Characteristics of hepatitis B virus genotype G coinfected with genotype H in chimeric mice carrying human hepatocytes

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ABSTRACT

Accumulated evidence indicated that hepatitis B virus genotype G (HBV/G) is present exclusively in coinfection with other HBV genotypes. In Mexico, HBV/G from 6 men who had sex with men were coinfected with HBV/H. Phylogenetically complete genomes of the 6 Mexican HBV/G strains were closely related to previous ones from the US/Europe. Using uPA/SCID mice with human hepatocytes, monoinfection with HBV/G did not result in detectable HBV DNA in serum, whereas superinfection with HBV/G at week 10 inoculated HBV/H when HBV/H DNA was elevated to \( N \sim 10^7 \) copies/mL has enhanced the replication of HBV/G. The HBV/G was enhanced in another 3 inoculated with a serum passage containing HBV/G with a trace of HBV/H. Coinfection of mice with HBV/G and H induced fibrosis in the liver. In conclusion, the replication of HBV/G can be enhanced remarkably when it is coinfected with HBV/H. Coinfection with HBV/G may be directly cytopathic in immunosuppressive conditions.

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Introduction

On the basis of the nucleotide sequence divergence exceeding 8% throughout the complete genome sequence, hepatitis B virus (HBV) has been classified into eight genotypes designated by capital letters A (HBV/A) through H (HBV/H) (Arauz-Ruiz et al., 2002; Norder et al., 1994; Okamoto et al., 1988; Stuyver et al., 2000). The genotypes have different geographical distributions, virological characteristics and clinical manifestations (Magnius and Norder, 1995; Miyakawa and Mizokami, 2003).

One of the less studied genotypes is the HBV/G. It was first described in 2000, among inhabitants of France and Georgia, USA (Stuyver et al., 2000). The isolated strains had 36 base-pairs’ insertion in the core gene and two stop codons in the precore region depriving the ability of the virus to translate HBeAg. Nevertheless, some of the carriers were HBeAg positive (Stuyver et al., 2000) that was shortly after explained by the coexistence of the “HBeAg-potent” HBV/A strains in coinfection (Kato et al., 2002a,b). Further studies reported circulation of the genotype in Thailand (Suwannakarn et al., 2005), Japan (Ozasa et al., 2006) and Mexico (Sanchez et al., 2007) indicating global distribution and association of the infection with specific risk groups, such as injection drug users (IDU) and men who had sex with men (MSM). The studies also demonstrated that throughout the world HBV/G strains possess unprecedented genetic homology and are mainly present in coinfection with another endemic genotype. However, little is known about peculiarities of interaction of the HBV/G with various genotypes as well as about virological and clinical concerns of the coinfection.

Produced by genetic engineering, a mouse with severe combined immunodeficiency, carrying urokinase-type plasminogen activator transgenes controlled by albumin promoter (uPA/SCID) with transplanted human hepatocytes (Heckel et al., 1990; Rhim et al., 1994) was recently shown as an appropriate animal model for studying HBV (Dandri et al., 2001; Tsuge et al., 2005). Using this model it was demonstrated that during monoinfection, HBV/G might be able to replicate in hepatocytes at low level; but its replication was...
significantly enhanced by coinfection with HBV/A or C (Sugiyama et al., 2007).

Our purpose is to determine the complete sequences of HBV/G coinfected with HBV/H in sera obtained from MSM in Mexico and to elucidate the characteristics of HBV/G in coinfection with HBV/H using recently developed in vivo model.

Results

Phylogenetic relatedness of HBV complete genome sequences in Mexico

Six HBV/G strains in coinfection with HBV/H among MSM in Mexico were described in our previous study (Sanchez et al., 2007). In the present study, the complete genome sequences of not only 6 HBV/G strains but also 6 HBV/H strains from the same patients were determined by each specific PCR. The phylogenetic analyses indicated that the 6 HBV/G strains were close to those previously reported from the United States (US), France and Germany, and the 6 HBV/H strains were related to the previously reported ones in the US (Fig. 1). All 6 coinfected patients were positive for HBeAg, and asymptomatic carriers of chronic HBV infection at the time of sample collection (the details unknown).

