In vitro infection of human hepatoma cells (HepG2) with hepatitis B virus (HBV): spontaneous selection of a stable HBV surface antigen-producing HepG2 cell line containing integrated HBV DNA sequences

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The degree of susceptibility of human hepatoma (HepG2) cells to direct hepatitis B virus (HBV) infection remains unknown. We previously observed a low level of Dane particle production and viral DNA replication after in vitro infection of HepG2 cells with serum-derived HBV. However, this culture system appeared to be affected by variations as human hepatocyte cultures. In the present study, HBV infection of HepG2 cells led to a significant increase in the secretion of three envelope antigens (HBsAg, preS2Ag and preS1Ag) at 4 days post-infection, and Northern blot analysis revealed the presence of both preS1 (2·6 kb) and preS2/S (2·2 kb) transcripts. Expression of preS1Ag and the corresponding viral RNA became undetectable on 21 days post-infection whereas the 2·2 kb RNA species persisted and was associated with secretion of subviral HBs particles expressing preS2-epitopes and banding between 30 and 35% sucrose. At 35 days post-infection (fifth passage), a sudden high level production of HBsAg and preS1Ag was observed, followed by a massive cell death (90%). A stable HBsAg-producing HepG2 cell line, designated HepG2-BV3, grew out of the surviving cells. HepG2-BV3 cells could integrate HBV DNA sequences and produce the three HBV surface antigens. Treatment with dexamethasone increased the HBsAg and preS1Ag secretion. Such a HBsAg-producing HepG2 cell line obtained by in vitro HBV infection seems to mimic events that occur in the naturally occurring persistent chronic infection, and therefore may be an efficient in vitro model for studying the contribution of viral integration in the dysregulation of HBV and liver-specific genes expression.

Introduction

Attempts have been made to establish an in vitro model of hepatitis B virus (HBV) that allows infection and replication of the virus to be examined. Indeed, previous studies have shown that HBV enters and replicates in primary fetal human hepatocytes (Ochiya et al., 1989) as well as in normal adult human hepatocytes in the presence of DMSO (Gripon et al., 1988). However, the differentiated state of the hepatocytes in these cultures can be maintained only for a limited amount of time. Propagation of HBV in cell culture has been achieved by transfection of closed circular HBV DNA in human hepatoma cell lines (HepG2 or HuH7) (Sureau et al., 1986; Sells et al., 1987; Tsurimoto et al., 1987). However, the latter system did not mimic the in vivo process of viral infection, including virus penetration and early biochemical events. Therefore, we have focussed on the problem of in vitro infection of HepG2 cells (Aden et al., 1979; Knowles et al., 1984) with HBV.

The mechanism of HBV entry is not yet understood, however, we have previously demonstrated that several factors are essential for successful in vitro infection of HepG2 cells with HBV (Bchini et al., 1990). In particular (i) morphological integrity of virion particles and (ii) high level expression of preS1 sequences (at positions 21 to 47) of the large HBs envelope protein (LHBs) are determinant for the binding of HBV to hepatocytes (HepG2) (Petit et al., 1991a and c). In addition, we have identified preS1-specific binding proteins at the HepG2 cell membrane (Petit et al., 1992) suggesting that HepG2 cells display a specific receptor for HBV attachment and penetration. Using this model, we have succeeded in infecting HepG2 cells with HBV (Bchini et al., 1990), and an HepG2 cell line (HepG2-BV) that produces complete virions with the viral DNA replication being measurable was obtained. However, similar to normal human hepatocyte primary cultures (Gripon et al., 1988), this latter in vitro infection model also appeared subject to important variations (Petit et al., 1991b).

In the present report, we investigated the course of production of HBV-associated particles, and of the relative expression of all three HBV envelope proteins following in vitro infection of HepG2 cells with serum-
derived viral particles. At 35 days post-infection, sudden increased production of HBs particles and preS1Ag was spontaneously observed, followed by c.p.e. A stable HepG2 cell line that produced high levels of preS2-positive HBs particles and containing integrated HBV DNA sequences was therefore identified through selection and designated the HepG2-BV3 cell line. Our results indicate that (i) in vitro HBV infection of HepG2 cells is subject to wide variations not only from one experiment to another (Bchini et al., 1990; Petit et al., 1991b) but also during the long-term follow-up of infection in the same experiment as observed in HBV-infected patients and also that (ii) dysregulation in surface gene expression probably resulting from integration of HBV contributes to HBV persistence.

