Hepatitis B viral replication influences the expression of natural killer cell ligands

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Abstract

Background Hepatitis B virus (HBV) is accounting for over one million deaths annually due to immune-mediated chronic liver damage. Natural killer (NK) cells are abundant in the liver and contribute in HBV persistence. NK cytotoxic effects are controlled by signals from activating and inhibitory receptors. HBV may circumvent host antiviral immunity via the regulation of NK receptors and their ligands. We investigated the effect of viral replication and HBeAg mutations on NK mediators expression in the livers of chronic HBV (CHB) patients and in cell cultures.

Methods HBV monomers bearing hotspot mutations in the basal core promoter and precore region were transfected into HepG2 cells using a plasmid-free assay. Serum viremia and liver HBV RNA were measured in 19 CHB patients. The expression of HBV RNA and of NKG2D ligands, B7H6, DNAX accessory molecule-1, lectin-like transcript 1 (LLT1), LFA-1 and TRAIL was measured in the livers of CHB patients and transfected cells.

Results In general, high HBV replication in CHB patients and cell lines upregulated the mRNA of all NK cell ligands and particularly the inhibitory NK cell ligand, LLT1. The exception was the NKG2D ligand, MICA, that was significantly decreased in patients with high serum viremia and intrahepatic HBV RNA levels.

Conclusions HBV replication has differential effects on NK cell ligands suggesting a potential escape mechanisms through up-regulation of LLT1 and down-regulation of MICA. A general trend towards upregulating NK cell ligands can be counteracted by decreasing MICA and hence weakening NK surveillance.

Keywords Hepatitis B virus, NK cells, virus, LLT1, NKG2D, mutations, precore, viral replication

Introduction

Hepatitis B virus (HBV) is a hepatotropic DNA virus infecting humans for more than 1500 years [1]. To date, HBV is a leading cause of liver diseases worldwide, with more than 350 million people being persistently infected and at high risk developing liver failure, cirrhosis or hepatocellular carcinoma (HCC) [2,3]. An increased proportion of chronic disease is due to the emergence of viral variants that either abrogate or reduce HBeAg production [4]. One of the most critical mutations is the double A1762T/G1764A substitution in the basal core promoter (BCP) region and its appearance is usually followed by the development of the triple mutant A1762T/G1764A/C1766T [5]. The most common mutation that results in an HBeAg-negative phenotype is the G1896A variant of the precore region and is usually accompanied by a second G1899A mutation [6]. Variants with these mutations associate with more severe forms of liver damage and the development
of HCC [7, 8]. Studies on the effect of BCP mutations on viral replication remain controversial. Among other studies we have shown that they induce low viral transcription and replication (unpublished data) [9-11]. Precore mutated variants associate with high viral replication or have no effect on viral load (unpublished data) [11-13].

The outcome of HBV infection depends on the intensity and type of anti-viral immunity produced by the host. In acute infection the adaptive immune system mounts a strong, multi-specific response, but its effectiveness depends on the quality of the earlier innate immune response. NK cells are important effectors of the innate antiviral immunity. In addition to the direct killing of viral-infected cells without antigen-specific priming, they regulate the adaptive immune response by producing interferon (IFN)-γ, tumor necrosis factor (TNF)-α and immunoregulating cytokines. NK cells are enriched markedly in the liver, the site of HBV replication, implying that HBV has to evade NK cell-mediated immune responses to establish a persistent infection. In addition the high NK activity early in infection and during the incubation time suggests that they are key players in viral clearance [14-17]. NK cells, however, can also negatively regulate specific antiviral immunity in CHB infection by contributing in the liver inflammation through TNF-related apoptosis-inducing ligand (TRAIL)- and Fas-mediated death [17, 18] and by the direct killing of HBV-specific CD8+ T cells, which triggers the recruitment of inflammatory cells that amplify the hepatic damage [19, 20].

