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Dysregulation of hepatocyte cell cycle and cell viability by hepatitis B virus

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ABSTRACT

Background/aims: Dysregulation of the cell cycle is frequently associated with tumor development. Hepatitis B virus (HBV) is associated with a significant risk of developing hepatocellular carcinoma but the effects of HBV on cell cycle regulation are not completely understood.
Methods: We have used a recombinant adeno-HBV model system to investigate the effect of infection with HBV and the replication defective lamivudine resistant mutant rtM204I mutant on hepatocyte cell cycle and cell viability.
Results: Huh7 cells synchronised at the G1/S phase of the cell cycle were arrested at the G2/M following infection with rAdHBV-wt and rAdHBV-M204I. This was accompanied by increased levels of $p21^{cip1}$, p-cdc2, cyclins D, A and B. Cell viability was reduced and cleaved caspase 3 levels were increased in HBV- and rtM204I-infected cells. rAdHBV-M204I-infected Huh7 cells also demonstrated significant up-regulation of phospho-ERK, phospho-Akt, p53 and phospho-Mdm2 compared to mock-infected cells. These changes were comparable to those following infection of Huh7 cells with rAdHBV-wt.
Conclusion: Our results suggest that HBV, regardless of phenotype, produces cell cycle arrest and reduced hepatocyte viability. Perturbations in these cellular processes are likely to underlie HBV-associated liver oncogenic transformation and may help explain the ongoing risk of developing hepatocellular carcinoma in individuals in whom the lamivudine resistant rtM204I mutant emerges.
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1. Introduction

Hepatitis B virus remains the most common cause of hepatocellular carcinoma globally and while treatment with antiviral agents is available the benefit of these agents in reducing the overall risk of developing HCC is uncertain. Lamivudine (LMV) has been widely used for the treatment of chronic hepatitis B infection but it has been associated with the emergence of drug resistant variants amino acid substitutions from methionine to isoleucine or valine in codon 204 of the polymerase protein (rtM204I/V), often in conjunction with a compensatory rtL180M or rtV173L substitution upstream of the YMDD motif of the viral polymerase (Delaney et al., 2003; Hoofnagle et al., 2007). The rtM204I/V HBV mutant has been associated with the development of fulminant hepatitis (Liaw et al., 1999; Wang et al., 2002), hepatic flares (Hadziyannis et al., 2000; Liaw et al., 1999), a reduced likelihood of ALT normalisation (Lampertico et al., 2005), worsening liver histology (Kim et al., 2001), and a decrease in the rate of hepatitis B e antigen (HBeAg) seroconversion (Leung et al., 2001) even though this virus displays a defective replication phenotype *in vitro* (Melegari et al., 1998). Although more potent agents are now available the widespread use of LMV has meant that LMV resistant HBV mutants are not infrequent in treatment-experienced populations.

Although there is compelling evidence that clinical and histological disease progression is related to the level of virus replication (Chen et al., 2006; Iloeje et al., 2006; Mommeja-Marin et al., 2003), it is not known if the rtM204I mutant is as likely to produce oncogenic transformation in the liver as wild-type virus. However, patients with cirrhosis and in whom lamivudine resistant HBV has

Abbreviations: HBV, hepatitis B virus; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases.

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emerged have a substantial risk of developing HCC regardless of level of HBV viremia (Andreone et al., 2004; Di Marco et al., 2004, 2005).

We have previously shown that replication of wild-type HBV in hepatocyte is associated with dysregulation of cellular signalling pathways associated with cell survival and cell cycle progression (Chin et al., 2007). We have also shown that suppression of HBV replication *in vitro* by lamivudine failed to reverse these intracellular changes within the hepatocyte (Chin et al., 2007). The oncogenic potential of HBV could be attributed to a 'hit and run phenomenon' where the malignant phenotype may persist following initial infection and oncogenic transformation may still occur despite the absence of continued viral replication (Bauer et al., 1992). This may establish a background cellular oncogenic lesion onto which the subsequent development of liver inflammation further promotes tumor development (Karin and Greten, 2005). The mechanism(s) by which lamivudine resistant HBV produces oncogenic transformation has not previously been investigated. In this work we sought to further elucidate the effect of HBV infection, rather than overexpression of single viral proteins, on cell cycle progression and as proof of principle whether infection with a single replication defective HBV mutant like rtM204I causes dysregulation in cell cycle regulation and associated regulatory pathways in a manner that is similar to wild-type HBV.

2. Methods

2.1. Plasmids and generation of recombinant adenoviruses

The recombinant adeno-HBV virus (rAdHBV) was produced using the AdEasy system as described previously (Chin et al., 2007). A control plasmid, pAdTrack alone, was also transformed into AdEasier-1 cells to generate AdEasy-GFP which was used to produce a recombinant adenovirus expressing green fluorescent protein only (rAdGFP). The M204I mutant was generated by site-directed mutagenesis (Stratagene, La Jolla, CA) using the plasmid phrGFPHBV1.5 and the primers 5′GGC TTT CAG CTA TAT CGA TGA TGT GGT ATT GGG GG 3′ and 3′CCG AAA GTC GAT ATA GCT ACT ACA CCA TAA CCC CC 5′ (GeneWorks, Adelaide, SA, Australia). The sequence of the mutant was verified by bi-directional automated DNA sequencing using BigDye Terminator Cycle Sequencing (PE Applied Biosystems, Foster City, CA). The AdEasy-HBVrtM204I mutant was then generated as described above.