Methods

HepG2 cell culture. HepG2 cells were grown in Dulbecco’s MEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 2 mM-L-glutamine and antibiotics (penicillin 100 units/ml, and streptomycin 100 µg/ml) (in complete medium). Cells were seeded at a concentration of 2 x 10^5 cells per ml in T-75 flasks. The medium was changed every 2 days. Cultures were observed daily using a phase-contrast microscope. At confluence, cell monolayers were trypsinized, and passaged weekly thereafter.

HBV isolate and infection of HepG2 cells. The HBV isolate (HBsAg strain Wie; subtype adw2, genogroup A; Norder et al., 1993) was obtained from a patient with envelope antigen HBeAg-positive chronic hepatitis B, who had elevated alanine aminotransferase levels (>100 UI/ml). HBV-associated particles were concentrated (150-fold) from serum by means of ultracentrifugation (230000 g for 18 h) through a sucrose-density gradient (30%, w/w). The final viral particle-enriched pellet was resuspended in TNE buffer (10 mM-Tris-HCl, pH 7.2, 140 mM-NaCl and 10 mM-EDTA) at a concentration of 1 mg of protein per ml and stored at −80 °C. HBV DNA content was of 6 ng/ml using the Abbott 125I-labelled HBV DNA hybridization assay (Abbott).

Four days after plating, HepG2 cells (about 5 x 10^6 per ml) were incubated for 16 h at 37 °C with serum-derived viral preparation diluted in complete medium to a final concentration of 30 µg of protein per ml (corresponding to 200 pg of HBV DNA per ml). The m.o.i. corresponded to approximately 100 viral genomes equivalent (v.g.e.) per cell. After removal of the inoculum, cells were washed three times in PBS (threefold), and then cultured in complete medium.

were performed every week. Results were expressed as c.p.m. measured in the culture supernatants (P). Negative controls (N) corresponded to the mean value of 150 c.p.m. (for many determinations) and the results were considered positive when values were higher than 315 c.p.m. (P/N = 2.1). (b) Northern blot analysis of total RNA from HBV-infected HepG2 cells at 1, 2, 4 and 21 days after infection (lanes 1 to 4, respectively). Hybridization was performed with 32P-labelled whole HBV DNA used as probe. (c) Sedimentation profile obtained after isopycnic centrifugation in sucrose gradient of HBV particles present in the supernatant of infected-HepG2 cells at day 21. The bulk of HBsAg activity sedimented between 30 and 35% sucrose.
Sucrose-density gradient fractionation. Viral particles in culture media were spun down at 200,000 g for 2 h, and the pellet was then resuspended in TNE buffer (concentrated 10-fold) and centrifuged at 230,000 g for 18 h in a sucrose density gradient that ranged from 20 to 60% (w/w) concentration. Fractions (1-2 ml) were collected, tested for sucrose concentration using refractometry, and then diluted 1:10 for the detection of HBsAg and preS antigens by radiomunnoassays as described below.

Polyclonal-monoclonal antibody radiomunnoassays (PAb-MAb RIAs). Quantification of three antigenic specificities of the HBV envelope epitopes (HBs, preS2 and preS1) was performed using an in-house PAb-MAb RIA system utilizing rabbit polyclonal anti-HBs IgGs and mouse monoclonal antibody (Behrini et al., 1990). The immune complex was made soluble in SDS-PAGE sample buffer, run on 12.5% polyacrylamide gels and assayed by immunoblotting using rabbit polyclonal anti-HBs IgGs that were detected by incubation with the 125I-labelled F(ab')2 fragment of anti-mouse immunoglobulins (Igs) (Amersham).

