Acta Pharmaceutica Sinica B 2014;4(4):270–276

Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

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ORIGINAL ARTICLE

Establishment of drug-resistant HBV small-animal models by hydrodynamic injection

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Received 22 May 2014; revised 6 June 2014; accepted 23 June 2014

KEY WORDS

Drug-resistance;
Animal models;
Hepatitis B virus;
Hydrodynamic injection

Abstract In antiviral therapy of hepatitis B virus (HBV) infection, drug resistance remains a huge obstacle to the long-term effectiveness of nucleoside/tide analogs (NAs). Primary resistance mutation (rtM204V) contributes to lamivudine (LAM)-resistance, and compensatory mutations (rtL180M and rtV173L) restore viral fitness and increase replication efficiency. The evaluation of new anti-viral agents against drug-resistant HBV is limited by the lack of available small-animal models. We established LAM-resistance HBV replication mice models based on clinical LAM-resistant HBV mutants. Double (rtM204V+rtL180M) or triple (rtM204V+rtL180M+rtV173L) lamivudine-resistant mutations were introduced into HBV expression vector, followed by hydrodynamic injection into tail vein of NOD/SCID mice. Viremia was detected on days 5, 9, 13 and 17 and liver HBV DNA was detected on day 17 after injection. The serum and liver HBV DNA levels in LAM-resistant model carrying triple mutations are the highest among the models. Two NAs, LAM and entecavir (ETV), were used to test the availability of the models. LAM and ETV inhibited viral replication on wild-type model. LAM was no longer effective on LAM-resistant models, but ETV retains a strong activity. Therefore, these models can be used to evaluate anti-viral agents against lamivudine-resistance, affording new opportunities to establish other drug-resistant HBV small-animal models.

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

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http://dx.doi.org/10.1016/j.apsb.2014.06.009

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1. Introduction

Hepatitis B virus (HBV) is the prototype member of the hepadnavirus family, which is a group of the smallest DNA-containing enveloped animal viruses known. HBV infection is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[1–4]. It is estimated that approximately 350 million people worldwide are chronic HBV carriers and 15%–40% of them eventually develop HBV-related cirrhosis or HCC[5–8]. Around one million deaths per year are due to HBV-related liver pathologies[9,10]. There are two classes of drugs available for the treatment of chronic HBV infection: first is the immune modulator interferon-α (standard or pegylated (PEG)-IFN-α), second are nucleoside or nucleotide analogs (NAs), which act as the HBV polymerase inhibitors, such as lamivudine (LAM), entecavir (ETV), tenofovir disoproxil fumarate (TDF), etc. IFN-α monotherapy has been the standard of treatment for chronic hepatitis B since the middle 1990s[11], but it is poorly tolerated and effective, only effective in 20%–40% patients. With the introduction of LAM, several oral HBV polymerase inhibitors were approved and accounted for the vast majority of therapies for chronic hepatitis B due to higher tolerance and more convenient administration, which reduced the occurrence of HCC and liver cirrhosis[12]. However, drug resistance resulting from HBV polymerase mutations with the NAs treatment is a huge obstacle to successful anti-viral therapy. Clinically, along with long-term LAM treatment, the emergence of lamivudine-resistant HBV was discovered in approximately 24% of patients after 1 year of therapy and in 70% after 5 years of therapy, so it was no longer considered a first-line agent in treatment of chronic HBV infection[13]. Therefore, it is important for designing and exploring new anti-viral agents to understand the mechanism of HBV drug resistance and avoid it.

To date, there are several animal models being described previously for studying the mechanism of HBV infection and exploring new anti-viral agents, but the evaluation of new anti-viral agents against drug-resistant HBV is limited by the lack of available small-animal models. Here we describe recently developed drug-resistant HBV mice models that alleviate many experimental constraints. Adapting the hydrodynamic-based procedure, we established convenient and replication-competent drug-resistant HBV NOD/SCID mice models based on clinic LAM-resistant mutants and evaluated the availability of these models in the assessment of anti-viral drugs[14].

