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Characterization of a Cell Line HFH-T2, Producing Viral Particles, from Primary Human Fetal Hepatocytes Infected with Hepatitis B Virus

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Abstract A primary culture of human fetal hepatocytes was obtained though a therapeutic abortion process at 26 weeks of gestation period. More than 10^8 cells were seeded on a plastic plate. These hepatocytes were infected with hepatitis B virus (HBV). The HBV was purified from serum of one chronic HBV carrier. Transformed hepatocytes were subcultured in a 10% FBS-supplemented medium. The morphology of the transformed cell was epithelial-like. The cells from the first pass showed signs of early proliferation and had a latent period of more than 3 months after 6–7 passages. After the rest period, the transformed cell proliferated actively and they were subcultured every three days. Transformed hepatocytes were characterized by detection of the HBV transcript by RT-PCR. The secretion of virions from transformed cells was investigated by PCR with the cell medium. Two types of virions secreted into the culture medium were examined by using the transmission electron microscope. Another approach to study the secretion of virions in to culture medium was carried out with HBV antibody. HBsAg was detected in the culture medium of transformed cells using ELISA and Western blot analyses. These data suggested that the human fetal hepatocyte cell line has been established by infection of HBV, in which this cell line secreted viral particles into the culture medium.

Key words: Fetal hepatocyte, natural infection, hepatitis B virus, hepatocellular carcinoma, HBsAg particle, HBsAg, Dan particle, enzyme-linked immunosorbent assay

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Hepatitis B virus (HBV) is a small DNA virus of the hepadnaviradae family. HBV causes both acute and chronic hepatitis, infecting over 300 million people worldwide. Chronic infection with HBV is associated with subsequent development of hepatocellular carcinoma (HCC). HBV produces a chronic infection that affects a substantial proportion of the world population resulting in death from chronic liver disease and hepatocellular carcinoma. About 5% of the human population is believed to be infected by HBV [7].

It was reported that Chimpanzees are infected *in vivo* by HBV [22] and Woodchucks, ground squirrels, and Peking ducks are infected by HBV-like viruses [2, 10, 12]. However, the entire animal systems have limitations for studying the mechanism of HBV infection. Major progress was made in hepatoma cell lines transfected with cloned HBV DNA [18, 19, 20, 23]. Also, primary human adult hepatocytes [3, 4] and fetal hepatocytes [13] may be actively infected by HBV but only with artificial agents such as dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG). While these systems are certainly a valuable source for studying the replicative cycle of HBV, describing how the hepatocytes were treated with substances such as DMSO or PEG will provide a means for understanding the mechanisms of HBV infection under natural conditions. To obtain viral particles, human diploid MRC-5 cells were transfected with hepatitis A virus, however, the cell was not hepatocytic, indicating that natural infection and production of hepatitis B viral particles are still unsolved [6]. Therefore, we need a convenient *in vitro* system to study HBV infectivity under natural conditions.

In the present study, we have expanded the infectivity of HBV into a primary human fetal hepatocytes under natural condition and, at first, established a human fetal hepatocyte cell line which secretes viral particles by the HBV infection. The established cell line, named as HFH-T1, constitutively produced viral particles in the medium, indicating that the cell line can serve as a viral host.

MATERIALS AND METHODS

Materials

DMEM, Dulbecco's Modified Eagle's Medium (DME/High Glucose) and FBS were obtained from Hyclone Laboratories Inc. (Salt Lake City, Utah. U.S.A.). Antibiotics, antibiotic-antimycotic were obtained from Life Technologies (New York, NY, U.S.A.). RNA Isolation Kit and LA PCR Kit were obtained from TaKaRa Shuzo Co., LTD. (Otsu, Kyoto, Japan). BSA and alkaline phosphatase conjugated anti-mouse IgG were obtained from Sigma-Aldrich Co. (St. Louis. MO, U.S.A.). Anti-HBsAg 58-32 was obtained from TaKaRa Shuzo Co., LTD. (Otsu, Kyoto, Japan).

