Original article

Failure of lamivudine to reverse hepatitis B virus-associated changes in ERK, Akt and cell cycle regulatory proteins

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Background: Chronic infection with hepatitis B virus (HBV) is a major factor associated with the development of hepatocellular carcinoma, but the mechanism by which this occurs is unknown. Treatment of chronic hepatitis B with lamivudine results in virological suppression and histological improvement; however, the role of lamivudine in preventing the development of hepatocellular carcinoma is less well defined. We recently reported that replication of HBV in a cell-culture system was associated with the upregulation of pERK, pAkt, pc-Myc, nuclear cyclin B1, p21cip1 and p53 together with G2 cell cycle arrest.

Methods: In order to determine whether lamivudine is able to reverse the HBV-induced changes on signal transduction and cell cycle, we infected Huh7 cells with a recombinant adeno-HBV virus in the presence of 0–50 μM of lamivudine. Signal transduction and cell cycle regulatory proteins were analysed by western immunoblot.

Results: Although lamivudine was able to inhibit HBV replication, it failed to reverse the changes on ERK and Akt phosphorylation. Correspondingly, levels of phospho-GSK3β and p21cip1/waf1 were increased, as were cyclin D1, cyclin B1, p53 and pc-Myc.

Conclusions: Lamivudine was ineffective in reversing the HBV-induced changes in signal transduction pathways and cell cycle regulatory proteins, indicating that the HBV-infected cells remained primed for oncogenic transformation despite viral suppression.

Introduction

Hepatitis B virus (HBV) is endemic in many regions of the world and is a major health concern in developing countries, where chronic carrier rates range from 10% to 20% [1]. Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer death worldwide, and the vast majority of HCCs are caused by chronic hepatitis B infection [2].

The viral X protein (HBx) has been implicated as the key contributor to hepatocellular transformation [3]; however, the development of HCC has been linked to other host and viral factors, including advanced fibrosis and cirrhosis, increasing age, high alcohol intake and the male gender [4,5]. Viral factors are also significant contributors to oncogenesis, with HCC being reported to be more frequently associated with HBV genotype C infection [6,7] and hepatitis C virus (HCV) coinfection [8,9]. In addition, the level of HBV replication and HBV DNA load have emerged as important risk factors for HCC [4–6,10], even in individuals who are seronegative for hepatitis B e antigen (HBeAg) with normal serum alanine transferase and in the absence of cirrhosis [5].

Hepatitis B infection is associated with alterations in the cell-signalling pathways. Much of the reported literature describes the oncogenic properties of the HBx protein, which has been shown to have several functions: to activate the Src family of tyrosine kinases [11], extracellular signal-regulated kinases (ERKs), stress-activated protein kinases/NH2-terminal-Jun kinases (SAPK/JNK) and the p38 kinase...
[12–14]; inactivate the tumour suppressor protein p53 [15]; to modulate Wnt/Frizzled/β-catenin signalling [16]; and to stimulate the transcription of NFκB [17]. Recently, we reported that replication of HBV in liver-derived cells (Huh7) and primary marmoset hepatocytes was associated with the upregulation of phosphorylated ERK (pERK), Akt (pAkt) and c-Myc (pc-Myc) together with increased p21\(^{waf1}\), p53 and nuclear cyclin B1, in a complex interplay that was associated with G2 cell cycle arrest [18]. These changes may provide an intracellular environment that is conducive to malignant transformation and contribute to other factors, such as inflammatory responses, that are associated with HCC development [19].

Nucleoside and nucleotide analogues such as lamivudine, entecavir and adefovir are effective in decreasing HBV replication, improving liver histology and slowing disease progression in patients with cirrhotic liver disease [20–22], including those who are HBeAg seronegative [23,24]. The role of nucleoside analogues in reducing the likelihood of developing HCC has not been extensively studied. In one multicentre randomised study, the progression to HCC was delayed by the use of lamivudine – HCC occurred in 3.9% of patients in the lamivudine-treated group compared with 7.4% in the untreated group [20]. Other studies have shown that antiviral therapy was not completely protective against the subsequent development of HCC [25]. This raises the question of whether chronic low-level persistence of HBV DNA during nucleoside analogue treatment will continue to contribute to dysregulation in cell-signalling pathways that may predispose to oncogenic transformation in hepatocytes, because persistence of serum HBV DNA is associated with an increased risk of developing HCC [5] and cirrhosis [26] across a wide range of HBV viral loads.

