

Hepatitis B Virus Infection of HepaRG Cells, HepaRG-hNTCP Cells, and Primary Human Hepatocytes

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Abstract

Investigations of virus-host interactions rely on suitable in vitro cell culture systems that efficiently support virus infection. Such systems should ideally provide conditions that resemble those of natural host cells, e.g., the cell-type specific signaling and metabolic pathways. For HBV infection, primary human hepatocytes (PHHs) are the most faithful system fulfilling these requirements but access to these cells is limited. Moreover, the reproducibility of experimental results depends on many factors including the preparation method or variability of the donors. The human liver cell line HepaRG, after differentiation, resembles PHHs with respect to many hepatocyte-specific markers including the expression of cytochrome P450 enzymes, liver-specific transcription factors, and transporter proteins like the HBV-specific receptor, sodium taurocholate co-transporting polypeptide (NTCP). HepaRG cells have also been shown to express key molecules of the innate immune system. So far, the HepaRG cell line is the only one allowing both studies on HBV/HDV infection and liver-specific drug toxicity and metabolism. The relative low susceptibility of HepaRG cells when compared with PHHs depends on various factors and can partially be overcome by constitutive expression of the receptor NTCP, allowing infection without full differentiation. Ectopic NTCP expression does not interfere with the ability of cell differentiation induced by DMSO. Here, we describe in detail how to technically perform HBV infection in vitro with these cells. The methods can be used to explore the mechanism of HBV infection and to build an antiviral screening platform suitable for evaluation of drug efficacy in cells that are metabolically close to primary human hepatocytes.

Key words HepaRG, NTCP, PHH, HBV infection, Authentic infection

1 Introduction

Human Hepatitis B virus (HBV) belongs to the family *Hepadnaviridae*, which comprises two genera: the genus *Orthohepadnavirus* infecting mammals and the *Avihepadnaviruses* infecting birds. Members of the *Orthohepadnaviruses* including WHV (Woodchuck Hepatitis virus) [1], GSHV (Ground squirrel hepatitis virus) [2], WMHBV (woolly monkey hepatitis B virus) [3], and HBV can efficiently replicate in the liver of their respective hosts showing a pronounced species specificity. This restriction is mostly determined by the differences in their envelope proteins

and recognition of receptors. Although in vitro cultured primary human hepatocytes (PHHs) had been successfully infected with HBV already in 1986 [4], the use of these cells for systematic studies became practical only after optimization of the cell culture conditions, namely the implementation of DMSO to the culture medium and the addition of polyethylene glycol (PEG) to increase the efficacy of infection [5, 6]. PHHs are accepted as the gold-standard in vitro model for HBV infection, in which nearly 100% of cells are reproducibly infectable under certain conditions (e.g., increasing multiplicity of genome equivalents) [7]. Since none of the commonly used immortalized hepatic cell lines (e.g., HepG2, HuH7) supported HBV infection, PHH for a long time was the only system to study the complete HBV replication cycle. This limitation was overcome after the discovery of a human hepatoma cell line called HepaRG. This cell line in culture behaves like liver progenitor cells bearing the potential to differentiate into hepatocyte-like granular cells and biliary cells following DMSO treatment [8]. In addition to their susceptibility to HBV and Hepatitis D Virus (HDV), HepaRG cells have been intensively investigated with respect to hepatocyte-specific functions, such as albumin secretion, formation of bile canaliculi, drug transporter activities, expression of cytochrome P450 and glutathione S-transferase, and other enzymes involved in drug metabolism [9, 10]. Compared to PHHs, the unlimited availability of HepaRG cells makes it a very important tool for pharmacological and toxicological studies besides in vitro HBV and HDV infection/coinfection. Interestingly, it turns out that sodium taurocholate cotransporting polypeptide (NTCP), one of the transporters expressed on HepaRG cells solely after differentiation, is the specific receptor for HBV [11, 12].