Cell Isolation and Cell Culture

Human hepatocytes were obtained from fetal liver at 26 weeks of gestation. The fetal liver tissues were obtained from abortions performed to safeguard mothers from harm incurred by pregnancy, with the provisions in the law of Maternal and Child Health Care of the Republic of Korea. The liver tissue was placed immediately in a cold 10% fetal bovine serum/Dulbecco's Modified Eagle's Medium (10% FBS/DMEM) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml), and then washed 2-3 times with the same medium. The tissues were cut with scissors into small pieces and transferred to a 50-ml centrifuge tube. The larger fragments were allowed to settle and the supernatant was saved. The cell suspension was filtered through a gauze and washed with DMEM three times by centrifugation at 50 ×g for 3 min. Freshly isolated hepatocytes were seeded to 10 ml of 10% FBS/DMEM supplemented with penicillin and streptomycin, at a density of 10^8 cell per plastic dish (diameter 100 mm). The cells were incubated at 37°C under 5% CO_2 in air.

Infection of Cell Culture with HBV and Selection of Transformed Cells

Serum from one chronic HBV carrier with a high level of HBV DNA was used as a single source of viral particles. Infection was performed by incubating cell monolayers for 72 h at 37°C in the culture medium containing the infectious serum. The medium was changed once every 3 days. The proliferation of outlived and transformed cells was detected in culture after 7- 10 days. The noninfected hepatocytes were removed mechanically during continuous cell culturing.

Subculture of Transformed Cells

When transformed cells became confluent, they were trypsinized with 0.05% trypsin and subcultured at a 1:4 dilution in 10% FBS/DMEM. Cells were replenished with the culture medium every third day.

Detection of Intracellular HBV DNA by RT-PCR

Aliquots of the transformed cells were washed twice with phosphate-buffered saline (PBS). Total RNA was extracted from the cells using RNA Isolation Kit, 2.11 (Iowa Biotechnology Corp., Rchmond, CA, U.S.A.) according to the manufacturer's recommendations. The pellet was dissolved in a 20 μl of diethyl pyrocarbonate (DEPC)-treated water. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using an RNA LA PCR Kit Ver. 1.1 (TaKaRa). For reverse transcription, 1 μg of the total RNA was dissolved in 9.5 μl of RNase-free water. Four microliter of 25 mM of $MgCl_2$, 2 μl of 10× RNA PCR buffer (100 mM Tris HCl (pH 8.3) and 500 mM of KCl), 2 μl of 10 mM dNTP mixture, 0.5 μl of RNase inhibitor (40 units/μl), 1 μl of avian myelomablastosis virus (AMV) reverse transcriptase XL (5 units/μl), and 1 μl of random 9 mers (50 pmol/μl) were added. The mixture was then incubated at 30°C for 10 min, and 42°C for 30 min. PreS1 5', 5'-CGAGAATTCATGGGAGGTTGGTCTTCC-3' and PreS2 3', 5'-GCTGGATCCGTTCGGTGCAGGGTCCC-3' corresponding to the PreS1 and PreS2 regions of the HBV gene were used to amplify the cDNA. DNA amplification was completed in a 20 μl reaction volume containing 2 μl of 10× reaction buffer, 1.2 μl of 25 mM $MgCl_2$, 1 μl of 200 M dNTP mixture, 1μl of each primer (10 pmol/μl), 0.1 μl of *Tag* polymerase (TaKaRa), 3.7 μl of purified water, and 10 μl of the sample. PCR was performed with a TaKaRa PCR Thermal Cycler 480 (TaKaRa). The amplification was performed by heating the samples for 5 min at 94°C then using 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and extending for 1 min at 72°C. The amplified product was analyzed by electrophoresis on a 2% (w/v) agarose gel.

Southern Blot Analysis

After washing with PBS, cells were lyzed in extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA, 20 μg/ml RNase A, and 0.5% SDS) and incubated with 1 mg/ml of proteinase K at 37°C overnight. All samples were extracted with phenol, followed by phenol-chloroform-isoamyl alcohol (25:24:1, vol:vol:vol) and chloroform-isoamyl alcohol (24:1, vol:vol). Then, 10 M of ammonium acetate was added to the aqueous phase, and nucleic acids were precipitated with 2 vol of ethanol. The pellet was dissolved in 10 mM of Tris-HCl (pH 8.0), 1 mM EDTA. Extracted DNA was digested with *Hin*dIII and subjected to electrophoresis in a 0.8% agarose gel and transferred to nylon membranes. Hybridization was performed with a

DIG-labeled HBV DNA probe at 68°C in 5× SSC, 0.15% *N*-lauroylsarcosine, 0.02% SDS, and 1% blocking reagent.

