Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Influence of mutations in hepatitis B virus surface protein on viral antigenicity and phenotype in occult HBV strains from blood donors

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Background & Aims: This study aimed at investigating mutations in the hepatitis B surface protein (HBsAg) in occult hepatitis B virus (HBV) infection (OBI) and their influence on viral antigenicity and phenotype.

Methods: The characteristics of 61 carriers with OBI (OBI group), 153 HBsAg(+) carriers with serum HBsAg ⩽100 IU/ml (HBsAg-L group) and 54 carriers with serum HBsAg >100 IU/ml (HBsAg-H group) from 38,499 blood donors were investigated. Mutations in the major hydrophilic region (MHR) of the viral sequences were determined. Thirteen representative MHR mutations observed in OBI sequences were antigenically characterized with a panel of monoclonal antibodies (MAbs) and commercial HBsAg immunoassays and functionally characterized in HuH7 cells and hydrodynamically injected mice.

Results: Of 61 OBI sequences, 34 (55.7%) harbored MHR mutations, which was significantly higher than the frequency in either the HBsAg-L (34.0%, $p = 0.003$) or the HBsAg-H group (17.1%, $p < 0.001$). Alterations in antigenicity induced by the 13 representative MHR mutations identified in the OBI group were assessed by reacting recombinant HBV mutants with 30 different MAbs targeting various epitopes. Four out of the 13 mutations (C124R, C124Y, K141E, and D144A) strongly decreased the analytical sensitivity of seven commercial HBsAg immunoassays, and 10 (G119R, C124Y, I126S, Q129R, S136P, C139R, T140I, K141E, D144A, and G145R) significantly impaired virion and/or S protein secretion in both HuH7 cells and mice.

Conclusions: MHR mutations alter antigenicity and impair virion secretion, both of which may contribute to HBsAg detection failure in individuals with OBI.

Keywords: HBsAg mutants; Occult HBV infection; Antigenicity; Secretion deficiency.

Received 5 December 2011; received in revised form 26 April 2012; accepted 12 May 2012; available online 23 May 2012

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; MHR, major hydrophilic region; HBeAg, hepatitis B e antigen; HBcAg, hepatitis B core antigen; L-HBsAg, large hepatitis B surface antigen; anti-HBs, antibodies against hepatitis B surface antigen; anti-HBc, antibodies against hepatitis B core antigen; OBI, occult HBV infection; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; CLEIA, chemiluminescent enzyme immunoassay; CLIA, chemiluminescent immunoassay; ECLIA, electrochemiluminescence immunoassay.

Introduction

Hepatitis B virus (HBV) persistently infects over 350 million people worldwide and leads to hepatitis, liver cirrhosis, and hepatocellular carcinoma. The hepatitis B surface protein (HBsAg) is an envelope glycoprotein that currently serves as primary target for diagnosis and immunoprophylaxis of HBV infection. The dominant epitopes of HBsAg, which are the targets of neutralizing B cell responses, reside in the "a" determinant (aa 124–147) within the major hydrophilic region (MHR). Amino acid substitutions in the MHR can cause reduced binding of anti-HBs antibodies, resulting in immune escape. The most common MHR mutation, G145R, was initially described in 1990 [1]. The emergence of single or multiple amino acid (aa) substitutions at this and other positions within the MHR has been observed in infants born to HBsAg(+) mothers who received the HBV vaccine with or without HBIG, in liver transplant recipients who received HBIG, in patients who experienced HBsAg loss after anti-HBV therapy, and in individuals with occult HBV infection (OBI) [2–4].

Occult HBV infection is an atypical form of HBV infection, which is defined as the long-term persistence of HBV DNA, despite undetectable HBsAg by regular immunoassays [5,6]. Since its initial description in the late '70s, OBI has been a challenge in hepatitis B research, with its unique virological and clinical characteristics. Evidence from different geographical regions has demonstrated that OBI was distributed worldwide and was potentially a major source of HBV transmission by transfusion

and organ transplantation [7]. Acute viral reactivation may occur in association with immunosuppressive therapies and/or immunodeficiency [8]. In addition, OBI virus maintains its pro-oncogenic properties [9]. High frequencies of MHR mutations have been observed in OBI strains of individuals from Europe and Africa [10,11]. Generally, the modified proteins produced by MHR-mutated S genes, which are not well recognized by current HBsAg immunoassays, are considered a key cause of detection failure of HBsAg in OBI. However, the influence of MHR mutations on the characteristics of viral antigenicity and phenotype, which is important for uncovering the mechanism of OBI, has not been systematically investigated.

In this study, we investigated the molecular characteristics of OBI strains from a large cohort of blood donors. Representative MHR mutations were detected and were functionally characterized both *in vitro* and *in vivo*.

Materials and methods

Specimens

From 2007 to 2010, 38,499 blood donations were collected by the Xiamen Blood Service (Xiamen, China). Among these specimens, 308 were HBsAg(+) and 38,191 were HBsAg(−) by ELISA (Murex-V3, Abbott-Murex, UK). The serum HBsAg levels of HBsAg(+) specimens were determined using CMIA (Architect HBsAg, Abbott Laboratories, USA). Of the 38,191 HBsAg(−) specimens, 70 yielded repeatable positive results by Cobas Taqscreen (Roche Inc., Switzerland) or nested PCR, as described elsewhere [4]. Anti-HBs and anti-HBc antibodies were measured using commercial kits (Wantai, Beijing, China). Serum HBV DNA levels, viral genotypes and viral S gene MHR sequences (aa 110–160) were determined as previously described [4]. Amplified products were directly sequenced and analyzed using MEGA5. The experiments conformed to the 1975 Declaration of Helsinki and were approved by the Ethics Committee of the Xiamen Blood Service.

