Research Article

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The Hepatitis B e antigen (HBeAg) targets and suppresses activation of the Toll-like receptor signaling pathway

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Background & Aims: Viruses target innate immune pathways to evade host antiviral responses. Recent studies demonstrate a relationship between hepatitis B disease states and the host's innate immune response, although the mechanism of immunomodulation is unknown. In humans, the innate immune system recognizes pathogens via pattern recognition receptors such as the Toll-like receptors (TLR), initiating anti-inflammatory responses. TLR expression and pro-inflammatory cytokine production is reduced in hepatitis B e antigen (HBeAg)-positive patients following TLR stimulation. The aim of this study was to investigate interactions between TLR signaling pathways and the mature HBeAg protein localized in the cytosol.

Methods: The ability of HBeAg to inhibit TLR signaling and association with TLR adapters was evaluated by immunoprecipitation, immunostaining, and reporter studies.

Results: Our findings show that HBeAg co-localizes with Toll/IL-1 receptor (TIR)-containing proteins TRAM, Mal, and TLR2 at the sub-cellular level, which was not observed for Hepatitis B core antigen. Co-immunoprecipitation analysis demonstrated HBeAg interacted with TIR proteins Mal and TRAM, while a mutated HBeAg ablated interaction between Mal and MyD88. Importantly, HBeAg also disrupted homotypic TIR:TIR interaction critical for TLR-mediated signaling. Finally, HBeAg suppressed TIR-mediated activation of the inflammatory transcription factors, NF-κB and Interferon-β promoter activity.

Conclusions: Our study provides the first molecular mechanism describing HBeAg immunomodulation of innate immune signal transduction pathways via interaction and targeting of TLR-mediated signaling pathways. These finding suggest the mechanism as to how HBeAg evades innate immune responses contributing to the pathogenesis of chronic hepatitis B infection and the establishment of viral persistence.

Keywords: Hepatitis B precore; Pathogen recognition receptors; Innate immunity; Immunomodulate; Immunotolerant; NF-κB.

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Abbreviations: HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; CHB, chronic hepatitis B; HBcAg, hepatitis B core antigen; NF-κB, nuclear factor kappa B; TNF-α, tumor necrosis factor alpha; TLR, Toll-like receptor; TIR, Toll/IL-1 receptor; IL, interleukin; IFN, interferon; IRF, interferon regulatory factor; HEK, human embryonic kidney; Pam_3Cys, Pam_3Cys-SKKKK; PBMCs, peripheral blood monocytes.

Introduction

Hepatitis B virus (HBV) chronically infects more than 350 million people worldwide and is a major cause of cirrhosis and hepatocellular carcinoma. The pathogenesis of chronic hepatitis B (CHB) is largely immune-mediated as the virus itself is non-cytopathic to hepatocytes. CHB is often characterized by a prolonged immunotolerant, hepatitis B e antigen (HBeAg)-positive phase, where individuals are persistently infected with very high viral loads [6]. Surprisingly, in this phase there is essentially no underlying inflammation or liver damage. It has recently been proposed that HBV establishes chronic infection via evasion of the innate immune response [10], which may subsequently limit maturation of the adaptive immune system for the efficient clearance of HBV [21].

HBeAg is a non-particulate version of the HBV nucleocapsid protein and is transcribed, translated, and secreted very early in the HBV replication cycle [16,25]. HBeAg is regarded as an accessory protein of HBV and is not required for viral replication or infection, although it is required for establishment of chronic infection and is associated directly with, and is probably responsible for immunomodulation of host immune responses during CHB infection [25]. There is increasing recognition that the course of HBV infection is influenced by the degree of innate immune response [10]. HBeAg is translated from the preC transcript and the immature HBeAg is targeted to the lumen of the endoplasmic reticulum (ER) where the 19 residue signal peptide is removed [6,14]. Subsequently, 34 C-terminal residues, including the Arg-rich region, are removed to produce the mature form of HBeAg, termed p17 [14]. Although the majority of this

signal-cleaved product is secreted, between 20% and 30% [14] of the mature protein is retained in the cytoplasm, although no specific role has been ascribed to the cytosol-localized form of HBeAg [15]. Interestingly, the HBeAg precursory genetic codes share remarkable sequence conservation in all mammalian-infecting hepadnaviruses, irrespective of host, genotype, or geographic origin [29]. Alternatively, hepatitis B core antigen (HBcAg) is a truncated protein highly homologous to HBeAg, sharing an open reading frame [6]. HBcAg, however, lacks a distinctive 10 additional amino acids found in the N-terminal of HBeAg, although no function has been attributed to this unique region.

We have previously demonstrated that patients with CHB display down-regulated Toll-like receptor (TLR)-2 expression, and diminished responses to multiple TLR ligands in HBeAg-positive PBMCs and hepatocytes as compared to HBeAg-negative and normal patients [30,36]. Critically, uninfected PBMCs pre-incubated with HBeAg and subsequently stimulated with synthetic TLR2 ligand Pam_3Cys and TLR4 ligand lipopolysaccharide (LPS), display significantly decreased production of pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) *ex vivo* compared to control and HBeAg-negative samples [36]. These findings suggest HBeAg contributes to the suppression of key inflammatory response genes. Furthermore, Schlaak and co-workers recently observed that HBV virions suppress TLR responses in hepatocytes and non-parenchymal cells [37], although no mechanism was proposed.