The identification of human NTCP (hNTCP) as the *bona fide* HBV receptor profoundly changed the field of HBV infection models. The permissive but non-susceptible HepG2 cell line can be infected with HBV upon expressing hNTCP. It is now widely used for infection studies including high-throughput drug screening approaches. The endogenous hNTCP level of HepaRG cells that can be achieved through differentiation is only ca. 10% of that in PHHs [13]. This may partially explain the observation that HepaRG cells cannot be infected to a similar percentage compared to PHHs. Accordingly, when hNTCP is stably expressed in HepaRG cells [12], the HBV infection efficacy is improved. Moreover, since the differentiation process that is required for hNTCP expression in naive HepaRG cells is no longer required, HepaRG-hNTCP cells can already be infected shortly after seeding although at less efficacy than those differentiated. In comparison to HepG2-hNTCP, fully differentiated HepaRG-hNTCP secretes higher levels of HBsAg upon infection, resembling the levels obtained in HBV-infected PHHs.

The addition of DMSO to the culture medium upregulates the expression of hNTCP during differentiation of HepaRG cells and enhances HBV replication in both infected cells [6, 12] and cells that express HBV transcripts from a chromosomal integrate [14]. Thus, DMSO apparently has multiple effects on HBV infection including receptor induction in HepaRG cells but also accelerating replication at post-entry steps. Although the underlying mechanisms are not well understood, the presence of DMSO in the medium is necessary for an efficient infection.

Although inoculation with less than ten virions established chronic HBV infection in chimpanzee [15], all the cell culture-based infection models, including the most susceptible PHHs, are not able to support unlimited virus spread (under certain conditions limited spread within the culture is observed). This is probably due to the lack of microarchitecture of hepatocytes in flat monolayer culture, where progeny viruses diffuse into large volume of culture medium instead of accumulating locally in the space of Dissé. In order to get higher infection rates it is necessary to enrich virus on the two-dimensional cell monolayer surface. The addition of 4% polyethylene glycol (PEG) during the inoculation with virus is one of these measures. PEG boosts the interaction of the virus with heparansulfate proteoglycans, which is a prerequisite for subsequent engagement of the NTCP receptor [16]. However, as a consequence, the viral inoculum is firmly associated with the cells within the first days after infection, leading to a strong background signal in many assays detecting viral nucleic acid or protein. This fact should be kept in mind for data interpretation, especially when aiming at quantification of early infection markers including cccDNA.

In this chapter, we describe the methods covering preparation of virus, infection, and immunofluorescence readout to judge the infection efficacy. The principle of this method can be used for study of infection or adapted to practical screening for antiviral drugs.

2 Materials

2.1 Virus Production

2.1.1 Preparation of HBV from HepAD38 Cells

1. HepAD38 cells [17].
2. Tet-off medium: DMEM/F-12 (1:1) medium supplemented with 10% heat-inactivated and selected fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin.
3. Tet-on medium: Tet-off medium supplemented with 0.3 µg/ml tetracycline.
4. 40% PEG: dissolve 400 g PEG (MW 8000) in water to a final volume of 900 ml and autoclave it. Add 100 ml sterile 10× PBS.

5. T-175 flask with a growth area of 175 cm².
6. Falcon® cell culture multi-flasks (5-layer with a growth area of 875 cm²) or CellSTACK® cell culture chambers (5-stack with a growth area of 3180 cm²).

2.1.2 Preparation of HBV from Transiently Transfected Cells

1. HuH7 cells.
2. Plasmids harboring over-length HBV genome, such as pCHT-9/3091 [18] or P26 [19].
3. Culture medium: DMEM medium supplemented with 10% heat-inactivated selected fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin.
4. 40% PEG: as mentioned above.

2.1.3 Preparation of HBV from Patient Serum

1. Patient serum with high viral load.
2. 20% sucrose/PBS (w/v): dissolve 20 g sucrose in PBS to a final volume of 100 ml, filtrate through 0.22 µm pore size filter.
3. Ultracentrifuge with SW28 rotor (or equivalents) and suitable centrifugation tubes.