Characteristics of HBV/G strains in Mexico

Sequence analyses revealed unique insertion of 36 nt in the core gene, two stop codons in the precore region and double mutation in the core promoter (CP) in all Mexican HBV/G strains, whereas no related mutations were found in the corresponding HBV/H strains (Fig. 2a and b). These data suggest that the HBeAg detected in serum of those patients had been produced by HBV/H. Additionally, several mutations, which might have affected the replication of the virus genome and amino acid substitutions of HBx, were found in the first half of the CP region, including the above double mutation (nt 1701–1765) (Fig. 2a).

Examining the genetic diversity and recombination of HBV/G

Comparing the complete sequences, both overall genetic distance among the HBV/G strains (0.0037 ± 0.0005 per site) and percent nucleotide homology (0.30 ± 0.24%) were much lower than those among the other intra-genotype groups. In consideration of previously reported recombination between HBV/A and HBV/G strains (i.e. AB056516) (Kato et al., 2002a), we have examined the Mexican HBV/G strains for possible event of intergenotypic recombination. Complete sequences of 3 to 5 clones isolated from each of the 6 HBV/G carriers revealed; no evidence of recombination by similarity and bootstrap scan (data not shown).

Intracellular expression of HBV DNA and antigens

Huh7 cells were transfected with a pUC19 vector carrying 1.24-fold the HBV genome. Three days post-transfection, they were harvested, lysed with NP-40 and tested for HBV DNA and antigens. The density of single-stranded (ss) HBV DNA was compared between HBV/G and H by Southern blotting. The expression of HBV DNA was higher for HBV/H than G, indicating that HBV/G had very low replication in vitro (Fig. 3a). As well, HBsAg, HBeAg and HBcrAg levels were much higher in HBV/H (Fig. 3b).

Superinfection with HBV/G on mice infected with HBV/H

Chimeric mice were infected with HBV/G and H particles propagated in Huh7 cells in order to confirm the infective efficiency. Monoinfection with HBV/G from the Huh7 cells culture medium did not result in detectable HBV DNA in mice serum (data not shown). Then, according to our previous method (Sugiyama et al., 2007), the dynamics of HBV DNA, HBsAg and HBeAg assessed in 3 chimeric mice (ChiM_H1–H3) with HBV/G on H superinfection, are shown in Fig. 4 (a–c). Initially each of the mice received inoculation of around 10^5 copies of HBV/H recovered from the Huh7 cells culture supernatants, and the dynamics of HBV/H DNA indicated approximately 2 logs elevation within the following 5 weeks. At week 10 when HBV/H DNA level exceeded concentration of ~10^7 copies/mL, the chimeric mice were superinfected by inoculation of HBV/G. The HBV/G DNA level increased within 5 weeks after the superinfection and plateaued around 10^7 copies/mL. Two HBV antigens (HBsAg and HBeAg) waxed and waned in profiles similar to that of HBV DNA.
Fig. 2. Nucleotide sequences spanning (a) core promoter and (b) precore gene in HBV genotypes A–H. The reference sequence is shown for the AB014370Ae. The precore region includes pregenome encapsidation signal. Positions of the double mutation (A1762T and G1764A), T1753C, and C1858T making a pair with G1896A are indicated by inverted triangles.

Two stop codons in the precore region are shaded.
Coinfection of mice with HBV/H and G by inoculation with a mouse passage of G-on-H superinfection

Another 3 chimeric mice (ChiM202-17, ChiM212-22 and ChiM314-12) received serum from sacrificed ChiM_H2 with G-on-H superinfection taken at week 34 when the HBV/G and H DNA was around 5×10^6, 10^8 copies/mL, respectively (Fig. 4b). Profiles of HBV/H and G, after inoculation with 10^8 copies of HBV DNA, were similar among the 3 chimeric mice. Despite receiving the inoculation with a mouse passage supposedly containing HBV/G strain, the HBV/G DNA was not detectable until week 4 after the passage. At the week 4 when HBV/H DNA level exceeded concentration of >10^7 copies/mL, HBV/G started to increase and plateaued around 10^8 copies/mL at week 16 (Fig. 4d).

