Establishment of A New HBV Cell Culture Model by Covalently Closed Circular DNA Direct Transfect

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Abstract. Hepatitis B virus (HBV) infection is a public health problem in China and worldwide. Covalently closed circular DNA (cccDNA) is the template for HBV replication and exists stably in hepatocytes, which is the main factor for persistent HBV infection and the key target for the cure of HBV infection. The establishment of a suitable cell model is helpful to research the HBV pathogenesis and antiviral drug screening. We have established a new cell culture model of HBV infection by direct transfect of cccDNA, which provides a practical model for further research of HBV virology and antiviral drug development.

1. Introduction

According to the World Health Organization, about 240 million people worldwide had chronic hepatitis B, caused by infection with Hepatitis B virus (HBV) [1], most of them lived in low- and middle-income countries, about 650,000 people died from fibrosis, cirrhosis and liver cancer caused by chronic hepatitis B each year. In China, chronic hepatitis B was one of the main diseases threatening people health. About 86 million people were infected with chronic hepatitis B, which about 20 million were suffering from chronic hepatitis B [2, 3].

HBV is a DNA virus belonging to the Hepadnaviridae family. The mature virion contains about 3.2 KB relaxed circular DNA (rcDNA) genome, which the negative chain is a complete ring, while the positive chain has a large gap and variable length [4]. Viral particles first enter the cytoplasm through specific receptors, then remove the envelope of the genome into the nucleus, and finally form a covalently closed circular DNA (cccDNA) with tight covalent bond in the role of HBV viral polymerase [5]. In the form of small supercoiled chromosomes, cccDNA persists in the nucleus of hepatocytes and serves as a template for the replication viruses, through the production of new rcDNA re-entry into the cell nucleus for their own cycle of renewal and replenishment [6, 7]. To sum up, cccDNA is not only the cause of the persistence of chronic hepatitis B infection, but also the crux of the problem that antiviral drugs difficult to eradicate the virus and relapse after drug withdrawal, cccDNA is an inherent sign of chronic hepatitis B, and a key target for antiviral drugs [8].

In the past decades, despite the remarkable achievements have made in the field of HBV research, due to the lack of appropriate experimental models and other reasons, there was still a lack of in-depth understanding of the formation, maintenance and degradation of HBV cccDNA metabolic mechanisms [9]. The representative Hepatitis B cell models mainly include primary human hepatocytes (PHH), HepaRG, HepG2, Huh7, HepG2.2.15 and HepAD38. PHH can be infected by HBV, which is an ideal natural cell model for studying HBV infection. However, PHH is difficult to obtain, slow to proliferate,
and difficult to maintain its differentiation in vitro [10, 11]. The HepaRG cell model supported HBV reinfection, but the experimental conditions required 4 weeks of culture differentiation and depended on a high number of viral cell infections to achieve infection [12]. HepG2 and Huh7 are the most common liver cancer cell lines, which are not infected by HBV because of a lack of viral entry receptor. Sodium taurocholate cotransporting polypeptide (NTCP) has been proved to be a highly affinity receptor for HBV, and the stable expression of exogenous NTCP enables HepG2 and Huh7 cells to be infected with HBV [13]. HepG2.2.15 and HepAD38 are commonly used as stable transfect hepatoma cell lines, the former integrated and stably expressed HBV genome in the host cell genome, while the latter used tetracycline, tet-off expression regulatory system to induce HBV pgRNA and HBeAg expression [14, 15]. The DNA plasmid or recombinant virus vector used for HBV infection in the above-mentioned cell models may have significantly different virological characteristics and host reactivity from 3.2 kb cccDNA.

In this study, cccDNA products which are identical with the natural cccDNA sequence were obtained easily and efficiently by PCR amplification, BspQI digestion, cyclization and purification and other molecular biological methods. We have established a new cell culture model of HBV infection by direct cccDNA transfect, which provides a rapid and sensitive in vitro test platform for HBV virology and high-throughput drug screening.

2. Materials and methods

2.1. Preparation of cccDNA
A simple and efficient procedure for preparation of cccDNA was established in our laboratory. Firstly, the full-length HBV DNA was amplified by PCR reaction used the pEASY-HBV plasmid as the template, which contains the full-length HBV DNA was cloned in our previous studies [16, 17]. The designed primers were modified from those used by Li et al. [18] The reaction mixture contains 0.5μl fusion DNA polymerase, 2μl of plasmid DNA diluted 100 times, 1μl of each of the forward and reverse primer, 10μl GC buffer, 4μl dNTPs, and double-distilled water (ddH2O) to reach a final volume of 50μl. Secondly, PCR product was purified with DNA fragment purification kit according to the manufacturer's instructions and digested with BspQI restriction enzyme. Thirdly, T4 DNA ligase was used to cyclize the purified BspQI digested product. Finally, the products of cyclization were purified and verified by EcoRI digestion. Agarose Gel electrophoresis was used to detect the products of each stage.

