Hepatitis B Virus Replication in Well Differentiated Mouse Hepatocyte Cell Lines Immortalized by Plasmid DNA

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ABSTRACT

The primary hepatocytes cultured from adult BALB/c mice were readily transfected by plasmid DNA and could be immortalized at a frequency of approximately 0.1 to 0.6 per 106 cells/μg of the transfected DNA. There was no detectable plasmid DNA at the tenth cell passages. A total of five mouse hepatocyte cell lines were established. Most of them were tumorigenic. Three of the established mouse hepatocyte cell lines were well differentiated, since they expressed liver-specific genes. Further transfection of these three well differentiated mouse hepatocyte cell lines with hepatitis B virus (HBV) DNA showed that the HBV-transfected cells had integrated HBV genomes, HBV-specific mRNA transcripts, and expression of hepatitis B surface and hepatitis B core antigens. One of the lines, ML-3Neo (HBV), even secreted HBV-like particles. Furthermore, circulating hepatitis B surface antigens were detected in the sera of BALB/c mice bearing ML-3Neo (HBV) tumors. These cell lines provide a convenient model for future studies on the host immune reaction against HBV and on the transformation of hepatocytes by HBV and other cellular oncogenes and the determination of their effects on hepatocellular differentiation.

INTRODUCTION

Expression of oncogenes has been characterized as an important step in the transformation of mammalian cells. Oncogenes are identified frequently by the introduction of foreign DNA into recipient cells by the calcium phosphate transfection procedure. Those genes which induce transformation of mammalian cells are classified as oncogenes. However, the process of the addition of foreign DNA into the recipient cells with the aid of calcium phosphate is quite drastic, and oncogenes may be activated during the transfection process. Previous studies demonstrated that transformed cells could be obtained from NIH3T3 cells transfected with DNA extracted from normal cells (1). Furthermore, plasmid DNA not containing oncogenes could also induce transformation of cell lines at a frequency of 10 to 100 × 10^-6 cells/1 to 10 μg of transfected DNA (2). Therefore, the addition of foreign DNA to an immortalized cell line may induce mutations which will lead to transformation of the recipient cell lines (3–5). However, these experiments were generally performed on immortalized cell lines known to have some background conditions prone to spontaneous transformation. Whether normal cells will be immortalized or even transformed by the transfected plasmid DNA has not been studied. Murine hepatocytes have been known to be very difficult to immortalize. Not only have the hepatocytes been difficult to culture for long periods but they also lose their differentiated hepatocyte-specific functions after establishment (6–8). Many efforts have been made previously to establish murine hepatocyte cell lines which still possess hepatocyte-specific functions. To our knowledge, only one such cell line was established from liver cells of a transgenic mouse carrying SV40 T-antigen (9). Therefore, we selected murine hepatocytes as recipient cells to study the immortalization and transformation effects due to the addition of foreign DNA. The established murine hepatocyte cell lines with differentiated hepatocyte functions serve as a good tool to study many interesting biological questions related to hepatocytes.

The HBV is a partially double-stranded DNA virus with a genomic length of about 3200 base pairs. Several lines of evidence indicate an apparent relationship between persistent HBV infection and the formation of HCC (for review see Ref. 10). Much effort has been invested in understanding the mechanisms of HBV-induced cell transformation at the molecular level (11–15). However, the transfection mechanism of HBV still remains elusive. Furthermore, the growth of HCC in vivo is a balanced result between tumorigenic activities and host immune responses. Recently, the host immune response against HBV infection has been extensively studied (16–20). Due to the fact that interactions between cells in the immune system are major histocompatibility antigen restricted, it is very difficult to analyze immune response activities in outbred animals. Therefore, progress toward a detailed understanding of the mechanisms of host immune responses against HBV-infected hepatocytes and HCC has been slow. To understand the tumorigenicity of and host immune reactions against HBV-infected hepatocytes and HCC, it is important to establish models in inbred animals. Unfortunately, the HBV has a very narrow host range; it infects only the human and chimpanzee (21). Recent studies have found that, in addition to well differentiated human hepatoma cell lines, a rat hepatoma cell line (Q7) could also produce HBV particles after HBV DNA transfection (22). These results suggest the possibility that well differentiated murine hepatocyte cell lines may also support HBV replication and be a convenient model to study the tumorigenicity of and immune mechanisms against HBV-infected hepatocytes and HCC. We, therefore, initiated our studies by transfecting perfused primary BALB/c murine hepatocytes with plasmid DNA. Five murine hepatocyte cell lines were successfully established. Three of them possessed hepatocyte-specific functions. Furthermore, after transfection with HBV DNA, these cell lines expressed HBV genes. One cell line, ML-3Neo (HBV), even secreted HBV-like particles into the culture medium.

