Research Article

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Viral Hepatitis

Decay of ccc-DNA marks persistence of intrahepatic viral DNA synthesis under tenofovir in HIV-HBV co-infected patients

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Background & Aims: In the presence of highly-potent antivirals, persistence of hepatitis B virus (HBV) is most well-characterized by covalently-closed circular DNA (cccDNA) and total intrahepatic DNA (IH-DNA). We sought to determine how antiviral therapy could affect their levels during human immunodeficiency virus (HIV)-HBV co-infection.

Methods: Sixty co-infected patients from a well-defined cohort with $\geqslant$1 liver biopsy were studied. HBV cccDNA and total IH-DNA were extracted from biopsies and quantified by real-time PCR. Factors associated with intrahepatic viral load were determined using mixed-effect linear regression and half-life viral kinetics during reconstructed follow-up using non-linear exponential decay models.

Results: At biopsy, 35 (58.3%) patients were hepatitis B "e" antigen (HBeAg)-positive and 33 (55.0%) had detectable plasma HBV-DNA (median = 4.58 $\log_{10}$ IU/ml, IQR = 2.95–7.43). Overall, median cccDNA was −0.95 $\log_{10}$ copies/cell (IQR = −1.70, −0.17) and total IH-DNA was 0.27 $\log_{10}$ copies/cell (IQR = −0.39, 2.00). In multivariable analysis, significantly lower levels of cccDNA and total IH-DNA were observed in patients with HBeAg-negative serology, nadir CD4$^+$ cell counts >250/mm^3, and longer cumulative TDF-duration, but not lamivudine- or adefovir-duration. In post-hoc analysis using reconstructed TDF-duration (median 29.6 months, IQR = 15.0–36.1, n = 31), average half-life of cccDNA was estimated at 9.2 months (HBeAg-positive = 8.6, HBeAg-negative = 26.2) and total IH DNA at 5.8 months (HBeAg-positive = 1.3, HBeAg-negative = 13.6). Intrahepatic viral loads remained detectable for all patients, even with prolonged TDF-exposure.

Conclusions: In co-infection, TDF-use is associated with lower levels of HBV replication intermediates and cccDNA. Slow decay of intrahepatic viral loads underscores that TDF is unable to completely block intracellular viral DNA synthesis, which possibly accounts for continuous replenishment of the cccDNA pool.

Lay summary: Chronic hepatitis B virus (HBV) is a persistent infection, while the only real way of knowing the extent of this persistence is through measuring levels of virus in the liver. In this study, we examine levels of HBV in the liver among patients with both HBV and human immunodeficiency virus, or HIV, infection. It would appear that the currently available medication, namely "tenofovir", works well to decrease virus levels in the liver, but it remains at low levels despite long periods of treatment.

Introduction

During chronic infection with hepatitis B virus (HBV), higher levels of serum HBV-DNA replication are known to increase the risk of liver fibrosis, hepatocellular carcinoma (HCC), and end-stage liver disease [1–3]. The aim of current antiviral therapy is then to suppress HBV-DNA levels whereby liver-related morbidity and mortality is reduced. Potent nucleoside/nucleotide analogues (NA), such as tenofovir (TDF) or entecavir (ETV), are able to effectively eliminate circulating HBV-DNA in the overwhelming majority of HBV-infected patients with little to no emergence of resistance mutations [4]. Furthermore, the clinical benefits of antiviral-induced HBV suppression have already appeared in preliminary evidence, where liver fibrosis regression and decreased risk of HCC were observed during treatment with potent anti-HBV treatment [5,6].

Keywords: Hepatitis B virus; cccDNA; Intrahepatic HBV-DNA; Nucleoside/nucleotide analogue; Exponential decay.

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Abbreviations: HBV, hepatitis B virus; cccDNA, covalently-closed circular DNA; IH-DNA, intrahepatic DNA; HIV, human immunodeficiency virus; HBeAg, hepatitis B "e" antigen; IQR, interquartile range; TDF, tenofovir; HCC, hepatocellular carcinoma; NA, nucleoside/nucleotide analogue; ETV, entecavir; HBsAg, hepatitis B surface antigen; ART, antiretroviral therapy; qHBsAg, quantification of hepatitis B surface antigen; LAM, lamivudine; ADV, adefovir; CTL, cytotoxic T lymphocytes; AIDS, acquired immunodeficiency syndrome; ALT, alanine aminotransferase.

