Research Article

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Decreased infectivity of nucleoside analogs-resistant hepatitis B virus mutants

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Background & Aims: To understand the mechanisms of emergence and selection of HBV polymerase variants, which may also harbor mutations in the overlapping envelope protein, we analyzed the *in vitro* virus production and infectivity of the main viral mutants resistant to lamivudine and adefovir.

Methods: HBV-resistant mutants (rtL180M + M204V, rtV173L + L180M + M204V, rtM204I, rtL180M + M204I, rtN236T, rtA181V, rtA181V + rtN236T, rtA181T + N236T, and rtA181T) were produced in HepG2 cells permanently expressing the respective viral genomes. Viral protein expression, secretion, and viral particle production were studied by ELISA, Western blot, and transmission electron microscopy. To study only the effect of surface gene mutants on virus infectivity, HepaRG cells were inoculated with HDV pseudo-particles coated with the mutant HBV envelopes. To evaluate infectivity and replication in a global fashion, HepaRG cells were inoculated with HBV mutants.

Results: HBeAg was expressed and secreted in cell supernatants in all mutant-expressing cell lines. As expected, mutants harboring a sW196Stop mutation in the surface gene did not express small envelope proteins. All mutants expressing HBsAg were able to produce viral particles. HDV particles coated with mutant envelopes were less infectious than WT in HepaRG cells. Finally, we found that resistant mutants exhibit lower infectivity and replication ability than WT virus.

Conclusions: Based on this study, we found that envelope substitutions modulate viral protein expression, HDV coating, and viral infectivity. These envelope modifications provide novel insights into the features of emerging HBV variants during antiviral therapies and suggest that such mutants are less prone to transmission than their WT counterpart.

Keywords: HBV; Nucleos(t)ide analog; Drug resistance; Selection; Mutants; Quasi-species.

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Abbreviations: HBV, hepatitis B virus; RT, reverse transcriptase; S, surface; HDV, hepatitis delta virus; PCR, polymerase chain reaction; WT, wild type; ORF, open reading frame; Ag, antigen; SVP, subviral particle; cccDNA, covalently closed circular DNA.

Introduction

Despite the development of new antivirals with a high antiviral potency and a high barrier to resistance, antiviral therapy of chronic hepatitis B remains a treatment challenge. Indeed, the persistence of the viral genome as covalently closed circular DNA in the liver of infected patients requires long-term antiviral therapy. Because of the high spontaneous error rate of the HBV polymerase (2×10^{-5} nucleotide substitution per site per year), virions circulate as a complex quasi-species which evolves over time under selective pressure. Antiviral therapy with nucleoside analogs generally selects for drug-resistant mutants harbouring mutations in the viral polymerase gene. The main resistant mutants that have been described are the following: rtV173L, rtL180M, rtM204V/I, and rtA181V/T for lamivudine, rtN236T, and rtA181V/T for adefovir, rtI169T, rtI184G, rtS202G/I, and rtM250V for entecavir [1]. The main lamivudine resistant mutants are cross-resistant to emtricitabine and telbivudine. The rtA181T/V and rtN236T mutations are also associated with a decreased susceptibility to tenofovir *in vitro*. Complex mutants such as rtA181V/T + rtN236T are also suspected to confer multidrug resistance, i.e. simultaneous resistance to lamivudine, adefovir, and reduced susceptibility to tenofovir [2,3].

The HBV genome is very compact and organised into overlapping open reading frames (ORF), with the envelope(s) gene overlapping a part of the polymerase gene. Thus, mutations, selected as a consequence of nucleoside analog therapy, within the polymerase gene, may result in sequence and structural changes in the three envelope proteins encoded by a single ORF. Indeed, mutations in the polymerase selected during the course of antiviral nucleoside analog therapy can induce changes in the HBs antigen (HBsAg). For example, lamivudine- or adefovir-resistance associated mutations occurring in codon 173, 181, and 204 of the polymerase ORF may also result in amino acids substitution in the S domain such as sE164D, sW172stop, sL173F, sI195M, or sW196L/S/stop. These changes may alter the main functions of the HBV envelope proteins including envelopment of HBV nucleocapsids in Dane particles, and binding or entry into hepatocytes. The use of the hepatitis delta virus (HDV) coated by HBV variant proteins represents an interesting tool to study the impact of mutations in the HBV surface gene on infectivity and uncouples it from the effect of the mutations on the overlapping polymerase gene on viral replication [4,5].

