Modulation of MAPK pathways and cell cycle by replicating hepatitis B virus: Factors contributing to hepatocarcinogenesis

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Background/Aims: Chronic infection with the hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma but the mechanism by which this occurs is unknown. Numerous studies have focused on the HBV X protein showing that it activates signal transduction pathways while few have investigated these changes in HBV-replicating hepatocytes.

Methods: We utilized the recombinant adenovirus system to deliver a replication competent HBV genome into Huh7 and primary marmoset hepatocytes (PMH) to examine the effects of active viral replication on the regulation of Ras-ERK signal transduction and related pathways.

Results: Huh7 cells and PMHs replicating HBV demonstrated significant upregulation in phosphorylated ERK, Akt, c-myc together with increased p53, cyclin B1 and p21cip1 expression and cell cycle progression to G2 phase in the absence of increased cell proliferation. Phosphorylation of the key cell survival kinase, Akt, was significantly increased, resulting in increased serine phosphorylation of the downstream target, GSK3-β.

Conclusions: These results demonstrated simultaneous activation of the MAP Kinase and Akt pathways in HBV-replicating hepatocytes that resulted in dysregulation in the control of cell cycle progression and which help explain the early pathogenic mechanisms that underlie malignant transformation associated with chronic hepatitis B infection.

Keywords: Hepatitis; Carcinoma; Cell signaling

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Abbreviations: HBV, hepatitis B virus; MAPK, mitogen activated protein kinases; ERK, extracellular signal-regulated kinases; SAPK/JNK, stress-activated protein kinases/NH2-terminal-Jun kinases.

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1. Introduction

The mechanism by which HBV results in HCC development is not well understood and the risk of developing HCC remains significantly elevated despite suppression of viral replication by antiviral therapy and after clearance of serum HBcAg and HBV viremia [1–3].

The HBx protein of HBV has been proposed as an important oncogenic stimulus for the development of HCC in chronically infected individuals [4–7]. However, several studies have reported divergent effects of HBx on p53-, Fas- and TNF-α [8–10], c-myc-, and Ras-mediated apoptosis [11–13] and transactivation of IL-8 [14–16]. Our understanding of how HBV causes HCC remains a challenge and the frequent discrepancies in experimental findings may reflect limitations in duplicating cellular changes that occur in natural infection. The HBV large (LHB) and middle (MHB) envelope proteins have also been shown to contribute to hepatocarcinogenesis of HBV [17,18], further emphasizing the role of viral factors, other than HBx, and active HBV replication as mediators of HCC development.

To address the role of active HBV replication on hepatocarcinogenesis we used a recombinant adenovirus system to deliver a replication competent HBV genome rather than single viral genes into primary marmoset hepatocytes, and contrasted the changes to cell signaling pathways induced by HBV infection with those seen in the human hepatoma cell line, Huh7.

2. Materials and methods

2.1. Plasmids

A 1.5x full length replication competent HBV (genotype A, subtype adw2) [17] was subcloned into pAdTrack (provided by B Vogelstein, Howard Hughes Medical Centre, Baltimore) which was pre-digested with HindIII and EcoRV (Promega). The plasmid pAdTrack-HBwt was digested with Pmel and transformed into AdEasier-1 cells by electroporation (Bio-Rad Gene Pulser). Clones (AdEasy-HBV) were transformed into Top 10F cells (Invitrogen) and confirmed by DNA sequencing. An AdEasy-GFP control expressing green fluorescent protein (GFP) only was also produced.

2.2. Antibodies

Primary antibodies used were total p44/p42 MAP Kinase, phospho-p44/p42 MAP Kinase (Thr202/Tyr204), total Akt, phospho-Akt (Thr308), total c-myc, phospho-c-myc (Thr58/Ser62), phospho-GSK3-β (Ser9), cyclin D1, cyclin A, cyclin B1, p21-Waf1/Cip1, cdc2, pan-actin (all by Cell Signaling Technology) and p53 antibody (DKZF, Heidelberg, Germany).

2.3. Cell lines

Huh7 and 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS) and streptomycin 50 μg/ml at 37 °C in 5% CO2.

Primary marmoset hepatocyte (PMH) cultures were prepared from the common marmoset, Callithrix jacchus, according to a previously described method [19].