Detection of Secreted HBV

One milliliter of the culture medium from transformed cell was centrifuged at a low speed to remove insoluble materials, and then heated to 99°C. Ten microliter was analyzed by PCR with primers 1 (5' CGAGGATCCATGGGAGGTTG-GTCTTCC 3') and 2 (5' CCGAATTCATTTCCGTCTGG-CCAGTG 3').

Enzyme-Linked Immunosorbent Assay (ELISA)

Anti-HBs antibody in mouse sera was quantified using ELISA. Microtitre plates (Nunc Immunoplate) were coated with 1 μg protein/ml of virus in a coating buffer (0.5 M carbonate-bicarbonate, pH 9.6) at 4°C for 24 h. The plates were washed and the site for unbound virus was blocked by adding 1% BSA in PBS for 1 h at room temperature. After washing, the first antibody (anti-HBs, anti-mouse sera) at a 1:500 dilution in PBS containing 0.05% Tween-20 was added, incubated for 2 h at room temperature, and washed three times with PBS-Tween-20 (0.05%). Alkaline phosphatase conjugated anti-mouse IgG (Sigma Co., St. Louis, MO, U.S.A.) at a 1:1,000 dilution in PBS containing 0.05% Tween-20, was added, incubated for 2 h at room temperature, and finally washed. Finally, 200 μl of p-nitrophenyl phosphate solution (1 mg/ml in 9.7% v/v diethylamine, pH 9.8) was added, incubated for 30 min, and the reaction was stopped by adding 50 μl of 3 M NaOH. The absorbances were read at 405 nm.

Purification of Virions

Viral particles produced and released from the transformed cells into the culture medium were purified using the sucrose density gradient centrifugation. After removal of cellular debris, viral suspension was adjusted to the final concentration of 0.5 M NaCl. Viral particles were recovered from the precipitates with 9% (w/v) polyethyleneglycol (MW 8,000, Sigma) followed by centrifugation at 6,000 rpm for 30 min (Hanil Supra22K, Inchon, Korea). Pellet was resuspended in a TNE buffer (0.01 M Tris, pH 8.3, 0.15 M NaCl, and 0.01 M EDTA, pH 7.5). Viral suspension was loaded on 20-60% sucrose for density gradient centrifugation, at 25,000 rpm for 16 h in a STS 60.4 rotor (Kontron, Itali). Virus fraction was collected with a syringe.

Electron Microscopy

One drop of the purified virus suspension was mixed in a depression slide with a drop of 2% aqueous solution of uranyl acetate. A drop of the mixture was picked up by touching the parmvor film side of the grid surface. After 30 s, excess fluid was drained from the grid by touching its edge to the filter paper. The grid was observed with an electron microscopy (JEOL Co., Tokyo, Japan).

Western Blotting of Viral Proteins

SDS-PAGE was performed by the method originally described by Laemmli [9]. Viral polypeptides were electrophoresed in a 10% polyacrylamide gel at 100 V for 1 h and 30 min. The molecular weights of the polypeptides were calculated from their electrophoretic mobilities relative to their standard proteins (Novagen, San Diego, CA, U.S.A.) which were run in parallel.

After electrophoresis, the bands were transferred from the gel onto a PVDF membrane at 40 mA for approximately 16 h by using a transfer buffer of 25 mM of Tris, 192 mM of glycine, 20% of methanol, pH 8.3. PVDF (polyvinylidene fluoride) membrane was washed briefly in PBS. After being saturated with protein by soaking in PBS with 1% bovine serum albumin (Sigma) for 1 h at room temperature, the membrane was probed with a 1:1,000 dilution of mouse anti-HBs sera in PBS with 0.05% Tween-20. Finally, the membrane was washed with PBS-Tween 20 (0.05%) and incubated in BCIP/NBT phosphatase substrate, consisting of 5-bromo-4-chloro-3-indolyl phosphate sodium salt, 0.15 mg/ml *p*-nitroblue tetrazolium choride, at 0.3 mg/ml in a carbonate buffer (0.1 M $NaHCO_3$, 1.0 mM $MgCl_2$, pH 9.8) for 15 min at room temperature. The reaction was stopped by washing with distilled water.

