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Hepatitis B virus replication causes oxidative stress in HepAD38 liver cells

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Abstract

We used human hepatoma HepAD38 cells, in which HBV production is under the control of a tetracycline-regulated promotor, to investigate changes induced in the host cell by HBV replication that could contribute to malignant transformation. Parameters of oxidative stress (malondialdehyde, glutathione) and cell proliferation were determined at different times after induction (0–96 h). In HBV-producing cells, the redox status peaked at 72 h. cDNA micro array analysis at 72 h post induction revealed 3 groups of genes that were up-regulated by HBV: (i) heat shock proteins, (ii) oxidative and metabolic stress and (iii) growth and apoptosis related genes. Continuous HBV production did not accelerate karyotypic changes in cells cultured for 4 months (18 passages). In conclusion: HBV replication modulates host gene expression and induces oxidative stress. In this HepAD38 model early events (0–4 days) in the host cell after induction of HBV replication can be studied under strictly defined conditions. (Mol Cell Biochem **290:** 79–85, 2006)

Key words: HBV, liver cancer, viral hepatitis, mutations, cell model, karyotype

Abbreviation: FCS, foetal calf serum; GSH, glutathione; GSSG, oxidized glutathione; HCC, hepatocellular carcinoma; MDA, malondialdehyde; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species, tet, tetracycline

Introduction

Hepatitis B virus (HBV) infection may result in acute or chronic hepatitis that can ultimately lead to the development of liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Worldwide, there are more than 350 million people carrying the hepatitis B surface antigen (HBsAg) and it is estimated that each year over 1 million people die because of HBV [2].

In general, between the moment of infection with HBV and the clinical diagnosis of HCC there is period of 15–30 years. Carcinogenesis is in many cases a multistep process in which a number of genetic alterations accumulate in a cell. In the case of HCC due to HBV infection, oncogenesis may be related to the HBV-induced chronic inflammation. Because of an enhanced cell turnover, the repair of damaged DNA may be compromised rendering the cells more susceptible to spontaneous or mutagen-induced alterations [3]. Another mechanism may involve the integration of viral DNA in the nuclear DNA. However, although HBV DNA can be detected integrated into the genome of cell lines derived from HCC,

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this integration is usually not specific nor clearly associated with activation of known cellular proto-oncogenes. Also, viral proteins may act as trans-activators of cellular genes [4] or induce cellular stress or oxidative stress leading to DNA damage [5].

To counteract the effect of oxidative stress, the cell has developed two important defence mechanisms: redox-active sulfhydryl systems [glutathione and thioredoxine (TRX)] and enzymatic systems [including superoxide dismutase, catalase and glutathione peroxidase] [6]. Glutathione (GSH) is the most abundant non-protein sulfhydryl-containing compound in the cell and constitutes the major thiol buffer. GSH plays a central role in controlling the cellular thiol/disulfide balance that is essential for normal redox signalling [7]. There is considerable evidence that redox signalling mechanisms function in cell regulation and growth control. Not only is cellular GSH involved in proliferation, in addition changes in the cellular GSH/Glutathione disulfide (GSSG) redox state (oxidative stress) have been implicated in cell cycle responses such as differentiation and apoptosis [8]. The redox status of the cells can be calculated by the Nernst equation for the couple GSSG/2GSH [9]. A second marker for oxidative stress is malondialdehyde (MDA) that is formed by lipid peroxidation and that was shown to accumulate in serum of chronic hepatitis patients [10]. An imbalance in the redox homeostasis results in an increased flux of reactive oxygen species (ROS) [11]. Under such pro-oxidant conditions, highly reactive radicals can damage DNA, RNA, proteins and lipids, which may lead to mutations or apoptosis [12]. Only a limited number of models are available to investigate which changes are induced in the host cell by HBV replication. Infection of primary cultures of human hepatocytes with HBV results in a low infection and replication rate. In addition, primary human hepatocytes are difficult to obtain for experimental studies. Cell lines such as HepG2.2.15 continuously produce virus particles [13]. As a consequence, there is no correct background to which the effect of virus replication can be compared. Therefore, we chose to address this matter in the HBV-inducible cell line HepAD38, which allows a direct comparison of cellular characteristics with or without HBV replication [14].

