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Novel stable HBV producing cell line systems for expression and screening antiviral inhibitor of hepatitis B virus in human hepatoma cell line

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is currently a major public health burden. Therefore, there is an urgent need for the development of novel antiviral inhibitors. The stable HBV-producing cell lines of genotype D are widely used to investigate the HBV life cycle and to evaluate antiviral agents. However, stable HBV-producing cell lines of different genotypes do not exist. To construct more convenient and efficient novel cell systems, stable cell lines of genotypes A, B, and C were established using a full-length HBV genome sequence isolated from chronic HBV patients in human hepatoma HepG2 cells. Novel HBV clones were identified and stable HBV-producing cell lines derived from these clones were constructed. HBV replication activities demonstrated time-dependent expression, and the novel cell lines were susceptible to several antiviral inhibitors with no cytotoxicity. Furthermore, infectious viruses were produced from these cell lines. In conclusion, we have established novel stable HBV-producing cell line systems of genotypes A, B, and C. These systems can provide valuable tools for screening antiviral agents and analyzing viral phenotypes *in vitro*.

1. Introduction

Chronic hepatitis B virus (HBV) infection is currently a major public health burden, affecting over 240 million individuals globally [1]. Patients with chronic HBV have an elevated risk of chronic active hepatitis, cirrhosis, or primary hepatocellular carcinoma [2,3]. Establishment of infection and viral persistence are both dependent on the formation of covalently closed circular DNA (cccDNA) [4]. Current treatments with interferon-α or nucleoside analogs do not clear cccDNA from infected hepatocytes, and their effects on clearing hepatitis B surface antigen (HBsAg) are limited [5,6].

HBV has a partially double-stranded circular DNA genome of approximately 3200 base pairs. To date, HBV has been classified into 10 genotypes 'A' through 'J' based on an intergroup divergence >7.5% across the complete genome [7,8]. These genotypes can be further classified into subgenotypes based on a divergence of >4%. HBV genotypes have different geographic distributions [9,10]; genotypes A through D are mainly found in America, Europe, Asia, and Africa, while genotypes E through J are found in restricted parts of the world and are less well studied [11,12]. A growing body of evidence suggests that genotypic differences are associated with severity of liver disease, response to antiviral therapies, and the time to seroconversion of hepatitis B e antigen (HBeAg) [13,14].

The stable HBV-producing human hepatoma cell line 2.2.15 stably integrated two head-to-tail copies of the D-genotype HBV genome into the genome of human hepatoma HepG2 cells [15]. HepG2.2.15 cells were often used to evaluate antiviral compounds [16–18]. However, stable HBV-producing cell lines of genotypes A, B, and C were not commonly used in the research field. Currently, transient transfection assay using the minimum length of HBV replication-competent genome is a valuable tool for evaluating antiviral activity against genotypes A, B, and C [19–22]. However, this method is not ideal for use in high-throughput screening with many antiviral agents.

In this study, we have established novel cell systems with a full-

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length HBV genome sequence of genotypes A, B, and C isolated from patients with chronic HBV. Furthermore, we investigated HBV replication activities and production of infectious viruses and evaluated their susceptibility to several antiviral inhibitors in these cell lines.

2. Material and methods

2.1. Patients

Sera were obtained from 9 patients with chronic HBV; patient characteristics are shown in Table 1. Genotypes A, B, and C were obtained from 2, 1, and 6 HBV isolates from patient serum, respectively. The sera had high HBV DNA levels in all patients and high HBeAg levels in 8 patients (excluding patient C-2). Written informed consent was obtained from all patients.

2.2. HBV genome sequencing from patient serum and plasmid construction

HBV DNA was extracted from serum using QIAmp DNA blood kit (QIAGEN, Hilden, Germany). Six primer sets were designed to amplify 3 fragments covering the entire HBV genome (Table S1). The HBV genome sequence was confirmed with the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA) (Table S2). Amplified fragments were cloned with the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad, CA). In each amplified HBV genome fragment, at least 4 clones were isolated. The most dominant sequences found in the clones were determined as consensus sequences. The plasmid used the clone containing the consensus sequence was constructed with a 1.24-fold copy of the HBV genome (approximately 4000 base pairs) to ensure minimal competence for replication [21,22].