2.4. Production of recombinant adenoviruses

The rAdHBV virions were produced by transfection of pAdEasy-HBVwt into semi-confluent 293T cells and amplified by serialpassaging in culture. For the initial production of rAdHBV virions, 293T cells were transfected with Pur-1-linearized pAdEasy-HBVwt DNA using Fugene-6 Transfection Reagent (Roche) according to manufacturer’s instruction and transfections monitored by GFP expression. Cells were collected at day 5 post-transfection, centrifuged at 1500g for 5 min, resuspended in 2 ml of fresh media and lysed by freeze-thawing. Lysates were centrifuged at 3000g for 15 min and supernatants containing rAdHBV virions were filtered with a 0.22 μm filter (Millipore) and stored in 500 μl aliquots at −70 °C. High titre virus stocks were prepared by serial passaging in 293T cells. The titre of the final virus stock was determined by infecting Huh7 cells and analyzing for GFP expression by flow cytometry 48 h post infection (PI) (see below). Titres were expressed as GFP expressing units (GEU) and an MOI of 1 was defined as the viral dilution that resulted in >99% GEUs. A recombinant adenovirus expressing GFP alone, rAdGFP, was produced in the same manner to serve as a control virus.

For infections with rAdHBV or rAdGFP virions, Huh7 cells grown in 6-well tissue culture plates (Nunc) and at 70% confluency were infected with rAdHBV or rAdGFP at a multiplicity of infection (MOI) of 1.0. For PMH cells, infections were performed with rAdHBV or rAdGFP at an MOI of 1.0 on confluent cells (day 3 post-plating). Mock-infected controls were treated with PBS alone.

2.5. Detection of HBV DNA

Cells and culture media supernatants were collected 24 or 72 h PI for PMH and Huh7 cells, respectively, for analyses of HBV DNA replicative intermediates as described previously [20]. Total cell lysates were collected 24 or 96 h PI for PMH and Huh7 cells, respectively, for analyses of HBV cccDNA using real time PCR as described by Bowden et al. [21]. The β-Globin gene was used as an internal control standard and levels of HBV cccDNA were expressed as copies of cccDNA/genome equivalent (cp/GEq).

2.6. Analysis by Western immunoblotting

Huh7 cells were harvested 72 h PI while PMHs were harvested 24 h PI using 200 μl of ice-cold protein cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM sodium EDTA, 1 mM EGTA, 1% Triton 2.5 mM sodium pyrophosphate, 1 mM β-glycero phosphate, 1 mM sodium vanadate, 1 mM sodium molybdate, 5 mM sodium fluoride) supplemented with complete protease inhibitor cocktail (Roche). To assess the effect of PDGF stimulation, cell monolayer was incubated with 10 ng/ml of PDGF for 30 min prior to harvest. Lysates were clarified by centrifugation and the supernatants, representing cytoplasmic and membrane fractions, were collected. The pellet, representing the nuclear fraction, was resuspended in protein lysis buffer containing 0.5% SDS. Total cellular fractions were prepared by adding SDS (final concentration 0.5%) to 100 μl of non-clarified cell lysates. Thirty micrograms of protein from the total cell and cytoplasmic fractions and total amount of the nuclear fractions were dissolved in Laemmli buffer (50 mM Tris (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 8% glycerol, 0.01% bromophenol blue), resolved by a denaturing 12% SDS-PAGE and transferred to Hybond-C Extra membrane (Amersham Biosciences). Membranes were blocked with 5% skim milk milk powder in TBST (20 mM Tris, pH 7.4, 50 mM sodium chloride, 0.5% Tween 20) at 4 °C overnight, followed by incubation with primary antibody in TBST-5% skim milk powder at 4 °C overnight. Membranes were washed with TBST followed by incubation with secondary antibody (1:1000 dilution of anti-mouse or anti-rabbit) (DakoCytomation) in TBST-5% skim milk milk powder at room temperature for 1 h, washed, followed by ECL (Amersham Biosciences).
Experiments were performed in triplicate, unless otherwise stated. Immunoblots were analyzed with a Bio-Rad GS710 Scanner with QUANTITY ONE 4.1.0 software package (Bio-Rad), and Prism 4.0 (GraphPad Software).

2.7. Flow cytometry for GFP expression, cell cycle analysis

Triplicate sets of PMH and Huh7 cells were infected with serial dilutions of rAdHBV or rAdGFP, collected at 24 and 48 h, respectively, washed twice with PBS and fixed in 1.0 ml of BD Cytofix Buffer (BD Pharmingen). Expression of green fluorescence was measured by flow cytometry using a FACS Calibur (Becton Dickinson).

