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The response to interferon is influenced by hepatitis B virus genotype *in vitro* and *in vivo*

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ABSTRACT

Purpose: To investigate the effectiveness of an interferon administration on different genotypes of hepatitis B virus (HBV) *in vitro* and *in vivo*.
Methods: In vitro, we transfected plasmids carrying different HBV genotypes including recently identified new genotype I into HepG2 and HuH7 cells, then treated with standard interferon alpha (IFN-α); *in vivo*, we treated mice with pegylated interferon alpha (Peg-IFN-α) after injection with HBV DNA of different genotypes. The culture supernatants from cell culture and sera from mice were collected and used in hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) assays by ELISA and HBV DNA measurement by PCR.
Results: Both in cell culture and in mouse model, it was observed that HBV genotypes A and B exhibited significantly better response to IFN-α2a or Peg-IFN-α2a in terms of reduced expression of HBsAg, HBeAg and the HBV DNA level as compared to HBV genotypes C and D. Moreover, the inhibitory effect of IFN-α2a or Peg-IFN-α2a on HBV genotype I was greater than on genotype C or D, but less than genotype A. However, there was no significant response difference between genotypes A and B, C and D, B and I, respectively.
Conclusion: The effectiveness of IFN/Peg-IFN to suppress HBV replication is dependent on different HBV genotypes. IFN/Peg-IFN is more effective on HBV genotype A or B than on genotype C, D or I. Treatment regimens are suggested to be adapted to HBV genotype.

1. Introduction

Worldwide, HBV affects about 350–400 million people and accounts annually for one million deaths from HBV associated disease including cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (Dienstag, 2008).

HBV replication is the key motor of HBV related disease progression, so HBV elimination or sustained HBV suppression may reduce the risk or slow the progression of life-threatening sequelae of virus persistence (Liaw, 2011).

Currently, there are two kinds of clinically available anti-HBV drugs: one is immunomodulatory drug (IFN-α and Peg-IFN-α), which could provide sustained off-treatment response; another is direct antiviral drug (nucleos(t)ide analogs) which could provide therapy-maintained response. Interferon alpha, with immunomodulatory, antiproliferative and viral inhibitory properties, is the first licensed drug for the treatment of chronic HBV infection. Compared to nucleos(t)ide analogs, it has the advantages of the finite treatment duration, the absence of viral resistance and the potential loss of HBsAg (Cooksley, 2010).

Several HBV factors, including HBV genotype, viral load and specific viral mutations have been documented to be associated with the response to IFN-α and Peg-IFN-α. Among them, HBV genotype is the strongest predictor of response (Erhardt et al., 2008). Several studies have shown that HBV genotype influence the outcome of the therapy with interferon, with genotype A better than genotype D (Erhardt et al., 2005) and genotype B better than genotype C (Cooksley et al., 2003; Wai et al., 2002; Zhao et al., 2007).

However, there are several intrinsic weaknesses of the clinical trials conducted so far which make it difficult to reach firm conclusions on the role of HBV genotypes in response to IFN therapy (Raimondi et al., 2010). Because of diverse geographical distributions of HBV genotypes, most clinical trials involving more than two genotypes were multicenter and pooled together patients of different ethnicities to reach a sufficient statistical power. Ethnicity and

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environmental differences can confound the differences between genotypes. Ethnicity is the most confound factor which should be excluded since overlap between ethnicity and genotype is very high. Without eliminating this factor, a false-positive association of effectiveness of IFN with the HBV genotype may be revealed. Therefore, the role of HBV genotype in tailoring treatment regimens needs further investigation in pure systems such as cell culture and mouse model. Moreover, there is no available report on antiviral response of HBV genotype I which is recently identified as a new genotype (Tran et al., 2008) so far.

The present study is aimed to clarify the association between different genotypes of HBV including new genotype I and response to IFN administration *in vitro* by cell transfection and *in vivo* using hydrodynamic injection mouse model.

2. Materials and methods

2.1. Plasmids and cell lines

The recombinant plasmids pUC19-HBV-A/B/C/D/I containing full length genome of HBV DNA clones were kindly provided by Professor Kenji Abe (Department of Pathology, National Institute of Infectious Diseases, Tokyo, Japan). These HBV DNA clones' names are: genotype A (HBV-RU204, accession #: AB126580; subgenotype A2), genotype B (HBV-IF720-1, accession #: AB205121; subgenotype B1), genotype C (HBV-TJ3-1-1, accession #: AB205124; subgenotype C2), genotype D (HBV-RU313-2, accession #: AB205127; subgenotype D2) and genotype I (HBV-VH24, accession #: AB231908; subgenotype I1). All HBV DNA clones used in this study showed wild type without any hot spot mutations such as pre-core stop codon mutation, double mutations in basal core-promotor and/or pre-S deletion mutation. These full-length HBV genomes were amplified according to the method of Parekh (Parekh et al., 2003) which was modified of Gunther's method (Gunther et al., 1995). These clones were ligated to the cloning sites of Hind III and SacI of pUC19 vector.