2.3. Cell culture

HepG2 cells and HepG2.2.15 cells were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Life Technologies, Carlsbad, CA) and supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, and 5 mg/mL insulin in the presence (HepG2.2.15) or absence (HepG2) of 400 μg/mL geneticin.

2.4. Transient transfection assay

HepG2 cells were seeded into 24-well plates at a density of 3.0×10^5 cells/well. After 24 h incubation, the cells were transfected with 0.5 μg of 1.24-fold HBV genome plasmids using the Lipofectamine 3000 transfection reagent (Invitrogen, Carlsbad, CA). In the preliminary experiment, there were no differences in transfection efficiencies among all HBV strains. HBV DNA in viral nucleocapsid (HBV core DNA) was extracted as described previously [23]. In short, cells were lysed and then the plasmids were digested by DNase. Viral nucleocapsids were digested by Protease and then DNA in the viral nucleocapsid was purified. HBV core DNA production in the cells and extracellular HBV DNA, HBeAg, and HBsAg secretions in the medium were evaluated 48 h after transfection [24]. We selected the HBV strains for the stable cell construction by the higher expression of HBV DNAs and viral antigens than the reference strain of each genotype.

2.5. Construction of stable HBV-producing cell lines

The plasmid was constructed with two head-to-tail copies of the HBV genome (2-fold copy, approximately 6400 base pairs) of genotypes A, B, and C. The full-length HBV genome sequence was amplified from the 1.24-fold HBV genome plasmid. Amplified fragments were purified and ligated using the In-Fusion HD Cloning Kit (TaKaRa Biochemicals, Shiga, Japan). Plasmids with two head-to-tail copies were identified by restriction analysis. HepG2 cells were then seeded into 10-cm dishes at a density of 3.0×10^6 cells/dish. After incubating for 24 h, the cells were transfected with 5 μg of plasmids with two head-to-tail copies of the HBV genome and 250 ng of plasmids bearing the coding sequence for the hygromycin resistance gene using the Lipofectamine 3000 transfection reagent. The cells were trypsinized 96 h after transfection and transferred into 4 × 10-cm dishes. Clones were selected using 250 μg/mL of hygromycin B and then were further re-cloned by a limiting dilution method. The clones secreting detectable HBeAg and HBsAg were selected as stable HBV-producing cells.

2.6. Integration of HBV DNA from stable HBV-producing cell lines

Stable HBV-producing cells were collected at a density of 8.0×10^6 cells. Genomic DNA was prepared as previously described [25]. For Southern blotting, the genome DNA was digested with *EcoT22*I and then separated on a 1% agarose gel. For the detection of viral DNA, the transferred membranes were probed with full-length HBV DNA of each genotype labeled with AlkPhos direct labeling reagents (GE Healthcare, Piscataway, NJ) [24].

2.7. HBV replication assay

Stable HBV-producing cells were seeded into 24-well plates at a density of 2.0×10^5 cells/well (day 0). HBV core DNA and HBV RNA productions in the cells and extracellular HBV DNA, HBeAg, and HBsAg secretions in the medium were evaluated at day 1, 4, and 7 after cell seeding. To determine antiviral activity, antiviral inhibitors were added to the plates 24 h after cell seeding and incubated for 6 days. HBV core DNA production in the cells and extracellular HBV DNA secretion in the medium were evaluated.

Table 1
Clinical characteristics of HBV patients.

Patient ID	Country of origin	Age (years)	Sex	ALT (IU/L)	Genotype	HBeAg	HBeAb	HBV DNA (log copies/mL)
A-1	Japan	39	Male	317	A	>1250	(−)	9.0
A-2	Philippines	23	Female	26	A	1166.6	(−)	9.7
B-1	China	26	Female	18	B	1371.4	(−)	8.4
C-1	Japan	34	Female	20	C	839.4	0.0	8.8
C-2	Japan	35	Male	157	C	NT	NT	9.1
C-3	Japan	26	Male	33	C	1604.8	(−)	9.4
C-4	China	26	Female	16	C	1357.8	(−)	8.8
C-5	Thailand	33	Female	33	C	1448.3	(−)	9.3
C-6	Japan	26	Female	39	C	1368.9	(−)	9.1

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBeAb, hepatitis B e antibody; NT, not tested.

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