For cell cycle analysis, triplicate sets of Huh7 cells were infected with rAdHBV or rAdGFP, collected at 24, 36 and 72 h PI, washed twice with PBS and fixed with 70% ethanol in PBS at −20 °C overnight. Cells were pelleted by centrifugation at 1500 g, resuspended in 0.1% BSA in PBS, incubated with 20 μg/ml RNAseA (Roche), at 37 °C for 30 min, then stained with 5 μg/ml propidium iodide (Sigma–Aldrich) for 30 min in the dark. Flow cytometry was performed with the FACS Calibur (Becton Dickinson), and cell cycle phases analyzed with FlowJo Version 4.6.2. Apoptosis was determined by scoring Sub-G1 cells displaying pyknotic nuclei using flow cytometry.

Fig. 1. Infection of Huh7 and PMH cells with rAdHBV virions results in active viral replication. Huh7 cells and PMH were infected with rAdHBV virions. GFP expression was determined by fluorescence microscopy (a and e) and flow cytometry (b and f). Autoradiographs of intracellular and secreted HBV replicative DNA intermediates, relaxed circular (RC), double stranded linear (L) and single stranded (SS) at day 3 PI for Huh7 cells and day 1 PI for PMH, respectively (c and g). Serum starvation of rAdHBV-infected cells resulted in inhibition of HBV replication (d).
2.8. Cell proliferation assay

Cell proliferation was determined by colorimetric assay [22]. Briefly, Huh7 cells were seeded in 24-well plates (10^4 cells/well), infected the following day with rAdHBV, rAdGFP or mock-infected and harvested every 12 h for 96 h. Cells were washed with PBS, fixed in methanol for 15 min before air drying and staining with 0.1% crystal violet (Sigma) for 5 min at room temperature. Plates were washed with distilled water and air dried. Bound dye was solubilized in 2% sodium deoxycholate (Sigma) for 30 min at 37 °C. Supernatants were collected and absorbance measured at 620 nm using the SmartSpec 3000 TM spectrophotometer (Bio-Rad).
3. Results

3.1. HBV replication in Huh7 cells and PMH

Although Huh7 cells were known to be permissive for HBV replication this was not known for PMH. Infection of both Huh7 cells and PMH with rAdHBV resulted in expression of GFP in >95% of cells (Fig. 1a, b, e and f). Intra- and extra-cellular HBV DNA replication intermediates (relaxed circular, double stranded linear and single stranded) were readily detectable 72 h PI for Huh7, increasing thereafter until day 6 (Fig. 1c), and 24 h PI for PMH cells (Fig. 1g). HBV cccDNA was detected by quantitative real time PCR in both Huh7 cells and PMH. The median (±SD) level of HBV cccDNA was higher in PMH (2459 ± 549 cp/GEq) compared to Huh7 cells (1962 ± 748 cp/GEq). Because FCS is a growth factor and may stimulate

Fig. 3. HBV infection increases phosphorylated Akt. Huh7 cells and PMH were mock (PBS) infected or infected with rAdGFP and rAdHBV. Thirty micrograms of total cellular fractions was analyzed for phospho-Akt (pAkt). (a) pAkt levels at days 1 and 3 PI in rAdHBV-infected Huh7. (b) pAkt levels at day 1 PI in rAdGFP- and rAdHBV-infected PMH. (c) pAkt levels were increased in rAdHBV-infected Huh7 cells, 206% (±SD 22) (P < 0.05) and PMH, 260% (±SD 120) (P < 0.01), respectively. Total Akt levels remained unchanged. Levels were expressed as % relative to mock controls. Experiments were performed in triplicate.

Fig. 4. HBV infection increases inactive GSK3-ß. Huh7 cells and PMH were mock (PBS) infected or infected with rAdGFP and rAdHBV. Thirty micrograms of total cellular fractions was analyzed for phospho-Ser-9-GSK3-ß (a and b) and levels expressed as a percentage relative to mock controls (c). Levels were increased by 67% (±SD 21) (P < 0.05) and 255% (±SD 63) P < 0.05, for rAdHBV-infected Huh7 cells and PMH, respectively. (d) PDGF stimulation of rAdHBV-infected Huh7 cells indicating decreased Ser-9-GSK3-ß phosphorylation compared to PDGF-stimulated mock controls. Experiments were performed in triplicate except for PDGF experiments, which were performed in duplicate.
signaling pathways, at 48 h PI, rAdHBV infected Huh7 cells were deprived of serum to assess the effect of serum starvation on viral replication. Deprivation of FCS for 24–48 h was associated with a significant decline in HBV DNA (Fig. 1d) and therefore all subsequent experiments were performed in the presence of 10% FCS and comparisons made between rAdHBV-infected cells and rAdGFP and mock-infected cells.

