Expression of Hepatitis B Surface Antigen in Chang Cells Transfected with Hepatitis B Virus DNA

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(Received for publication on December 8, 1989)

Abstract. To investigate hepatitis B virus (HBV) biology in vitro, the transfection of recirculized HBV DNA into Chang cell line was performed. Linear HBV DNA was isolated from recombinant HBV DNA, pHBRI05, which includes the whole genome of HBV and was recirculized. Chang cells were transfected with this recirculized HBV DNA by the two different procedures of calcium/phosphate coprecipitation and electroporation. After the transfection, the presence of large nucleated cells with multinuclei and ground-glass cytoplasma were noticed and these cells seemed to proliferate faster than untreated Chang cells. Transient expression of hepatitis B virus surface antigen (HBsAg) was demonstrated in cytoplasma of transfected cells by indirect immunofluorescence. HBsAg was not detected in the culture supernatants by radioimmunoassay. The extra-chromosomal HBV DNA was detected in the transfected cells by both procedures 7 weeks after the transfection by Southern blot analysis but it was lost 4 weeks after that. It was demonstrated that it was possible to transfect Chang cells with HBV DNA and that DNA was functioning to express HBsAg transiently. (Keio J Med: 39 (2): 79–85, June 1990)

Key words: hepatitis B virus, transfection, cell line, hepatocellular carcinoma

Introduction

Hepatitis B virus (HBV) infection is one of global health problems which affects many people especially in Asia and Africa. The number of HBV carriers is estimated at 2 to 3 hundred millions in the world. An ominous characteristic of HBV is that it causes not only a transient infection, i.e., acute hepatitis, but also chronic liver disease. It is now well known that hepatocellular carcinoma (HCC) arises after a long period of persistent infection of HBV. Recent advances of molecular biology have shed a light to basic understanding of the relationship between hepatocarcinogenesis and the integration of HBV DNA into liver cell genome, although precise mechanisms of HCC development have not yet been known.

In vitro model system is considered to be required for better understanding about HBV proliferation in liver cells. Since the successful molecular cloning of the whole length of HBV DNA in 1980, HBV DNA transfection experiments have been performed as a substitute of HBV infection, hoping that it would permit the analyses of viral gene transcriptions, viral protein productions, viral replication in liver cells and moreover liver carcinogenesis. Fibroblasts, HCC cell lines and cancer cell lines of other tissues have been used as recipient cells. An ideal model might be to use a primary culture of normal human hepatocytes. But this is technically difficult and the cell damage caused by transfection process may make this model unpractical. Therefore we chose a steadily growing human normal liver cell line, Chang liver cell, as a recipient of HBV DNA transfection. In the present study we investigated the transfection of Chang cell with recirculized HBV DNA and the production of the viral surface coat. To our knowledge this is the first report to describe the HBV DNA transfection to Chang liver cell.
Materials and Methods

Cells

Chang cell which was derived from normal human adult hepatocytes was used as a recipient of HBV DNA transfection. Chang cells were cultured in Eagle’s minimum essential medium (MEM) (Nissui Seiyaku Co. Ltd., Tokyo, Japan) supplemented with 10% calf serum (Nakarai Chemicals Co. Ltd., Kyoto, Japan) and 100IU/ml of penicillin and 100ug/ml of streptomycin (Flow Laboratories, Inc., McLean, CA, USA). PLC/PRF/5 cells which was demonstrated to excrete hepatitis B surface antigen (HBsAg) into the culture supernatant was used as a positive control in the detection of HBsAg production and in DNA analysis. The cells were cultured in Eagle’s MEM (Nissui Seiyaku) containing 10% fetal bovine serum (FBS) (Grand Island Biochemicals, GIBCO, Grand Island, NY USA) and antibiotics as mentioned above. These cells were regularly passed a new culture flask when they became fully confluent.