In order to address whether nucleoside analogues are able to reverse these HBV-induced changes in signal transduction, we examined the \textit{in vitro} effects of lamivudine in HBV-infected liver-derived cells. We found that lamivudine was not able to reverse the effects of HBV on signal transduction and cell cycle regulatory proteins. These findings provide important insight in to the reason why these agents may not be completely effective in preventing the progression of chronic hepatitis B infection to HCC development.

**Methods**

**Plasmids**

The recombinant adeno-HBV (rAdHBV) was produced using the AdEasy system as described previously [18]. Briefly, the rAdHBV was produced using the AdEasy system [27]. A 1.5× full-length replication competent HBV (genotype A, subtype adw2) was excised from plasmid phrGFPHBV1.5 by digestion with \textit{Hind}III and \textit{XhoI} (New England Biolabs, Arundel, Queensland, Australia) and ligated with T4 DNA Polymerase (400 U/μl; Promega, Annandale, NSW, Australia) overnight at 4°C into pAdTrack, which was pre-digested with \textit{Hind}III and \textit{EcoROV} (Promega) [18]. The replication competent HBV genome was not placed under the control of a CMV promoter to ensure that viral replication was only driven by the HBV promoters. The plasmid pAdTrack-HBVwt was digested with \textit{Pmel} and transformed into AdEasier-1 cells (BJ5183 cells containing the E1- and E3-deleted AdEasy-1 plasmid) by electroporation using a BioRad Gene Pulser (BioRad Laboratories, Regents Park, NSW, Australia). Transformants (AdEasy-HBV) were screened by \textit{Pci} restriction digest and clones of the correct size were subcultured. DNA was extracted using UltraClean™15 DNA Purification Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) and then subjected to heat-shock transformation into calcium/rubidium competent Top 10F\(^{′}\) cells (Invitrogen, Mt. Waverley, Victoria, Australia). Clones were confirmed by DNA sequencing.

A control plasmid (pAdTrack) was also transformed into AdEasier-1 cells to generate AdEasy-GFP, which was used to produce recombinant adeno-green fluorescent protein (rAdGFP).

**Antibodies**

Primary antibodies used were total p44/p42 mitogen-activated protein kinases (MAPK), phospho-p44/p42 MAPK (Thr202/Tyr204), total Akt, pAkt (Thr308), pc-Myc (Thr58/Ser62), phospho-GSK3β (Ser9), cyclin D1, cyclin B1, p21\(^{waf1/cip1}\), panactin (all by Cell Signaling Technology, Arundel, Queensland, Australia) and total p53 antiamoese antibody (DKZF, Heidelberg, Germany).

**Cell culture and infection**

Huh7 and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with heat-inactivated 10% fetal calf serum and streptomycin 50 μg/ml at 37°C in 5% CO\(_2\). The rAdHBV-wild type (wt) virions were produced by transfection of pAdEasy-HBVwt into 293T cells and amplified by serial passaging in culture as previously described [18].

For infection with rAdHBV-wt or rAdGFP virions, Huh7 cells were seeded onto six-well tissue culture plates (Nunc, Noble Park, Victoria, Australia) 1 day prior to infection to ensure that cells were 70% confluent at the time of infection. Cells were infected with rAdHBV-wt or rAdGFP at a multiplicity of infection (MOI) of 1.0. Mock-infected controls were treated with phosphate-buffered saline alone. Lamivudine (0–50 μM) was added to the infected or control cell culture 12 h post-infection (PI). Cells were harvested 72 h PI for protein and DNA analyses by western and Southern blotting, respectively.
Detection of HBV DNA

Huh7 cells and media supernatant were collected 72 h PI for analysis of HBV replicative intermediates and after 96 h for HBV covalently closed circular (CCC) DNA as described previously [18,28].