3.2. HBV stimulates the MAPK signaling pathway

In order to examine the effects of HBV on the Ras-ERK pathway cell lysates of mock (PBS), rAdGFP and rAdHBV-infected Huh7s were examined for expression of total and phospho-ERK1/2. Infection of Huh7 cells with rAdHBV resulted in a 205% (±SD 13) upregulation of ERK1/2 phosphorylation (pERK) compared to mock controls (P < 0.01) while the levels of total ERK remained unchanged (Fig. 2a and d). The upregulation of pERK was sustained over a 3-day period (154% ± SD 46; P = 0.0023) (Fig. 2c). To determine that the observed effects were not simply a consequence of HBV replication in malignantly transformed Huh7 cells we repeated the experiments in PMH. Infection of PMH with rAdHBV resulted in a 402% (±SD 71) increase in the level of pERK compared to mock controls (P < 0.01) (Fig. 2b and d). Infection with rAdGFP did not result in increased expression of total or pERK.

rAdHBV-infected Huh7 cells were also more sensitive to PDGF stimulation which resulted in a 119% (±SD 57) increase in pERK compared to mock controls (P < 0.01) (Fig. 2e). Phosphorylated p38 MAPK was not significantly altered in rAdHBV-infected Huh7 (14% ±SD 22) and PMHs (−7% ±SD 14) (P = ns) (Fig. 2f and g).
3.3. HBV stimulates the Akt pathway

Upregulation of Ras-ERK also feeds into the PI3K effector pathway. We therefore investigated the effect of HBV replication on PI3K-Akt signaling. Infection of Huh7 cells with rAdHBV resulted in a 206% (±SD 22) increase in the level of phospho-Akt (pAkt) compared to mock controls ($P < 0.05$) (Fig. 3a and c). There was no difference in pAkt expression in rAdGFP-infected cells compared to mock controls (results not shown). Similarly, infection of PMH with rAdHBV resulted in significant increase in pAkt levels of 260% (±SD 120) ($P < 0.01$) (Fig. 3b and c). In order to determine that the upregulation of pAkt had a biologically functional role, we investigated the effect of pAkt on the phosphorylation of GSK3-β, a downstream substrate of Akt. In both Huh7 cells and PMH replicating HBV the phospho-Ser9-GSK3-β increased by 67% (±SD 21) ($P < 0.05$) (Fig. 4a and c) and 255% (±SD 63) ($P < 0.05$) (Fig. 4b and c), respectively, compared to mock controls. Furthermore, stimulation with the growth factor, PDGF, a known activator of GSK3-β, resulted in an increase in pGSK3-β levels in mock-controls but not in rAdHBV-infected Huh7 cells (Fig. 4d).

We also investigated the effect of HBV induced upregulation of Akt on p53 and found that p53 levels were increased in rAdHBV-infected Huh7 cells (43% ±SD 21) and PMH (63% ±SD 7) ($P = 0.0038$) compared to mock controls (Fig. 5a and b). Total intracellular levels of p21 were also increased both in rAdHBV-infected Huh7 cells, 42% (±SD 20) ($P = 0.02$) and PMHs, 83% (±SD 30) ($P = 0.014$) (Fig. 5c–e).

3.4. HBV modulates c-myc

Induction of c-myc protein phosphorylation is Ras-dependent [23], and can be rapidly induced in response to mitogenic stimulation [24]. In rAdHBV-infected Huh7 cells and PMH, we found increased expression of phospho-c-myc (p-c-myc), 63% (±SD 4) ($P = 0.05$) and 125% (±SD 35) ($P = 0.004$), respectively, compared to mock controls (Fig. 6). There were no differences in total c-myc expression. Experiments were performed in triplicate.