HBV DNA

Recombinant HBV DNA, pHBR105, including the whole genome of HBV (HBsAg subtype of adr) which had been inserted into a BamHI site of plasmid pBR322 was a kind gift from Dr. Takeshima at Kitasato Institute, Tokyo, Japan. The plasmid was propagated in Escherichia coli 101 in a liquid LB (Luria-Bertani) medium with 50ug/ml of ampicillin and was isolated by a large-scale isolation method. HBV DNA sequence was then isolated from pHBR105 by separation with low melting point agar electrophoresis after digesting with BamHI.

Recirculation of HBV DNA

Linear HBV DNA was self-ligated or recirculated in a buffer consisting of 0.066M Tris-HCl (pH7.5), 5mM MgCl₂, 5mM dithiothreitol and 1mM ATP with 1 unit T4 DNA ligase (Takara Biomedicals, Takara Shuzo Co., Ltd., Kyoto, Japan) per μg of DNA. The solution was mixed well and then incubated 30 min at 16°C. It was demonstrated by agarose gel electrophoresis that monomeric recirculized HBV DNA was the predominant component in the reaction mixture.

Transfection of recirculized HBV DNA into Chang liver cells

Two methods were used for DNA transfection. DNA transfection was performed with two methods. One was calcium/phosphate (CaCl₂) coprecipitation method. Chang liver cells, grown in Eagle’s MEM containing 10% FBS, at a density of 7 × 10⁶ cells per 100-mm culture dish (Nunc, Roskilde, Denmark), were transfected with 10μg of monomeric recirculized HBV DNA by the method of Wigler, with the exception that the cells were exposed to the CaCl₂-coprecipitated DNA for 20 h at 37°C before fresh medium was added.

The other method was the electroporation method. Chang liver cells were harvested with 0.25% trypsin (Nakarai Chemicals, Kyoto, Japan). Cells were washed twice in ice cold Ca²⁺Mg²⁺-free phosphate buffered saline (PBS, 0.15M, pH 7.4), then resuspended in the PBS at a concentration of 1 × 10⁷ cells/ml. Five μg of monomeric recirculized HBV DNA and 0.8ml of cell suspension were placed in a Gene Pulser cuvette (Bio-Rad Laboratories, Richmond, CA, USA) and thoroughly mixed by pipetting. The cell suspension and HBV DNA were incubated together for 10 min on ice prior to electroporation. The cuvette containing cells and HBV DNA was placed in the Gene Pulser chamber, pulsed once, and then returned to ice and incubated for an additional 10 min at 0°C before plating. Voltage, duration and capacitance were set at 1200 V, 0.6 msec and 25 μFD, respectively. The cells were then washed in Eagle’s medium twice and seeded in culture dishes (Nunc). Cell viability was examined by Trypan blue dye exclusion.

Observation of the morphological change

Transfected cells were cultured in the above medium in the culture dish (Nunc). When the cells grew to an almost confluent state about 3 weeks after the transfection, all cells were harvested with trypsinization and divided into 24 aliquots of 1ml and cultured in a 24-well plate (Falcon, Becton Dickinson, Cockysville, MD, USA) in the above medium. The cell morphology was observed with a phase-contrast microscope every day and cells of which morphology was different from that of original Chang cells were selected and trypsinized them all in the well. The harvested cells were divided into 96-well microtiter plate (Nunc) and subcloned three times until cells with the same morphology were obtained.