MATERIALS AND METHODS

Establishment of Mouse Hepatocyte Cell Lines by DNA Transfection. Hepatocytes were obtained from the livers of 1-month-old BALB/c mice (animal breeding center, Veterans General Hospital, Taipei, Taiwan, Republic of China) by perfusion as described by Klaunig et al. (23). Dispersed mouse hepatocytes were cultured at a density of 10^6 cells/6-cm Petri dish in DMEM, supplemented with 10% fetal calf serum.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyl transferase; HBsAg, HBV surface antigen; HBcAg, HBV core antigen.

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serum, antibiotics (penicillin 100 IU/ml, and streptomycin 50 µg/ml) and 2 mM L-glutamine at 37°C under 5% CO₂. After 5 days in culture, the mouse hepatocytes were transfected with the DNA of various plasmids. These plasmids were: (a) pSV2HBxEN, containing SV40 early promoter, the HBV X gene, and the HBV enhancer region (the HBV genome promoter); (b) pSV2CAT, containing the CAT gene and SV40 early promoter; (c) pSV2Neo containing SV40 early promoter and the neomycin-resistant gene; (d) pUE21E containing the HBV enhancer I fragment from HindIII 963 to SpeI 1236 was inserted into the pUC18 HindIII site. Circular-form plasmid DNA (8 µg) was used to transfect 10⁶ primary hepatocyte cells in a Petri dish (6 cm in diameter) by the calcium phosphate method described previously (24). The cultured medium was replaced by fresh medium after incubation at 37°C for 8 h. Thereafter, medium was replaced with fresh completed DMEM medium twice a week. Approximately 2 months later, cells were subcultured with 0.25% trypsin, 1 mM EDTA, and 3 mM glucose in Hank’s balanced salt solution. Eventually, immortalized cell lines were established. Three murine hepatocyte cell lines, ML-1Ex, ML-2CAT, and ML-3Neo, were further cotransfected with 14 µg plasmid pSHH2.1 (the plasmid containing tandem dimmer HBV DNA) (25) and pSV2neo to 5 × 10⁶ cells, followed by testing of HBV gene expression in these mouse cell lines.

RNA isolation and Northern blot analysis. Total RNA was extracted by the guanidinium/cesium chloride method (26). The RNA was dehydrated with formamide, electrophoresed, and transferred to nitrocellulose paper, after which Northern blot analyses were performed with 32P-labeled probe as described before (27). The probes were HBV DNA and liver-specific genes, i.e., albumin, transferrin, and α₁-antitrypsin (28).

Assay for secreted HBs and HBc/e. Cultured fluids of mouse hepatocyte cell lines were collected at intervals of 3 days. The secretion of HBsAg and HBc/e antigens was measured by enzyme immunoassay kits according to the manufacturer's instructions (Ever New Biotech, Inc., Hsinchu, Taiwan, Republic of China).

Growth of cells in methylcellulose suspension. The growth of mouse hepatocyte cell lines in methylcellulose was performed as described previously (32). Briefly, a feeder layer consisting of 4 ml of 0.9% agar (Bacto-Agar; Difco Laboratories, Detroit, MI) in DMEM, 10% fetal bovine serum, 100 units/ml penicillin, 50 µg/ml streptomycin, and 2.5 µg/ml fungizone was allowed to solidify in plastic petri dishes (6 cm in diameter). Completed DMEM culture medium (4 ml) containing 10⁵, 10⁶, 10⁷, and 10⁸ cells, respectively, and 1.3% methylcellulose (Dow Chemical Co.; 4000 centipoises) was poured onto the agar layer, and triplicate dishes were prepared for each dilution. The number of visible colonies (>0.5 mm in diameter) was scored at 14 days after initial cell seeding.

Determination of tumorigenicity in BALB/c mice. To determine the tumorigenicity of established mouse hepatocyte cell lines, the 6-week-old inbred BALB/c mice were given s.c. injections of 10⁶ cells of each established mouse hepatocyte cell line after 1 month to observe tumor formation.

