response by HBV was mainly evidenced on Toll-like receptor 3 and RIG-I/MDA5 signaling pathways upon engagement with exogenous agonist, leading to a decreased expression of several pro-inflammatory and antiviral cytokine genes. Finally, we demonstrate that this early inhibition of dsRNA-mediated response is due to factor(s) present in the HBV inoculum, but not being HBsAg or HBeAg themselves, and does not require *de novo* viral protein synthesis and replication.

Conclusions: Our data provide strong evidence that HBV viral particles themselves can readily inhibit host innate immune responses upon virion/cell interactions, and may explain, at least partially, the “stealthy” character of HBV.

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Keywords: Hepatitis B virus; Hepatocytes; Early host/virus interaction; IFN response; dsRNA-mediated innate immunity.

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Abbreviations: Bac, baculovirus; DMSO, dimethyl sulfoxyde; GFP, green fluorescent protein; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; ISG, interferon stimulated genes; KC, Kupffer cells; LSEC, liver sinusoidal endothelial cells; MDA, melanoma differentiation-associated gene; MOI, multiplicity of infection; NLR, NOD-like receptor; PEG, polyethylene glycol; PHH, primary human hepatocytes; PRR, pathogen recognition receptor; RIG, retinoic-acid-inducible protein; RLR, RIG-like receptor; SV, Sendai virus; TLR, toll-like receptor; VGE, virus genome equivalent.

Introduction

Viral hepatitis represents a major health problem worldwide, with hundreds of millions of chronic carriers who have a high risk of developing liver cirrhosis and hepatocellular carcinoma [1]. To establish and maintain persistent replication, hepatitis B virus (HBV) has evolved multiple strategies to evade the host innate and adaptive immune responses [2]. To restore immune control of the virus, virus-mediated inhibitory mechanisms could be defeated/unlocked in an interventional therapeutic perspective. A better knowledge of the underlying molecular mechanisms responsible for virus-induced blockade of immune responses is crucial before envisaging such strategies to improve the success of current antiviral treatments [3].

Most viruses are detected early after infection by both immune and/or infected cells via pathogen recognition receptors (PRR), including Toll-like receptors (TLR) and RIG-I like receptors (RLR) and NOD-like receptors (NLR) [4]. It is still unclear if HBV is recognized by the innate immune system and/or if the virus can actively suppress or avoid early antiviral responses that drive the control of HBV infection [2,5,6]. The few data obtained in acutely infected humans, chimpanzees and woodchucks have shown that during the natural course of HBV infection, the activation of innate responses is predominantly weak or absent [7–10]. In particular, a seminal work performed in chimpanzees, has shown that HBV does not induce a strong modulation of gene expression in the liver of an infected animal as compared to hepatitis C virus (HCV) [10]. Following this work HBV was qualified as a “stealth virus” [11] as opposed to HCV. A stealth virus can be a virus that

does not induce measurable innate responses because of a lack of detection by PRR, or a virus that is able to actively inhibit nascent innate responses. In this respect, some other data suggest that, during chronic infection, HBV could negatively regulate host immune responses by interfering with TLR expression and signaling pathways [12–14], or by inhibiting interferon (IFN) response [15–19]. Underlying molecular mechanisms could involve several distinct HBV proteins as recently reviewed [2,5,6].

However to date, there are no very early kinetic interaction study between HBV and hepatocytes that have been performed to determine if the virus could; i) be initially detected by host cells; ii) modulate host immune gene expression; and then iii) inhibit innate responses. Only one study, performed in primary human hepatocyte (PHH) cultures showed that HBV could induce the transient production of interleukin (IL)-6, thus suggesting that the virus could be initially "sensed", at least by liver macrophages (i.e. present in PHH cultures), and lead to the production of pro-inflammatory cytokines. However, HBV could, 24 hour (h) after the onset of infection, inhibit the production of IL-6 by a yet unknown mechanism [20].

To better characterize the early interplay between HBV and hepatocyte innate immunity in terms of recognition and evasion, we used a non-transformed human hepatocyte cell line, differentiated HepaRG (dHepaRG), which is permissive for persistent HBV infection and is devoid of contamination by immune cells [21–23]. We showed that hepatocytes themselves could "sense" HBV and initiate an antiviral response, when delivered to cells by baculoviral transduction, as previously observed [24], but also during a more physiologic infection with HBV virions. While an efficient antiviral response was observed against HBV replication, when launched by baculovirus, this response was abortive in the context of a physiological HBV infection. In this case, the suppression of innate responses was exerted by viral component(s) already present within the inoculum not requiring *de novo* viral synthesis. This active suppression of pathogen-sensing pathways in the very early phase of infection, which prevented the establishment of a competent innate immunity, correlated with the development of a persistent infection *in vitro*.