2.2 Prepare Cells for Infection Assay

1. HepaRG [8], HepaRG-hNTCP cells [12] or primary human hepatocytes (PHHs).
2. Growth medium: William E medium supplemented with 10% heat-inactivated fetal bovine serum (*see Note 1*), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 50 µM hydrocortisone.
3. Differentiation medium: Growth medium supplemented with 1.5–2% DMSO (*see Note 2*).
4. Collagen solution, type I from rat tail.

2.3 HBV Infection of HepaRG, HepaRG-hNTCP, or PHH Cells

1. Prepared HBV virus.
2. Differentiation medium: as mentioned above.
3. 40% PEG: as mentioned above.

2.4 Immunofluorescence Assay (IF) to Quantify Intracellular Viral Core/L-Protein

1. 4% PFA solution: Add 4 g of paraformaldehyde to 100 ml PBS, heat it up while stirring to approximately 60 °C. Adjust the pH to 7 after it is completely dissolved.
2. Rabbit anti-core polyclonal antibody (DAKO).
3. Purified MA18/7 antibody (1 mg/ml): mouse monoclonal antibody against preS1 [20].
4. Blocking buffer: dissolve BSA in PBS to a final concentration of 2%.
5. Permeabilization buffer: freshly prepare 0.5% Triton-100 in PBS (v/v).

6. Fluorescent-labeled secondary antibodies, such as Alexa Fluor 488 goat anti-rabbit or Alexa Fluor 546 goat anti-mouse antibody (ThermoFisher).
7. Hoechst stock solution (5 mg/ml): dissolve 100 mg Hoechst in 20 ml of water, store at -20°C .
8. Fluoromount-G, if the cells are seeded in wells with cover slips.

3 Methods

3.1 Preparation of HBV Stock for Infection

3.1.1 Preparation of HBV from HepAD38 Cells

For virus preparation, two factors have to be taken into consideration: scale and convenience. The protocol below using Falcon® multi-flasks with the culture medium of 150 ml produces 2–3 ml concentrated virus stock every 3 days. It can be simply scaled up to bigger sizes such as CellSTACK® cell culture chambers in proportion to their growth area.

1. Expand HepAD38 cells in Tet-on medium by splitting them every 2–3 days to get 2 T-175 flasks with 70% confluence ($\sim 1 \times 10^8$ cell in total) (*see Note 3*).
2. Trypsinize and seed all cells into one Falcon® multi-flasks with five layers, grow for 3–5 days until reaching 80% confluence (*see Note 4*).
3. Change to Tet-off medium (150 ml) and refer as day 0 post induction (*see Note 5*).
4. Change medium every 3 days (*see Note 6*).
5. Collect the supernatant starting from day 15 until day 60 (*see Note 7*).
6. Every time (or every second time) of collecting supernatant, start to concentrate the virus immediately since the infectivity might get gradually reduced overtime.
7. Clarify the supernatant by sterile filtering through a $0.45\ \mu\text{m}$ pore size filter (*see Note 8*).
8. Add 27 ml 40% PEG into 150 ml supernatant (final 6% PEG), mix by inverting 30 times, and store at 4°C overnight.
9. Centrifuge at $10,000 \times g$ for 1 h with fixed angle rotor at 4°C (*see Note 9*).
10. Remove the supernatant and use 1/50 to 1/100 of the original volume of PBS/10% FCS to suspend the pellet (*see Note 10*) and collect all suspension.
11. Shake or rotate the virus suspension at 4°C overnight.
12. Centrifuge at $3000 \times g$ for 10 min at 4°C , transfer the supernatant to a new tube, and centrifuge again at $3000 \times g$ for 10 min at 4°C to remove insoluble precipitate.
13. Aliquot and freeze the supernatant at -80°C after quantification.