RESULTS

Establishment of mouse hepatocyte cell lines by plasmid transfection. Three independent experiments were performed to obtain hepatocyte suspension from livers of 1-month-old BALB/c mice by perfusion. In each liver perfusion, approximately 2.5 × 10⁷ hepatocytes with viability of about 70% were obtained. They were then equally distributed into and cultured in 25 petri dishes (6 cm in diameter). After culture for 5 days, 36 µg of each plasmid DNA (pSV2HBxEN, pSV2CAT, pSV2Neo, pUE21E) and 4 µg of pSV2Neo plasmid were used to cotransfect five dishes of the mouse hepatocyte primary culture. The transfected cells were fed with fresh completed DMEM medium twice each week. One month later, most of the hepatocytes appeared to be dead. However, in a few dishes some hepatocytes remained viable and started to grow. After culturing for 2 months, those viable hepatocytes continued to grow and form foci. Usually, there were 4 to 10 foci in each dish. In the first experiment, viable and growing cells were found only in a dish containing hepatocytes transfected with pSV2HBxEN, but not in those transfected with pSV2CAT, pUE21E, or pSV2Neo (Table 1). In the second experiment, cell growth and foci formation were found in one dish containing hepatocytes transfected with pSV2CAT. In the third experi-

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Total no. of viable hepatocytes seeded (×10⁵)</th>
<th>Established cell line</th>
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<tbody>
<tr>
<td>pSV2HBxEN</td>
<td>3</td>
<td>ML-1HBxEN</td>
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<tr>
<td>Exp. 1</td>
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<td>Exp. 2</td>
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<td>Exp. 3</td>
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<tr>
<td>pSV2CAT</td>
<td>3</td>
<td>ML-2CAT</td>
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<tr>
<td>Exp. 1</td>
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<tr>
<td>Exp. 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUE21E</td>
<td>3</td>
<td>ML-5CAT</td>
</tr>
<tr>
<td>Exp. 1</td>
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<tr>
<td>Exp. 3</td>
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<tr>
<td>pSV2Neo</td>
<td>3</td>
<td>ML-3Neo</td>
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<td>Exp. 2</td>
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<td>Exp. 3</td>
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In all three experiments, no cell growth was found in the mock transfected (calcium phosphate alone) dishes. The average immortalization efficiency was approximately 0.1 to 0.6 × 10⁻⁷ cells/μg transfected DNA, assuming that each foci would be immortalized. Cells were then subcultured by trypsinization. Only cells from those Petri dishes containing foci could survive subculturing. Presently, over 50 passages of cell culture have been completed in each cell line. These immortalized cell lines were designated as ML-1EX (mouse hepatocyte cells transfected with pSV₂HBxEN), ML-2CAT (mouse hepatocytes transfected with pSV₂CAT), ML-3Neo (mouse hepatocytes transfected with pSV₂Neo), ML-4En (mouse hepatocytes transfected with pUE21E), and ML-5CAT (mouse hepatocytes transfected with pSV₂CAT). As shown in Fig. 1, all five cell lines exhibited typical epithelial-like morphology and had a doubling time of approximately 24 h.

Expression of Liver-specific Genes in Mouse Hepatocyte Cell Lines. In order to study the expression of liver specific genes in the five newly established mouse hepatocyte cell lines, Northern blot analyses were performed using mouse liver specific genes.

Fig. 1. Morphology of established mouse hepatocyte cell lines. A, cultured ML-1EX cells; B, cultured ML-2CAT cells; C, cultured ML-3Neo cells; D, cultured ML-4En cells; E, cultured ML-5CAT cells. × 200.
as probes. As shown in Fig. 2, ML-1EX, ML-2CAT, and ML-3Neo cells were all found to express albumin, transferrin, and αr-antitrypsin genes, which was characteristic of well differentiated hepatocytes. The expression of albumin, transferrin, and αr-antitrypsin genes was not detectable in the other two cell lines, ML-4En and ML-5CAT (data not shown). These results indicated that, among the five newly established cell lines, three of them (ML-1EX, ML-2CAT, and ML-3Neo) retained their hepatocyte-specific functions and belonged to well differentiated hepatocyte cell lines, while the other two (ML-4EN and ML-5CAT) were poorly differentiated hepatocyte cell lines.

Absence of Transfected DNA in the Genome of Mouse Hepatocyte Cell Lines. At the 10th subculture passage, the presence of transfected plasmid DNAs in these five cell lines was tested by Southern blot analyses using pSV2HBxEn, pSV2CAT, pSV2Neo, and pUE21E as probes. No hybridizing band could be detected (data not shown), indicating the absence of transfected plasmid DNAs in these cell genomes. Northern blot analyses were also performed to determine the expression of transfected genes. As expected, no specific mRNA could be detected (data not shown).