Cloning and sequencing HBV DNA in chimeric mice coinfected with HBV/H and G

HBV DNA clones from sera of the ChiM_H1 and ChiM_H2 sampled at 26 and 34 weeks, respectively (Fig. 4a and b) included those of HBV/H and G invariably. At least 5 clones were propagated and completely sequenced in each serum; but no mutation was observed when the clones were compared to the original inoculum of either genotype. No evidence of recombinations was detected between HBV/H and G inevitably. At least 5 clones were propagated and completely sequenced in each serum; but no mutation was observed when the clones were compared to the original inoculum of either genotype. No evidence of recombinations was detected between HBV/H and G on the basis of complete genome analyses.

Pathology in the liver of chimeric mouse infected with HBV/G and H

Fig. 5a shows histology of liver of a chimeric mouse 26 weeks after superinfection with HBV/G on H (ChiM_H1). The mouse coinfected with HBV/G and H revealed fibrosis of stage 1 (F1) and inflammation of grade 2 (A2) with Hematoxlin–Eosin and Masson’s trichrome stain (Fig. 5a), whereas the mouse monoinfected with HBV/H had no fibrosis (Fig. 5b). ChiM_H2 also had F1A1 at week 34, but ChiM_H3 was not available for histological examination due to sudden death.
actively (Sugiyama et al., 2007), suggesting that the replication of HBV/G might be also enhanced by core protein of HBV/H. Two other functional analyses of HBV/G had been already reported. Kremsdorf et al. have proposed the involvement of polymerase encoded by HBV/G in active replication (Kremsdorf et al., 1996) and Li et al. showed that lack of HBeAg expression rather than a replication defect could be the primary determinant for the rare occurrence of HBV/G monoinfection (Li et al., 2007). Hence, possibility remains for other viral elements beyond core protein from coinfecting genotypes to enhance the replication of HBV/G. Further studies are needed to elucidate the mechanism of HBV/G replication by cotransfection of other proteins beyond core protein of HBV/H or construction of domain-switch experiments between the genome of HBV/G and H.

Considering that coinfection with HBV/G may be associated with pathological manifestations, liver histology was investigated in tissue obtained from the mice used in this study. The chimeric mouse co-infected with HBV/G and H had developed fibrosis and inflammation (F1–2, A1–2) in the liver that was not observed in mice with HBV/G or H monoinfection. This might be supported by clinical data in Mexico; Mexican patients infected with the most prevalent HBV/H have milder liver damage when it is in monoinfection rather than in coinfection with other genotypes (personal communication). Our recent study also showed fibrosis of F1–F2 stage in the majority of the mice superinfected with HBV/G on A or C (Sugiyama et al., 2007). Clinically, Lacombe and her colleagues reported more severe fibrosis in human immunodeficiency type-1 (HIV)-positive French patients who were infected with HBV/G than the others (Lacombe et al., 2006). Taken together, clinical and experimental observations indicate that in immunodeficient conditions HBV/G possesses stronger disease-inducing capacity when it is coinfected with other genotype. Unfortunately, studied patients did not have exact diagnosis due to neither histopathological examination nor abdominal ultrasonography, although they were asymptomatic.
Further prospected studies are required to investigate whether HBV DNA levels (viral replication) and/or other factors would affect liver fibrosis in immunosuppressive conditions.

Two remarkable viral genomic characteristics of the HBV/G have been established by previous reports; one of them is the unique insertion of 36 bp in the core gene, which is shared by all HBV/G strains studied herein and previously (Fig. 2). Although screening of the entire DNA Genome Bank (BLAST search) did not reveal any homologous to the HBV/G strains, the peak of HBV DNA was around 10^8 copies/mL. (c) Another chimeric mouse (ChiM202-17) received serum from ChiM_H2 with G-on-H superinfection revealed F2A2 at week 24.

In conclusion, the replication of HBV/G can be enhanced remarkably when it is coinfected with HBV/H prevalent in Mexico. Coinfection with HBV/G may be directly cytopathic in immunosuppressive conditions. Further epidemiological, clinical and in vitro studies are required to confirm the clinical manifestation of the HBV/G coinfection with various genotypes and evaluate its genotypic peculiarities.

**Methods**

**Serum samples**

Nineteen HBsAg-positive sera were obtained from previously described MSM cohort (age range 22–30 years) in Mexico (Sanchez et al., 2007). HBeAg was detected with chemiluminescent enzyme immunoassay (Ortho Clinical Diagnostics, Tokyo, Japan).