Virus and antibodies production

HBV replicons containing MHR mutations detected in OBI strains were constructed by site-directed mutagenesis (Supplementary Table 1). Recombinant viruses were produced in HuH7 cells and purified by ultracentrifuge. The S protein concentrations of virus samples were measured using an in-house HBsAg mass-ELISA kit, which utilizes monoclonal antibodies (MAbs) targeting aa 57–79 of HBsAg. Thirty mouse MAbs against HBsAg were produced by standard hybridoma technology and were characterized by strip immunoblot assay (SIA) and peptide epitope mapping, as described in Supplementary Materials and methods.

Antigenicity analysis

Quantitative purified viruses were tested with anti-HBs MAbs by using an indirect chemiluminescent immunoassay as described in Supplementary Materials and methods. Moreover, these viruses were tested with seven HBsAg immunoassays: Assay A (Murex-V3, Abbott-Murex, UK), Assay B (Monolisa-Ultra, Bio-Rad, France), Assay C (Hepanostika-Ultra, BioMerieux, France), Assay D (XC-HBsAg, InTec, China), Assay E (WT-HBsUltra, Wantai, China), Assay F (Architect-HBsAg, Abbott-Laboratories, USA), and Assay G (Elecsys-HBsAg, Roche-Diagnostics, USA) according to the manufacturers' recommendations.

In vitro and in vivo HBV phenotyping

The capability of HBV transcription, replication, protein expression, and virion secretion of MHR mutants was evaluated by transfection in HuH7 cells and hydrodynamic injection in mice, as previously described [12,13]. Methodological details are provided in Supplementary Materials and methods.

Statistical analysis

The unpaired *t*-test and the Kruskal–Wallis ANOVA were used to compare continuous variables, while the Mantel–Haenszel χ^2 test or the Fisher's exact test was used for categorical variables. Differences were considered significant at a 2-tailed $p < 0.05$. Calculations were performed with SPSS 17.0.

Results

MHR mutants in blood donors with OBI

The MHR (aa 110–160) of the viral S gene was successfully amplified and sequenced in 61 out of 70 OBI samples (OBI group) and in 207 out of 308 HBsAg(+) samples. These specimens were used for further molecular characterization. When HBsAg(+) individuals were divided into two groups according to their HBsAg level by a cut-off value of 100 IU/ml, which was widely recognized as a predictor of HBsAg seroclearance in CHB [14–16], 153 donors had a HBsAg level ⩽100 IU/ml (HBsAg-L group) and the remaining 54 donors had a HBsAg level >100 IU/ml (HBsAg-H group). The serological and virological characteristics of the three groups with different serum HBsAg levels are shown in Table 1. Molecular analyses revealed that the viral MHR mutation frequency (amino acid substitution) in the OBI group (34/61, 55.7%) was significantly higher than that of the two HBsAg(+) groups ($p = 0.003$ *vs.* the HBsAg-L group and $p < 0.001$ *vs.* the HBsAg-H group). Moreover, among the sequences obtained from HBsAg(+) individuals, the viral MHR mutation frequency was significantly higher ($p = 0.003$) in the HBsAg-L group (52/153, 34.0%) than in the HBsAg-H group (7/54, 13.0%). Using the HBsAg level of 200 IU/ml or 10 IU/ml as cut-off, results were similar to those obtained at 100 IU/ml (Supplementary Tables 2 and 3).

This result, observed with sequences of both genotype B and C, suggests a strong association between MHR mutations and decreased serum HBsAg.

All the mutations at different positions, observed in the three groups, are illustrated in Table 1 (the MHR amino acid variability of the sequences from the OBI group is shown in Supplementary Fig. 1). We selected G119R, P120T, C124R, C124Y, I126S, Q129R, S136P, C139R, T140I, K141E, D144A, G145A, and G145R for further functional analysis, according to the following criteria: (1) with a high prevalence in the OBI and/or HBsAg-L group; and (2) associated with OBI as described in previous studies (Fig. 1A and Supplementary Table 4). These mutations were rarely observed in the HBsAg-H group. Additionally, the M133T/L mutation, which was found to be more frequent in the L-HBsAg group than the OBI group (12.4% *vs.* 3%, $p = 0.04$), was also investigated *in vitro*.

Antigenicity of MHR mutants to an MAb panel

Recombinant viruses carrying the above mentioned representative mutations were produced in cells and were subjected to reaction with an anti-HBs MAb panel. The panel contained 30 MAbs, selected from over 300 clones, including 19 MAbs against linear epitopes and 11 MAbs against conformational epitopes (Fig. 1C). According to a cluster analysis of MAbs' reactivity profiles toward various mutants (Fig. 1D), MAbs could be grouped into seven classes (A–G). Epitope mapping revealed that class A MAbs bound epitopes on the "first loop N-terminus" (aa 113–127), class D was specific for epitopes on the internal loop of HBsAg (aa 57–79), class F bound epitopes surrounding the