We have previously shown that the *in vivo* evolution of a viral quasi-species composed of resistant mutants not only depends

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on their replication capacity in infected cells in the presence of antivirals but also on their infectivity modulated by the presence of mutations in the overlapping surface gene [4].

To better understand the kinetics of emergence of resistance to antiviral drugs that have been widely prescribed worldwide, i.e. lamivudine and adefovir, we have performed a systematic analysis of the replication capacity of the main resistant mutants selected, including virus particle release in HepG2 cell lines permanently expressing these mutants. Furthermore, a detailed study of their infectious capacity in HepaRG cells was performed. The biological and clinical relevance of our findings is discussed.

Materials and methods

Mutant cell lines

HepG2 stable cell lines permanently expressing the main HBV mutants resistant to lamivudine and adefovir were as follows: rtL180M + rtM204V (called LMMV), rtV173L + rtL180M + rtM204V (VLLMMV), rtM204I (MI), rtL180M + rtM204I (LMMI), rtN236T (NT), rtA181V (AV), rtA181V + rtN236T (AVNT), rtA181T (AT), rtA181T + rtN236T (ATNT) [6,7]. The rtA181V + rtN236T and the rtA181T + rtN236T are suspected to be multidrug resistant strains. HBV mutants belong to genotype A and wild type HBV belongs to genotype D [8]. Cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle medium (Eurobio, Courtaboeuf, France) supplemented with 10% fetal bovine serum, 200 mM of L-glutamine, 100 U/ml penicillin, 10 μg/ml streptomycin, 110 μg/ml sodium pyruvate, and non-essential amino acid mixture. In addition, cell lines were grown in the presence of 400 μg per ml of G418 (Sigma–Aldrich) to maintain selective pressure for proper HBV expression.

Western blot analysis of viral proteins

Cells were lysed in lysis buffer (50 mmol/L Tris–HCl [pH 8], 1 mmol/L ethylenediaminetetraacetic acid [EDTA], 1% NP-40). 20 μg of total proteins from the cell lysates and 2×10^6 g.e. of HBV present in the cell culture supernatant concentrated were subjected to electrophoresis in 12% sodium dodecyl sulfate–polyacrylamide gels. Proteins were then transferred to nitrocellulose membrane (Amersham enhanced chemiluminescence membrane) and incubated with anti-HBs (gift from M.A. Petit; 1:500 dilution) or anti-Pre-S2 (Ab8635, Abcam Cambridge Science Park, Paris, France; 1:1000 dilution) or anti-EAA1 (Early Endosomal Antigen 1; BD Biosciences, Franklin Lakes, NJ, USA; 1:2000 dilution) used as loading control antibody. Corresponding secondary antibodies labeled with horseradish peroxidase were used for detection by enhanced chemiluminescence.

Intra- and extracellular detection of viral proteins by ELISA

The intracellular expression of hepatitis B surface antigen was analyzed in cell lysate samples corresponding to 1 μg of total proteins by the Monolisa HBs ULTRA kit (Bio-Rad), as well as secreted HBsAg using 10 μl of cell culture supernatant. Both the intra- and extra cellular expression of HBeAg was analyzed by the Monolisa HBe Ag-Ab PLUS kit (Bio-Rad) using 1 μg of total proteins from cell lysate or 50 μl of cell culture supernatant, respectively. Monolisa HBe Ag-Ab PLUS kit is also able to detect HBcAg (data not shown).

HBV particle production

Culture media from mutant-expressing cell lines were collected at days 5, 7, and 9 post-plating. Clarified media were subjected to ultracentrifugation on a 20% sucrose cushion in Tris 10 mM; NaCl 0.2 M; EDTA 1 mM; (pH 7.4) buffer for 2 h at 44,000 rpm in a Ti50.2 fixed-angle rotor (Beckman, Paris, France). Sedimented particles were resuspended in an appropriate volume of phosphate-buffered saline to achieve a 500-fold concentration factor. HBV particle production was standardized by quantitative PCR using standard made from a full-length HBV genome cloned into a Puc vector (Promega) and quantified by spectroscopy [9]. Then, due to the possible production of non-infectious naked nucleocapsids, we performed a native gel agarose assay where 10 μl of the concentrated viral preparations were directly loaded onto a 1.2% agarose gel and were electrophoresed. After migration, HBV DNA was transferred onto a nylon membrane (Hybond N+, Amersham, United Kingdom). Membranes were then incubated in denaturation buffer containing 1 M NaCl and 0.2 M NaOH during 20 min, neutralized by 0.5 M Tris, pH 7.4; 1 M NaCl during 5 min and washed with a solution consisting of 0.3 M sodium chloride and 30 mM trisodium citrate (adjusted to pH 7.0 with HCl). Membranes were fixed for two hours at 80 °C and hybridized with a full-length nick translation-generated ^{32}P-labeled HBV probe. Signals of enveloped or naked nucleocapsids were quantified using PhosphorImager analysis (GE Healthcare, Velizy, France). The ratios between naked and enveloped particles were used to standardize precisely the concentration of infectious particles in each viral preparation.