Colony Formation Efficiency and Tumorigenicity of Mouse Hepatocyte Cell Lines. To determine the tumorigenic potential of these cell lines, cells were tested for their ability to form colonies in methylcellulose. All five cell lines had very low efficiency (<0.01%) of colony formation in methylcellulose (Table 2). As a control, a human hepatoma cell line, HA22T/VGH, had more than 10% cells which formed colonies on methylcellulose agar (33). Since all five cell lines were derived from inbred BALB/c mice, their tumorigenicity was examined in BALB/c mice (Table 2). Two lines, ML-1EX and ML-3Neo, induced tumors in all mice tested at the dosage of 10⁷ cells/mouse. In contrast, the ML-4En and ML-5CAT were not tumorigenic. The ML-2CAT was barely tumorigenic since only 1 of 12 mice given injections formed a tumor. Furthermore, this tumor did not appear until 2 months later and grew very slowly (tumor size 3 mm).

Transfection of Mouse Hepatocyte Cell Lines with HBV DNA and Establishment of Stable HBV Transfectant Cell Lines. The three well differentiated hepatocyte cell lines, ML-1EX, ML-2CAT, and ML-3Neo, were further cotransfected with DNA of plasmids pSHH2.1 and pSV2Neo. The transfected cells were selected by G418 for cells resistant to neomycin, and stable HBV transfectant cell lines were established. They were designated as ML-1EX (HBV), ML-2CAT (HBV), and ML-3Neo (HBV), respectively. The transfection efficiency was around 10⁻³ to 10⁻⁴.

Analysis of HBV Genes and Their Expression in Stable HBV DNA-transfected Cell Lines. To analyze the presence of HBV genomes in these HBV DNA-transfected cell lines, total cellular high molecular weight DNAs of ML-1EX (HBV), ML-2CAT (HBV), and ML-3Neo (HBV) were isolated. Southern blot analyses using HBV whole genome as a probe were performed. As shown in Fig. 3A, HindIII digestion (no digestion site in HBV genome) yielded multiple HBV-specific DNA fragments larger than 3.2 kilobases in all three cell lines. With EcoRI digestion, a 3.2-kilobase HBV-specific band (expected size of HBV monomer) appeared in all three cell lines. An additional 3.0-kilobase band was found in ML-2CAT (HBV) (Fig. 3B). These results clearly indicated that all three cell lines had integrated HBV genomes. The analyses on the extrachromosomal DNA showed three HBV-specific bands in the ML-3Neo.
HBV replication in mouse hepatocytes immortalized by plasmid DNA

Fig. 3. Southern blot analyses on mouse hepatocytes transfected with HBV dimer plasmids, using HBV whole genome as probes. A, the high molecular weight DNA digested with HindIII; B, the high molecular weight and extrachromosomal DNA digested with EcoRI; C, the undigested low molecular weight extrachromosomal DNA. Twenty μg of cellular DNA were applied to each lane. kb, kilobases.

Fig. 4. A, Northern blot analyses on HBV gene expressions. The RNA was isolated from parent cell lines ML-1Ex, ML-2CAT, ML-3Neo and stable HBV DNA transfectant cell lines ML-1Ex (HBV), ML-2CAT (HBV), ML-3Neo (HBV). Twenty-five μg total cellular RNA were loaded per lane, using HBV whole genome as probe. B, endogenous polymerase activity in the viral particles secreted by the stable HBV-transfected cell lines ML-1Ex (HBV), ML-2CAT (HBV), and ML-3Neo (HBV). Approximately 70-ml supernatants from each cell line were collected, and the precipitated viral and core particles were used for the assay of endogenous polymerase activity. kb, kilobases.

