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ORIGINAL ARTICLE

Hepatitis B virus infection and replication in a new cell culture system established by fusing HepG2 cells with primary human hepatocytes

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Background: Hepatitis B virus (HBV) infection is strictly species and tissue specific, therefore none of the cell models established previously can reproduce the natural infection process of HBV *in vitro*. The aim of this study was to establish a new cell line that is susceptible to HBV and can support the replication of HBV.
Methods: A hybrid cell line was established by fusing primary human hepatocytes with HepG2 cells. The hybrid cells were incubated with HBV-positive serum for 12 hours. HBV DNA was detected by quantitative fluorescence polymerase chain reaction (QF-PCR). HBsAg (surface antigen) and HBeAg (extracellular form of core antigen) were observed by electrochemiluminescence (ECL). HBcAg (core antigen) was detected by the indirect immunofluorescence technique. HBV covalently closed circular DNA (cccDNA) was analyzed by Southern blot hybridization and quantified using real-time PCR.
Results: A new cell line was established and named HepCHLine-7. The extracellular HBV DNA was observed from Day 2 and the levels ranged from 9.80 ($\pm$ 0.32) $\times$ 10^2 copies/mL to 3.12 ($\pm$ 0.03) $\times$ 10^4 copies/mL. Intracellular HBV DNA was detected at Day 2 after infection and

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the levels ranged from 7.92 (± 1.08) × 10^3 copies/mL to 5.63 (± 0.11) × 10^5 copies/mL. HBsAg in the culture medium was detected from Day 4 to Day 20. HBeAg secretion was positive from Day 5 to Day 20. HBcAg constantly showed positive signals in approximately 20% (± 0.82%) of hybrid cells. Intracellular HBV cccDNA could be detected as early as 2 days postinfection and the highest level was 15.76 (± 0.26) copies/cell.

Conclusion: HepCHLine-7 cells were susceptible to HBV and supported the replication of HBV. They are therefore suitable for studying the complete life cycle of HBV.

Introduction

Hepatitis B virus (HBV) is a member of the hepadnaviridae family that poses a significant health threat to millions of people worldwide. It has been estimated that two billion people have serologic evidence of past or present HBV infection and 360 million people suffer from chronic HBV infection.[1,2] People infected with HBV have a much higher risk of developing liver diseases, such as cirrhosis, liver failure, and hepatocellular carcinoma.[3,4]

HBV has a small, circular, partial double-stranded DNA genome of 3200 bases. The life cycle of HBV is believed to begin when the virus attaches to the host cell membrane. Then the viral membrane fuses with the cell membrane and the viral genome is released into the cell.[5,6] After the viral genome has entered the host cell, the partially double-stranded DNA converts into covalently closed circular DNA (cccDNA). The cccDNA acts as the template for further propagation of pregenomic RNA and mRNA.[7]

HBV infection is characterized by a narrow species and tissue tropism. Therefore, a reliable and sensitive *in vitro* infection cell model is still lacking. Currently, there are two main cell models used for the study of HBV *in vitro*, hepatoma-derived cells and primary human hepatocytes. The hepatoma-derived cells, such as HepG2, Huh-6, and Huh-7 cells, have been confirmed to support HBV transcription and replication. However, in these processes of infection, the integrated and transfected HBV genome acts as the template or the cells are added to chemical reagents to improve their susceptibility.[8–11] This processing destroys the complete life cycle of HBV. Although primary human hepatocytes are susceptible to HBV,[12,13] limited resources and technical difficulties associated with culture *in vitro* restrict their use as a model for HBV infection.

In this study, we attempted to establish an *in vitro* infection cell culture system by fusing primary human hepatocytes with HepG2 cells, which could support the natural infection of HBV.

Methods

Isolation and culture of primary human hepatocytes

Liver tissue was obtained from the surgical removal of liver tissue of a female patient with hepatic hemangioma who had no serological evidence of past or present HBV infection. This study was approved by the Medical Ethics Committee of the Shandong University Medical School, Ji'nan, China.

Hepatocytes were isolated using a two-step collagenase perfusion technique as previously reported.[14,15] The isolated hepatocytes were resuspended in hepatocyte medium containing Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA), supplemented with 200 mL/L (20%) fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), 20 mg/L dimethyl sulfoxide (DMSO, Roche, Basel, Switzerland), 20 mg/L polyethylene glygol (PEG, Roche, Basel, Switzerland), 10 μg/L insulin, 0.1 μmol/L dexamethasone, and 4 mmol/L glutathione. The cells were cultured in an incubator at 37°C in a humidified atmosphere with 5% CO_2 in air. The viability of hepatocytes was determined by trypan blue (Gibco, Carlsbad, CA, USA) dye exclusion.