HDV particle production

In HBV mutant-expressing cell lines, HDV particle production was achieved by transient transfection of pSVLD3 plasmid [10]. One percent dimethyl sulfoxide was added to cells 24 h post-transfection. Culture medium was harvested at days 5, 7, 9, and 12 post-transfection. Clarified medium was subjected to ultracentrifugation and resuspension in phosphate-buffered saline as described above for HBV production. HDV particle production was standardized by reverse-transcription quantitative PCR HDV genome-specific with a full-length HDV plasmid quantified by spectroscopy.

Electron microscopy studies of secreted mutant viral particles

For electron microscopy studies, HBV and HDV viral suspensions were adsorbed onto formvar-coated, carbon-stabilized, nickel grids (200 mesh) for 2 min. Grids were then directly stained with 4% phosphotungstic acid for 2 min. Grids were examined using a JEOL (Croissy-sur-Seine, France) JEM 1400 electron microscope, a Gatan Orius camera (GATAN, Evry, France), and the "Digital micrograph" software.

Analysis of in vitro infectivity

HDV is a viral satellite of HBV because its transmission occurs only with HBV infection. HDV utilizes HBV extra production of envelope proteins to be coated, to be secreted out of the co-infected cell, and to infect hepatocytes. The fact that HBV and HDV harbor the same envelope prompts one to hypothesize that they may share the same entry pathway. This analysis was performed by using HepaRG cells, which is the only cell line susceptible for HBV/HDV infection if initiated using virions [4,5].

HDV particles were produced in HBV mutant-expressing cell lines transfected with the pSVLD3 plasmid encoding the HDV genome as a trimer. Cell culture supernatants were collected, concentrated by ultracentrifugation and standardized by reverse-transcription quantitative PCR HDV genome-specific.

Concentrated supernatants containing WT or mutant HBV particles as well as HDV pseudo-particles enveloped with WT or mutant HBV surface proteins were used to inoculate HepaRG cells after normalization of their titers based on PCR quantification of HBV or HDV genomes. Differentiated HepaRG cells (5×10^5 cells/20-mm diameter well) were exposed to 10^8 HBV or 5×10^7 HDV virions per well for 16 h in the presence of 4% polyethylene glycol 8000. Cells were harvested at day 12 or 8 post-inoculation for HBV and HDV, respectively. Total cellular RNA was purified by the Nucleospin RNA II kit (Macherey Nagel). RNA were analyzed after reverse transcription and quantification of cDNA by real time PCR using specific conditions for HBV or HDV, as previously described [4].

Immunostaining was also performed to detect HBsAg or small HDVAg (p24) in infected HepaRG cell. Twelve or 8 days after infection into glass chambers for HBV or HDV infection, cells were fixed with 4% paraformaldehyde for 10 min at room temperature or cold methanol, washed twice, and then permeabilized with PBS supplemented with saponin 0.1%. Cells were incubated first for 2 h at room temperature with anti-HBsAg (1:500 dilution) or anti-HDVAg antibodies (a gift from A. Kay; 1:500 dilution), and after three washes for 1 h with Alexa Fluor 555–conjugated goat anti-rabbit (A31630; Invitrogen, 1:1000 dilution). Finally, cell nuclei were stained with PBS 4.6-diamidino-2-phenylindole (1:1000 dilution) and saponin (0.1%) for 5 min. Photographs were taken by using a fluorescence microscope (Nikon eclipse TE2000-E; Nikon, Champigny sur Marne, France) and processed using the NIS-Element BR 3.0 software (Nikon).