3.1.2 Preparation of HBV from Transiently Transfected Cells

Both pCHT-9/3091 and P26 constructs contain 1.1 mer over-length HBV genomes driven by the CMV promoter. The protocol described here might need to be modified when using different constructs according to the kinetics of virus production.

1. Seed 3×10^6 HuH7 cells (*see* **Note 11**) in 10-cm dish with Culture medium.
2. On the next day, transfect proximally 70% confluent HuH7 cells (*see* **Note 12**) with transfection reagents such as lipofectamine 2000 or JetPrime according to the manufacturer's protocol and refer as day 0.
3. Refresh the medium at days 3 and 8.
4. Collect culture supernatant between day 3–8 and day 8–12. Centrifuge the collected supernatants at $3000 \times g$ for 15 min at 4 °C to remove cell debris.
5. Add 40% PEG to a final concentration of 6% (w/v) and prepared virus as mentioned above for HepAD38 cells.

3.1.3 Preparation of HBV from Patient Serum

Some patient sera with high titers of HBV ($>10^9$ IU/ml) might be used directly for infection. However, sera could contain unknown inhibitors interfering with infection. Therefore, pelleting of virus through a sucrose cushion is strongly recommended, which not only concentrates virus but helps to remove inhibitory substances from human serum.

1. Place 10 ml 20% sucrose/PBS at the bottom of an ultracentrifugation tube for an SW28 rotor.
2. Carefully add serum on the top of the sucrose layer without disturbing the sucrose cushion (*see* **Note 13**), fill the tube with PBS if the serum volume is less than 23 ml.
3. Centrifuge at 28,000 rpm ($140,000 \times g$) for 4 h at 4 °C.
4. Carefully remove the supernatant and add 1 ml PBS with 10% FCS to suspend the pellet. Cover the centrifugation tube with parafilm.
5. Shake the tube at 4 °C overnight to allow resuspension of the virus.
6. Pipette up and down for >30 times, transfer virus to an Eppendorf tube, and centrifuge at $3000 \times g$ for 10 min at 4 °C to remove insoluble precipitate.
7. Aliquot and freeze the supernatant at -80 °C after quantification.

3.2 Prepare Cells for Infection Assay

3.2.1 Differentiate HepaRG or HepaRG-hNTCP Cells for Infection

The following protocol describes differentiation of HepaRG cells in a 24-well plate. It is also applicable for HepaRG-hNTCP cells and can be proportionally adjusted to different plate formats.

1. HepaRG cells are maintained in Growth medium with weekly splitting at a ratio of $\sim 1:8$.

2. Trypsinize and seed HepaRG cells at a density of 10^5 cells/well in Growth medium and refer as day 0.
3. Replace the Growth medium with 500 μ l Differentiation medium at day 14 (*see Note 14*).
4. Refresh the Differentiation medium every 2–3 days (*see Note 15*).
5. The HepaRG cells are ready for infection at day 28 (*see Note 16*).

3.2.2 Plating of PHHs for HBV Infection

PHHs can be prepared from the liver tissue if the respective infrastructure and technique are available on site. It might also be available from commercial vendors (e.g., Biopredic) providing plated or cryopreserved platable human hepatocytes. The cell viability should be above 75% before seeding. The following protocol describes the seeding of PHHs in a 24-well plate.

1. Dilute collagen in PBS to a final concentration of 100 μ g/ml.
2. Add 200 μ l diluted collagen solution to each well. Allow the collagen to bind for 1 h at room temperature.
3. Remove collagen solution. Leave the plate open in the culture hood for 10–30 min until it gets dry (i.e., there is no liquid visible on the plate).
4. If needed, wash PHH with Growth medium by centrifugation at $50\times g$ for 5 min at 4 °C (*see Note 17*).
5. Dilute PHH to 3×10^5 living cells/ml in Growth medium, and add 500 μ l to each well.
6. Four hours later, remove unattached cells and add 500 μ l Differentiation medium (*see Note 18*).
7. The next day the PHHs are ready for infection or they can be kept in serum-free Differentiation medium for up to 3 days with daily medium change (*see Note 19*).

