Research Article

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Immune cell responses are not required to induce substantial hepatitis B virus antigen decline during pegylated interferon-alpha administration

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Background & Aims: Pegylated interferon-alpha (PegIFNα) remains an attractive treatment option for chronic hepatitis B virus (HBV) infection because it induces higher rates of antigen loss and seroconversion than treatment with polymerase inhibitors. Although early HBsAg decline is recognised as the best predictor of sustained response to IFN-based therapy, it is unclear whether immune cell functions are required to induce significant antigenemia reduction in the first weeks of treatment. Aim of the study was to investigate whether PegIFNα can induce sustained human hepatocyte responsiveness and substantial loss of circulating and intrahepatic viral antigen loads in a system lacking immune cell functions.

Methods: HBV-infected humanized uPA/SCID mice received either PegIFNα, entecavir (ETV), or both agents in combination. Serological and intrahepatic changes were determined by qRT-PCR and immunohistochemistry and compared to untreated mice.

Results: After 4 weeks of treatment, median viremia reduction was greater in mice treated with ETV (either with or without PegIFNα) than with PegIFNα. In contrast, levels of circulating HBeAg, HBsAg, and intrahepatic HBcAg were significantly reduced ($p = 0.03$) only in mice receiving PegIFNα alone or in combination, as compared to mice receiving ETV monotherapy. Progressive antigen reduction was also demonstrated in mice receiving PegIFNα for 12 weeks (HBeAg = Δ1 log; HBsAg = Δ1.4 log; $p < 0.0001$). Notably, repeated administrations of the longer-active PegIFNα could breach the impairment of HBV-infected hepatocyte responsiveness and induce sustained enhancement of human interferon stimulated genes (ISG).

Conclusions: The antiviral effects of PegIFNα exerted on the human hepatocytes can induce sustained responsiveness and trigger substantial HBV antigen decline without claiming the involvement of immune cell responses.

Keywords: cccDNA; HBsAg; HBeAg; Interferon stimulated genes; Hepatocyte responsiveness; Humanized mice.

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Introduction

Chronic hepatitis B virus (HBV) infection is the most difficult to cure hepatitis worldwide and is considered the main risk factor for the development of hepatocellular carcinoma [1]. HBV displays unique replication strategies and the presence of the viral covalently closed circular DNA (cccDNA) molecule is responsible for failure of viral clearance and relapse of viral activity after antiviral therapy with polymerase inhibitors in chronically infected individuals. The cccDNA minichromosome utilizes the cellular transcriptional machinery [2,3] to generate all transcripts necessary for protein production and viral replication, which takes place in the cytoplasm after reverse transcription of an over-length pregenomic RNA (pgRNA). While the pgRNA provides all components required for the production of HBV-DNA containing nucleocapsids, the production of envelope proteins, needed for virion secretion and production of non-infectious subviral particles (SVPs), depends on the transcription of distinct viral genes (preS/S). Indeed, it is the large amount of empty SVPs, which accounts for most of the circulating surface antigens (HBsAg) detected in infected individuals, while a distinct (precore)-RNA species is responsible for the production of a non-particulate form of the nucleoprotein, the HBeAg. Both HBsAg and HBeAg are implicated in exerting immune-modulating functions contributing to viral persistence [4,5]. Because nucleos(t)ide analogues (NUCs) efficiently suppress HBV replication without affecting cccDNA transcription [1,2], monotherapy with NUCs has modest effects on the levels of HBsAg and HBeAg and immunological control is rarely achieved [6]. In contrast, faster HBsAg

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decrease is more frequent in patients treated with IFNα alone or in combination with NUCs [7–13]. Since HBsAg seroconversion represents the closest outcome to clinical cure [14], despite poor tolerability and therapeutic responses limited to a subset of patients, a finite course of IFNα treatment remains an important anti-HBV strategy [9].

Quantitative HBsAg assays revealed that early HBsAg decline achieved during IFN treatment represents the best biomarker for assessing treatment response [15]. Despite such clinical evidence, the underlying mechanisms are not fully elucidated, though both viral characteristics and host factors, such as *IL28B* genotype, may influence the probability of IFN response [9]. Persistence of high antigen levels is considered a major factor driving functional exhaustion of HBV-specific immune cells. Thus, antigen removal appears essential to allow functional reconstitution of antiviral T cell responses. Furthermore, the poor restoration of immune cell functions recently observed in the early phases of IFN treatment [16,17] and the limited rates of cccDNA reduction determined in patients receiving IFN-based therapy [7,18] do not explain the early kinetics of HBsAg decline. In particular, it is not clear whether the involvement of immune cells is required to induce significant early antigenemia decline.

IFNα was shown to accelerate pgRNA and core particle degradation in transgenic mice [19,20], as well as to induce epigenetic repression of the cccDNA in human hepatocytes [21]. This latter antiviral function indicates that IFNα can affect the levels of viral antigens by acting directly on the hepatocytes. However, previous studies showed that the responsiveness of HBV-infected human hepatocytes is short-lived and impaired upon administration of conventional IFNα [22]. To compare the impact of conventional *vs.* pegylated IFNα, and of treatment regimens involving combination of PegIFNα with the potent polymerase inhibitor entecavir (ETV), in lowering viral loads and inducing hepatocyte responsiveness in a system lacking adaptive immune responses, we employed humanized uPA/SCID mice stably infected with HBV.

