Research Article

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Viral Hepatitis

Intrahepatic innate immune response pathways are downregulated in untreated chronic hepatitis B

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See Editorial, pages 884–885

Background & Aims: Hepatitis B virus (HBV) persistence and the pathobiology of chronic HBV (CHB) infections result from the interplay between viral replication and host immune responses. We aimed to comprehensively analyse the expression of intrahepatic host genes as well as serum and liver HBV markers in a large cohort of untreated CHB patients.

Methods: One-hundred and five CHB patients untreated at the time of liver biopsy (34 HBeAg[+] and 71 HBeAg[−]) were analysed for the intrahepatic expression profile of 67 genes belonging to multiple innate immunity pathways. Results were correlated to serological (quantification of HBsAg [qHBsAg] and HBV DNA) and intrahepatic viral markers (total HBV DNA, pre-genomic RNA and covalently closed circular HBV DNA).

Results: Intrahepatic gene expression profiling revealed a strong downregulation of antiviral effectors, interferon stimulated genes, Toll-like and pathogen recognition receptor pathways in CHB patients as compared to non-infected controls, which was not directly correlated to HBV replication. A subset of genes [*CXCL10*, *GBP1*, *IFITM1*, *IFNB1*, *IL10*, *IL6*, *ISG15*, *TLR3*, *SOCS1*, *SOCS3*] was more repressed in HBeAg(−) respect to HBeAg(+) patients (median of serum HBV DNA 7.9×10^3 *vs.* 7.9×10^7 IU/ml, respectively). Notably, HBeAg(−) patients with lower qHBsAg ($<5 \times 10^3$ IU/ml) showed a relief of repression of genes belonging to multiple pathways.

Conclusions: Our results show a strong impairment of innate immune responses in the liver of CHB patients. The association of low levels of qHBsAg with gene repression, if confirmed, might prove useful for the identification of patients who would most benefit from immune-modulators and/or HBsAg targeting agents as strategies to restore immune responsiveness.

Lay summary: Chronic hepatitis B virus (HBV) infections represent a major public health problem worldwide. Over 200 million people are chronically infected and at risk of developing chronic hepatitis, liver cirrhosis and cancer. Our work aimed to understand the molecular consequences of chronic hepatitis B in the infected liver. It was conducted in a large cohort of untreated chronically infected HBV patients and analysed the expression of immunity and liver disease-related genes in the liver, with respect to markers of viral replication and persistence. Our results indicate that chronic HBV infection has a suppressive effect on immune responses, which was more pronounced with high levels of hepatitis B virus surface antigen (HBsAg). These data provide novel insight into the mechanisms of HBV persistence in the liver and suggest that approaches aimed at reducing HBsAg levels, may restore immune responsiveness against the virus.

Keywords: HBV; Intrahepatic innate immune response; cccDNA; HBsAg.
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Introduction

More than 240 million people are chronically infected with hepatitis B virus (HBV) worldwide [1]. Chronic hepatitis B (CHB) can evolve towards liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC), which is responsible for over 0.5–1 million deaths per year [2]. Viral persistence is mostly ascribed to the intrahepatic pool of covalently closed circular DNA (cccDNA), which represents the unique template for HBV replication [3–5]. Currently available therapies fail to clear intrahepatic cccDNA. As a consequence, life-long treatments are necessary to avoid relapse after treatment cessation [6–8]. The mechanisms at the basis of the establishment of HBV long-lasting infection are still a matter of debate, but the active inhibition of host immune responses by HBV might play a role in favouring chronicity. Indeed, there are several lines of evidence that HBV interferes with the host immune response by multiple mechanisms, including the

Research Article

impairment of interferon (IFN)α signalling [9–11], HBx-dependent downregulation of innate signalling proteins [12] and the induction of immune tolerance by decreasing Toll-like receptors (TLRs) activity or by inducing immunosuppressive cytokines [13]. The interplay between viral replication and host immune responses in CHB patients translates into distinct stages of disease, typically divided into four phases: immune-tolerant (IT), HBeAg(+) active chronic hepatitis or immune-active (IA), HBeAg(−) active chronic hepatitis and the HBeAg(−) inactive carrier status (IC), reflecting the host immune control. The long-term course is variable, ranging from virological remission after decades of IC status to the development of cirrhosis, and progression to HCC [14]. Quantification of serum HBs antigen (qHBsAg) and serum HBV DNA, reflecting viral load, have been recently proposed to define the status of IC, predict viral reactivation [15–17] and to guide the management of current antiviral therapies [18,19]. The role of qHBsAg and serum HBV DNA as surrogate markers of intrahepatic cccDNA activity is debated [20–22]. Indeed, several *in vivo* studies indicate that serum HBV DNA is strongly correlated to intrahepatic cccDNA and total HBV DNA in both HBeAg-positive (HBeAg(+)) and −negative (HBeAg(−)) patients, while qHBsAg level is correlated to intrahepatic HBV replication markers only in HBeAg(+) individuals [20–22]. These data suggest that the relationship between serological markers and intrahepatic HBV replicative status is complex and probably affected by host factors. Due to the lack of specific animal models that recapitulate the complexity of the natural history of CHB over several decades and the limited availability of human liver samples, no exhaustive information is available about the interaction between the virus and host immune response modulation *in vivo* in chronically HBV-infected individuals. Furthermore, it has been clearly shown that HBV specific host immune responses should be analysed in the liver compartment, because immune cells in the blood circulation do not accurately reflect their status within the infected liver [23]. Studies performed in transgenic mouse models [24,25], and those of experimental acute infection in chimpanzees [26] and woodchucks [27] have contributed to uncover immunological mechanisms involved in the establishment of infection and clearance, but virus-host interactions during persistent infection are still ill defined. Indeed, one study has addressed this issue taking advantage of the woodchuck model of chronic infection [28], but results obtained recently in the woodchuck and chimpanzee models of chronic infection with TLR7 agonists have been difficult to translate to the human situation [29–31]. This emphasizes the need for translational studies to be performed in chronically infected patients. Only very recently, a first analysis of intrahepatic immune genes signatures has been performed in a small number of HBeAg(+) CHB patients, including three patients in the immune tolerance phase and 16 in the immunoactive phase [32].