2.2. Establishment of the cell model
cccDNA was transfected into HepG2 cells by lipofectamine 3000 reagent. Prepared the cells the day before transfect and made cell suspension at a concentration of 5×10⁴ cells/ml, cccDNA treatment group and transfect reagent control group were set, and each group was inoculated with 21 multiple wells and 200μl cell suspension per well in 96 well plates. The cells were cultured in a carbon dioxide incubator with 5% CO₂ at 37°C, culture medium was changed every day, and transfect was carried out when the cell concentration reached 90%-95%. Transfect was carried out according to the instructions, and 0.2μg cccDNA was added to each well in the experimental group. After transfect, the cells were cultured at 37°C, 200μl supernatant was collected and 200μl fresh medium was added per well per day.

2.3. Detection of the HBV-specific markers in supernatant
The expression of HBsAg and HBeAg was detected by Elisa Kit. The supernatants collected from 1 to 6 days after transfect were detected simultaneously with HBsAg standard samples of 2IU / ml, 1IU / ml, 0.5IU / ml, 0.2IU / ml, 0.1IU / ml and 0IU / ml and HBeAg standard samples of 2 NCU / ml, 1 NCU / ml, 0.5 NCU / ml, 0.2 NCU / ml, 0.1 NCU / ml and 0 NCU / ml, and positive and negative control samples included in the kit. Firstly, the samples, standard or positive-negative control 50μl and enzyme complex 50μl was added to each hole, then incubated at 37°C for 30 minutes. Secondly, wash the board with lotion 5 times, and shake dry, pat dry. Thirdly, each 50μl of colouration solution A and
B were added to each well, incubated for 15 minutes. Then termination solution 50μl was added and mixed to each well. Finally, the wavelength at 450nm and 630nm in each well sample was read by full wavelength multifunctional enzyme marker thermo varioskan flash.

2.4. Detection of the HBV DNA and cccDNA in cell samples
The expression of HBV DNA and cccDNA was detected by qPCR in cells from 1 to 6 days after transfect. Firstly, the total DNA in the cell was extracted by cell DNA extraction kit. Secondly, the nucleic acid concentration of the total DNA was determined using NanoDrop microvolume spectrophotometers. Two 150ng of the total DNA were taken from each sample, one was treated with T5 exonuclease as the template for cccDNA qPCR detection and the other one was not as the template for HBV DNA qPCR detection [19]. The forward primer 5'-TTCTCCGCTGTCGTACC-3' and the reverse primer 5'-GGTTTCTGTTGGCGTTC-3' were used for HBV DNA qPCR. The forward primer 5'-CTTCTCATCTCGGACC-3' and the reverse primer 5'-CACAGCTTTGGAGGCTTG-3' were used for cccDNA qPCR [20]. The cccDNA prepared in the laboratory was treated with T5 exonuclease, after purification and recovery, the OD value was determined by NanoDrop microvolume spectrophotometers, and the sample was diluted to 5×10^8 copies/μl, 5×10^7 copies/μl, 5×10^6 copies/μl, 5×10^5 copies/μl, 5×10^4 copies/μl, 5×10^3 copies/μl, 5×10^2 copies/μl calculated by the following formula, which were used as the standard samples for the detection of cccDNA and HBV DNA by qPCR.

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\frac{(x \text{ ng/μl}) \times 10^9}{(2692+1140) \times 660} = \frac{6.022 \times 10^{23} \text{ copies/μl}}{}
\]

2.5. MTT Assay at one day after transfect
After transfect one day, MTT cell proliferation and cytotoxicity test kit were used to detect the difference of cell proliferation between the experimental group, the lipofectamine 3000 control group and blank group. Firstly, the supernatant of each group was carefully discarded and added with a new solution containing 90μl fresh culture medium and 10μl MTT solution, continue to culture for 4 hours. Then the supernatant was removed carefully, 110μl formazan solution was added into each hole, and the 96-hole plate was placed on the shaker at low speed for 10 min to make the crystal fully dissolved. Finally, the absorption values of the holes were measured at 490nm by full wavelength multifunctional enzyme marker.