HBV cells. Their molecular weights were 5.0, 4.0, and 2.5 kilobases (Fig. 3C). After prolonged exposure, an additional HBV-specific band of 3.2 kilobases was observed (data not shown). After digestion with EcoRI, these different forms of HBV-specific DNA were all shifted to 3.2 kilobases (Fig. 3B). These results suggested that 4.0- and 2.5-kilobase HBV-specific bands may be the relaxed circular form and the closed circular form DNA, respectively. The nature of 5.0-kilobase HBV-specific DNA remains to be identified. There was no detectable extrachromosomal HBV DNA in ML-1Ex (HBV) and ML-2CAT (HBV) cells. For the detection of HBV-specific transcripts, Northern blot analyses revealed two major HBV specific RNA species: a 3.5- to 3.8-kilobase and a broad 2.1- to 2.5-kilobase band (Fig. 4A). These RNA sizes corresponded to the HBV pregenomic RNA (3.5 to 3.8 kilobases) and HBsAg mRNA (2.0 to 2.3 kilobases). A HBV-specific band with a molecular size of 7.9 kilobases appeared in ML-2 CAT (HBV), which might be the transcript initiated from a cellular promoter flanking the HBV integration site. The cultured fluids of HBV DNA-transfected mouse hepatocyte cell lines were assayed for the secretion of HBs and HBe by immunoassay. As shown in Table 3, all three cell lines secreted a significant amount of HBs and HBe antigens. Among them, ML-3Neo (HBV) apparently secreted the highest levels of HBs and HBe/antigens.

Table 3 Detection of secreted HBV antigens in HBV-transfected cell lines

<table>
<thead>
<tr>
<th>Mouse cell line</th>
<th>HBV antigen (P/N ratio)</th>
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<tr>
<td></td>
<td>e/core antigen</td>
</tr>
<tr>
<td>ML-1Ex (HBV)</td>
<td>3.9</td>
</tr>
<tr>
<td>ML-2CAT (HBV)</td>
<td>5.4</td>
</tr>
<tr>
<td>ML-3Neo (HBV)</td>
<td>14.9</td>
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</table>

*P/N ratio, ratio of positive results to negative controls at the absorbance of 492 nm after enzyme immunoassay.
Detection of Circulating HBs Antigen in Sera of Tumor-bearing Mice. The HBV DNA-transfected cell line ML-3 Neo (HBV) was tumorigenic in BALB/c and secreted HBV-like particles. After s.c. injection of 10^7 ML-3Neo (HBV) cells, all 12 BALB/c mice produced tumors. The secretion of HBsAg into the mice sera was tested by enzyme immunoassay. It was found that mice bearing ML-3Neo (HBV) tumor cells had circulating HBs antigen in the sera. Time course analyses showed that the secretion of HBs antigen peaked at approximately 45 days (P/N ratio = 8.7) after tumor injection, and then the level of HBs antigen started to decline. HBc antigen and endogenous polymerase positive HBV core particles were not detectable in the sera of these tumor-bearing mice (data not shown).

DISCUSSION

It has long been known that adult murine hepatocytes are difficult to culture, and to our knowledge spontaneous immortalization of adult murine hepatocytes has never been reported. However, we have demonstrated that adult murine hepatocytes could be immortalized and that some of them even were tumorigenic by transfection with plasmid DNA. Apparently, the immortalization was not due to the integration or expression of any specific genes in the plasmids pSV2HBxEN, pSV2CAT, pUE21E, and pSV2Neo, since there was no detectable DNA or mRNA of these plasmids, even at very early subculture passages (passage 10) of these cell lines. A similar phenomenon was found by Lau et al. (2) using plasmid DNA to induce tumorigenic foci in the immortalized CHEF/18 cell line. They suggested that a 'hit and run mechanism' was involved in the transformation process. The estimated frequency of the immortalization of mouse hepatocytes is 0.1 to 0.6 × 10^{-7} cells/μg of transfected DNA, which is lower than the frequency of induction of tumorigenic focus from CHEF/18 cell lines, 10 to 100 × 10^{-6} cells/μg of transfected DNA. Since most of our immortalized mouse hepatocyte cell lines were tumorigenic, our results indicated that plasmid DNA not only can immortalize primary mouse hepatocyte cultures but it can also induce tumorigenic transformation in those cells, at a frequency about 1000-fold lower than that of the induction of tumorigenic foci from the immortalized cell line. The frequency of 0.1 to 0.6 × 10^{-7}μg DNA is still in the range of a single mutation. Our study suggests that a “single hit” may be enough to immortalize and sometimes even to induce tumorigenic transformation in mouse hepatocytes. Whether a “hit and run” mechanism plays a role in our transformation process remains to be unknown.

Three of our immortalized mouse hepatocyte cell lines, ML-1EX, ML-2CAT, and ML-3Neo, expressed liver-specific genes, albumin, transferrin, and α1-antitrypsin. These results indicate that these three cell lines are well differentiated mouse hepatocyte cell lines. These cell lines will provide a good and also quite needed model to study the gene regulation of mouse hepatocytes and also to study the transformation of hepatocytes by HBV and other cellular oncogenes.

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