Obtainment of HGPRT(−) HepG2 cells

The original HepG2 cells were supplied by the China Centre for Type Culture Collection (Wuhan, China). Hypoxanthine-guanine phosphoribosyltransferase null [HGPRT(−)] HepG2 cells were selected from the original HepG2 cells in the present of ethyl methane sulfonate (EMS, Sigma Aldrich, St Louis, MO, USA) and 6-mercaptopurine (6-MP, Sigma Aldrich, St Louis, MO, USA). The cells were resistant to 0.3 g/L EMS and 10 mg/L 6-MP but could not grow in hypoxanthine–aminopterin–thymidine (HAT, Gibco, Carlsbad, CA, USA) medium (DMEM supplemented with 5% FBS, 100 μmol/L hypoxanthine, 0.4 μmol/L aminopterin, and 16 μmol/L thymidine), which suggested a complete defect in inosinic acid pyrophosphorylase (IPP).[16] In addition, reversion to 6-MP was not detected. The characteristics of HGPRT(−) HepG2 cells were similar to those of the original HepG2 cells, such as morphology and the mean chromosome number.

Cell fusion and selection of the hybrid cells

Hepatocytes (1 × 10^6 cells) and HGPRT(−) HepG2 cells (2 × 10^6 cells) were mixed in a tube in the present of 50% PEG 1500 and stirred gently. Then, the mixed cells were washed twice with DMEM. The final mixture was plated into a 96-well plate in HAT medium. The medium was changed every 2–3 days for 2 weeks. When the cells could stably grow, they were diluted into 10 cells/well, two cells/well,

one cell/well, and 0.5 cell/well by using the limited dilution method. After several rounds of limiting dilution cloning, one hybrid cell clone was obtained and named HepCHLine-7. Thereafter, the culture medium was changed to HT medium for 1 week. Then, the obtained hybrid cells were maintained in DMEM supplemented with 5% FBS.

Analysis of the chromosomes in hybrid cells

Chromosome numbers were counted in original HepG2, HGPRT(−) HepG2, and HepCHLine-7 cells by the trypsin G-banding method as described previously.[17] Twenty cells of each group were analyzed.

Infection with HBV *in vitro*

Serum containing HBV DNA 7.5×10^8 copies/mL was obtained from a chronic hepatitis B patient who was positive for HBsAg (surface antigen) and HBeAg (extracellular form of core antigen). About 3×10^6 hybrid cells were incubated with 4 mL DMEM supplemented with 10% HBV serum in a water bath for 12 hours at 37°C with gentle shaking. The ratio of cell and HBV DNA copy was 1:100. HGPRT(−) HepG2 cells were used as a control. Following 12 hours of exposure, the cells were washed five times with phosphate-buffered saline (PBS) to remove the unabsorbed virus, and the PBS used in the fifth wash was collected for detection of the presence of HBV DNA. Then, the infected cells were cultured in DMEM supplemented with 10% FBS. The start of this incubation was taken as time zero.

Detection of HBV DNA in cells and culture medium

Postinfection, the infected cells were harvested and their culture supernatants were collected in order every day. All samples were stored at −70°C until used. To avoid potential interference from the residue of the infectious source, the detection of HBV DNA was performed from the 2nd day after infection. Total DNA was extracted from infected cells and culture medium according to the method described previously.[18] The HBV DNA was detected by quantitative fluorescence polymerase chain reaction (QF-PCR). HBV PCR Fluorescent Quantitative Detection Kit (Piji Biotec, Shenzhen, China) was used according to the manufacturer's instructions.

HBV-infected HGPRT(−) HepG2 cells and uninfected HepCHLine-7 cells were used as a control.

Detection of HBsAg, HBeAg, and HBcAg

The HBsAg and HBeAg levels in the culture medium were measured from the 2nd day postinfection by electrochemiluminescence (ECL) using an Elecsys 2010 fully-automated ECL analyzer (Roche, Basel, Switzerland).