Materials and methods

HBV and Sendai viral inocula

HBV inoculum was either concentrated from filtered HepG2.2.15 (wild-type virus) or K6 (HBx negative virus) [25] supernatants by polyethylene glycol (PEG) precipitation as previously described [22], or partially purified by heparin chromatography [26], then concentrated using centrifugal filters devices (Amicon Ultracel 100K, Millipore). A mock "HBV-negative" inoculum (mock control) was generated by depletion of Dane particles, HBsAg and HBeAg using centrifugal filters devices (Amicon Ultracel 10K, Millipore). After DNA extraction (QIAmp Ultrasens Virus kit, Qiagen), HBV inoculum was titrated by qPCR with forward 5′-GCTGACGCAACCCCCACT-3′ and reverse 5′-AGGAGTTCCGCAGTATGG-3′ probes using a standard curve from a quantified HBV encoded plasmid. All preparations were tested for the absence of endotoxin (Lonza Verviers, Belgium). Sendai virus (SV; Cantell strain; titer: 4000 HAU/ml) was obtained from Charles River Laboratories (Bois des Oncins, France) and used according to recommendations.

HBV virion and viral protein purification

Dane particles were purified from the PEG precipitated HBV inoculum by sequential ultracentrifugation through a cushion of sucrose first, then on a sucrose density gradients at 35,000 rpm for 16 h at 4 °C in a Beckman SW41Ti Rotor. Collected fractions were tested for sucrose density, HBV DNA (qPCR), HBcAg (Western blot with anti-HBc (Dako)), HBeAg and HBsAg (ELISA). The overall purity of preparation was investigated by SDS-PAGE and SYPRO-Ruby Protein Gel Staining (Life Technologies). The concentrations of HBsAg and HBeAg were measured by commercial immunoassay kits, according to the manufacturer's protocols (Autobio Diagnostics Co., China). One NCU, i.e. unit used in "HBeAg detection and relative quantification ELISA" from Autobio, is equivalent to 13.33 ng.

Human hepatocyte culture and HBV infection

The human liver progenitor HepaRG cells were cultured and infected as previously described [21,22]. PHHs were prepared from surgical liver resections as previously described [27]. They were infected similarly to differentiated HepaRG.

Baculovirus vectors and cell transduction

Two baculoviruses were used in this study: a 1.1x genome-length HBV recombinant baculovirus vector (Bac-HBV) and a control baculovirus expressing GFP instead of HBV pgRNA. Baculoviral transduction of mammalian cells was performed as previously described [24].

Cell stimulation

Cells (10^6 per well) were stimulated with TLR agonists (Invivogen) and harvested after 6 h (except for RIG-I/MDA5 after 24 h) for the analysis of *IL-6* protein production by ELISA:TLR1/2 (pam3CSK4, 0.8 μg/ml), TLR3 (poly(I:C), 10 μg/ml), TLR4 (LPS 0.4 μg/ml), TLR5 (flagellin, 0.1 μg/ml), TLR6 (FSL-1, 0.1 μg/ml), TLR7/8 (ssRNA, 10 μg/ml), RIG-I/MDA5 (transfection of poly(I:C) with the reagent lyovec, 0.2 μg/ml). For the cytokine gene expression analysis following poly(I:C) stimulation, cells were harvested after 3 h of stimulation.

Nucleic acid purification, RT-qPCR, and qPCR

Total RNA or DNA were respectively purified with the Nucleospin RNA II or MasterPure™ DNA Purification kits according to manufacturer's instructions (Macherey Nagel or Epicentre). cDNA was obtained after reverse transcription using the SuperScript® III Reverse Transcriptase (Life technologies) and real time quantitative qPCR (the sequence of primer pairs are listed in Table 1) was performed using the EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Life technologies), and run on the MyiQ Biorad machine. Relative mRNA expression was analyzed with q-base software (Biogazelle, Belgium) using the comparative cycle threshold ($2^{-\Delta\Delta Ct}$) method with two housekeeping genes (*RPLP0* and *β-actin*) previously tested for their stability in the HepaRG cells and the PHH, and normalized to the control conditions (=1). The relative HBV mRNA level was quantified using the same primer pairs used for the HBV PCR quantification.

Analysis of cytokine production

At selected time points, cell culture supernatants were harvested and tested for the secretion of IL-6, IFN-λ1/3 (R&D system), IFN-α, and IFN-β (PBL Interferon Source) according to the manufacturer's instructions.

UV inactivation

HBV and mock inocula were irradiated or not at room temperature on a UV Transilluminator (Appligene) delivering 3.3 mw/cm^2 for 30, 60, or 90 min. The efficacy of HBV replication with UV-inactivated inocula was analyzed after exposure to HepaRG cells by RT-qPCR and secretions of HBeAg and HBsAg were analyzed by ELISA (see Supplementary Fig. 3A).

Table 1. Sequences of human primer pairs used for RT-qPCR.

Gene	Forward	Reverse
IFN-α	gtgaggaaatacttccaaagaatcac	tctcatgatttctgctctgacaa
IFN-β	gccgcattgaccatgtatgaga	gagatcttcagtttcggaggtaac
IL-29	gtggtgctggtgactttgg	ctcctgtggtgacagagatttg
IL-6	acccctgacccaaccacaaat	agctgcgcagaatgagatgagtt
IL1-β	aatctgtacctgtcctgcgtgtt	tgggtaatttttgggatctacactct
ISG56	agccaacatgtcctcacagac	cttctaccactcgtttcatgc
OAS1	aggtggtaaagggtggctcc	acaaccaggtcagcgtcagat
b-ACTIN	tggcattgccgacaggatgc	tctgctggaggtggacagcga
RPLP0	caccattgaaatcctgagtgatgt	tgaccagcccaaaggagaag

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