Western blot analysis. HepG2 cell culture supernatants (over 3 days at subconfluence) were immunoprecipitated with HBs(F39.20)-specific monoclonal antibody (Behrini et al., 1990). The immune complex was made soluble in SDS-PAGE sample buffer, run on 12.5% polyacrylamide gels and assayed by immunoblotting using rabbit polyclonal anti-HBs IgGs that were detected by incubation with the 125I-labelled F(ab')2 fragment of anti-rabbit IgGs (Amersham).

Immunocytochemical staining. Subconfluent HepG2 cells were trypsinized, harvested, counted, resuspended in cold PBS and placed onto slides (2 × 10⁶ per slide) with a Cytospin 3 instrument (Shandon). Immediately after preparation, the slides were air-dried for 2 h, fixed in acetone for 10 min, in chloroform for 30 min and washed in PBS for 5 to 10 min. The slides were then stored at −80 °C until used. A three-step immunoperoxidase technique was performed (Mason & Sammons, 1979) using HBs(F39.20)-, preS2(F376)- and preS1(F35.25)-specific monoclonal antibodies. Non-specific protein staining was reduced by diluting monoclonal immunoglobulins in PBS containing 50% of normal human serum.

Analysis of HBV DNA sequences by Southern blot. After trypsinization and washings in PBS, subconfluent HepG2 cells were lysed in 0.5% SDS (50 mm-Tris–HCl, pH 8, 100 mm-NaCl and 1 mm-EDTA), and incubated with 50 µg of proteinase K per ml for 12 h at 37 °C. The DNA was first extracted with phenol/chloroform and then with chloroform. The DNA solution was adjusted to a concentration of 0.3 m with NaCl, precipitated with 2 vol of cold ethanol, and then chilled for 12 h at −20 °C. The precipitate was resuspended in TNE buffer. High M₉ DNA was digested for 5 h at 37 °C with restriction endonucleases, EcoRI and HindIII (Boehringer Mannheim) (45 U of enzyme for 15 µg of DNA). DNA was then analysed by electrophoresis on a 0.8% agarose gel and transferred to a Hybond-N membrane (Amersham; Southern, 1975). Filters were hybridized with a 32P-labelled HBV DNA probe (HBsAg: subtype ayw3, genegroup D; Charnay et al., 1979). The probe was labelled by nick-translation (sp. act. 1 × 10⁶ c.p.m. per µg). Hybridization was performed at 65 °C in the presence of 10% dextran sulphate. Filters were washed, dried and then exposed for 12 h to 1 week at −70 °C to Cronex film (Dupont) with an intensifying screen.

RNA analysis. Total RNA was extracted from subconfluent HepG2 cells using RNAβ solution (Bioprime systems). Twenty µg of RNA were diluted in 1 × MOPS buffer containing formaldehyde and deionized formamide, incubated for 15 min at 65 °C and chilled on ice. RNA samples were electrophoresed through 1% agarose gels containing 1% M-formaldehyde, and transferred to a nylon membrane (Hybond-N, Amersham). Filters were prehybridized for 2 h at 65 °C in 6 × SSC, 5 × Denhardt's solution, 0.1% SDS, and 100 µg/ml of denatured herring sperm DNA, and then were allowed to hybridize with the nick-translated full-length HBV DNA as described above.

Dexamethasone treatment of cell cultures. HBV-infected HepG2 cells (10⁶/ml) were cultured for 3 days in complete medium containing 10⁻⁴ M-dexamethasone. Culture medium was removed and assayed for HBsAg, preS2Ag and preS1Ag by PAb-MAb RIAs.

Identification of HBsAg particles by electron microscopic examination. HepG2 cell culture supernatants (about 40 ml) were centrifuged at 200,000 g for 2 h. The pellet was dissolved in TNE buffer (0.1 ml), mixed with 0.1 ml of HBs(F39.20)-specific monoclonal antibody (100 µg of IgG per ml) and incubated at 37 °C for 1 h, then at 4 °C for 16 h. The mixture was then diluted in 10 ml of TNE buffer and centrifuged at 48,000 g for 1 h. The pellet was resuspended in 0.1 ml of TNE buffer, dropped on a carbon-coated grid, and observed after negative staining using a JEOL 100 CX electron microscope.