The expression vector pIRES2-EGFP, HepG2 cells, HuH7 cells and *Escherichia coli* (*E. coli*) *JM*109 were maintained in our laboratory.

2.2. Construction of the recombinant plasmids pIRES2-EGFP-HBV-A/B/C/D/I

For the expression of viral genes, the pIRES2-EGFP was reconstructed by adding a 18-nt sequence encoding the precore initiation site after the CMV promoter and a 160 base-pair HBV DNA fragment encoding the carboxy terminus of the HBV X gene as well as the polyadenylation signal for HBV mRNA according to H. Yang et al. (2004). At first, the DNA fragment including the 18-nt and 160 base-pair HBV DNA fragment joined by a linker with BglII and SacI restrict enzyme sites was synthesized, then one NheI and one XmaI were added to 5′ and 3′ end, respectively. Then the fragment was cloned into pIRES2-EGFP vector through NheI and XmaI sites. After digestion with HindIII, 5′ overhangs of pUC19-HBV-A/B/C/D/I plasmids were filled in to form blunt ends with DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs, USA), then purified and extracted using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). After digestion with BglII, 5′ overhangs of the reconstructed pIRES2-EGFP plasmid was filled in to form blunt ends, then purified and extracted. The above extracted fragments were digested with SacI, and the interest fragments were purified and extracted. Finally, the insert fragments (HBV DNA clones), coming from pUC19-HBV-A/B/C/D/I plasmids digested with HindIII and SacI, were cloned into the reconstructed pIRES2-EGFP expression vector digested with BglII and SacI by T4 DNA ligase (TaKaRa, Dalian, China). After insertion of different genotype-specific HBV sequences, the final product was a hybrid 1.1 unit HBV (1.0 HBV isolate + 0.1 adw2 HBV sequence necessary for viral transcription and replication) driven by the pIRES2-EGFP CMV promoter. The products of ligation reaction were transformed *E. coli* competent cells according to the manual. Clones were inoculated into LB broth with kanamycin and shaken overnight at 37 °C. Then after mini-prep, restriction analyses were executed to identify positive clone. Finally, sequencing analysis (Invitrogen, Beijing, China) was used to validate the sequences of inserts.

The recombinant plasmids were extracted and purified with QIAGEN Plasmid Purification Midi Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The concentration and purity of these plasmids were determined with micro-volume spectrophotometer of thermo Scientific NanoDrop 2000c (NanoDrop, Wilmington, USA).

2.3. Cell culture and transfection

Human hepatocellular carcinoma cells (HepG2 and HuH7) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Beijing, China), supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 °C in a CO_2 humidified incubator. The cells were transfected with recombinant plasmids pIRES2-EGFP-HBV-A/B/C/D/I respectively using Lipofection 2000 (Invitrogen Carlsbad, CA, USA) following the manufacturer's guidelines. Medium were changed 6 h after transfection, and in the "IFN-treated group", 1000 U/ml of recombinant human IFN-α2a (PBL InterferonSource, Piscataway, NJ) was added to the fresh media, two times at 24 h interval. The reconstructed pIRES2-EGFP vector was used as a mock transfection control. 72 h after transfection the supernatants were harvested. All experiments were performed in triplicate.

2.4. Determination of HBsA g and HBeAg

The expression levels of HBsAg and HBeAg secreted into supernatants were separately assayed using an enzymed-linked immunosorbent assay kits (Rongsheng Biochemical Laboratory, Shanghai, China). As recommend in the instructions, a ratio of S/CO (signal/cutoff) value ≥ 2.1 was considered a positive response for HBsAg or HBeAg.

2.5. Measurement of HBV DNA

100 μL of supernatant was pre-heated at 50 °C for 20 min and then treated with 1 U DNase I (TaKaRa, Dalian, China) and $MgCl_2$ at a final concentration of 10 mol/L for 1 h to eliminate residual plasmids. The reaction was terminated with EDTA at a final concentration of 25 mmol/L by incubating at 70 °C for 10 min. Then the HBV DNA was extracted using QIAamp DNA blood kits (QIAGEN, Hilden, Germany) and HBV DNA quantification assays were performed using a commercial real-time fluorescence quantitative PCR kit (Kehua, Shanghai, China). As recommended in the instructions, an HBV DNA level $\geq 5.0 \times 10^2$ copies/mL was considered a positive response.

2.6. Mice experiments

Male BALB/C mice at specific-pathogen-free (SPF) level, weighing 18–20 g, were provided by the Animal Center of the Third Affiliated Hospital of Harbin Medical University. All animal experiment protocols were approved by the Committee for Ethics in Animal Experimentation of Harbin Medical University. HBV infection animal model was established by hydrodynamic injection of HBV DNA (Liu et al., 1999; Yang et al., 2002). Briefly, mice were injected *via* the tail vein with 15 μg purified plasmid DNA