3.3 HBV Infection of HepaRG, HepaRG-hNTCP, or PHH Cells

The genome equivalence of virus stock should be quantified by qPCR or DNA dot-blot [19] (*see Note 20*), which is not described in this chapter. The DNA quantification result should be carefully interpreted, since transfected cells might contain input DNA and cell culture might secrete large amount of naked nucleocapsid. A sucrose or CsCl gradient of prepared virus can be useful to precisely quantify the enveloped virus fractions. Typically, at least 100 MGE (multiplicity of genome equivalents) is required for an efficient HBV infection. Here, we describe the infection of HepaRG, HepaRG-hNTCP, or PHH cells in a 24-well plate.

1. For each well, mix 50 μ l 40% PEG with 450 μ l Differentiation medium. The final PEG concentration is 4% (*see Note 20*). Vortex for 5 s.
2. Add desired amount of HBV, and vortex again for 5 s. Usually, 20 μ l concentrated virus from HepAD38 can result in a well-detectable infection.

3. Aspirate cell culture medium, then add inoculum, and refer as day 0.
4. Four- to twenty-four-hours after infection (*see* **Note 21**), wash the cell with PBS for two times and then add 500 μ l Differentiation medium.
5. Refresh and/or collect medium every 2–3 days.
6. Monitor the viral markers and choose the end point of infection as needed (*see* **Note 22**).

3.4 IF Against Viral Core or L Proteins to Analyze Infected Cells (See Note 23)

The following protocol describes the IF staining of infected cells in a 24-well plate.

1. At 6–12 days after infection, wash cells once with PBS.
2. Add 250 μ l 4 % PFA for 10 min at room temperature.
3. Aspirate the fixation buffer and wash once with PBS, then the cells in PBS can be stored at 4 °C (*see* **Note 24**) or stained as the following.
4. Add 250 μ l permeabilization buffer for 10 min at room temperature.
5. Remove permeabilization buffer and wash once with PBS.
6. Add 250 μ l first antibody (anti-core or Mal8/7 antibody, 1:1000 diluted in blocking buffer) for 1 h at room temperature or overnight at 4 °C.
7. Remove antibody and wash cells three times with PBS.
8. Add 250 μ l fluorescent labeled secondary antibody (diluted as recommended by manufacturer in blocking buffer) and Hoechst (1:1000 diluted) for 1 h at room temperature.
9. Wash cells three times with PBS, then the cells are ready for examination under a fluorescent microscope.
10. If cells are seeded in wells with cover slips, carefully take it out and mount it to a slide with Fluoromount-G. Leave it dry for 10 min before microscope analysis.

4 Notes

1. The differentiation process of HepaRG cells strongly depends on the serum used. Serum from different batches or manufacturers should be tested for differentiation with 2 % DMSO.
2. 2 % DMSO is preferred if it is tolerated. The sensitivity of cells to DMSO relies on the quality of serum more than that of DMSO. If a severe toxic effect is observed, the concentration of DMSO can be reduced to 1.5 %.