RESULT AND DISCUSSION

Primary Cultures and Selection of Human Fetal Hepatocytes

It has been known that *in vivo* infection of HBV in humans involves the uptake of viral particles into hepatocytes, since the data supporting either a direct fusion mechanism with the outer cell membrane or an energy-dependent receptor-mediated endocytotic pathway have both been reported [14].

A primary culture of hepatocyte *in vitro* is a useful method to study liver disease. However, the non-durability of primary culture cells limits the usefulness of this system. HBV causes both acute and chronic hepatitis which occur frequently in East Asia, including Korea. It is necessary to make an immortalized hepatocyte producing HBV for further studies of liver. This will indeed provide a system for investigating the mechanism related to HBV. Many attempts have been made to infect hepatocytes to investigate the mechanism of HBV infection. Unfortunately, these studies have brought limited success. Some systems have provided valuable sources for studying the mechanism of infection [1, 3, 4, 13, 15], but these systems were treated with the substances such as DMSO or PEG. To date, there are no reports describing the *in vitro* natural infection in cultured hepatocytes. HBV-host interaction will be explained in fundamental basis when a natural infection system is established. Thus, we have made an effort to

construct a cell line producing HBV by natural infection. An experiment was designed to provide natural infection to hepatocytes. Hepatocytes were prepared from fetal liver at 26 weeks of gestation and over 10^8 cells were seeded on a plastic plate (100 mm diameter). Highly confluent monolayers could only be obtained 24 h after plating. The cells were incubated with serum from one chronic HBV carrier with a high level of HBV DNA. The infection was performed by an overnight incubation process. After 10 days post-infection, only a few cells changed in morphology. In fact, these cells resembled epithelial cells in morphology. The size was much larger than uninfected hepatocytes (Fig. 1A). Based on the data, the hepatocytes appeared to be transformed by HBV infection. Interestingly, although fetal hepatocytes from early gestation (12–13 weeks) were previously used for HBV infection, these hepatocytes were not infected by HBV (data not shown). However, in 1989, Ochiya *et al.* [13] used human fetal hepatocytes prepared from fetal liver at 20–24 weeks of gestation to infect hepatocytes co-cultured with HB611 cells [13]. Based on these findings, an appropriate gestation stage appeared to be very important for getting HBV infection. At this point, we do not know when the critical stage is during fetal development for HBV infection. It is true that the cell density of hepatocytes might also affect the infectivity. In our case, the HBV infection was tried with 10^6 or 10^7 cells. The infection did not occurr in this cell density (data not shown). Over 10^8 cells appeared to be essential for acquiring a successful infection. Therefore, it is likely that both cell density and fetal developmental stage are important factors to HBV infection.

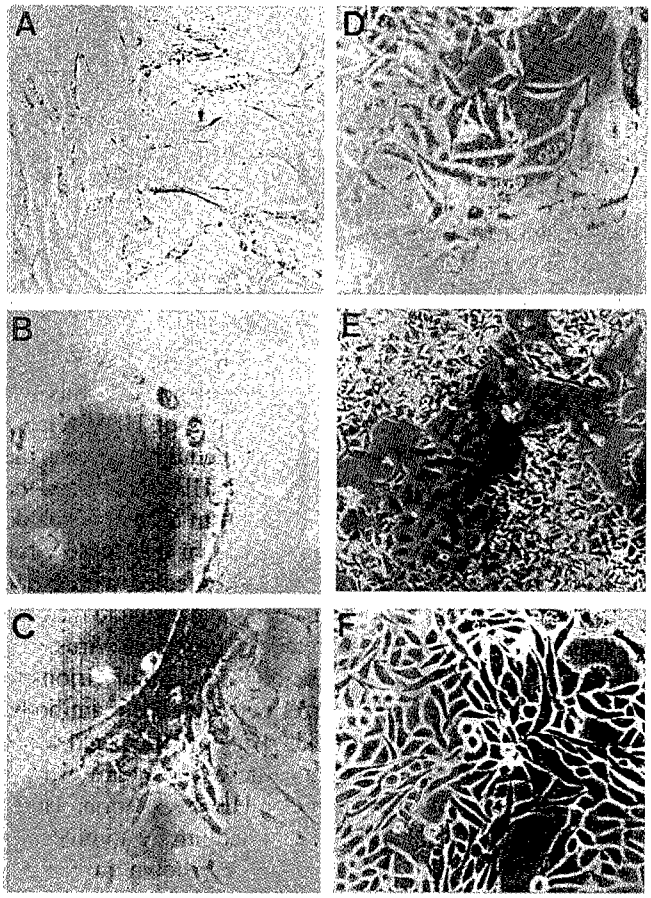

Fig. 1. Morphology of transformed human fetal hepatocytes. (A) Human fetal hepatocytes transformed by HBV infection. (B)-(E) indicates change of transformed cell after the latent period. (F) Stably transformed cell. (A)-(D), (F) ×400, (E) ×100.