Analysis by western immunoblotting

Huh7 cells were harvested 72 h PI as previously described [18]. Briefly, 200 μl of 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-glycerophosphate, 1 mM sodium vanadate, 1 mM sodium molybdate and 5 mM sodium fluoride) supplemented with complete protease inhibitor cocktail (Roche, Castle Hill, NSW, Australia) was added to the cell monolayer. Lysates were clarified by centrifugation and the cytoplasmic fractions were collected. Total cell lysates were prepared by adding SDS (final concentration 0.5%) to 100 μl of non-clarified lysates. Proteins were analysed by SDS-PAGE and immunoblotting as described previously [18]. Experiments were performed in triplicate, unless otherwise stated. Immunoblots were scanned with a BioRad GS710 densitometer and analysed using Quantity One 4.1.0 software package (BioRad) and Prism 4.0, a statistical package (GraphPad Software, San Diego, CA, USA). The level of cellular signalling and cell cycle regulatory proteins in HBV-infected cells treated with various concentrations of lamivudine were expressed relative to untreated HBV-infected cells. The relative levels of expression were corrected for activation induced by AdGFP infection by subtracting this background activity from the levels induced by AdHBV-wt infection. In general, infection of Huh7 cells with rAdGFP resulted in an increase in relative levels of various proteins by no more than 5% above mock-infected cells.

Results

Lamivudine sensitivity for wt-HBV

We first established that Huh7 cells infected with rAdHBV-wt were responsive to the antiviral effects of lamivudine. Huh7 cells were infected with rAdHBV-wt (MOI of 1.0) in the presence of lamivudine (3TC; 0–50 μM). Intracellular hepatitis B virus (HBV) DNA replication intermediates (relaxed circular [rc], double-stranded linear [ds] and single stranded [ss]) were detected by Southern blot analysis 72 h post infection. (A) Intracellular hepatitis B virus (HBV) DNA replication intermediates (relaxed circular [rc], double-stranded linear [ds] and single-stranded [ss]) were detected by Southern blot analysis 72 h post infection. (B) Dose-response curve shows the median inhibition concentration (IC50) of lamivudine of 0.75 μM. (C) HBV covalently closed circular (CCC) DNA copies/ genome equivalent (copies/GEq) by real-time PCR. Error bars represent standard error of the mean.
Lamivudine concentrations of 0.5 μM and 50 μM, 112 (SD ±5.7) and 110 (SD ±3.5) HBV CCC DNA copies/genome equivalents (GEq) were detected, respectively, compared with 70 (SD ±12.2) CCC DNA copies/GEq in the untreated controls (P = not significant [NS]; Figure 1C).

Lamivudine fails to reverse rAdHBV-wt induction of pERK1/2 and pAkt
In order to determine if lamivudine was able to reverse the HBV-induced changes in ERK-, c-Myc- and PI(3)K/Akt signalling pathways [18], we examined the effects of HBV on the phosphorylation of these signalling intermediates in the presence of increasing concentrations of lamivudine. Infection of Huh7 cells with rAdHBV-wt resulted in an increase in pERK1/2 and pAkt compared with mock controls, whereas the levels of total ERK and Akt remained unchanged (Figures 2A & 3A). The increased levels of both pERK and pAkt were not reversed by lamivudine treatment of cells. In contrast to mock-infected cells, pERK levels in HBV-infected cells were increased by 30% (SD ±14%; P<0.05) in the absence of lamivudine and remained upregulated at 0.5 μM (43% SD ±9%) to 50 μM (50% SD ±15%) of lamivudine (P=NS; Figure 2B). At low concentrations, lamivudine also failed to reverse the HBV-induced upregulation of pc-Myc (69% SD ±28%) at 0 μM lamivudine compared with 56% (SD ±21%) at 0.5 μM (Figures 2A & B); however, at 50 μM, lamivudine partially reversed the upregulation of pc-Myc (37% SD ±10%; P<0.05).

Similarly, in contrast to mock-infected cells, pAkt levels were increased by 152% (SD ±75%; P<0.5) in rAdHBV-wt-infected cells. Like pERK, the levels of pAkt in HBV-infected cells were not significantly reduced in the presence of increasing concentrations of lamivudine from 0.5 μM (188% SD ±17%) to 50.0 μM (153% SD ±73%; P=NS; Figure 3B).