Materials and methods

Cell cultures and production of HBV by HepAD38 cells

HepAD38 cells are stably transfected with a cDNA copy of HBV wild-type pregenomic RNA [14] and in which HBV replication is under the control of a tetracycline inducible promoter. The cells are cultured as described previously [15]. In short, HepAD38 cells were seeded on 25 cm^2 flasks at a density of 5×10^6 cells in 5 ml of seeding medium (+tetracycline). After 2–3 days the cultures were induced for viral production by changing to medium without tetracycline. At various time points supernatant (5 μl) was taken to determine HBV production. A 523bp fragment from the HBV *core* gene was amplified by a HBV specific PCR and the reaction products were run on a 1% agarose gel and DNA, visualized by ethidium bromide staining and densitometric quantified [15].

Twelve hours after withdrawing of tetracycline from the medium HBV DNA can be detected. The amount of viral DNA increased gradually with time until 156 h after induction (time the cells need to be trypsinized). In control experiments HepG2 cells (HB-8065, ATCC, Rockville MD, USA) were used, culture conditions were as described for HepAD38 cells.

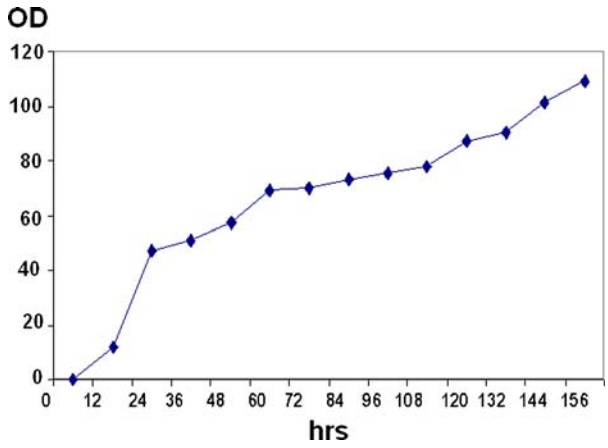

Fig. 1. Production of HBV particles by HepAD38 and release into the culture supernatant by HepAD38 cells. HBV core DNA in culture medium (samples obtained at different time point after induction) was detected by means of a HBV core specific PCR. The reaction products were separated on a 1% agarose gel and stained with ethidium bromide. Corresponding densitometric data were plotted.

Determination of GSH and of MDA

Cells were grown in culture flasks to near-confluency (90–100%) as described above. Cells were trypsinized and the cell viability and number was determined using the Trypan blue exclusion test. For GSH: cells were lysed by 3 cycles of repeated freezing in liquid nitrogen and thawing at 37 °C, the supernatant was collected after centrifugation (15 min 13.000 × g 4 °C). In the supernatant, the total glutathione content $(GSH)_{tot}$, as the sum of reduced glutathione (GSH) and oxidized glutathione (GSSG), as well as GSSG were determined according to the recycling method [16]. For MDA: pellets of 15×10^6 cells were stored at –80 °C until analysis. The determination of thiobarbituric acid reacting substances and

the subsequent separation of malondialdehyde by reversed-phase HPLC (Waters 996 HPLC photodiode array detector with Millenium software; Milford, MA, USA) were carried out according to the method described by Jentzsch *et al*. [17]. For internal standard 1,1,3,3-tetraethoxypropane was used. Results for GSH and MDA were obtained from 4 separate experiments each performed in duplicate.

Gene expression

The expression of mRNA of multiple genes of HepAD38 cells, 72 h after induction of viral replication, was analyzed by hybridization to a cDNA expression array (GEArray Q series Human stress & Toxicity PathwayFinder Gene Array (HS-012), SuperArray Bioscience Corp., Frederick, MD USA). As control, mRNA obtained from parallel cultures in which HBV replication had not been induced was used. Briefly, HepAD38 cells were grown in tissue culture flasks with medium containing tetracycline to near-confluency (90–100%). All cultures were washed twice with PBS, divided in two groups and were further incubated with fresh medium. For the induction group the medium did not contain tetracycline whereas the medium for the control group was supplement with tetracycline. Cells were harvested at 72 hr, their number determined and samples stored in Trizol (Invitrogen, Merelbeke, Belgium) for further analysis (5×10^6 cells/ml Trizol). RNA was extracted and ^{32}P-labelled cDNA was prepared using RT-labelling kit (SuperArray, Frederick, USA) according to the manufacture's instructions. cDNA's (from induced and non-induced HepAD38 cells) were hybridized overnight to the cDNA expression array followed by radioactive detection according to the manufacturer's suggestions (SuperArray). After digital recording of the image, a densitometric analysis of the signals was performed and the intensity was calculated relative to the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are the mean of 3 separate cell culture experiments and the subsequent array analysis.