The transformed cells from the first passage showed signs of early proliferation. However, these cells needed a long rest period of more than 3 months after 6–7 passages. After this rest period, the transformed cells proliferated actively and they were subcultured every three days. However, the size and morphology of the cells changed compared to those of the cells before the rest period. Our observed lag time of 3 months to stabilize the transformed cells was longer than previously reported [5, 15]. Since it is not clear why it took an exceptionally long lag period, it is likely that the origin of the cell and mechanism of transformation are important factors. After the lag time, the cell line actively proliferated and it was quite stable. Therefore, the cell line may not differentiate before the lag period, and completes differentiation following the intracellular changes during a certain period.

Detection of HBV Transcript by RT-PCR

Transformed hepatocytes after 2 passages were characterized by detection of HBV transcript by reverse transcriptase and subsequent PCR of the cDNA. As shown in Fig. 2, HBV RNA (525 bp in size) was detectable. A positive control with Hep3B cells in which HBV gene was integrated showed the same size of the transcript. HBV RNA was undetectable in NIH3T3 cells as a negative control. For the fidelity of RT-PCR data, another primer pair which covers the S1 gene of HBV was used. As expected, a 170 bp band was detected as well (data not shown).

Detection of HBV DNA Produced by Transformed Cells

It has been reported that cells infected with HBV show different replication genomic structures [8, 11, 18, 21]. To determine whether or not various genomic structures of HBV DNA are detected in transformed cells, total DNA was prepared from the cells, digested with restriction endonuclease *Hin*dIII, and resolved in a 1% agarose gel. Southern blot analysis was performed. The membrane was hybridized with DIG-labled HBV probe. Restriction enzyme *Hin*dIII does not cleave within the HBV genome, thus it was used to digest host chromosomal DNA (20 μg) to load large amounts of cellular DNA [11]. During the synthesis of progeny viral genomes, cells infected with HBV showed a different pattern from the virion in genomic

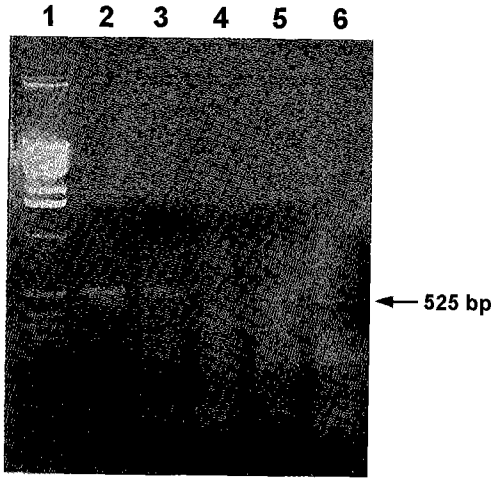

Fig. 2. Detection of HBV RNA by RT-PCR in transformed cell.
RNA was extracted with RNA isolation kit, 2.11 by following the manufacturer's recommendation. RNA was reverse transcribed with a random primer and cDNA was amplified by PCR. Plasmid containing HBV DNA sequences (pHBV315) was used as a positive control for PCR. Hep3B and NIH 3T3 cell served as positive and negative controls, respectively. Lanes 1, 2, 3, 4, 5, and 6 indicate molecular weight markers, pHBV315, Hep3B cell, NIH3T3 cell, transformed cell before the latent period, and transformed cell after the latent period, respectively.

structures [8, 11, 18, 21]. As shown in Fig. 3, the result indicated two genomic forms of viral DNA. A more slowly migrating form was assumed to be a linear circle, while the faster-migrating form was probably a single-stranded form of HBV DNA. In our data, the relaxed circle