The HBcAg (core antigen) within the infected cells was observed by indirect immunofluorescence analysis. First, infected cells were fixed with 4% paraformaldehyde for 20 minutes at 4°C and permeabilized in Triton X-100. Then, cells were blocked by incubation with PBS containing 10% goat serum for 30 minutes at 37°C. Cells were then incubated with a specific mouse monoclonal antibody against HBcAg at 4°C overnight. Thereafter, cells were incubated with the secondary antibody, a fluorescein isothiocyanate (FITC)-tagged goat antimouse immunoglobulin G (IgG), for 30 minutes at 37°C. Finally, cells were incubated in 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature and examined using an inverted fluorescence microscope (Olympus, Tokyo, Japan).

HBV-infected HGPRT(−) HepG2 cells and uninfected HepCHLine-7 cells were used as a control.

Detection of HBV cccDNA

The cells were washed with PBS and lysed in lysis buffer (50mM Tris, 100mM NaCl, 0.1% Triton X-100, 5mM $MgCl_2$, pH 8.0) for 10 minutes at 4°C. Nuclei were separated from the cytoplasmic fraction by centrifugation at 270 *g* for 2 minutes at 4°C. Episomal DNA was extracted from nuclei as previously described.[19] Cells were lysed in lysis buffer without pronase, and protein−detergent complexes were precipitated by the addition of 0.25 mL of 2.5M KCl. The lysates were mixed by shaking and centrifuged to remove most of the cellular DNA. Viral cccDNA in the supernatant was extracted with an equal volume of phenol. HBV cccDNA was further purified using the method of Yang et al.[20] The purified HBV cccDNA were detected by Southern blot hybridization and quantified using real-time PCR as described previously.[21] The DIG High Prime DNA Labeling and Detection Starter Kit I (Roche, Basel, Switzerland) was used.

HBV-infected HGPRT(−) HepG2 cells and uninfected HepCHLine-7 cells were used as a control.

Blocking test

The viral serum from a hepatitis B patient was preincubated with various antibodies against the pre-S1, pre-S2, and S domains of HBV surface proteins in a water bath for 30 minutes at 37°C. Then, approximately 3×10^6 hybrid cells were infected by the treated viral serum in a water bath for 12 hours at 37°C with gentle shaking. The ratio of cell and HBV DNA copy was 1:100. HBV DNA, HBsAg, HBeAg, HBcAg, and cccDNA were detected from Day 2 to Day 18 postinfection.

Results

Establishment of hybrid cell line

A cell line named HepCHLine-7 was established from one colony. The morphology did not change after the 30th subculture. To confirm that the HepCHLine-7 cell line was a hybrid line, its chromosome number was analyzed by the G-banding method. The mean modal chromosome numbers were 51, 53, and 99 in original HepG2, HGPRT(−) HepG2, and HepCHLine-7 cells. The results indicated that the HepCHLine-7 was a hybrid cell line by fusing HGPRT(−) HepG2 cells (53 chromosomes) with human hepatocytes (46 chromosomes).

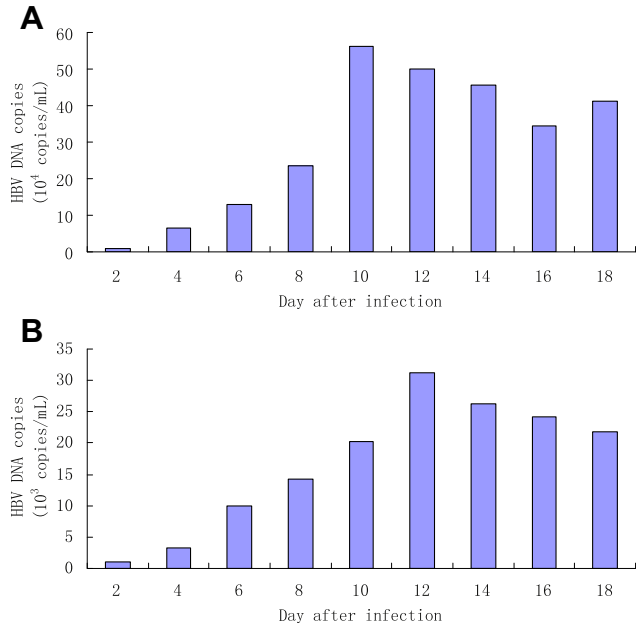

Figure 1. Detection of HBV DNA within cells and in culture medium. (A) HBV DNA was extracted from infected cells and observed from Day 2 to Day 18 postinfection. The levels ranged from 7.92 (± 1.08) × 10^3 copies/mL to 5.63 (± 0.11) × 10^5 copies/mL. (B) HBV DNA was isolated from culture medium and detected from Day 2 to Day 18 postinfection. The levels ranged from 9.80 (± 0.32) × 10^2 copies/mL to 3.12 (± 0.03) × 10^4 copies/mL. HBV = hepatitis B virus.