2. Materials and methods

2.1. Reagents and animals

Female NOD/SCID mice at 6–9 weeks of age were purchased from Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. Animals were bred and cared under specific pathogen-free conditions in the Experimental Animal Center of the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. Our studies on mice were carried out in strict accordance with the recommendations in the Code of Ethics of the World Medical Association.

Lamivudine (LAM) and entecavir (ETV) tablets were purchased from GlaxoSmithKline Pharmaceuticals (Suzhou) Co., Ltd. and Sino-American Shanghai Squibb Pharmaceuticals Co., Ltd., respectively.

2.2. Construction of lamivudine-resistant mutations

pTmcs-HBV1.3 and the Sleeping Beauty transposase expression plasmid (pCMV-SB) were generously provided by F.V. Chisari's laboratory. pTmcs-HBV1.3 is an HBV replication-competent plasmid, which encodes a wild-type, terminally redundant (1.3-unit-length) HBV genome (Genbank accession number V01460). The supergenomic DNA was flanked by the inverted repeat (IR) recognition sequences of the Sleeping Beauty transposase. pTmcs-HBV1.3-3TCR and pTmcs-HBV1.3-3TCR-V173L are two different lamivudine-resistant mutants carrying rtL180M–rtM204V double mutations and rtL180M–rtM204V–rtV173L triple mutations, respectively. For their generations, rtL180M, rtM204V and rtV173L mutations were introduced into pTmcs-HBV1.3 by site-mutagenesis with the appropriate primers using QuickChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) as per the manufacturer's recommendations. The primer pairs of 5′–TTGGCTTTCAGTTATGTGGATGATGTGGTATTG–3′ and 5′–CAATACCACATCATCCACATAACTGAAAGCCAA–3′ were for rtM204V mutation, 5′–GTGGGCCTCAGCCCGTTTCTCATG-GCTCAGTTTACTAGTGCC–3′ and 5′–GGCACTAGTAAACTGA-GCCATGAGAAACGGGCTGAGGCCCAC–3′ were for rtL180M mutation, 5′–AAAATTCCTATGGGATTGGGCCTCAGCCCG-TTT–3′ and 5′–AAACGGGCTGAGGCCCAATCCCATAGGAA-TTTT–3′ were for rtV173L mutation. All constructs were sequenced to confirm that no additional mutations had been introduced.

2.3. Hydrodynamic injection of plasmid

The method of hydrodynamic injection of plasmids has been previously described[15]. In brief, a total of 13.5 μg of pTmcs-HBV1.3, pTmcs-HBV1.3-3TCR or pTmcs-HBV1.3-3TCR-V173L and 4.5 μg of pCMV-SB were co-injected into the tail vein of 6- to 9-week-old NOD/SCID mice in a volume of saline equivalent to 8% of the mouse body weight (*e.g.*, 1.6 mL for mouse of 20 g). The total volume was delivered within 5–8 s. The mouse serum samples were taken from the retro-orbital vessels on day 5 after injection to measure the HBV surface antigen (HBsAg) level by using the Diagnostic Kit for Hepatitis B Virus Surface Antigen (ELISA) (Shanghai Kehua Bio-Engineering) according to the manufacturer's instructions. HBsAg/Cut-off value in serum serves as an estimate of transfection efficiency in these experiments.

2.4. Evaluation of the availability of mouse models

The HBsAg-positive mice were randomly divided into three groups with 10 mice each on day 5 after hydrodynamic injection. Briefly, LAM (20 mg/kg) and ETV (0.1 mg/kg) were given twice per day by oral gavage administration in a medication course lasting 12 days, and the equivalent volume of saline was used as a control. To assess the efficacies of LAM and ETV against HBV replication, the mouse serum samples were collected every four days after drug administration. On the last day, the mice were sacrificed and the liver tissues were harvested and stored at -70 °C until assayed.

2.5. Quantitation of HBV DNA in mouse sera

The mouse serum samples were collected on days 0, 4, 8 and 12 after drug administration, and the method of purifying encapsidated viral DNA has been previously described[15]. Briefly, 50 μL of isolated serum was first digested with DNase I to eliminate

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