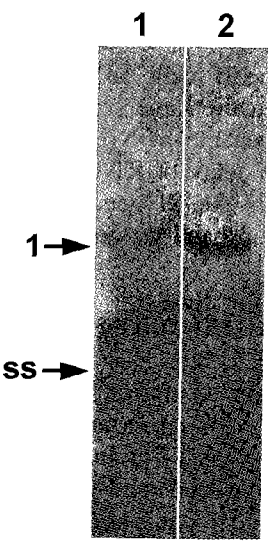

Fig. 3. Southern blot analysis of HBV DNA produced by transformed cells.
DNA was extracted from transformed cells as described in Materials and Methods. Extracted DNA was digested with *Hin*dIII, resolved by electrophoresis, transferred to nylon membrane, and probed with a DIG labeled HBV cDNA. Lanes 1 and 2 indicate NIH3T3 cell (negative control) and transformed cell, respectively. l, linear HBV DNA; ss, single-stranded HBV DNA.

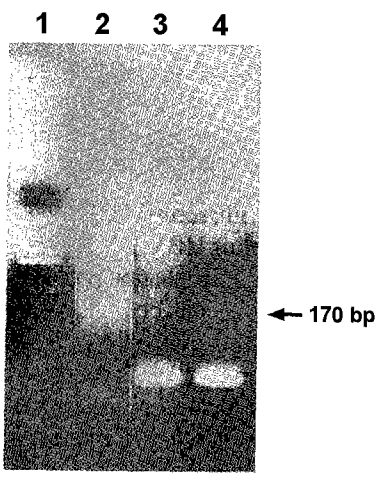

Fig. 4. Detection of HBV DNA by PCR in the culture medium of transformed cell.
Culture medium of transformed cells was centrifuged at low speed to remove insoluble materials, and then heated to 99°C. Ten microliter was analyzed by standard PCR which generated the expected 170 bp DNA. Lanes 1, 2, 3, and 4 indicate molecular weight markers, pHBV315, culture medium of transformed cell, and 170 bp DNA, respectively.

was not observed, because there seemed to be a low copy number of the replication of HBV.

Detection of HBV Secretion in Culture Medium

To investigate whether the transformed cells secrete virions after establishing the cell line, we performed several experiments such as PCR, electron microscope observation, ELISA, and Western blot analysis with the culture medium. As shown in Fig. 4, a 170 bp band corresponding to the pre S1 region of HBV was detected in the culture medium by PCR. In order to confirm the secretion of viral particles, virions were isolated from the culture medium by sucrose density gradient centrifugation and examined using transmission electron microscopy (Fig. 5). Two types of HBV-related particles were secreted in the culture medium; one was the Dan particle (40 nm in size) that contains HBV DNA, and the other was subviral HBsAg particles (20 nm in size) which do not contain the viral genome. It is known that these virus particles are shown in patient's serum infected with HBV. Therefore, the presence of Dan particles in the culture medium indicates that the cell line secreted the infectious virus.

Another approach to examine the secretion of virions to the culture medium can be performed with HBV antibody. Specific antigens of HBsAg and HBeAg are detected when cells are infected by HBV. Thus, these factors are usually monitored as a method to detect HBV secretion. Here, HBsAg was assayed in the culture medium by commercial enzyme immunoassay (EIA) kits. As shown in Fig. 6, HBV antigen was not well detected in the HepG2 cell culture medium. However, the HBV-specific protein was secreted from the transformed cell line. To further confirm the secretion of HBV into the medium, HBsAg was

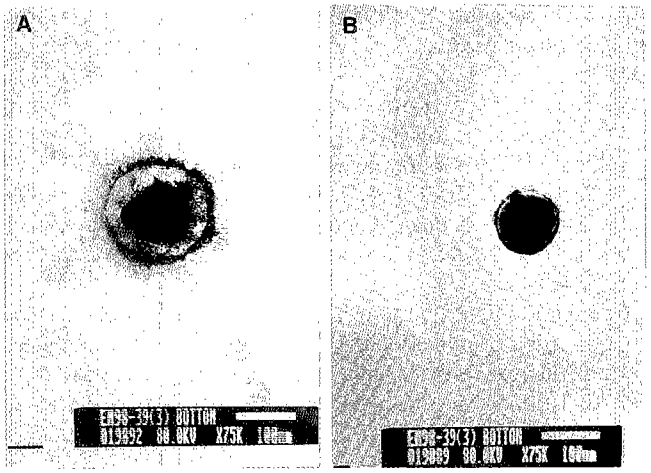

Fig. 5. Morphology of virions examined using electron microscopy. Virions were purified from culture medium of transformed cells by sucrose density gradient centrifugation, stained with uranyl acetate, and examined using electron microscopy. (A) Dan particles with DNA. (B) Subviral particles without DNA. Bar; 100 nm.