Western blot analysis

HepAD38 cells cultures in which HBV replication was induced ($n = 3$) or that were not induced ($n = 3$) were carefully detached by trypsin/EDTA and cells collected in 1 mL lysis buffer (2% SDS, 50 mm Tris-HCl, 10% glycerol, pH = 6.8) containing 36 μl protease inhibitor (Complete Protease Inhibitor Cocktail Tablets, Roche). Samples were homogenized, centrifugation (10.000 × g, 15 vmin, 4 °C) and the protein concentration in the supernatant was determined by the standard Bradford method. Protein samples were loaded onto a 4–12% bis-tris NuPage gel (Invitrogen) and separated using the Xcell II module (Invitrogen) according to the manufacturer's instructions. After blotting the proteins onto a PVDF membrane (Bio-Rad Laboratories, Nazareth, Belgium) and blocking of non-specific binding the membranes were incubated with primary antibodies (Annexin V mouse U4C8, [18], HSP70 goat sc-1060, Santa Cruz Biotechnology, Inc, California, United States or HSP105 rabbit N-187, Santa Cruz Biotechnology) followed by corresponding horseradish peroxidase-conjugated secondary antibody. Immunoreactivity was visualized using chemiluminescence detection (ECL-plus, Amersham Biosciences).

Proliferation characteristics of HepAD38 cells

To study the effect of induction of HBV replication following removal of tetracycline from the medium, growth curves and calibration curves were determined for HepAD38 cells cultured in medium with or without tetracycline (MTT, Roche, Mannheim, Germany). For induced and non-induced HepAD38 cells, the growth curve over a period of 6 days and the calibration curves were determined in three separate experiments.

Karyotyping of HepAD38 cells at different passages with or without virus production

Karyotyping was performed on chromosome preparations of cultured cell lines according to standard methods. In short, cells were treated with 10 μg/ml colcemid for 120 or 240 min before trypsinization. Cells were treated hypotonically (0.75 M KCl) at 37 °C and fixed with 3:1 methanol:acetic acid. Twenty G-banded metaphases for each cell line were analyzed and karyotyped.

Results

Effect of virus induction on markers of oxidative stress in HepAD38 cells

To monitor whether cellular stress was induced in HepAD38 cells following induction of HBV replication, the formation of MDA in cells was determined in parallel cultures with or without virus replication (during the first 96 hr after tetracycline withdrawal) (Fig. 2A). For both conditions a temporary rise in the amount of MDA at 24 hr, which returned to basal levels at 96 hr, was noted.

In addition, levels of GSHtot and GSSG were determined. A temporary rise in GSHtot levels was noted during the first 24 hr. Induction of HBV replication did not influence the total level of GSH in the cells (Fig. 2B).

The redox status of the cells can be presented by the ratio: [GSSG]/[GSH]$_t$ × 100%. When this ratio was plotted for HBV producing cells and for non-producing cells, an increase was observed in both conditions. The increase was significantly higher in cells with HBV replication and showed a peak at 72 hr (Fig. 2C). When HepG2 cells were cultured under the same conditions (first in medium with tetracycline followed by medium without tetracycline) no increase in the redox status was observed. Thus, HBV replication induces oxidative stress in HepAD38 cells.