3.5. HBV stimulates cyclins A and B

In order to investigate the effects of HBV replication on the cell cycle, intracellular levels of cyclins D1, A and B1 were determined in rAdHBV-infected Huh7 cells and PMH compared to those of rAdGFP-infected and mock controls. In the total cell lysates of rAdHBV-infected Huh7 cells, cyclin D1, A and B1 levels were increased, 28% (±SD 18) ($P = 0.05$), 53% (±SD 30) ($P = 0.05$), and 110% (±SD 26) ($P = 0.013$), respectively (Fig. 7a–c). In rAdHBV-infected PMHs, neither cyclin D1 nor cyclin A was significantly increased compared to mock controls (Fig. 7d). However, intranuclear cyclin B1 was strongly upregulated in both rAdHBV-infected Huh7 as well as PMH, 105% (±SD 25) ($P = 0.05$) and 419% (±SD 138) ($P = 0.0015$), respectively (Fig. 7b, c, e and f), while cytoplasmic cyclin B1 levels were reduced...
by 29% (±SD 6) \( (P = 0.0015) \) in PMH (Fig. 7e) and Huh7 cells (data not shown) compared to mock controls (Fig. 7e and f). In addition to the increase in cyclin B1 we also observed an increase in cdc2 levels in rAdHBV-infected (69% ±SD 5) compared to mock-infected cells \( (P < 0.0001) \) (Fig. 7b). There were no significant
differences in cyclin D1, A or B1 observed in rAdGFP-infected cells compared to mock controls.

### 3.6. HBV stimulates cell cycle progression to G2 phase

The effect of active viral replication on total cellular DNA synthesis in rAdHBV-, rAdGFP- and PBS (mock)-infected Huh7 cells was determined at days 1, 2 and 3 PI (Fig. 8). The proportion of rAdHBV-infected cells in G1 and S phases over days 1–3 was not significantly different from mock or rAdGFP-infected controls. However, the proportion of cells in G2 phase at day 3 was significantly increased in rAdHBV-infected Huh7 cells, 32% (±SD 1.0) compared to mock controls, (8% ±SD 5.0, P < 0.03) and rAdGFP infected cells, (15% ±SD 1.0) (Fig. 8c), indicating that HBV replication stimulated initiation of cell DNA synthesis. However, despite this cellular proliferation was not significantly increased (Fig. 9) and the sub-G1 cell population indicative of pyknotic nuclei characteristic of

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**Fig. 8.** HBV infected Huh7 cells have increased progression to G2 phase. Flow cytometry histograms of DNA content of mock (PBS)-, rAdGFP- and rAdHBV-infected Huh7 cells. Total DNA content from triplicate experiments was determined by propidium iodide staining at days 1 (a), 2 (b) and 3 (c) PI, and the % of cells in G1, S and G2 phases of the cell cycle was determined using the FlowJo software program. Experiments were performed in triplicate and data presented include means and SD.

**Fig. 9.** Cell proliferation is not increased in rAdHBV-infected cells. Huh7 cells were infected with either rAdGFP or rAdHBV and cellular proliferation determined over a 90 h time course by colorimetric assay. Proliferation of AdHBV-infected cells was not significantly different to rAdGFP or mock-infected cells.
apoptosis was reduced (Fig. 10) in HBV infected cells compared to rAdGFP and mock infected cells. Together with the increase in the levels of p21cip1 (Fig. 5b), cdc2 (Fig. 7b) and intranuclear cyclin B1 (Fig. 7b and e) these findings are compatible with G2 arrest in rAdHBV-infected cells.

4. Discussion

Dysregulation of the signal transduction pathways such as Ras-MAPK signaling, IRS1/IGF pathways, NFκB, cell cycle, Wnt/β-catenin and apoptotic pathways have all been implicated in the development of HBV-associated carcinogenesis [7,25–30]. However, it is difficult to derive a unifying theme to explain the precise mechanism(s) linking the initiating oncogenic stimulus with subsequent changes in signal transduction. We sought to examine how HBV infection, rather than the expression of a single viral protein, affected the cell cycle and also influenced related pathways in an integrated manner to trigger a succession of cellular events that might promote oncogenesis. To achieve this we utilized the recombinant adenovirus system used to deliver the HBV genome as infection with rAdenoGFP did not significantly alter any of the above cellular parameters, consistent with the findings of others who have utilized recombinant adeno-viral delivery systems to study the effects of viral proteins on cell signaling events [22,31,32] and cell cycle regulatory proteins including p21cip1, p27kip1, cyclins or the activity of cyclin dependent kinases [33]. Although Huh7 cells are a transformed cell line the pattern of changes in MAPK and Akt signaling and cell cycle regulatory proteins was remarkably similar to that we observed in HBV infected PMH, suggesting that Huh7 cells are a suitable cell line for further studies of the mechanisms of HBV associated hepatocarcinogenesis and apoptosis.