Results

Expression of viral proteins and RNAs during 3 weeks following HBV infection

HepG2 cells were infected with the viral preparation (approx. 10⁸ v.g.e. per ml and per 10⁶ cells) for 16 h at 37 °C. After removal of the inoculum, virus weakly associated with the cells was eluted by extensive washings. No HBV surface antigens were detected in the third washing (Fig. 1a, day 1 post-infection). Culture medium was collected at days 2, 4, 7, and 21 after infection, initially clarified and then centrifuged at 200,000 g for 2 h. The resulting supernatants and pellets were tested for HBsAg, preS2Ag and preS1Ag. All HBV surface antigens were recovered in the particle-associated fraction, indicating that infected HepG2 cells secreted HBsAg.

Fig. 2. Kinetic analysis of HBsAg (+), preS2Ag (●) and preS1Ag (■) secreted by HBV-infected HepG2 cells from day 35 to day 75 after infection. A massive cell death (90%) was observed from day 55 to day 75.
Fig. 3. For legend see opposite.
viral antigens as particulate forms. Fig. 1(a) shows the kinetic analysis of HBsAg, preS2Ag and preS1Ag produced by HepG2 cells during the first 3 weeks following infection (including three passages of cells after treatment with trypsin). Maximal secretion of three surface antigens was observed at day 4 post-infection. HBsAg and preS2Ag production then slightly decreased but remained significantly positive after three successive subcultures (at day 21 post-infection). In contrast, preS1Ag rapidly dropped at day 7 post-infection and then became negative at 21 days (Fig. 1a).

The presence of HBV-specific transcripts in infected-HepG2 cells at days 1, 2, 4 and 21 after infection was analysed by Northern blot. As shown in Fig. 1(b) HBV RNAs at 2.2 kb and 2.6 kb rose from nearly undetectable levels at day 1 to high levels at 2 and 4 days post-infection. The former could be the transcript for the major (SHBs, p24/gp27) and the middle (MHBs, gp33/gp36) surface proteins, whereas the latter could be the transcript for the large (LHBs, p39/gp42) surface proteins. The 2.6 kb/LHBs RNA could not be seen at day 21 whereas the 2.2 kb/SHBs–MHBs RNA persisted. These results are in agreement with previous immunoblot analysis suggesting the presence of HBV surface antigens (Fig. 1a). Of note, the 3.6 kb RNA corresponding to pregenomic RNA that codes for core proteins (HBcAg) was not detected (Fig. 1b).

HBV-infected HepG2 cell cultures were tested for HBeAg and for the presence of HBV DNA by RIA and hybridization assay (Abbott Laboratories). HBV replication markers were not detected (results not shown). The latter results indicate early active transcription of HBV DNA that leads to synthesis of envelope proteins alone, and absence of virus replication.

Sedimentation analysis of secreted viral particles

To characterize the viral particles released from HBV-infected HepG2 cells, culture media collected at day 21 post-infection were subjected to isopycnic centrifugation in a sucrose density gradient (Fig. 1c). By using PAb-MAb RIAs, only one population of viral particles positive for HBsAg and preS2Ag was found in fractions 8 to 11 between 30 and 35% sucrose. Neither HBV DNA nor the major core protein (determined by Southern or Western blotting, respectively) could be detected in fraction 10, confirming the secretion of viral particles only composed of HBs proteins, but not of mature complete virions.

Spontaneous selection of a stable HBsAg-producing HepG2 cell line

At day 35 after infection (fifth passage), a fourfold increase in c.p.m. measured for HBsAg and a strong positive signal (P/N > 100) for preS1Ag were observed (Fig. 2). Even after concentration, neither HBeAg nor HBV DNA could be detected in supernatants of HBV-infected HepG2 cells (results not shown). From day 40 to day 75 post-infection, the amounts of HBsAg and preS1Ag that were released into the culture medium progressively decreased. Cell death began at day 50 post-infection and rapidly increased thereafter (about 90% of cells died at day 75 post-infection). The remaining viable HepG2 cells were grown first in culture plate wells, and then propagated in flasks as described in Methods. A stable HBsAg-producing HepG2 cell line, designated HepG2-BV3, was thus spontaneously selected. The HepG2-BV3 cells did not morphologically differ from the parental HepG2 cells as observed by phase-contrast microscopy. However, HepG2-BV3 grew one and a half times faster than the parental HepG2 cells did.