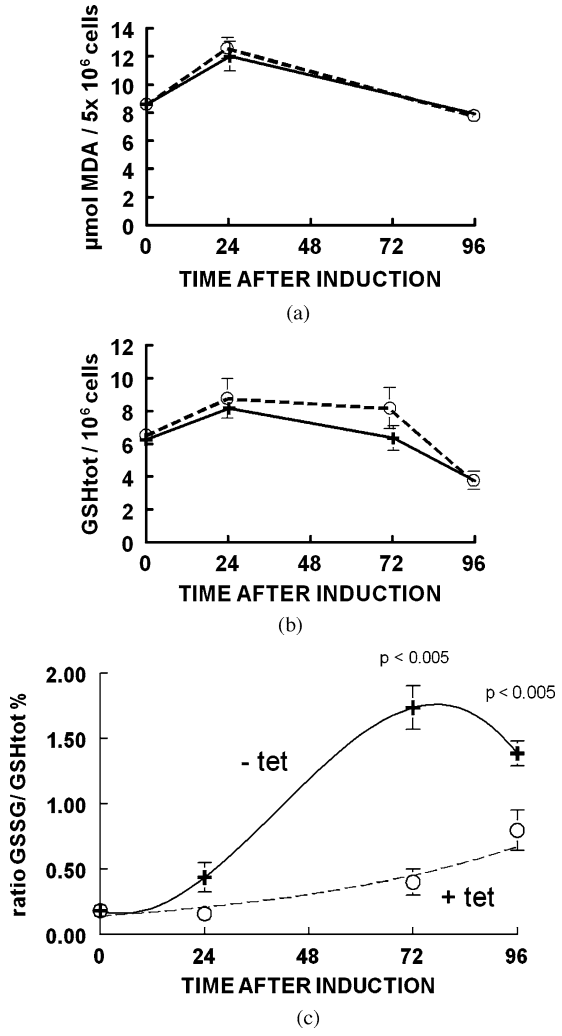

Fig. 2. Effect of induction of HBV replication on oxidative stress parameters in HepAD38 cells. Cells were cultured in monolayer to near confluency in medium with tetracycline (no HBV replication). At $t = 0$, the medium was replaced by either medium with tetracycline (+ tet, broken line) or medium without tetracycline (−tet, full line) (induction of HBV replication). At the indicated time points cells were collected. (A) Malondialdehyde (MDA) (μM/5 × 10^6 cells). (B) Total glutathione (GSH_{tot}) in the cells. (nM/10^6 cells). (C) Oxidative stress was assessed by the ratio of $(GSSG)/(GSH)_{ttot}$ × 100%.

Effect of virus induction on host cell gene expression

The mRNA expression of a selected group of stress related genes was determined by GEArray Q array 72 hrs after induction of HBV production, i.e. at the peak of oxidative stress. Signal RI (relative intensity) was calculated relative to the intensity of the GAPDH signal (I_G). The induction is represented by the ratio $RI_{HBV}/RI_{control}$. Analysis of the hybridization results revealed that 20 of the 96 genes investigated were up-regulated with a cut-off of twofold increase in 3 experiments. The differentially expressed genes identified in this study can be functionally grouped (see Table 1).

In order to confirm the results obtained by microarray we performed Western blot analysis for selected proteins using antibodies against Annexin V, HSP70 and HSP105. Densitometric quantitation of the protein bands showed a HBV expression-related increase of + 180% for HSP70, + 190% for HSP105 and + 240% for Annexin V which is in agreement with results obtained for mRNA expression (Table 1).

Effect of virus induction on cell proliferation

The proliferation rate of HepAD38 cells was determined following induction of HBV replication and was compared with proliferation of non-induced cells. Cell numbers were calculated from the corresponding MTT calibration curve (linear relationship between cell number (0 and 100.000 cells/well) and Absorbance (A_{595}–A_{655}); data not shown). No difference in increase of cell number was observed during the first 6 days (Fig. 3) for both culture conditions.