The effector functions of NK cells are tightly regulated by the dynamic and coordinated balance of activating and inhibitory signals. In the present study, the NK cell receptors were selected on the basis of being known to control key cell functions. The main NK cell activating receptors studied in this respect include C-type lectin-like NK group 2D (NKG2D) receptor and immunoglobulin-like receptor NKp30. NKG2D is a potent activating receptor expressed in all NK cells, NKT cells and activated CD8+ T lymphocytes [21, 22]. It binds to a family of ligands (NKG2DL) including the major histocompatibility complex class-I polypeptide-related chain (MHC) protein family that contains MICA and MICB; and the cytomegalovirus UL16-binding proteins (ULBP) family, which consists of five members, ULBP1-4 and RAET1G [23]. Another activating receptor involved in the direct regulation of NK cell-mediated recognition of cellular stress is the DNAX accessory molecule-1 (DNAM-1, CD226), which is constitutively expressed on NK cells, T cells, macrophages and a small subset of B cells [24]. Similar to NKG2D, DNAM-1 not only promotes adhesion and activation of NK cells and CTLs but also greatly enhances their cytotoxicity toward ligand-expressing targets [24]. Furthermore, lectin-like transcript 1 (LLT1, gene clec2d) is a type II transmembrane receptor that belongs to the C-type lectin like (CTL) superfamily of NK cell receptors. Six alternatively spliced transcripts of clec2d exist, with the isoform 1 (designed as LLT1) being the only one presented on the cell surface and hence the only isoform able to participate in the cell-to-cell transmission [25]. LLT1 is expressed mainly on activated lymphocytes, including NK, T cells and B cells, as well as on antigen presenting cells (APC) [26]. However, a decade ago, LLT1 was also identified as a physiological ligand of the NKR-P1 (CD161), expressed on all NK cells and subsets of T cells and represents the only member of the human NKR-P1 subfamily [27, 28]. NK cell cytotoxicity and IFN-γ production is inhibited upon engagement of NKR-P1 on NK cell with LLT1 on target cell [27, 28]. Upon viral infection, LLT1 expression is upregulated on surface of epithelial cells, while IFN-γ has been shown to increase LLT1 expression on B cells and APC [29]. Hence, LLT1-mediated signaling is thought to represent a system that regulates both innate and adaptive responses. For activated NK cells to achieve successful lysis, strong adhesion to target cells that is required is mediated by the β2 integrin LFA1 [30].

The objective of this study was to investigate the modulation of ligands for cell surface NK receptors in chronic HBV (CHB) patients and HepG2 cells transfected with BCP and precore mutants. Furthermore, we aimed to correlate patient’s viral replication and the differential viral capacity of hotspot mutants with the expression of NK receptors and their ligands at the RNA level.

**Patients and methods**

**Patients**

Nineteen patients (11 men and 8 women; mean age 44.5±12 years) with CHB were studied (Table 1). Tissue samples were obtained by percutaneous needle biopsy, snap-frozen and stored in liquid nitrogen. From all the individuals included in the study a serum sample was collected at the time of liver biopsy, stored at -80°C and HBV DNA levels were measured using real-time polymerase chain reaction (qPCR). Individuals with a history of excess alcohol, or positive for HCV, HDV or HIV were excluded. Informed consent was obtained from all the patients (The department of Hepatology St Mary's Liver Tissue Bank, 06/Q0509/39). 1mg of liver tissue was processed using the gentleMACS Dissociator (Miltenyi Biotec SAS, Paris, France) prior to RNA extraction.

**Transient transfection experiments**

The G1896A, G1899A, G1896A/G1899A, A1762T/G1764A and A1762T/G1764A/C1766T mutations were generated by splice extension mutagenesis using as a template a plasmid (p3.8II) containing a 1.2 genome length of HBV subtype adw (genotype C) [31] in a pBluescript II KS (+) background (Stratagene, CA, USA) (Table 2). Full-length HBV genomes were amplified from wild-type (WT) or mutant HBV genomes according to the method described by Günther, using the P1 sense (HBV positions: nt1821-1841) and P2 antisense primers (HBV positions: nt1823-1806) modified to contain the HindIII/ SapI sites and the SacI/SapI sites, respectively (Table 2) [32]. PCRs were performed using the FastStart High Fidelity PCR System (Roche Applied Science, Germany) according to the manufacturer's instructions. Each of the 3.2kb amplified HBV
Hepatitis B virus modulates natural killer ligands