Previous studies, mainly utilizing the isolated expression of HBx protein in cell culture systems and the HBx gene in transgenic mice, have been invaluable in determining which signaling pathways may be involved in the multi-step carcinogenic process. However, numerous discrepancies have been reported in these studies and the use of neoplastic cell lines to study oncogenic mechanisms also seems counter-intuitive. We were able to overcome this by using primary primate-derived hepatocytes.

Akt is a serine/threonine kinase whose principal function is to facilitate growth-mediated signal transduction pathways involved in cell cycle and is involved in extensive cross-talk between several signaling pathways [34] (Fig. 11). The PI(3)K/Akt signaling pathway may in fact have a central role in driving hepatocyte transformation [35]. We found, in both Huh7 cells and PMH infected with HBV, that phosphorylated Akt levels were significantly increased. Also, of the numerous targets that Akt acts upon, we examined the effects on GSK3-β phosphorylation and p21cip1. Consistent with the upregulation of pAkt and pERK, HBV-infected cells demonstrated a significant increase in p-Ser9-GSK3-β (the inactive form of GSK3-β).

GSK3-β is constitutively active in resting cells and is a key enzyme responsible for the regulation and turnover of cyclin D1 [24,36]. In HBV-infected cells, therefore, the Akt induced inhibition of GSK3-β would be expected to increase cyclin D1 levels. Furthermore, the presence of increased levels of inactive GSK3-β (i.e., p-Ser9-GSK3-β) would also be expected to prevent the degradation of c-myc [23,24]. The increased p-c-myc levels in HBV-replicating cells may reflect not only Ras-ERK-mediated stimulation but also decreased degradation resulting from the inactivation of GSK3-β (Fig. 11).

In both HBV infected Huh7 cells and PMH we found elevations in both p53 and its downstream substrate p21cip1 levels. p21cip1 has an important role in mediating cell cycle arrest at the G1/S and G2/M boundaries of cell cycle by both p53 dependent [37,38] and independent [33,39] mechanisms. It is absent in cells during S phase...
phase [38] but in G2 phase the intranuclear accumulation of cyclins A and B1 is accompanied by the de-novo synthesis and accumulation of p21cip1[39]. The accumulation of p21cip1 in G2 is ERK dependent and serves to delay cell cycle progression to mitosis in order to protect cells against DNA damaging agents and apoptosis [33,39,40]. The increased p53, p21cip1, cdc2 levels and intranuclear accumulation of cyclin B1 observed in Huh7 cells are all consistent with G2 arrest. This was further supported by the cell cycle analysis of infected Huh7 cells which showed that cells infected with HBV did not progress beyond G2 phase (Fig. 9). In contrast to Huh7 cells PMH have a limited capacity to divide. Consequently, comparative cell cycle analyses with HBV infected PMH as G2 arrest in these primary hepatocytes may have represented little more than the inability of these cells to readily enter mitosis rather than an effect of HBV infection. Nevertheless, like HBV infected Huh7 cells infected PMH revealed changes consistent with G2 arrest, namely increased p53, p21cip1, cdc2 levels and intranuclear cyclin B1. The ability of HBV to produce G2 arrest may provide the virus with a survival advantage since viral replication is increased in both G1 and G2 phases of cell cycle and reduced during S phase [41]. Interestingly, HBx protein has also been shown to increase the levels of p21cip1 in hepatocytes and thereby reduce cell cycle progression [42]. However, the role of HBx on p21cip1 expression in the context of HBV replication is not clear. We have therefore generated a recombinant adenovirus encompassing an infectious clone of HBV that harbors an X-gene deletion (data not shown) in order to clarify the role of HBx on ERK and Akt signaling pathways and cell cycle progression.

Although dysregulated cell signaling events are a feature of HBV-infected cells and provide a catalyst for malignant transformation, the precise mechanisms are likely to be far more complex than our findings alone.

Fig. 11. Simplified schematic representation of the effect of HBV replication on the Ras and PI3K/Akt pathways. The diagram shows how integration of multiple cellular networks might contribute to cell growth and survival. Arrowheads in black (→) indicate either pathway activation or increase/decreased protein expression. The symbol (↑↓) indicates pathway inhibition. [This figure appears in colour on the web.]
would suggest. Defining the role of HBV replication in hepatocarcinogenesis will be essential for the development of effective therapeutic strategies.

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