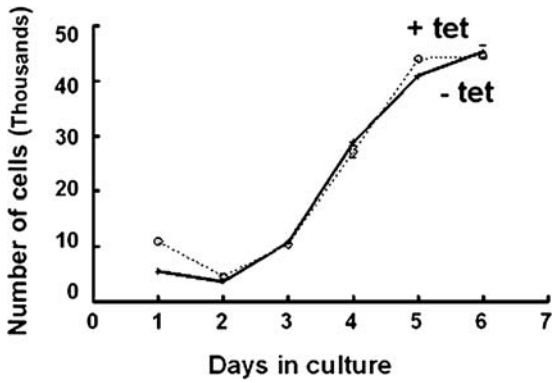

Fig. 3. Proliferation of virus producing and non-induced HepAD38 cells. The growth rate of HepAD38 cells was determined following induction of HBV replication and was compared with that of non-induced cells. Cell numbers were calculated from the corresponding MTT-calibration curve (linear relationship between 0 and 100.000 cells/well and Absorbance (A_595–A_655)). No differences in cell number were observed for both culture conditions during the first 6 days.

Table 1. The mRNA expression in HepAD38 cells 72 h after induction of HBV replication was determined for a selected group of genes using GEArray Q series Human stress & Toxicity PathwayFinder Gene Array (HS-012) and compared with the expression at 72 hrs in HepAD38 cells of the same batch cultured in the presence of tetracycline (= no HBV replication). After hybridization and recording of the signal the relative intensity (RI) was calculated to GAPDH (IG)

Function	Description	Symbol	Genebank	$RI_{HBV}/RI_{control}$
Oxidative or Metabolic stress				
	Glutathione reductase	GSR	NM_000637	3,1
	Superoxide dismutase 2, mitochondrial	SOD2	NM_000636	2,7
	Metallothionein 2A	MT2A	NM_005953	3,1
	Metallothionein 1A (functional)	MT1A	NM_005946	3,4
Heat shock				
	Heat shock 10 kDa protein 1 (chaperonin 10)	HSPE1	NM_002157	2,2
	Heat shock 27 kDa protein 1	HSPB1	NM_001540	4,3
	DnaJ (Hsp40) homolog, subfamily A, member 1	DNAJA1	NM_001539	2
	Heat shock 60 kDa protein 1 (chaperonin)	HSPD1	NM_002156	2,3
	Heat shock 70 kDa protein 1A	HSPA1A	NM_005345	5,6
	Heat shock 70 kDa protein 4	HSPA4	NM_002154	2,8
	Heat shock 70 kDa protein 8	HSPA8	NM_006597	2,3
	Heat shock 70 kDa protein 9B (mortalin-2)	HSPA9B	NM_004134	2,8
	Heat shock 90 kDa protein 1, alpha	HSPCA	NM_005348	2,9
	Heat shock 90 kDa protein 1, beta	HSPCB	NM_007355	2,1
	Heat shock 105 kDa/110kDa protein 1	HSPH1	NM_006644	5,2
Growth and apoptosis				
DNA damage and repair	RAD50 homolog (S. cerevisiae)	RAD50	NM_005732	3
	X-ray repair complementing defective repair in Chinese hamster cells 5	XRCC5	NM_021141	1,9
Apoptosis signaling	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	NM_020529	2,6
	Annexin A5	ANXA5	NM_001154	3,8
Growth arrest and senescence	Growth arrest and DNA-damage-inducible, alpha	GADD45A	NM_001924	3,6

The induction is represented by the ratio RI HBV/RI control. Analysis of the hybridization results revealed that 20 of the 96 genes investigated were significantly more than twofold upregulated 72 hr after induction of HBV replication. The differentially expressed genes identified in this study can be functionally grouped in 3 classes: (A) Heat shock proteins, (B) Oxidative and metabolic stress and (C) Genes related to growth and apoptosis. Results are the mean of 3 separate cell culture experiments and subsequent array analysis.

Effects of continuous HBV replication on the karyotype of the cells

To investigate whether continuous virus replication in HepAD38 cells may result in an accumulation of mutations, karyotype analysis was performed. To this end HepAD38 cells were grown in the absence (and parallel cultures in the presence) of tetracycline for 18 consecutive passages (4 months). Chromosome analysis of the HepAD38 cell line at zero passages revealed that the main karyotype of the cell line was 50,XY, −1, +2, +20, −21, del(1)(p2), del(1)(q21), +3mar. Changes in the karyotype at passage 6 were a loss of chromosome 6 and an extra chromosome 8 and 16 in all cell lines and a *t*(5;6) in 30% of the cells. Other changes appeared sporadically and were observed in only a single cell. Surprisingly, at 18 passages the main karyotype was the same as the karyotype at 0 passages. In 25% of the cells a 12p- chromosome was observed. Other sporadic changes were observed in single cells. In cells that had been cultured without tetracycline and thus in which HBV replicated, similar changes were observed as in not-induced HepAD38 cells.