fragments were purified from agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific, Life Science Research, Lutterworth, UK) and were cloned into a pCRII TA-vector (Invitrogen, Paisley, UK). All constructs were sequenced to confirm the presence of the mutations and to ensure that no additional mutations were introduced in the process.
Linear HBV monomers were released by SapI restriction digestion (New England Biolabs, Beverly, MA), gel purified, and then used for transient transfection of HepG2 by lipofection. HepG2 cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal bovine serum, 2 mM L-Glutamine, 100 IU penicillin mL−1 and 100 μg streptomycin mL−1. Cells were seeded at a density of 4x10^5 cells in 60 mm diameter dishes (Corning, Flintshire, UK) and the following day were transfected with 500 ng HBV linear monomers using Lipofectamine Plus (Invitrogen) according to manufacturer’s instructions. The culture medium was changed 24 h after transfection, and cells were harvested at 48 and 72 h.

All transfections included 1 μg of reporter plasmid expressing a green fluorescent protein to monitor transfection efficiency by fluorescence-activated cell sorting analysis and ranged 43-50% while the empty pUC19 vector used as a negative control.

**RNA quantification**

**Northern blot analysis**

For Northern blot analysis, 10 μg of total RNA per sample was separated on a 1% formaldehyde-agarose gel and blotted onto a Zeta- Probe GT membrane (BioRad, Bio-Rad Laboratories, Hercules, CA). Radioactive probes were prepared by random priming, using either full-length HBV DNA or 18S rRNA probes labeled with αdCTP (Amersham). After hybridization the membrane was washed and exposed to X-Omat film (Kodak, Rochester, NY) at -80°C.

**Relative quantification by real-time (RT) PCR**

Total RNA was extracted from HepG2 cells 48 h after transfection with SapI-digested HBV DNA monomers and liver biopsies using Trizol reagent (Invitrogen) as recommended by the manufacturer. The RNA samples were treated with DNase (Qiagen, Crawley, UK) for 10 min at 25°C and were then purified using the RNeasy mini kit (Qiagen). RNA integrity was confirmed by agarose gel electrophoresis under UV. RNA concentrations were monitored by using the nanodrop, and RNA purity was confirmed by agarose gel electrophoresis under UV. RNA samples were treated with DNase (Qiagen, Crawley, UK) for 10 min at 25°C and were then stored at -80°C until further use.

Two μg RNA from each transfection and 500 ng RNA from each liver biopsy were reverses transcribed in 20 μL reactions using the RT2 First Strand kit (Qiagen). There were three biological replicates in each group for each transfection experiment and duplicates for each liver biopsy samples. The resulting cDNA samples were stored at -80°C until further use.

The relative levels of total HBV RNA transcripts concentrations were analyzed by real-time PCR with a TaqMan (Life technologies Paisley, UK) in a Light-Cycle (Roche Diagnostics, West Sussex, UK). Each PCR reaction contained cDNA synthesized from 125 ng of total RNA. The thermocycler parameters were 95°C for 10 sec, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. Relative changes in gene expression for both viral and cell RNA transcripts were calculated using ΔΔCt (threshold cycle) method. Threshold cycle numbers (Ct) above 35 were considered below detection level. The β-ACTIN housekeeping gene was used to normalize the RNA amounts. Results are expressed relative to β-ACTIN with NK markers as 2-ΔΔCt, where ΔΔCt = ΔCt_targetgene - ΔΔCt_control and where ΔCt = Ct_targetgene - Ct_ACT.

**Statistical analysis**

Two group comparisons of continuous variables were performed using the non-parametric Mann-Whitney test with two tailed values (GraphPrism 6). P values below 0.05 were considered statistical significant.

**Results**

**Liver and serum viremia in CHB patients**

We determined the expression of total HBV RNA in the liver of infected patients by RT PCR and compared this to the levels of serum viremia. It is well known that intrahepatic viremia does not correlate with serum viremia since the liver sampled cannot be representative. Indeed the levels of intrahepatic HBV RNA did not correlate with serum HBV viremia (r=0.34, P>0.1) (Fig. 1). We therefore divided the patients into four groups: 1) Low Serum Viremia patients, included those with serum viremia 1x10^4 – 1x10^6 viral copies/mL; 2) High Serum Viremia group, included patients with serum viremia 1x10^-6 – 1x10^-4 viral copies/mL; 3) Low Liver HBV RNA patients, included those liver samples with 1x10^9 – 1x10^10 HBV RNA levels relative to β-ACTIN expression; and 4) High Liver HBV RNA samples with 1x10^9 – 1x10^10 HBV RNA levels.
Expression of NK regulatory molecules