TLRs are widely expressed on both immune-specialized and non-specialized cells. TLRs are defined by the presence of a cytosolic Toll/IL-1 receptor (TIR) motif which facilitates inflammatory signaling pathways via homotypic TIR:TIR interactions. TLRs are expressed either extra- or intra-cellularly, commensurate with their respective ligands [1]. All TLRs, with the exception of TLR3, recruit MyD88 to their receptor complex; as do members of the IL-1 receptor family [1,5]. Recruitment of MyD88 to the receptor complex initiates a well characterized signal transduction pathway that results in the activation of NF-κB, resulting in the expression of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 [1]. While TLRs induce common signaling pathways, there is critical specificity in recruitment of TIR-containing adapter proteins leading to distinctive inflammatory gene expression [26]. MyD88-adapter like (Mal)/TIRAP also induces NF-κB activation and is responsible for signaling selectively via TLR4 and TLR2. TIR-domain containing adapter protein inducing IFN-β (TRIF, also known as TICAM1) mediates the MyD88-independent pathway leading to TLR4-mediated activation of the transcription factor IRF3, which regulates interferon (IFN)-β production. Importantly, TRIF also mediates downstream signaling from TLR3, independent of MyD88. TRIF-related adapter molecule (TRAM, also known as TICAM2) specifically bridges TLR4 with TRIF, where TLR4-mediated responses are ablated in TRAM-deficient macrophages, but not TLR3 [26].

Previous studies have shown that TLR-induced IFN-α/β and IFN-induced MyD88 expression inhibit HBV replication [19,39], implying the importance of a robust TLR response in facilitating seroconversion during CHB. Furthermore, recent studies have demonstrated that reconstituting the TLR response by exogenous addition of the TLR2 ligand Pam_3Cys inhibits HBV replication [33]. TLRs may, therefore, represent a viable HBV target for inhibiting innate immune responses due to their role in suppressing viral replication. However, the mechanism whereby HBV evades host innate responses is unknown [10]. We hypothesized that HBeAg may antagonize TLR-signaling pathways and suppress innate responses, particularly in CHB infected individuals. In this study, we have found that HBeAg specifically inhibits TIR-mediated activation of NF-κB and IFN-β. The inhibitory effects of HBeAg are dependent upon the unique 10 additional precore specific codons located at the N-terminal which we have called the precore specific sequence (PSS). The PSS was identified as similar to the TIR motif, found only in the mature HBeAg (p17), distinguishing it from HBcAg. Furthermore, mutations within the putative N-terminal TIR region of HBeAg (p17) ablate inhibitory effects previously observed. We identified HBeAg-p17 co-localization with TIR-containing proteins exclusively at the plasma membrane, while HBcAg appears diffuse throughout the cytosol, not localized with TIR-containing proteins. This study provides, for the first time, a mechanism whereby HBeAg suppression of TLR signaling pathways may explain HBeAg immunomodulation of the host innate immune response.

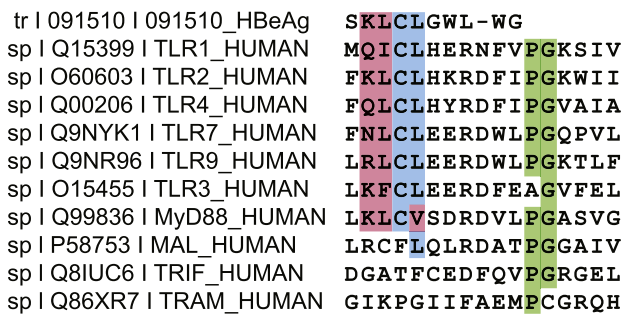

Fig. 1. Alignment of HBeAg with TIR-containing proteins. Clustal alignment of HBeAg ((−)10-(−)1) genotype D PSS with human TIR-containing proteins consisting of the region associated with the TIR BB loop important for signaling to NF-κB activation. The PG (green) motif is critically required for TIR-mediated signaling (4, 20) which is absent in the PSS.

Materials and methods

Cell culture and reagents

Huh7, HEK293, and HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) with 10% heat inactivated fetal calf serum (FCS) and 2 mM glutamine in a humidified incubator at 37 °C, 5% CO_2. TLR ligands Pam_3Cys (TLR2) were obtained from EMC Microcollections (Tuebingen, Germany) and poly (I:C) (TLR3) purchased from Invivogen (San Diego, USA). Human TNF-α and human IL-1 were purchased from PeproTech. Glutathione–agarose beads (Amersham Biosciences) and anti-GST antibody were sourced from Santa Cruz. The anti-Flag M2-HRP conjugated beads, anti-Flag epitope tag antibodies, and EZview Red anti-HA affinity gel beads are from Sigma.

Plasmids

Flag tagged Mal, TRAM, TRIF, TLR2, TLR3, and RIG-I were kind gifts from K. Fitzgerald (University of Massachusetts). Flag-MyD88 was provided by J. Tschopp (Lausanne University). pCI-HBcAg and pCI-HBeAg were subcloned from the infectious genotype D, HBV construct [31] using standard methodology. These were then used as a template for generating the HBeAg-(p17)-HA ((−)10–148aa) and HBeAg-(A(−8)-S(−7)A(−6)) (HBeAg p17Δ3x-HA) (pCMV-HA, Clontech), HBeAg-(p17)-DS Red plasmid (adapted from pIRES2-DsRed-Express, Clontech), p17-GFP ((−)10–148aa), and truncated HBcAg-GFP (1–148aa) plasmids (pEGFP-N1, Clontech). pETGEXCT was a kind gift from A. Sharrocks (University of Manchester) and used in the generation of the HBeAg-((−)10–19aa) and HBeAg triple mutant (A(−8)-S(−7)A(−6))-GST plasmids (HBeAg(Δ3x)-GST).