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Unfortunately, the current arsenal of NA therapy only targets HBV polymerase activity during the replication cycle. The formation of HBV covalently-closed circular DNA (cccDNA) within the hepatocyte, essential for the production of viral RNAs encoding polymerase, core, and envelope proteins [7]; remains intact during treatment. Studies have demonstrated modest declines in intrahepatic viral loads of roughly one $\log_{10}$ copies/cell in the first year after administrating NA-based therapy. Yet these findings are largely attributed to the reduction of viral DNA synthesis in the cytoplasm, leading to reduced intracellular recycling of cccDNA and slower rate of infection of new hepatocytes [8,9]. Even after patients achieve hepatitis B surface antigen (HBsAg) seroconversion, which is considered the most important therapeutic goal for chronic HBV infection, detectable levels of intracellular replication are still observed [8].

Few studies have examined the levels of intrahepatic viral load specifically in treated patients co-infected with HBV and the human immunodeficiency virus (HIV). This is concerning given that many of the immune mechanisms purportedly governing control of cccDNA are also mitigated by HIV-infection, while the common causes of deficiencies in intrahepatic anti-HBV T cell responses are also generally observed in HIV-infected patients [10–12]. Only one study to date has looked at intracellular levels of HBV-DNA replication in co-infected patients during short-term antiretroviral therapy (ART) containing an anti-HBV active agent [13]. However, the small numbers of patients, homogeneous clinical characteristics of the study population (i.e., severely immunocompromised, high HIV-RNA viral loads, and Asian), and matched design make it difficult to determine therapeutic differences or other clinical risk-factors associated with levels of intrahepatic HBV replication.

The overall aim of the study herein was to examine how antiviral therapy affects intrahepatic HBV viral loads in HIV-HBV co-infected patients. We first analyzed levels of cccDNA and total intrahepatic (IH)-DNA with respect to different antiviral agents, while accounting for determinants linked to both HBV- and HIV-disease status. Particular attention was placed on the evolution of intrahepatic replicative intermediates during potent anti-HBV therapy, namely by estimating the viral decay of intrahepatic viral loads *in vivo* among patients receiving TDF. Second, we aimed to study the relationship of intrahepatic viral loads and serological markers. cccDNA and total IH levels were correlated with quantifiable HBsAg and hepatitis B "e" antigen (HBeAg). One time cccDNA and total IH-DNA levels were also used to predict seroclearance later during follow-up, when treatment with potent anti-HBV agents became increasingly common.

Patients and methods

Study design

Patients were selected from the French HIV-HBV cohort study as described previously [14]. Briefly, this prospective study recruited 308 patients from seven centers located in Paris and Lyon, France during May 2002–May 2003. Patients were included if they had HIV-positive serology confirmed by western blot and HBsAg-positive serology for at least six months. All data collection continued until 2010–2011.

Subjects were selected for this study if they had a snap-frozen liver biopsy for at least one time-point. In total, 60 patients were included, 47 and 13 with one and two liver biopsy(ies), respectively. All patients provided written informed consent to participate in the cohort and the protocol was approved by the Hôpital Pitié-Salpêtrière and Hôpital Saint-Antoine Ethics Committees (Paris, France) in accordance with the Helsinki Declaration.

Virological and serological parameters from blood

Plasma HBV-DNA viral load was quantified using a commercial PCR-based assay (COBAS® AmpliPrep/COBAS TaqMan®, detection limit: 12 IU/ml; or COBAS Amplicor HBV Monitor, detection limit: 60 IU/ml; Roche Diagnostics, Meylan, France). HBV genotypes and presence of LAM or adefovir (ADV)-resistant mutants were determined using DNA chip technology from follow-up visits during the cohort study (bioMérieux, Marcy l'Etoile, France) [15]. In the event of missing data, we assumed that patients harbored the same HBV strain from the last available sequence and if no prior sequencing was possible, genotype was considered missing and antiviral resistance absent.