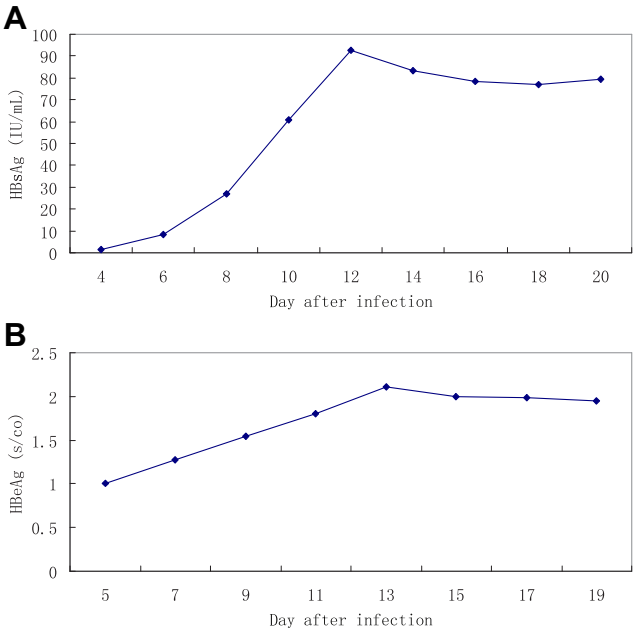

Figure 2. Detection of HBsAg and HBeAg in culture medium. (A) HBsAg was detected from Day 4 to Day 18 postinfection. The levels ranged from 1.670 (± 0.031) IU/mL to 92.450 (± 0.735) IU/mL. (B) HBeAg was observed from Day 5 to Day 18 postinfection. The levels ranged from 1.001 (±0.002) s/co to 2.103 (±0.007) s/co. HBsAg = surface antigen of hepatitis B virus; HBeAg = extracellular form of the core antigen of hepatitis B virus; s/co = absorbance rate/cut-off ration.

Analysis of HBV DNA in cells and culture medium

The total DNA was extracted from infected cells and quantified by QF-PCR. The levels of intracellular HBV DNA ranged from 7.92 (± 1.08) × 10^3 copies/mL to 5.63 (± 0.11) × 10^5 copies/mL and the highest level appeared at Day 10 (Fig. 1A). To investigate whether HBV DNA could be released into culture medium, viral DNA was extracted from the medium and quantified. The copy number of viral load ranged from 9.80 (± 0.32) × 10^2 copies/mL to 3.12 (± 0.03) × 10^4 copies/mL with a secretion peak at Day 12 (Fig. 1B). These HBV DNA molecules were not detected in the culture medium at time zero and in the fifth washing with PBS. HBV DNA was not observed in the controlled cells.

Detection of HBsAg, HBeAg, and HBcAg

To investigate whether infected cells could secrete HBV-specific antigen into the culture medium, the culture medium was collected and detected for the presence of HBsAg and HBeAg by ECL analysis. HBsAg was observed from Day 4 postinfection and the levels ranged from 1.670 (± 0.031) IU/mL to 92.450 (± 0.735) IU/mL (≥ 1.0 IU/mL was considered positive; Fig. 2A). HBeAg was detected from Day 5 postinfection and the levels ranged from 1.001 (± 0.002) s/co to 2.103 (± 0.007) s/co (absorbance rate/cut-off ration, ≥1.0 s/co was considered positive; Fig. 2B). HBsAg and HBeAg were not detected in the culture medium of controlled cells.

To estimate the percentage of infection cells, indirect immunofluorescence was used to detect the presence of HBcAg within the infected cells. HBcAg expression was first observed at Day 4 after infection and constantly showed positive signals in approximately 20% (± 0.82%) of hybrid cells. However HBcAg did not appear positive within controlled cells (Fig. 3).

Detection of HBV cccDNA

HBV cccDNA was detected by Southern blot hybridization. A slight band of 2.0 kb was observed that corresponded to the expected position for the HBV cccDNA. However, no cccDNA was detected in controlled cells (Fig. 4). The formation of HBV cccDNA was detected from Day 2 postinfection. By quantification using real-time PCR, the copy number ranged from 0.02 (± 0.002) copies/cell to 15.76 (± 0.26) copies/cell.