Detection of hepatitis B surface antigen (HBsAg)

After obtaining uniform cells as described above, the dilution was repeated 3 times, the transfected cells were propagated and trypsinized. A part of them was cultured in Lab-Tek chamber slides (Miles Scientific, Naperville, IL, USA). The culture of the rest of the cells were continued in 100-mm culture dish (Nunc). Two days after seeding in Lab-Tek chamber slides, the slides were air-dried and fixed with methanol. One hundred μg/ml of a rabbit anti-HBsAg IgG (Protogen AG., Lanfeldingen, Switzerland) or a normal rabbit IgG as a control
were added to the slides and incubated for overnight at 4°C. Then the slides were washed 4 times with PBS, followed by the incubation with 50 μg/ml of FITC-labeled goat anti-rabbit IgG (Nihon Koutai Kenkyujo, Tokyo, Japan) for 30 min at 37°C. And the slides were washed 4 times with PBS. The slides were mounted with a buffered-glycerol mounting medium (Fujirebio Inc., Tokyo, Japan) and observed under a fluorescence microscope (Olympus, Tokyo, Japan). Untreated Chang cells were used as a negative control and PLC/PRF/5 cells were used as a positive control.

HBsAg in the culture supernatant of the transfected cells was assayed by the Ausria 11-125 radioimmunoassay (Abbott Diagnostics, North Chicago, IL, USA). The supernatants of Chang cells and PLC/PRF/5 cells were also examined.

Detection of HBV DNA in the transfected cells

Most of the cultured cells were frozen at -80°C until they were used as a source of DNA and a little part of the cells were used for further culture. DNA from the transfected cells, Chang cells and PLC/PRF/5 cells was isolated by extraction with guanidine thiocyanate followed by centrifugation through a cesium chloride cushion as described by Chirgwin et al. DNA phase was obtained and dialyzed against TE buffer (10 mM Tris-HCl (pH 8) and 1 mM ethylenediaminetetraacetic acid (EDTA)) for overnight and then treated with 500 μg/ml of proteinase K (Boehringer, Mannheim, FRG) followed by phenol/chloroform extraction. DNA was resuspended in an adequate volume of TE buffer and stored at 4°C until use. The plasmid pHBR105 was labeled with 32P-dCTP (800 cpm/nmol, Amersham, London, UK) by nick translation procedure of Rigby et al. to a specific activity of 2 × 106 cpm/μg. Ten μg DNA from cells was subjected to electrophoresis in 0.8% agarose horizontal gel at 35 V for 20 h. Another 10 μg DNA from PLC/PRF/5 cells was digested with Hind III (Takara Shuzo Co., Ltd) and was subjected to the same electrophoresis. After the electrophoresis the gel was soaked in 0.25N HCl for 10 min and rinsed thoroughly in distilled water and then soaked in denaturing solution (1.5M NaCl and 0.5M NaOH) for 30 min. After washing with distilled water, the gel was soaked in the neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, pH7.2 and 0.001M Na2EDTA) for 60 min. DNA in the gel was then transferred to a nylon membrane (Hybond-N, Amersham International plc., UK) with 20×SSC (3.0M NaCl and 0.3M Na3citrate) using a capillary blotting. Prehybridization and hybridization were performed according to the method described elsewhere. The filter was exposed to a Kodak XAR-5 film at -80°C for 48 h with intensifying screens.

Results

Morphological changes

Most of Chang cells which were seeded into culture dishes after the DNA transfections did not attach the dishes, because cells were damaged during the trans-
fection procedures. Cell viability after the both transfection procedures, the calcium/phosphate coprecipitation method and electroporation method, ranged from 0 to 10%. Several trials of transfection were made. When cells attached to the dishes and grew, culture was continued. We obtained one dish from 10 trials of calcium/phosphate coprecipitation method and 5 trials of electroporation method. During the first week, it was noted large nucleated cells with multinuclei and ground-glass cytoplasm were present. These cells grew faster than other cells which appeared similar in morphology to original Chang cells. Three weeks after the last subcloning, when 7 weeks had passed since the transfection procedures, cells grew constantly in a microtiter plate and it was noted that the contour of each cell was unclear (Fig. 1) compared with original Chang cells. There was no obvious morphological difference between the cells transfected by the calcium/phosphate coprecipitation procedure and the cells transfected by the electroporation procedure.