3. HepG2-derived HepAD38 cells are prone to clump together during culturing and might be difficult to be trypsinized into single cells. Too many cell clusters after splitting impair the long-run viability of cell culture. Shortening the splitting period such as every 2 days helps to prevent this problem. Cell strainer with 40 μm pore size can be used to remove big clusters after trypsinization. If it is still unsatisfactory, add 5 $\mu\text{g}/\text{ml}$ insulin and 50 μM hydrocortison to the Tet-on medium during cell propagation.
4. Cells growing in multilayer can hardly be examined under microscope. Homogenous distribution is usually a good sign. When using a CellSTACK chamber, it is important to leave the chamber in a horizontal position so that all cells are completely covered by culture medium.
5. HepAD38 cells grow slowly in Tet-off medium since the transcription of viral pregenomic RNA is strongly induced.
6. Occasionally changing the medium after 4 days of culturing is tolerable. In this case, the volume of culture medium should be increased.
7. The secreted HBeAg should be monitored over the whole culturing, which is a good indicator for the virus production. HepAD38 cells produce high amount of naked nucleocapsids that may interfere with quantification of virions.
8. This step aims at clarifying the virus-containing medium from cell debris. Vacuum-driven filtration system facilitates this step. Centrifugation can be used as well, such as $5000 \times g$ for 15 min at 4 $^{\circ}\text{C}$.
9. Centrifugation in a fixed rotor results in a thin layer of precipitate at the distal side of the centrifugation tube. This pellet can be then suspended more easily compared to a thick pellet formed at the bottom of the tube.
10. After pipetting up and down, the suspension is quite turbid.
11. HepG2.2.15 cells containing stably integrated HBV genomes can be seeded and cultivated in Culture medium as well. HepG2.2.15 cells constitutively produce virus after reaching confluence. The supernatant of HepG2.2.15 cells can be collected and viral particles can be concentrated by PEG precipitation as well. However, ~ 50 -fold lower virus concentration in comparison to HepAD38 cells should be expected.
12. HuH7 cells can be transfected with higher efficiency than HepG2 cells and produce higher levels of HBV.
13. Cut the end of 1 ml pipette tips and use them to slowly add the first 10 ml serum to avoid disrupting the sucrose solution.

14. It is not necessary to change the medium during the first 2 weeks. If there is a significant evaporation of medium, add 0.5 ml of Growth medium at day 7 post seeding.
15. During the DMSO-induced differentiation process, some cells inevitably undergo apoptosis; the majority differentiates into hepatic or biliary epithelial cells. The formed hepatic cell region, characterized by the formation of canaliculi between cells, should be resistant to DMSO. If the hepatic islands are not well formed or quickly disrupted, different sources of HepaRG cells or serum should be considered.
16. Over-expression of NTCP has no apparent impact on the differentiation of HepaRG cells. Bile canaliculi should be easily recognized when HepaRG-hNTCP cells get differentiated, which can also be stained with anti-MRP2 antibody [7].
17. PHHs are fragile and should be centrifuged at low force.
18. The unattached PHHs 4 h post seeding will not be able to attach firmly even after overnight incubation.
19. PHHs will be dedifferentiated in serum-containing medium and therefore down-regulate the NTCP expression and reduce their susceptibility to HBV infection. However, serum-free condition usually reduces the lifetime of PHH in culture and is not necessary as long as the cells are infected.
20. PEG enhances the infection efficacy by ~10-fold via promoting virus attachment to heparan sulfate proteoglycans [16].
21. HBV infection is a “slow” process. Overnight inoculation results in ~5-fold higher infection rate than 2–4 h inoculation.
22. cccDNA reaches plateau at day 4; core protein can be detected at day 5 p.i.; Envelope protein can be detected at day 7 p.i.; HBeAg can be measured between day 4–10; HBsAg can be measured between day 7–13.
23. The HBV X protein and polymerase are difficult to be detected by IF postinfection, which might be due to their very low expression level in the context of authentic infection.
24. The PFA-fixed cells are stable in PBS at 4 °C for at least 2 weeks.

Acknowledgments

The HepAD38 cell line was kindly provided by Dr. Christoph Seeger. This work was supported by the German Center for Infection Research (DZIF), TTU Hepatitis, Project 5.807 and 5.704.

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Hepatitis B Virus

Methods and Protocols

Guo, H.; Cuconati, A. (Eds.)

2017, XII, 297 p. 57 illus., 31 illus. in color., Hardcover

ISBN: 978-1-4939-6698-1

A product of Humana Press