Detection of the results of the blocking test

HBV DNA, HBsAg, HBeAg, HBcAg, and cccDNA were not detected as they were in the control infection.

Discussion

Primary human hepatocytes and human fetal hepatocytes have been reported to be susceptible to HBV infection.[12,13]

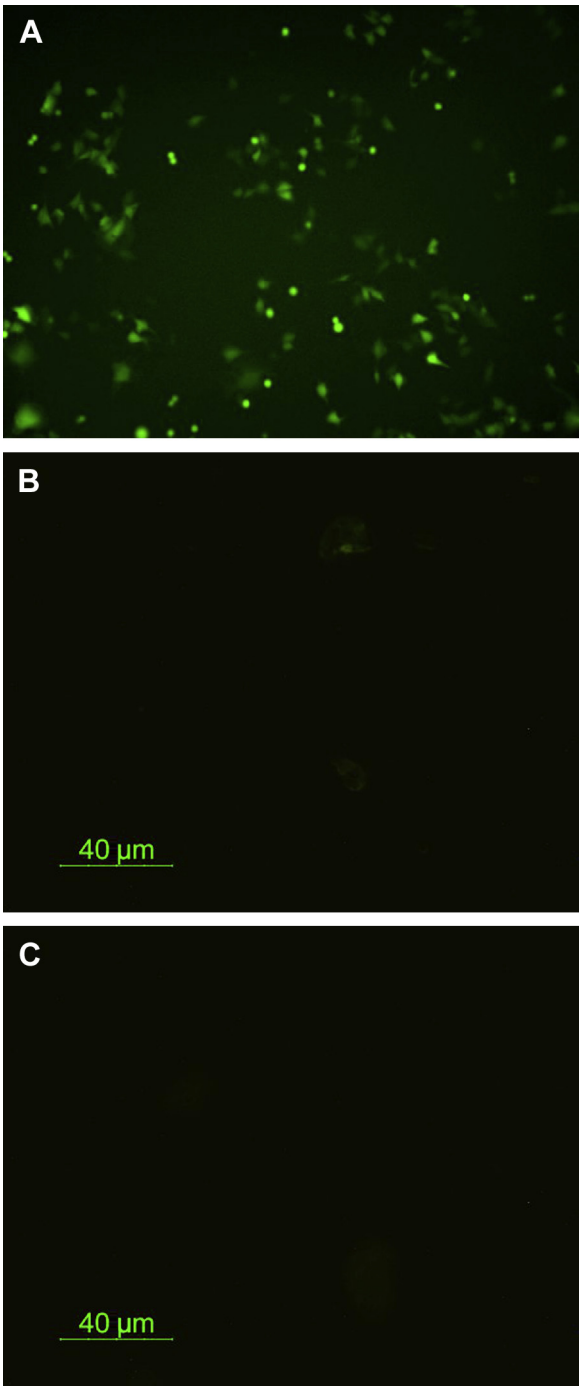

Figure 3. HBcAg was analyzed by indirect immunofluorescence. HBcAg was detected in (A) HBV-infected HepCHLine-7 cells, but was not found in (B) HBV-infected HGPRT(−) HepG2 cells and (C) uninfected HepCHLine-7 cells. HBcAg = core antigen of hepatitis B virus; HBV = hepatitis B virus; HGPRT = hypoxanthine-guanine phosphoribosyltransferase.