3. Results

3.1. Simple and efficient preparation of cccDNA
Using the standardized process established and optimized by our laboratory, such as PCR reaction, PCR product purification, BspQI digestion reaction, BspQI digestion product purification, T4 DNA ligase cyclization reaction, cyclization product recovery, EcoRI digestion verification, etc., finally, enough cccDNA was obtained (Fig 1). The loading of each sample is comparable in agarose gel electrophoresis, which adjusted according to each reaction system. The results showed that the superhelical cccDNA was successfully prepared by using simple molecular biological methods.
3.2. Persistence of virus-specific markers in supernatant
Levels of HBsAg and HBeAg in supernatant were detected at predetermined time points by ELISA (Fig 2). HBsAg and HBeAg could be detected at 1 to 6 days after cccDNA transfec in the experimental group, while the HBsAg expression level increased gradually from 1 to 4 days, reached the peak on the 4th day and began to decrease on the 5th and 6th day (Fig 2A), and the HBeAg expression level increased gradually from 1 to 3 days then decreased, reached the peak on the 3th day (Fig 2B). The expression of HBsAg and HBeAg in the lipofectamine 3000 control group remained negative throughout (Fig 2).

3.3. Persistence of the HBV DNA and cccDNA in cells
Levels of HBV DNA and cccDNA in cells were detected at predetermined time points by qPCR (Fig 3). The expression of HBV DNA and cccDNA in the experimental group were significantly higher than that in lipofectamine 3000 control group although the level of expression decreased gradually at 1 to 6 days after cccDNA transfec. The reason for the gradual decline in HBV DNA and cccDNA may be the rapid proliferation of untransfected cells, whereas we used a whole genome of 150 ng in each
sample. Nevertheless, we detected persisted expression of HBV DNA and cccDNA, indicating that we have successfully established a new HBV cell model.

![Persistence of the HBV DNA and cccDNA in cells. (A) Levels of HBV DNA in cells after cccDNA transflect. (B) Levels of cccDNA in cells after cccDNA transflect.](image)

**Figure 3.** Persistence of the HBV DNA and cccDNA in cells. (A) Levels of HBV DNA in cells after cccDNA transflect. (B) Levels of cccDNA in cells after cccDNA transflect.

3.4. **No obvious cell injury in the cell model**

One day after transfect The HepG2 cell in different group were detected by MTT, in contrast to the positive biochemistry for viral biomarkers expression of HBV in supernatant and cells did not lead to serious cell injury. In conclusion, the new cell culture model of HBV infection we have establishment are successful.

![The detection of cell injury in the cell model by MTT.](image)

**Figure 4.** The detection of cell injury in the cell model by MTT.

4. **Conclusion**

Cell models of HBV serve as important tools to study pathogenic mechanisms and evaluate drugs before clinical treatment [21]. There are three kinds of cell models about HBV infection: HBV DNA integrated stable cell models, HBV infection cell models, HBV recombination cccDNA transflect cell models [9]. The DNA plasmid or recombinant virus vector used for HBV infection in the above-mentioned cell models may have significantly different virological characteristics and host reactivity from 3.2 kb cccDNA.

In the current study, using of specific restriction site BspQI in HBV sequence, we have prepared the cccDNA products which is identical to natural sequence by using simple molecular biological method. We have established an optimized, simple and efficient standard operating procedure in the laboratory, and can obtain cccDNA products of more than 100 μg in a single preparation. cccDNA was transfected into HepG2 cell via the lipofectamine method. In doing this, a new HBV cell model was established successfully. The HBV infection persisted and HBV markers, including HBV DNA,
HBsAg, HBeAg and cccDNA, were detected at up to 6 days using this model and cell injury in the cell model was minimal.

It is believed that the key factor of HBV persistent infection is the failure to eradicate cccDNA [22, 23], the therapeutic strategy targeting cccDNA directly is considered to be the most likely method to eliminate HBV, and is an important direction for the development of new drugs [24]. However, the formation and degradation of cccDNA are still poorly understood at present. Our HBV cell model established by direct transfec of cccDNA will change this dilemma. This will provide new insights into the biochemistry of cccDNA, the molecular mechanisms by which the body's immune system processes cccDNA in acute HBV infection, and new techniques for manipulating cccDNA; these will contribute to the ultimate achievement of a complete eradication of cccDNA.

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