Qualitative HBsAg and HBeAg were detected using a commercial enzyme-linked immunoassay [14]. HBV serology was available at the time of liver biopsy and every yearly visit thereafter. HBsAg quantification (qHBsAg) and HBeAg quantification (qHBeAg) were performed using the Architect assay with Architect i2000 analyzer (Abbott Laboratories, Rungis, France). qHBeAg was reported in Paul Ehrlich Institute Units (PEIU)/ml [16].

Anti-hepatitis C virus (HCV) and anti-hepatitis D virus (HDV) antibodies in serum were detected using commercially-available enzyme immunoassays.

Liver biopsies and quantification of intrahepatic HBV-DNA levels

Liver biopsies were obtained based on concomitant guidelines from the European Association for the Study of the Liver [17] with the intention of evaluating liver fibrosis using METAVIR criteria [14].

From snap-frozen biopsies, DNA was extracted using the MasterPure DNA purification kit (Epicentre, Biozym, Germany) according to manufacturer's instructions. Briefly, 500 µl of lysis buffer was added to liver tissue and then gently ground using a plastic grinder. After an overnight proteinase K digestion at 42 °C and RNase digestion (30 min at 37 °C), 500 µl of precipitation buffer was added to each sample. Samples were well homogenized prior to a 10 min centrifugation (10,000 g). The adequate volume of isopropanol was added to the supernatants and DNA was precipitated. Pellets were rinsed with 70% ethanol and resuspended in 50 µl of Tris-EDTA buffer.

cccDNA and total IH-DNA were amplified using the Light Cycler System (Roche Diagnostics, Mannheim, Germany) with HBV-specific primers and fluorescence hybridization probes [8]. Forward and reverse primers were 5′-CTCCCCGT CTGTGCCTTCT-3′ (NCCC1 nucleotides (nt) 1548–1566) and 5′-GCCCCAAAGCCACC CAAG-3′ (CCCAS2 nt 1903–1886), respectively, for cccDNA amplification and 5′-CTCGTGGTGGACTTCTCTC-3′ (2RC/CCS nt 256–274) and 5′-CTGCAGGATGAAGAG GAA-3′ (2RC/CCAS nt 421–404), respectively, for total IH-DNA amplification. Fluorescence resonance energy transfer hybridization probes were 5′-GTTCACGGT GGTCTCCATGCAACGT-FL-3′ and 5′-R640-AGGTGAAGCGAAGTGCACA CGGACC-p-3′ for cccDNA quantification and 5′-CACTCACCAACCTCCTGTCCTCCAA-FL-3′ and 5′-R640-TGTCCTGGTTATCGCTGGATGTGTCT-3′ for total IH-DNA quantification. Probes for cccDNA might have performed less efficiently with certain strains of HBV genotype G and hence data on cccDNA were assumed to be missing for patients harboring this genotype. When quantifying cccDNA levels, plasmid-safe deoxyribonuclease (Plasmid-safe DNase, Epicentre by Illumina) was added to eliminate contaminating relaxed circular DNA. All intrahepatic HBV-DNA values were normalized for cellular DNA content using the beta-globin gene kit (Roche DNA Control Kit; Roche Diagnostics) and the average cell number used to determine viral loads in liver biopsy samples was 972 (range = 167–3250) beta-globin gene copies. Serial dilutions of plasmids containing an HBV monomer (pHBV-EcoR1) served as the quantification standard from which cccDNA and total IH-DNA levels were determined (lower limit of quantification = 0.001 copy/cell).

Statistical analysis

In a risk-factor analysis, various determinants of intracellular replication were first examined in univariable analysis using a mixed-effect linear regression model, accounting for within-individual correlation of patients with more than one biopsy. With a solely predictive objective in mind, a multivariable model was constructed, where variables with a p value <0.1 in univariable analysis were included in a forward-stepwise approach while any variable above this threshold was removed. Due to highly skewed distributions, cumulative treatment therapy was divided in tertiles for lamivudine (LAM) and by 0, 0–36, and >36 months for ADV and TDF.

In an analysis of viral decay kinetics during TDF-use, cccDNA and total IH DNA levels were evaluated on reconstructed TDF-duration. Two models were employed to estimate viral levels over time, $V(t)$ – the first represents only one continuous phase of exponential decay (single-phase) and the second represents an initial phase of exponential decay followed by no decay (two-phase):