Detection of HBsAg in transfected Chang cells

The transfected Chang cells 7 weeks after the transfection were subcultured onto Lab-Tek chamber slides and examined for the presence of HBsAg by indirect immunofluorescence. Both cells transfected by the calcium/phosphate coprecipitation procedure and the electroporation procedure showed cytoplasmic fluorescent staining (Fig. 2-a,b), but the fluorescence of the cells transfected by calcium/phosphate coprecipitation procedure was somewhat weak (Fig. 2-a) compared with the cells transfected by electroporation procedure (Fig. 2-b). These stained cells consisted approximately 20% of the whole cells in terms of cell number. The fluorescence intensity of PLC/PRF/5 was as strong as the cells transfected by electroporation procedure (Fig. 2-c), while that of Chang was negligible (Fig. 2-d). Eleven weeks after the transfection, the fluorescence became undetectable in the both transfected Chang cells.

HBsAg was not detectable in the culture supernatants of the both transfected cells, while the culture super-

Fig. 2  Micrographs of the fluorescence-positive cells in the transfected cells. HBsAg was expressed in the cytoplasm of the transfected cells. The cells of the rest area were not stained. a: the transfected cells with a procedure of calcium/phosphate precipitation, b: the transfected cells with a procedure of electroporation, c: PLC/PRF/5 cells (positive control), d: Chang cells (negative control).
The accumulated HBsAg in the culture supernatants of cells.

<table>
<thead>
<tr>
<th>Cells</th>
<th>HBsAg concentration in the culture supernatant (cpm/ml/10^6 cells/day)</th>
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<tbody>
<tr>
<td>PLC/PRF/5</td>
<td>9150</td>
</tr>
<tr>
<td>Transfected Chang cells with a procedure of calcium/phosphate precipitation (7 weeks after the transfection)</td>
<td>32</td>
</tr>
<tr>
<td>Transfected Chang cells with a procedure of electroporation (7 weeks after the transfection)</td>
<td>41</td>
</tr>
<tr>
<td>Chang</td>
<td>17</td>
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**Detection of HBV DNA in the transfected Chang cells**

The result of Southern blot analysis of DNA from PLC/PRF/5, the transfected Chang cells and untreated Chang cells is shown in Fig. 3. The DNA from the transfected cells were examined 7 and 11 weeks after the transfection. Hind III-digested DNA from PLC/PRF/5 cells revealed 6 bands corresponding to the integrated HBV genomes (Fig. 3) and uncut DNA from PLC/PRF/5 cells revealed one band which was seen at a high molecular site. DNA from Chang cells transfected with HBV DNA by calcium-phosphate coprecipitation procedure was examined 3 weeks after the transfection. A 3.2kb band was seen. DNA from Chang cells transfected with HBV DNA by the electroporation method 3 weeks after the subcloning showed 3.2kb band and another lower band, whereas no band with molecular size higher than 3.2kb was observed. On the other hand, 11 weeks after the transfection, no bands hybridizing with HBV DNA were demonstrated.

**Discussion**

It was demonstrated that it was possible to transfet Chang cells with a recombinant recirculized HBV DNA by the method of electroporation or calcium/phosphate coprecipitation. The transfected HBV DNA was demonstrated to be functioning since the production of HBsAg was observed in the transfected Chang cells. HBV DNA was detectable in DNA from Chang cells 7 weeks after the transfection but no integration form was observed. HBV DNA became undetectable 11 weeks after the transfection. These results suggest that Chang liver cells transfected with HBV DNA serve as a model for studying HBsAg production in hepatocytes.