We have previously shown that the HBV-induced upregulation of pAkt was accompanied by increased phosphorylation of GSK3β [18]. Therefore, we wanted to determine whether treatment of HBV-infected Huh7 cells with lamivudine could reduce phospho-Ser9-GSK3β to levels comparable to uninfected cells. Levels of phospho-Ser9-GSK3β remained increased in rAdHBV-wt-infected Huh7 cells in comparison with mock-infected cells (112% SD ±70%; P<0.01) and at 0.5 μM (107% SD ±85%; P=NS) and 50 μM lamivudine (81% SD ±56%; P=NS; Figures 3A & B), consistent with the upregulation of pAkt.

Effect of viral suppression on p21<sup>cip/waf1</sup>, p53 and cyclins D1 and B1 expression
HBV infection of hepatocytes results in increased expression of p21<sup>cip/waf1</sup>, p53, cyclin D1 and B1 [18].
Therefore, we wanted to determine whether suppression of HBV replication by lamivudine could restore cyclin D1, B1, p21\(^{cip/\text{waf1}}\) and p53 to levels comparable to uninfected cells. At lamivudine concentrations from 0.5 to 50 μM, the levels of cyclin D1, B1, p21\(^{cip/\text{waf1}}\) and p53 remained upregulated in HBV-infected cells compared with uninfected controls (Figures 4 & 5). Levels of p21\(^{cip/\text{waf1}}\) were increased by 172% (SD ±15%; \(P<0.05\)) in the absence of lamivudine and were only moderately reduced at lamivudine concentrations of 0.5 μM (129% SD ±9%; \(P=0.007\)) and 5 μM (147% SD ±12%; \(P=0.005\)); however, at the highest concentration of lamivudine (50.0 μM), p21\(^{cip/\text{waf1}}\) levels were slightly increased (179% SD ±30%; \(P=\text{NS}\); Figure 4B).

Similarly, p53 levels were increased by 39% (SD ±10%; \(P<0.05\)) at 0 μM lamivudine and were not significantly reduced at lamivudine 0.5 μM (33% SD ±7%; \(P=\text{NS}\)), 5 μM (40% SD ±10%; \(P=\text{NS}\)) or 50 μM (33% SD ±4%; \(P=\text{NS}\); Figure 4).

Infection of Huh7 cells with rAdHBV-wt also resulted in an increase in total levels of cyclin D1 (44% SD ±27%) and B1 (69% SD ±19%) compared with mock controls (\(P<0.05\); Figure 5). However, these changes were not reversed by lamivudine in increasing concentrations ranging from 0.5 μM (cyclin D1 38% SD ±5%; cyclin B1 47% SD ±23%) to 50 μM (cyclin D1 67% SD ±20%; cyclin B1 62% SD ±25%; \(P=\text{NS}\); Figure 5).

Discussion

In this study we attempted to demonstrate whether suppression of HBV replication by lamivudine was able to reverse the changes in the MAPK and Akt signalling and cell cycle proteins. We found that despite the suppression of viral replication, lamivudine not only failed to reverse the upregulation in ERK and Akt phosphorylation but the expression of cyclins D1 and B1 remained upregulated. Furthermore, levels of p53 and pGSK3\(^{\beta}\) were also unaffected by lamivudine. The levels of p21\(^{cip/\text{waf1}}\) were modestly reduced, but did not return to levels comparable to mock-infected cells (Figure 6). Recently Choudhari and coworkers also demonstrated that lamivudine failed to reverse the activation of Akt and STAT3 in HBV stably transfected HepG2Ad38 cells despite suppression of viral replication [29], reinforcing the significance of our findings.

Over-expression of p21\(^{cip/\text{waf1}}\) has been associated with poorly differentiated HCC [30] and may also be a marker for progression from chronic hepatitis to cirrhosis and HCC [31], and resistance to chemotherapy and radiation. The p21\(^{cip/\text{waf1}}\) protein acts to stabilize and activate cyclin D1-CDK4/6 complexes [32], and thereby promotes cell cycling. It also inhibits apoptotic cell death [33] and promotes...
Figure 4. Viral suppression is not associated with down-regulation of p21cip/waf1 and p53 expression

(A) Immunoblot of total cell lysates of recombinant adenovirus B virus-wild type (rAdHBV-wt)-infected Huh7 cells treated with lamivudine (3TC; 0, 0.5, 5.0 and 50 μM). Thirty micrograms of total protein was analysed for p21cip/waf1 and p53. Panactin was used as an internal standard.