Discussion

In the present study we investigated what happens in HepAD38 cells after induction of HBV replication. Different from other *in vitro* models, the HepAD38 cells allow us to monitor the effects on the host cell solely triggered by HBV replication. Metabolic and expressional characteristics can be compared directly in these cells whereas in other cell models

one has to take into account differences in age, genetic origin and/or culture history of the cells [19, 20].

A number of studies have linked hepatitis virus proteins or viral hepatitis to the development of oxidative stress [21, 22]. Elevation of reactive oxygen species (ROS) above the buffering capacity of the cell results in a shift of the cells from active proliferation to a quiescent state. In addition damage to the DNA was noted which might represent one of the triggering factors initiating mutations [23]. Two prominent markers to monitor redox status or oxidative stress are Malondialdehyde (MDA) and cellular Glutathione/Glutathione disulfide (GSH/GSSG). ROS generated as a result of HBV replication did not significantly affect lipid peroxidation as reflected by the level of MDA (Fig. 2A). However, induction of HBV replication resulted in oxidative stress as reflected by a more than 3 fold increase in the ratio GSSG/GSHtot at 72 hours post induction (Fig. 2C).

Using the microarray technique specifically focussed on stress-related genes we could indeed identify a number of genes linked to cellular stress that were up-regulated at 72 h post induction in HBV producing cells: A) Heat shock proteins, B) Oxidative and metabolic stress genes and C) Genes related to growth and apoptosis (Table 1).

Heat shock proteins are known to be up-regulated under stress conditions and during virus infection [24]. They function mainly as molecular chaperones facilitating protein folding and are linked to HBV replication [25]. In addition, elevated expression of HSP's is significantly correlated with disease progression in HBV infection [24]. The array observations are in agreement with these studies and are further validated by our demonstration of parallel changes in protein levels of selected HSP's, by Western blot analysis.

We find that induction of HBV replication stimulates oxidative stress (Fig. 2C). We also found that a number of enzymes (oxidative stress related genes: GSR, SOD, MT1 and MT2) are up-regulated by HBV replication. From this combination we can conclude that HBV replication in itself leads to oxidative stress, which is rapidly (within 72–96 hr) counteracted by the up-regulation of these oxidative stress-related genes and not through a decline of virus production (Fig. 1).

Oxidative stress on its one is a condition that will lead to an increase of mutations in the cells. This condition would correspond to the long-term culturing of HepAD38 cells in the absence of tetracycline. However, we could not demonstrate an increased mutation rate over a period of 4 months. On the contrary, the karyotypic analysis of HBV expressing cells and non-induced control cells showed that both cultures acquire limited and similar chromosomal changes upon continuous culture. Changes from the karyotype present at the start of the experiment in cells expressing HBV and in non-expressing cells are most likely the result of random genetic drift. This absence of a higher number of mutations supports the finding of activation of several compensatory mechanisms that could counteract the effects of (oxidative) stress. We can further speculate that also in the natural situation the stimulation of SOD, GSR and MT-gene expression by HBV can, to some extent, be protective for the liver cell.

Finally, we also demonstrate significant changes in mRNA expression of related to growth and apoptosis. (Table 1) And, although over a period of 6 days post induction, HepAD38 cells produce HBV (Fig. 1) and were (temporarily) under oxidative stress (Fig. 2C), no difference in cell proliferation was observed for induced and non-induced cells during the same period (Fig. 3). The absence of a difference in proliferation rate is another argument against lasting differences in redox status that are known to be able to elicit a shift in the rate of cell growth [8].

In conclusion, in short-term experiments with the HepAD38 model we have shown that HBV replication causes increased oxidative stress and the up-regulation of stress-related mRNA and proteins in the host cell. These processes could be involved in the development of malignant transformation of the host cell. The HepAD38 model however, does not seem useful to investigate long-term effects of HBV replication.

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