There are several reports of HBV DNA transfection into cultured cells. A variety of cell lines have been used as recipients, including L cell, 3T3 cell, HeLa cell, HepG2 cell, and other hepatoma cell lines which had been derived from mouse fibroblast, human uterus carcinoma, human hepatoblastoma, HuH-7 cell and other hepatoma cell lines which had been derived from human hepatoma. Although these cell lines are different in many aspects from normal human hepatocytes, HBV DNA was shown to function at various levels. Even virus particles were found after transfection in some cell lines. However, in all these cases, the way for HBV DNA to get into the cells is completely different from that of the natural virus infection and the host cells are different in many aspects, possibly lacking most of liver cell functions. In the natural infection of HBV to hepatocytes it is speculated that HBV gets access to hepatocytes by polymerized serum albumin bridging HBV surface coat and the polymerized serum albumin receptor on liver cell membrane. And HBV proliferates in the liver cell. During a long time of persistent infection HBV DNA integration into host cell genome could occur and it is postulated that the change to a malignant cell could occur as a result of HBV DNA integration. Comparing the artificial HBV DNA introduction into cultured cell lines with this natural infection, HBV DNA transfection seems to represent only a part of the natural infection.
Chang cell has been in culture for long period and it is suggested that it does not have all functions of normal hepatocytes. But it was demonstrated by immunohistochemistry that the cells used in our study was still producing albumin (data not shown). Chang cell is different from the normal liver cell because it grows permanently but it can not be transplanted in nude mice and can not grow in soft agar. So it is thought that Chang cell is not a cancer cell. It is clear that the best way for studying the carcinogenesis related to HBV is to use a primary culture of normal human hepatocytes infected with HBV itself. But nobody has succeeded in such an experiment. The alternative is to transfect normal fresh hepatocytes with HBV DNA. But it is difficult to obtain sufficient number of normal human liver cells and it is unrealistic to transfect normal hepatocytes with HBV DNA because most cells die during the transfection procedures and it is very difficult to grow cells to reach enough number which will provide enough amount of RNA and DNA. Therefore, at present we think that it is best alternative way to transfect Chang cells with HBV DNA for studying an aspect of HBV infection.

HBsAg was not detected in the culture supernatant of transfected cells even when HBV DNA existed in the cells. It is possible that the expression of HBsAg was not sufficient to be detected in the culture supernatant of these cells. But the immunofluorescence study, the intracellular production of HBsAg seemed to be sufficient even compared with that of PLC/PRF/5 cells. So it is indicated that the transport mechanism through cell membrane do not work in these cells. Recently, it was demonstrated that the large HBsAg protein which contained preS1 was not secreted through cell membrane unless an excess of smaller HBs protein is co-expressed. HBsAg protein that we detected in the cells might be preS1 or PreS2, since HBsAb which we used was a polyclonal antibody. If this is the case, transfected HBV DNA would work to produce large or middle S protein but not small S protein in this system.

Southern blot analysis showed that HBV DNA disappeared from the cells 11 weeks after the transfection and that HBV DNA existed as an extrachromosomal form. In case of electroporation method, the band lower than 3.2kb was observed. According to the papers of Shafririz6 or Brechot8, this band may indicate the supercoiled form of HBV DNA. If HBV DNA replicated well, the smear pattern of autoradiogram lower than 3.2kb would appear. From these observations, it is thought that the replication of HBV DNA was not sufficient whereas the expression of HBsAg was sufficient in these transfected cells. Further study may be necessary to elucidate the mechanisms of the integration of HBV DNA into the DNA of host cells. If there are some factors that inhibit the replication or integration of HBV DNA into Chang cells, it might be possible to use these factors for preventing the occurrence of HBV carrier state.

Acknowledgement: This work was supported in part by a grant from Viral Hepatitis Research Foundation of Japan and a grant from Ministry of Health and Welfare of Japan. We wish to thank Dr. H. Takeshima for his generosity in providing us with pHB105.

References

virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. Proc Natl Acad Sci USA 84: 2678–2682, 1987

17. Tsurimoto T, Fujiyama A, Matsubara K: Stable expression and replication of hepatitis B virus genome in an integrated state in a human hepatoma cell line transfected with the cloned viral DNA. Proc Natl Acad Sci USA 84: 444–448, 1987