**Determination of HBV genotypes and the complete sequences of HBV**

Initially, HBV genotypes were determined by EIA with monoclonal antibodies directed to the preS2 epitopes (Usuda et al., 1999, 2000), with use of commercial kits (HBV GENOTYPE EIA, Institute of Immunology Co., Ltd., Tokyo, Japan), allowing the determination of HBV/A–F and HBV/H. HBV/G was confirmed by the G-specific PCR with one of the primers deduced from the sequence of 36 nt. insertion in the core gene (Kato et al., 2001). The complete genomes were further determined. In brief, two partially overlapping fragments were amplified by nested PCR using two sets of primers with LA Taq (TaKaRa Bio Inc., Tokyo, Japan) (Table 1). Similarly, the complete genomes of HBV/H strains were also amplified as two overlapping fragments using different set of primers shown in the Table 2. Thereafter, the PCR products were cloned in a plasmid pGEM-T easy vector (Promega Corp., Madison, WI, US) and sequenced using sequencing primers (Tables 1 and 2) with Prism Big Dye (Applied Biosystems, Foster City, CA, US) on the ABI 3100 DNA automated sequencer. Reference sequences for comparative analyses were retrieved from the DDBJ/EMBL/GenBank database.

**Plasmid constructs of HBV DNA**

HBV DNA from a Mexican patient (MEX33) was extracted from 100 μL of serum using the QIAamp DNA blood kit (QIAGEN, GmbH, Hilden, Germany). Two overlapping fragments, fragment A and fragment B approximately 1700 bp long, covering the entire genome

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5′–3′)</th>
<th>Positiona</th>
<th>Polarity</th>
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<tr>
<td>1) Fragment A</td>
<td>HBVG3157F CTCCTGACCTCCAAGATCG 3157–3176</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBVG3178R AGCCAAAAAGGCCATATGGCA 1937–1957</td>
<td>Antisense</td>
<td></td>
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<td></td>
<td>HBVG3187F AGGCACGTACTTCCTACTCT 3178–3208</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBVG1797R CATGGTCGCTTGTCGACAGAC 1817–1947</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td>2) Fragment B</td>
<td>HBVG1630F CTCATCATCTGCCAGCTGTC 1631–1651</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBVG1601F ACGTACATTAAAAGCCGCGA 1946–1966</td>
<td>Antisense</td>
<td></td>
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<tr>
<td></td>
<td>HBVG1013R TGGGTAAAAGGAGCAGCGAAAC 1034–1054</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBVG894F AAGTTGGGGTACTTTGCCAC 894–913</td>
<td>Antisense</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HBVG56R GAACTGGAGCCACCAGG 75–95</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>3) Sequencing primers</td>
<td>HBVG2491F TTCCTTGGACTCACAAGGTG 2249–2270</td>
<td>Sense</td>
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<tr>
<td></td>
<td>HBVG2052F GGGAATCCTTAGAGTCCTCTG 2052–2072</td>
<td>Antisense</td>
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<td>HBVG2510F AAATGTGAGCCACAGG 55–75</td>
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<td></td>
<td>HBSF2 CTTCATCCTGCTGCTATGCCT 407–427</td>
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<td>HBVG894F AAGTTGGGGTACTTTGCCAC 894–913</td>
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<td></td>
<td>HBVG1013R TGGGTAAAAGGAGCAGCGAAAC 1034–1054</td>
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<td></td>
<td>HBVG2052F GGGAATCCTTAGAGTCCTCTG 2052–2072</td>
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<tr>
<td></td>
<td>HBVG2510F AAATGTGAGCCACAGG 55–75</td>
<td>Sense</td>
<td></td>
</tr>
</tbody>
</table>

* Nucleotide position of reference sequence (AB056513).
of HBV, were amplified by nested PCR (primers sequences shown in Table 2). Primers used for fragment A were HBVH55F and HBVH1801R for 1st PCR and HBVH55F and HB6R for 2nd PCR. Primers used for fragment B were HBVH1611F and HBVH285R for 1st PCR and HBVH1611F and HBVH229R for 2nd PCR. Then these fragments were ligated into pgEM-T vector (Promega, Madison, WI) and cloned in DH5α cells. Ten clones each (pGEM-fragA-1 to 10, pGEM-fragB-1 to 10) were obtained and the nucleotide sequences were determined. As reported previously (Fujiwara et al., 2005; Sugiyama et al., 2006), these fragments were constructed into the plc19 vector deprived of promoters (Invitrogen Corp., Carlsbad, CA) by digestion with HindIII and EcoRI, resulting in 1,24-fold the HBV genome, just enough to transcribe over-sized pregenome and precore mRNA. Cloned HBV DNA sequences were determined with Prism Big Dye (Applied Biosystems, Foster City, CA) in the ABI 3100 automated sequencer. Based on our previous report (Sugiyama et al., 2007), a plasmid of HBV/G with 1,24-fold the HBV genome was also constructed in this study.