HBV proteins and DNA analysis in infected HepG2-BV3 cells

The production of HBV envelope antigens (HBs, preS2 and preS1) was followed using PAb-MAb RIAs after every passage during 16 weeks (results not shown). Nine months after infection (36th passage), the levels of HBsAg secretion by HepG2-BV3 cells were stabilized to reach a concentration between 50 and 100 ng of HBsAg per ml of supernatant over a 3-day culture period (determined using our in-house PAb-MAb RIA test as
Fig. 4. Southern blot analysis of DNA extracted from HepG2-BV3 cells. Lane 1, Control (parental HepG2 cell line); lane 2, undigested DNA; lane 3, EcoRI-digested DNA; lane 4, HindIII-digested DNA. The mass of DNA loaded was 15 μg. Size markers (kb) for HindIII-digested bacteriophage λ DNA are indicated on the right.

Discussion

HBV studies have been considerably limited because of the virus’ restricted host and tissue range and the lack of an in vitro culture system. Previously, we successfully infected well-differentiated HepG2 human hepatoma cells with serum-derived HBV particles (Bchini et al., 1990). Furthermore, we identified HepG2 cell preS1-binding proteins as receptor candidates that were required for initiation of infection through the preS1 domain of virions (Petit et al., 1992). Therefore, we believe that hepatoma-derived HepG2 cells are permissive and also susceptible to direct HBV infection. However, this in vitro infection model has its limitations: (i) the reproducibility of the infection process, as well as that of models based on primary cultures of human hepatocytes (Gripon et al., 1988; Ochiya et al., 1989), and (ii) hepatoma cells seem particularly susceptible to important dysregulation of HBV gene expression which often results in a loss of their ability to produce progeny virus particles, leading in some cases to a non-productive infection (Petit et al., 1991b).

In the present report, we describe spontaneous development and selection of a stable HBsAg producing HepG2 cell line, named HepG2-BV3, which was obtained by in vitro HBV infection. We examined the levels of surface gene transcripts and surface proteins in infected hepatoma cells. As well as the reference PLC/PRF/5 cell
line derived from human hepatocellular carcinoma (Alexander et al., 1976), the HepG2-BV3 cell line contains HBV genomes in integrated forms. The cascade of events leading to a clonal growth of hepatocytes that have integrated virus are unknown. Therefore, we focussed our investigations on the pathway leading to changes in the HBV gene expression during chronic infection, in a model that bypassed the cellular immune response.

At an early stage of HepG2 cell infection with HBV (during week 1 post-infection), all three envelope antigenic specificities (HBsAg, preS2Ag and preS1Ag) were synthesized and secreted into the medium by infected hepatoma cells. Both the preS1 and S promoter transcripts were increased within the cells, whereas neither the 3.5 kb pregenome RNA nor HBV DNA replicative intermediate forms were detected in this infection assay.

Expression of the large HBsAg transcript in HepG2 cells without extensive virus replication at an early stage of infection is in agreement with the report of Yuasa et al. (1991). This finding suggests that the preS1 promoter could be regulated by unknown cellular factors that probably enhanced its expression. The amount of preS1 transcripts in the cells and preS1 antigen in the medium rapidly dropped below undetectable levels (3-weeks post-infection). HBV-infected HepG2 cells continued, however, to express the S promoter transcript and to secrete HBsAg particles expressing few preS2-specific epitopes.