CHB patients. We examined the expression of the key NK cell ligands MICA, ULBP2, ULBP3, B7H6, the NK receptors DNAM-1, LFA-1, TRAIL and LLT1 in the livers of CHB patients (Fig. 2). In general, individuals with higher liver viral loads had higher expression of NK cell ligands and receptors. The exception to this was MICA, which was significantly downregulated in patients with high intrahepatic HBV RNA levels (P=0.02) and decreased in high viremic patients (P=0.05). We also compared the gene expression in 7 HBeAg(+) patients and 12 HBeAg(-) patients (Fig. 3). The expression of ULBP2, ULBP3, LLT1, LFA-1, and DNAM-1 was upregulated in HBeAg(+) patients, the majority of which had increased serum and liver HBV levels, while MICA was downregulated in this group of patients. Interestingly, LLT1 is a ligand for the inhibitory receptor CD161, expressed on NK cells and intrahepatic T cells. Thus, this together with the down-regulation of MICA, may represent potential escape mechanisms for HBV, countering the trends observed for the other NKG2D ligands and also B7H6 the ligand for NKp30. Consistent with the lack of correlation between serum and liver HBV viremia, we observed no consistent trends of NK cell ligand expression and serum viremia.

HBV transfection experiments. We had previously constructed using site directed mutagenesis, the replication-competent plasmids bearing the BCP mutations 1762/64 and 1762/64/66 and the precore 1896, 1899 and 1896/1899 (unpublished data) (Table 2). Using the same HBV constructs and plasmid-free transfection system, we have previously demonstrated that compared to the WT and precore variants, BCP mutations result in lower viral replication capacity while the precore variants show similar replication fitness to the WT. BCP mutants consistently produced the decreased accumulation of all HBV RNA transcripts (Northern blotting) (Fig. 4); the viral replicative intermediates, including cccDNA (Southern blotting); and the culture HB surface antigen (HBsAg) levels (ELISA) (unpublished data).

To investigate whether the differential replication of HBV induces changes in the expression of ligands for NK cell receptors on transfected HepG2 we performed RT qPCR (Fig. 5). Transfection of HepG2 cells induced the up-regulation of all NK cell ligands tested. MICA, ULBP3 and B7H6 transcript levels were not significantly altered after exposure to the WT and HBeAg mutant variants examined (Fig. 5). However we observed a weak trend towards an association of ULBP2 with viremia in that there were reduced levels of ULBP2 mRNA with the BCP mutants compared to WT and increased ULBP2 levels expression with the pre-core variants. However, the expression of LLT-1 in HepG2 transfected with the WT genome was significantly decreased compared to 1762/74 (P=0.01) and 1762/64/66 (P=0.04). Thus, LLT-1 correlated with the BCP mutations and their low replication efficiency. This is consistent with our findings on intrahepatic LLT1 RNA levels.

Discussion

Accumulating evidence suggest that activation of the NKG2D-ligand pathway contributes to the outcome of HBV infection. The unpredictable natural history of CHB infection makes it difficult to sample the immune correlates of viral replication activity longitudinally. The level of HBV DNA in serum is commonly used as a surrogate marker of HBV replication in the clinical setting but it does not reflect the levels of HBV in the liver [33]. To monitor HBV levels, in addition to serum viremia we assessed HBV RNA expression in the same liver sample that we determined NK ligand RNA expression. We found that MICA transcripts were decreased in highly viremic liver samples of CHB patients whereas there was a trend towards increased expression of ULBP2 and ULBP3 with HBV liver viremia. Thus, a general trend towards upregulating expression of NK cell ligands, could be counteracted by decreasing expression of MICA and hence weakening NK surveillance of the infected cells. These patients had minimal inflammation on their liver biopsies and therefore downregulation of MICA could be both harmful by inhibiting NK-induced viral clearance but also protective by preventing the infected hepatocytes from becoming targets for NK cell-mediated killing and hence preventing the development of liver injury. The NK markers B7H6, DNAM-1, LFA-1 and TRAIL were also slightly elevated in high viremic liver samples. Our results indicate that high HBV replication induces the upregulation of gene expression
of these NK ligands. A recent study by Lunemann showed lower levels of NKG2A, DNAM-1 and TRAIL on the NK cells of CHB patients as compared to acute patients [34]. It has been previously shown that higher expression of NKG2D ligands on cancer cells is associated with down-regulation of NKG2D on NK cells related to soluble NKG2D ligand secretion [35,36]. We therefore propose that a similar process occurs in CHB.