Material and methods

Generation of humanized mice, infection, and drug administration

Human liver-chimeric UPA/SCID/beige mice were generated by transplanting 1×10^6 thawed human hepatocytes and housed under specific pathogen-free conditions in accordance with the European Communities Council Directive (86/EEC) and protocols approved by the Ethical Committee of the city and state of Hamburg. Human hepatocytes obtained from two donors, one C/C variant for the *IL28* locus and one C/T variant (experiments in Supplementary Fig. 4) were used for the study. Levels of human chimerism were determined by measuring human serum albumin (HSA) in mouse serum (Human Albumin ELISA kit, Immunology Consultants Lab, Portland, USA) as reported [22]. HBV-infected mice received either PegIFNα alone (Pegasys, Hoffmann-La Roche Inc., Basel, Switzerland) or entecavir (Baraclude Solution; Bristol-Myers-Squibb, Munich, Germany), or both drugs in combination, as indicated in results. Some mice received a single injection of conventional IFNα (1350 IU/g body weight). Blood was withdrawn at indicated times. Liver specimens removed at euthanasia were snap-frozen in 2-methylbutane for histological and molecular analyses.

Serological and intrahepatic measurements

Viral DNA was extracted from serum samples using the QiAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) and quantitated in a Light Cycler (Roche Applied Science, Mannheim, Germany) using HBV-specific primers and FRET-hybridization probes [22]. Intrahepatic nucleic acids were isolated using the MasterPure DNA purification kit (Epicentre, Madison, USA) and the RNeasy RNA purification kit (Qiagen) [23]. Total HBV-DNA and cccDNA amounts were normalized for cellular DNA contents using the human beta-globin gene kit (Roche Applied Science) [22]. RNAs were reverse transcribed using oligo-dT primers and the Transcriptor Kit (Roche Applied Science) and quantified using pgRNA- or total HBV-RNA-specific primers [23]. To determine gene expression levels, human-specific primers from the TaqMan® Gene Expression Assay System were used and samples analyzed in the ViiA™ 7Real-Time PCR System (Life Technologies, Carlsbad, California, USA). The mean of the human housekeeping genes GAPDH and ribosomal protein L30 was used for normalization [22]. HBsAg and HBeAg quantification were performed on the Abbott Architect platforms (quantitative HBsAg kit, and HBeAg kit, Abbott, Ireland, Diagnostic Division) after diluting the mouse serum (1:150 for HBsAg and 1:3–1:10 for HBeAg) in the manual dilution serum (Abbott) as recommended by manufacturer [24]. HBeAg results were displayed as signal *vs.* noise (SC/O).

Immunohistochemistry

Human hepatocytes were identified in frozen mouse liver sections using a human cytokeratin-18 monoclonal mouse antibody (Dako Diagnostika, Glostrup, Denmark), while HBcAg staining was detected with a polyclonal rabbit anti-HBcAg antibody (Dako) and Stat1 proteins using a monoclonal mouse antibody (BD Biosciences, Franklin Lakes, USA) [22]. Specific signals were visualized with Alexa 488 or 546 labeled secondary antibodies (Invitrogen, Darmstadt, Germany) or the TSA Fluorescein System (Perkin Elmer, Jügesheim, Germany). Nuclear staining was achieved by Hoechst 33258 (Invitrogen, Eugene, USA). Stained sections were analyzed by fluorescence microscopy (Biorevo BZ-9000, Keyence, Osaka, Japan) using the same settings for all groups.

Statistics

The non-parametric Mann Whitney U Test was used to evaluate pairwise comparisons with p values <0.05 being considered statistically significant. To compare HBsAg decline within treatment groups, the Friedman test was used as non-parametric test for comparison of repeated measurements and the Dunns test was applied to compare all pairs as post-test. To compare HBeAg decline between baseline and end of treatment measurements, non-parametric Wilcoxon matched pair test was used. The Spearman r test was used to assess correlation between intrahepatic HBV-RNA levels and concentrations of circulating HBsAg and HBeAg. p values <0.05 was considered statistically significant.

Results

Serological viral changes induced by treatment of HBV infected humanized mice with PegIFNα and ETV alone or in combination

Mice with high levels of human hepatocyte chimerism (1–8 mg/ml HSA) were used to establish HBV infection. Twelve weeks after viral inoculation mice displaying viral titres ranging between 1×10^8 and 1×10^9 HBV-DNA copies/ml were included in the first set of experiments. Dose-finding experiments were first performed using different concentrations of PegIFNα, (2.5 or 25 ng/g body weight) (Supplementary Fig. 1). In line with other studies and dose scaling commonly used to convert human doses to mouse equivalent doses [22,25], the higher mouse-adapted dose formulation led to clear induction of human interferon-stimulated-genes (ISGs) in uninfected humanized mice and was chosen for all experiments. As schematically depicted in Fig. 1A, the mice were split into groups whereof one group (n = 6) received PegIFNα, one group (n = 6) ETV, and another group (n = 5) received both agents in combination. The serological analyses revealed that viremia decreases across individual animals were strongest in the groups receiving ETV alone or in combination with PegIFNα (median Δ3.4 log), while viremia reduction in the PegIFNα group was lower (median Δ1.4 log) at week 4 (Fig. 1B). In contrast, the reduction in HBV antigen levels, determined in individual mice, was more pronounced in mice