At a late stage of HepG2 cell infection with HBV (2-months post-infection), production of HBsAg and preS1Ag spontaneously greatly increased and this was followed by an hepatocytotoxic effect. This dysregulation of surface gene expression suggests disruption of the HBV genome, as observed during chronic infection by HBV in humans when viral core protein expression and viraemia is at low or undetectable levels (Dienes et al., 1990). HepG2-BV3 cell line stably producing high levels of HBsAg (6 to 9 months postinfection) was thus selected, and the state of the HBV DNA further analysed by the Southern blot technique. The lack of replicative intermediates and circular forms of the HBV genome, and the presence of HMW HBV DNA in the undigested DNA pattern suggested that HBV envelope antigens were produced from integrated copies. Digestion with HindIII revealed the presence of three DNA fragments with a major band at 22 kb, reflecting the existence of a limited number of integration sites in the cellular DNA. The uncut cellular DNA pattern and the HindIII restriction pattern differed demonstrating that HBV DNA integration occurred, rather than free oligomers were present. Additional information was provided by the use of EcoRI restriction endonuclease. The presence of a major band at the 2.6 kb position suggested that the majority of HepG2-BV3 cells had integrated several incomplete HBV DNA sequences (2.6 kb) in tandem with a head-to-tail orientation. This would correlate well with only the expression of HBV envelope proteins and the loss of an ability to express core proteins and to produce complete virions by the HepG2-BV3 cell line. However, cloning of the integrated viral sequences is necessary to establish this conclusively. Further investigation is now in progress.

It cannot be excluded that undetectable minute amounts of complete virions may be produced by a few cells. In support of this hypothesis, we demonstrated the presence of HBV DNA in untreated and Dex-treated HepG2-BV3 culture supernatants using a highly sensitive PCR amplification technique (results not shown), indicating that HBV replication occurred at very low levels in the HepG2-BV3 cell line.

Sudden increased production of both HBsAg and preS1Ag could be explained by HBV genomic fragmentation and rearrangement resulting from integration into the host cell genome. Such a dysregulated surface gene expression was followed by a massive cell death. This phenomenon is not well documented, but abnormal transcription of the LHBs probably resulted in intracellular retention of all forms of the surface protein (Ou & Rutter, 1987), and this has been shown to be cytotoxic (Chisari et al., 1987). This supports the idea that liver cell injury during HBV infection may be due to dysregulated surface gene expression (Huang & Yen, 1993), and that human liver-specific factors are likely to be critical for viral pathogenesis. In addition, the preS region of HBV could induce direct suppression of cellular gene expression through its binding capacity for hepatocyte nuclear factor-1 (Courtois et al., 1988). Thus, the virus itself may exert c.p.e.s during chronic in vivo infection along with the HLA class I restricted cytotoxic T lymphocyte response to HBV-encoded antigens, as well-documented by the Chisari’s group (Penna et al., 1991; Nayersina et al., 1993). Dysregulation of surface gene expression due to viral DNA integration seems to be a predominant phenomenon occurring during in vitro infection of hepatoma cells (HepG2 or HuH7) with HBV. This model appears therefore adequate not only for the study of the mechanisms implicated in chronic hepatitis B but also for the location of integrated viral sequences and their contribution in hepatocarcinogenesis. Therefore, we are investigating the expression by HepG2-BV3 cell line of the X gene and 3' end-truncated middle surface genes (preS2/S) which have been found to have a transcriptional transactivator function (Kékulé et al., 1993; Lauer et al., 1994).

Dexamethasone is known to exert a positive regulatory influence on HBV replication via glucocorticoid responsive elements within the HBV genome (Tur-Kaspa
et al., 1986; Farza et al., 1987). Increased secretion of HBsAg particles by Dex-treated HepG2-BV3 cells as previously described for HBV-transfected HepG2 cells (Tur-Kaspa & Laub, 1990) indicates that the glucocorticoid-dependent enhancer element in the HBV genome is present and functionally intact in the integrated DNA fragment. Therefore, the HepG2-BV3 cell line may be of use in investigations of the response to various drugs, biological stimuli and infections with other viruses in modifying HBV gene expression during chronic infection.

In conclusion, the HepG2-BV3 cell line obtained by in vitro HBV infection of HepG2 cells mimicks events which occur in naturally infected hepatocytes of patients at a late stage of chronic HBV infection (HBeAg-negative), when the DNA of HBV is present in an integrated form with viraemia occurring at low or undetectable levels. This system may serve as an efficient model for studying the sequence of events leading to hepatocellular carcinoma and assist us to delineate the contribution of viral integration in the dysregulation of HBV and liver-specific genes expression.

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References


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