In this context, our aim was to comprehensively analyse HBV replicative markers both in serum and liver, concomitantly with the intrahepatic host gene expression response in a large cohort of untreated CHB patients in different phases of the disease.

Patients and methods

Patients

One-hundred and five untreated CHB patients (30 women and 75 men) with concomitant liver biopsy and serum samples, followed at the Hepatology Unit of the "Hospices Civils de Lyon" and at the Gastroenterology and Hepatology Unit of the "Ospedale Maggiore Policlinico" in Milan, were retrospectively enrolled for the study. All patients provided written informed consent and the protocol was approved by the Lyon and Milan institutional Ethic Committees. Patients underwent needle liver biopsy for routine histology and part of the sample was stored at −80 °C. All patients were negative for serological markers of hepatitis C virus (HCV), human immunodeficiency virus and hepatitis D virus infections. Age ranged from 18 to 66 years (median 41 years) (Table 1). CHB patients were classified according to the typical phases of the natural history of CHB, following EASL recommendations [2,33]. Three patients out of 105 received a course of IFN therapy more than 10 years before enrolment in the study. One additional patient out of 105 was treated with lamivudine for few months 4 years before the liver biopsy and the enrolment in the study. All other patients were treatment naive, without a history of IFN or nucleos(t)ide analogues (NUC) therapy. Non-infected control liver samples were collected at the University of Rome "La Sapienza" (Ethical committee authorization n. 738/05) and were chosen among patients without chronic liver disease and for which liver samples did not show any sign of liver histological alteration: six patients underwent liver surgery for gallbladder stones (four males, two females); three patients underwent abdominal surgery for non-liver-related diseases (one female with ovarian cystadenoma; one male and one female with non-metastatic colon cancer).

Quantification of HBV virological and serological parameters in blood

HBeAg status was determined by using Abbott ARCHITECT® HBeAg assay (Abbott Diagnostic, Chicago, IL, United States). Serum HBV DNA quantification was performed by quantitative PCR using COBAS® AmpliPrep/COBAS TaqMan® HBV test (Roche Diagnostics, Manheim, Germany). The detection range was between 20 IU/ml and 1.7×10^8 IU/ml. Serum quantitative HBsAg (qHBsAg) was quantified with Elecsys® HBsAg II kit/COBAS e411® (Roche Diagnostics, Manheim, Germany) according to manufacturer's instructions.

Histologic analysis

Fibrosis and necroinflammatory activity were scored using METAVIR classification [34] by the pathology services of Hospices Civils de Lyon and Ospedale Maggiore Policlinico in Milan. According to EASL practice guidelines for HBV treatment [2], patients were divided into two groups for both necroinflammatory activity and fibrosis scores: none or mild (below or equal to 1) *vs.* moderate to severe (above 1).

DNA and RNA extraction from liver biopsies

DNA and RNA were extracted from snap frozen human liver needle biopsies [20,35]. Liver samples were first homogenized on ice using a TissueRuptor (Qiagen®, Hilden, Germany) in homogenization buffer (Tris HCl pH 8, 50 mM; EDTA 1 mM; NaCl 150 mM), then divided in two pieces and processed for DNA and RNA purification, respectively. After proteinase K digestion, DNA was isolated using MasterPure DNA Purification Kit (Epicentre®, by Illumina, Madison, United States). RNA was isolated according to the manufacturer's protocol (NucleoSpin® Total RNA and Protein Isolation Kit, Macherey-Nagel, Düren, Germany). Complementary DNA synthesis was performed using the SuperScript™ III first-Strand Synthesis SuperMix for qRT-PCR (Invitrogen by Life Technologies©, Carlsbad, United States). Quantity and integrity of the extracted DNA and RNA and synthetized cDNA were assessed by NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Quantification of HBV DNA and pre-genomic (pg)RNA in liver samples

Quantification was performed using the LightCycler® 480 Real-Time PCR System (Roche diagnostics, Manheim, Germany) with primers and fluorescence dual hybridization probes specific for total HBV DNA or covalently closed circular (ccc)DNA [8]. Before cccDNA amplification, DNA was treated with Plasmid-safe DNase (Epicentre by Illumina) to get rid of contaminating rcDNA. Serial dilutions of a plasmid containing an HBV monomer (pHBV-*Eco*R1) served as quantification standard. To normalize the number of viral copies per cell content, the number of cellular genomes was determined by using the β-globin gene kit (Roche Diagnostics, Manheim, Germany). The range of quantification was comprised between 10^1 and 10^7 copies of HBV genome/well for both cccDNA and total HBV DNA assays. Patients' samples were independently analysed in duplicate. cccDNA quantification fell under the limit of detection for 6/105 patients (all were HBeAg(−)). The intrahepatic cccDNA productivity was defined and calculated as the ratio of intrahepatic total HBV DNA over cccDNA. For pgRNA detection,