Cell culture and transfection

HuH7 cells were transfected with plasmids equivalent to 5 μg of HBV DNA constructs with use of the Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN), and harvested after 3 days in culture. Transfection efficiency was monitored by enzymatic activity of secreted alkaline phosphatase (SEAP) in the supernatant of culture with addition of 0.5 μg of reporter plasmids expressing the SEAP.

Determination of HBV markers

HBsAg and HBeAg were determined by chemiluminescent enzyme immunoassay (CLEIA) with commercial kits in a fully automated Lumipulse f CLEIA analyzer (Fujirebio Inc., Tokyo, Japan), and the HBeAg concentration was estimated by comparison to a standard curve generated using recombinant HBeAg. In the present study, the cutoff value was tentatively set at 3.0 log U/ml mL. Sera containing over 7.0 log U/ml mL of HBeAg were diluted 10–100-fold in normal human serum and re-tested to obtain the end titer.

Detection and quantification of serum HBV DNA

For HBV/G-specific real-time detection PCR (RTD-PCR), previously reported set of primers was used, where one of the primers contained sequence of the HBV/G unique 36-bp insertion (Sugiyama et al., 2007). For HBV/H DNA quantification, following primers were applied; HBVH29F: 5′-GGT CCA CCA ACT GTG CGG-3′, HBV229R: 5′-CGA TGC TAG ACT CTT GGG TAT-3′. Amplification and detection were performed using SYBR Green PCR Master Mix (Applied Biosystems) on the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The quantification standard was prepared by serial dilution of a known amount of the cloned plasmid of HBV/G or H. The specificity of these primers was confirmed in every PCR run by dissociation curve analysis (ABI Prism 7700 dissociation curve software; Applied Biosystems). In assays for HBV DNA in mouse sera, in which only 10 μL of sample is used, the sensitivity of the assay allowed detection of 1000 copies/mL of HBV/G or HBV/H DNA.

Molecular evolutionary analysis

Nucleotide sequences of HBV were aligned by the program CLUSTAL X, and the genetic distance was estimated with the 6-parameter method (Gojobori et al., 1982) in the Hepatitis Virus Database (Robertson et al., 1998). Based on these values, a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). To confirm the reliability of the phylogenetic tree, bootstrap resampling test was performed 1,000 times (Felsenstein, 1985).

Examination for recombination

Intergenotypic recombination was searched for using the method of Robertson et al. (Robertson et al., 1995) with use of the SimPlot program and bootscanning analysis (Lole et al., 1999). The mean genetic distances were calculated with a window size of 200 bp and a step size of 50 bp in this study.

Southern blot hybridizations

Southern blot hybridizations were performed with a full-length probe of HBV/G or H by previous methods (Fujiwara et al., 2005). No significant differences were observed in the detection between internal control HBV DNA and each probe.

Inoculation of chimeric mice with the liver repopulated for human hepatocytes

uPA+/+SCID+/+ mice with the liver repopulated for human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co., Ltd. (Hiroshima, Japan). The human hepatocytes were obtained from a single donor (female, 6 years, African American). Human serum albumin was measured by ELISA with commercial assay kits (Eiken Chemical Co., Ltd., Tokyo, Japan). They were inoculated with HBV recovered from culture supernatants of HuH7 cells transfected with plasmids containing 1,24-fold HBV genome constructs of the HBV/G or H (Sugiyama et al., 2006, 2007).
Histopathological examination

Liver tissues were fixed in formaldehyde, embedded in paraffin and stained with hematoxylin–eosin (H–E) or Masson’s trichrome. The fibrosis stage and inflammation grade were evaluated by independent expert pathologists.

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References