The rAdHBV-wild type (wt), rAdHBV-M204I mutant and rAdGFP virions were produced by transfection of pAdEasy-HBV-wt, pAdEasy-HBV-M204I mutant or pAdEasy-GFP into 293T cells and amplified by serial passaging in culture as previously described (Chin et al., 2007). The virus titres were expressed as GFP expressing units (GEU) in recombinant adenovirus-infected Huh7 cells (Chin et al., 2007). To determine GFP expression of rAdHBV-wt, rAdHBV-M204I or rAdGFP-infected Huh7 cells, triplicate sets of cells were infected with serial dilutions of virus, collected at 48 h, washed twice with PBS and fixed in 1.0 ml of BD Cytofix Buffer (BD Biosciences, San Jose, CA). Expression of green fluorescence was measured by flow cytometry using a FACS Calibur (BD Biosciences, San Jose, CA).

2.2. Antibodies

Primary antibodies used were total p44/p42 MAP Kinase, phospho-p44/p42 MAP Kinase (Thr202/Tyr204), total Akt, phospho-Akt (Thr308), phospho-GSK3β (Ser9), cyclin A, D1, cyclin B1, $p21^{Waf1/Cip1}$, phospho-cdc2 (Tyr15), caspase 3, pan-actin (Cell Signaling Technology, Beverly, MA) and total p53 anti-mouse antibody (DKZF, Heidelberg, Germany).

2.3. Cell culture and infection

Huh7 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% fetal calf serum (FCS), penicillin 10 units/ml and streptomycin 10 μg/ml (Invitrogen, Carlsbad, CA) at 37 °C in 5% CO_2. For infection with rAdHBV-wt, rAdHBV-M204I and rAdGFP, Huh7 cells were seeded onto 6-well tissue culture plates (Nunc, Roskilde, Denmark) 1 day prior to infection to ensure that cells were 70% confluent at the time of infection. Cells were infected with rAdHBV-wt, rAdHBV-M204I or rAdGFP at a multiplicity of infection (moi) of 1.0, defined as the viral dilution that resulted in >99% GEUs. Mock-infected controls were treated with PBS alone.

2.4. Analysis for HBV-DNA

For analysis of viral DNA, Huh7 cells and media supernatant were collected 72 h post-infection (pi) Huh7 cells for DNA extraction and detection by Southern blotting using ^{32}P-labelled HBV probe as described previously (Chin et al., 2007). HBV cccDNA was determined from intracellular extracts 96 h pi (Bowden et al., 2004).

2.5. Cell cycle synchronisation, infection and cell cycle analysis

Huh7 cells were seeded onto 80 cm^2 cell culture flasks (Nunc, Roskilde, Denmark) at a confluency of 40% in DMEM 10% FCS and allowed to adhere overnight at 37 °C with 5% CO_2. The following day, cells were treated with 2 mM thymidine in DMEM 10% FCS (first synchronisation). Twelve hours later, cell were washed three times with PBS and cultured in thymidine-free media (first synchronisation release) for 15 h, then washed again three times with PBS, and re-cultured in 2 mM thymidine-DMEM 10% FCS (second synchronisation) for 12 h. Cells were finally washed three times with PBS, trypsinized and 1,00,000 cells were plated onto 12-well plates (Nunc, Roskilde, Denmark) in DMEM 10% FCS media and infected with PBS (mock uninfected control), rAdeno-GFP control, rAdHBV-wt and rAdHBV-204I mutant at a (moi) of 1.0. Cells were harvested at times 0, 3, 6, 9, 24, 30, 48 and 72 h pi for cell cycle analysis. Cells were washed twice in PBS, trypsinized and collected by low-speed centrifugation (1500 × *g*) for 3 min. The cell pellet was resuspended in 200 μl of staining solution containing 0.1% Triton X-100 (Sigma, Castle Hill, NSW, Australia), 40 μg/ml propidium iodide (Sigma–Aldrich, Castle Hill, NSW, Australia), 100 μg/ml RNAase A (Roche Diagnostics, Castle Hill, NSW, Australia) and 5 mM EDTA in PBS. The cell suspension was incubated for 20 min at room temperature and analysed by flow cytometry. Flow cytometry was performed with the FACS Calibur (BD Biosciences, San Jose, CA) and the quantitative measurements of cell cycle phases were analyzed with FlowJo Version 4.6.2.

2.6. Cell viability assay

Huh 7 cells grown to semi-confluency were infected with rAdHBV-wt, rAdHBV-M204I or rAdGFP at a (moi) of 1.0. Three days pi the cells were passaged at 1:50 and incubated a further 4 days before washing and fixing cells with 1% glutaraldehyde (Merck, Whitehouse Station, NJ) for 10 min at room temperature followed by staining for 20 min with 0.1% crystal violet (Sigma, Castle Hill, NSW, Australia) and washing with deionized distilled water. Bound dye was solubilized in 0.1 M sodium citrate (Sigma) pH 4.2 in 50% methanol (Sigma) for 30 min at room temperature on a horizontal shaker. Supernatants were collected and absorbance measured at 550 nm (Spectramax 250 plate reader; GMI, Ramsey, MN).

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