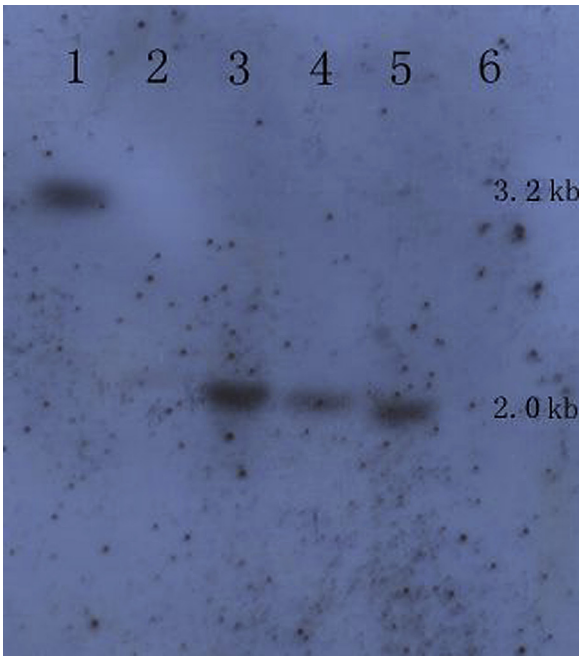

Figure 4. Southern blot analysis of HBV cccDNA. Lanes: 1, whole HBV DNA probe; 2, HBV-infected HGPRT(−) HepG2 cells; 3, Day 2 postinfection; 4, Day 10 postinfection; 5, Day 16 postinfection; 6, uninfected HepCHLine-7 cells. cccDNA = covalently closed circular DNA; HBV = hepatitis B virus; HGPRT = hypoxanthine-guanine phosphoribosyltransferase.

However, both hepatocytes are hampered by limited resources and technical difficulties in culture. Hepatoma-derived cell lines such as HepG2 cells were used for the *in vitro* study of HBV and were confirmed to support the replication of HBV. However, the viral genome was introduced by integration or transfection rather than infection.[8,9] In some previous studies, to improve their susceptibility, cells were subjected to treatment with DMSO or PEG.[10,11] However, these chemicals may induce an artificial mechanism for viral entry. The lack of efficient virus—cell interactions may be the major reason for HBV insusceptibility. Therefore, these cell lines could reveal some important details of HBV replication, but they were not suitable for studying the early steps of the HBV life cycle, such as attachment and penetration.

In this study, a new cell line named HepCHLine-7 was established by fusing primary human hepatocytes with HGPRT(−) HepG2 cells. To confirm that HepCHLine-7 cells were hybrid cells, the trypsin G-banding method was used to count the mean chromosome numbers. The results showed that the modal chromosome numbers in HepCHLine-7 cells, HGPRT(−) HepG2 cells, and primary human hepatocytes were 99, 53, and 46, respectively. Therefore, HepCHLine-7 cells were considered hybrid cells containing genomes from the parental cells.

Initial evaluation of the infection system was carried out using QF-PCR to detect HBV DNA. HBV DNA could be observed both within cells and in the culture medium, and the levels increased with the culture time. HBV DNA was not detected in controlled cells and the fifth washing PBS, which suggested that these DNA molecules were not from the infectious source.

In addition to assay of HBV DNA, secretory HBV antigens were detected in the culture medium and HBcAg was detected throughout the cytoplasm. The synthesis of HBV-specific antigens provided indication that replication and expression of the HBV genome occurred in infected HepCHLine-7 cells, whereas no significant replication or

expression of the HBV genome was detected in controlled cells. However, only about 20% (± 0.82%) of the cells constantly expressed HBcAg on Day 4 after HBV infection, which may be associated with the viral load and virulence. In the early step of infection, the cells were infected and supported the replication and expression of HBV when a sufficient number of virus particles adhered to the surface of the cells.

HBV cccDNA is a critical intracellular replicative intermediate that acts as the template for transcription of viral pregenome RNA and mRNA.[7] Therefore, cccDNA is considered as the resource of new HBV DNA. In order to provide further evidence that HepCHLine-7 cells can support the replication of HBV, we detected the intracellular HBV cccDNA. In the present study, HBV cccDNA was observed as early as Day 2 postinfection. This phenomenon is consistent with the view that the formation of cccDNA is a prerequisite for entering the replication cycle of HBV and is considered to be the earliest marker of HBV infection.

To determine whether the infection was blocked by specific antibodies, the virus was pre-incubated with various antibodies against the pre-S1, pre-S2, and S domains of HBV surface proteins. The infection was prevented because HBV DNA and specific antigens were not detected in the culture medium as they were in the control infection (data not shown). The results indicated that the uptake of HBV by HepCHLine-7 cells required specific attachment sites on the surface of HBV.

In conclusion, detection of HBV viral load, specific antigens, and cccDNA levels provided direct evidence that the HepCHLine-7 cell line was susceptible to HBV and supported the replication of HBV in a natural way. The HepCHLine-7 cell line inherited the high susceptibility to HBV from human hepatocytes and immortality from HepG2 cell. This *in vitro* infection system will be a useful tool to study the complete process of HBV infection and test the efficacy of antiviral drugs.

Conflicts of interest

All authors declare no conflicts of interest.

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