In the course of CHB infection, HBeAg-negativity concurs with the emergence of the precore mutation 1896 and 1899 while the BCP mutation 1762/64 and 1762/64/66 reduces HBeAg production. We have previously showed that the BCP mutations result in lower viral replication capacity, whereas the major precore mutations have no effect on viral replication. The expression of all NKG2DL was increased when HepG2 cells were transfected by HBV suggesting that HBV induces NKG2DL upregulation. This finding is consistent with our observation that NK cell ligands, with the exception of MICA, are increased in HBV infection. However, consistent trends for the ligands for activating receptors were not observed between in vivo and in vitro assays nor between HBeAg(+) and HBeAg(-) patients, with the possible exception of ULBP2. In general, NKG2D is a system thought to be important for clearing viral infection, and especially in cytomegalovirus infection, in which the virus has developed multiple mechanisms to down-regulate different NKG2D ligands [37]. Consistent with previous studies, we found that MICA expression was reduced in the
Hepatitis B virus modulates natural killer ligands

Liver of CHB patients [38,39]. Transfection of HepG2 cells with all HBV constructs induced its expression suggesting that early in infection MICA is upregulated but in the persistent infection HBV weakens the immune surveillance of NK cells by inhibiting MICA expression.

Conversely, expression of LLT1 was increased in patients with high serum and intrahepatic viremia. Likewise, in HepG2 transfections we found low but detectable levels of LLT1 that were positively correlated with the differential viral replication of the HBeAg mutated variants. Upregulation of

Figure 3 The RNA expression of natural killer regulators in liver biopsies from 7 HBeAg(-) and 12 HBeAg(+) chronic hepatitis B patients. Gene expression was normalized with β-ACTIN levels and data represents the mean ± standard error of mean (SEM) from 3 independent experiments of the relative gene expression between patients. The ALT levels are shown as the mean ± SEM.

LVL, low viral load; HVL, high viral load; HBeAg, HBV e antigen; HBeAb, ALT, alanine aminotransferases.
LLT1 and its subsequent engagement to their CD161 receptor expressed on NK cells have been shown to inhibit NK-mediated cytotoxicity and IFN-γ production by T cells [27]. LLT1 expression has been reported to be absent in normal liver tissue but upregulated in HCC. Here we show for the first time that LLT1 is expressed in HBV infected liver and its levels associate with viral replication in HBV infection. Thus, HBV may circumvent the immunity of the host by inhibiting NK cytotoxicity and IFN-γ production via the upregulation of LLT1.

A detailed characterization of the molecular players that link the HBV stimuli to the regulation of NK cells will be critical to advance our knowledge on how HBV circumvents the host's immunity. The manipulation of ligand expression can provide new insight on CHB immunopathogenesis and can lead to possibilities of developing effective treatment strategies.
Hepatitis B virus modulates natural killer ligands

Summary Box

What is already known:

- Natural killer (NK) cells play a key role in the outcome of hepatitis B virus (HBV) infection.
- HBV can circumvent host's immunity by modulating the expression of NK receptors and their ligands.
- MICA is ligand of the activating NKG2D receptor and is important in clearing viral infection.
- LLT1 is expressed on NK cells and is a ligand of the NKR-P1 receptor of NK cells.

What the new findings are:

- The gene expression of ULBP2, ULBP3, B7H6, DNAM-1, LFA-1 and TRAIL was elevated in high HBV replication in vivo.
- HBV infection repressed MICA expression in the liver of chronic HBV patients whereas HBV transfection induced its expression, suggesting that early in infection MICA is upregulated.
- LLT1 expression was increased in patients with high serum and intrahepatic viremia.
- In HepG2 transfections LLT1 levels were positively correlated with the differential replication of HBeAg mutated variants.
- A general trend towards upregulating expression of NK ligands can be counteracted by decreasing expression of MICA and hence weakening NK surveillance of the infected cells.

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References