analyzed by SDS-PAGE and an immunoblot. As shown in Fig. 7B, a specific band of HBsAg was detected in the transformed cell line. The band was not detected in a negative control (Fig. 7A).

As mentioned above, the transformed cells changed in shape and size after 3 months of the latent period. In fact, the cell line seems to be very stable at this time. We need to check the cell line for having the same characteristics even though the shape and size of the cell are changed after the latent period. RT-PCR and observation made by electron microscopy were performed for this purpose. HBV transcript and virus particles were detected in the cell line (data not shown). The experiments to confirm this result are currently under investigation. Also, to investigate whether the cells still remained as hepatocytes after the lag time, synthesis of alpha fetoprotein and albumin, which is

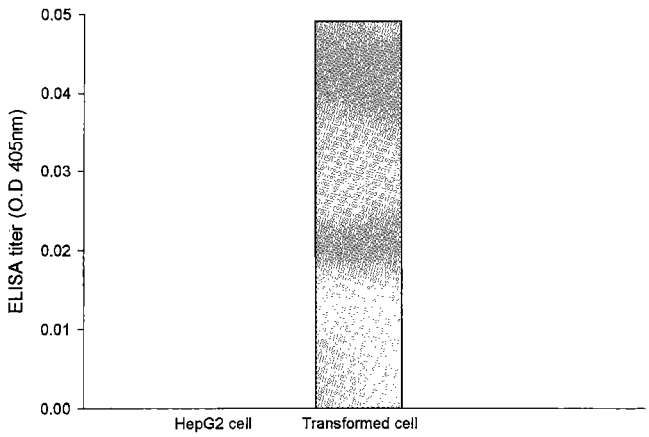

Fig. 6. Detection of HBV by ELISA using monoclonal antibody (HBsAg).

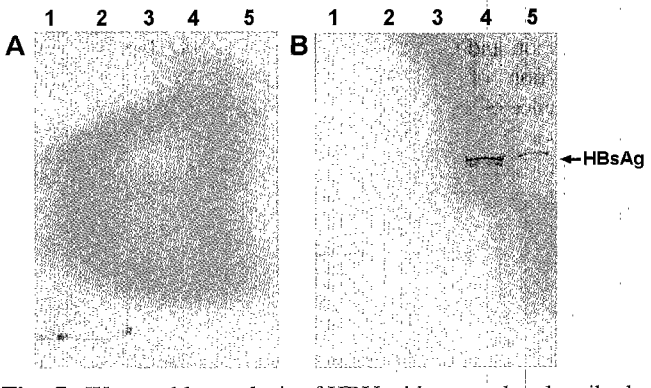

Fig. 7. Western blot analysis of HBV with monoclonal antibody to HBsAg.
Proteins were extracted from virions, electrophoresed in a 10% SDS-PAGE, and then transferred onto a PVDF membrane. The membrane was blocked with 1% of bovine serum albumin, incubated with mouse anti-HBsAg, and reacted with 2nd antibody (alkaline phosphatase-conjugated). The membrane was stained with BCIP and NBT as substrates. (A) and (B) indicate without antibody (negative control) and with antibody, respectively. Lanes 1 and 2 indicate transformed cell before the latent period and transformed cell after the latent period, respectively. Lanes 3-5 indicate virus particles from sucrose density gradient.

the indicator of functional hepatocytes, was measured by using the immunocytochemical stain. Transformed cells showed staining for alpha fetoprotein and albumin in the cytoplasm around the nucleus (data not shown).

In conclusion, we obtained a human fetal hepatocyte cell line by directly infecting HBV to the cell line, which produced viral particles into the culture medium. Our cell line should be very useful not only for further studies of the HBV infection mechanism, but also for experiments conducted to investigate the efficiency of HBV vaccines as well.

Acknowledgments

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