(B) p21cip/waf1 (upper graph) and p53 (lower graph) of rAdHBV-wt infected Huh7 cells were expressed as a percentage of mock controls (error bars represent the standard error of the mean). Levels of p21cip/waf1 and p53 levels were increased in the absence of 3TC and remained increased throughout the dose range of lamivudine (0.5–50 μM). All experiments were performed in triplicate and corrected for background activation induced by recombinant Ad-GFP infection. MWM, molecular weight markers.

Figure 5. Viral suppression is not associated with down-regulation of cyclins D1 and B1

(A) Immunoblot of total cell lysates of recombinant adenovirus B virus-wild type (rAdHBV-wt)-infected Huh7 cells treated with lamivudine (3TC; 0, 0.5, 5.0 and 50 μM). Thirty micrograms of total protein was analysed for cyclins D1 and B1. Panactin was used as an internal standard.

(B) Cyclin D1 (upper graph) and cyclin B1 (lower graph) of rAdHBV-wt infected Huh7 cells were expressed as a percentage of mock controls (error bars represent the standard error of the mean). Total cyclin D1 and cyclin B1 levels were increased in the absence of 3TC and remained increased throughout the dose range of lamivudine (0.5–50 μM). All experiments were performed in triplicate and corrected for background activation induced by recombinant Ad-GFP infection. MWM, molecular weight markers.
DNA synthesis through loss of interaction with proliferating cell nuclear antigen [34]. It could be postulated that the increased levels of p21cip1/waf1 may have resulted either from p53-driven transcription or post-translational phosphorylation, although we are yet to establish the precise underlying mechanism.

In addition to the cell-cycle-promoting effects of p21cip1/waf1, pAkt directs the cell cycle through phosphorylation of GSK3-β to downregulate the degradation proteins necessary for maintenance of active cell cycling such as cyclin D1, c-myc and p21cip1/waf1 [35], which appear not to have been affected by the presence of inhibitory levels of lamivudine.

Although we have focused on the intracellular events in infected hepatocytes, the mechanism underlying the development of HCC in whole liver in vivo is multifactorial and involves a complex interplay linking the initiating oncogenic stimulus (HBV infection) with the associated changes in cell-signalling pathways and cell cycle regulation, together with an inflammatory and cytokine reaction driven by antigen-presenting cells in response to degradation products of apoptotic cells, chemical carcinogens and viral antigens [19]. For example, Akt is known to activate the NFκB pathway, and thus provides an important link between the cytokine signalling, the cell proliferative processes and tumour development [36]. HBV-mediated NFkB activation is also associated with the release of inflammatory cytokines resulting in a paracrine effect in adjacent Kupffer cells, which release mitogenic factors that promote proliferation and survival of hepatocytes and ultimately neoplastic transformation following in vivo challenge with diethylnitrosamine [37].

Figure 6. Schematic representation of the effect of HBV replication on key intermediates in PI3K/Akt pathway and cell cycle

Lamivudine fails to reverse hepatitis B virus (HBV)-induced upregulation of pAkt, p21cip1/waf1 and cyclin B1, thereby maintaining an intracellular environment conducive to oncogenic transformation. pGSK3β, phospho-GSK3β.
Profound changes in hepatocyte metabolism also contribute to the development of focal preneoplastic lesions long before the development of HCC [38]. Two conspicuous features that develop early in the preneoplastic stage are excessive glycogen storage (glycogenolysis) followed by the accumulation of mitochondria in glycogen poor basophilic hepatocytes [38,39]. The development of focal abnormalities during hepatic glycogenesis has also been reported in human HCC [40]. These changes are associated with overexpression of IRS-1 [41,42], a change that has also been reported in human HCC [43]. The concept of progressive hepatocyte dedifferentiation is supported by the observation that HCC commonly arises in foci of altered hepatocytes. Hepatic preneoplastic lesions develop in response to several oncogenic stimuli, which include chemicals (for example, diethylnitrosomine), hormones (dehydroepiandrosterone), radiation, hepadnaviral infection and oncogenic transgenes. Even after a brief exposure to chemical hepatocarcinogens, preneoplastic lesions will undergo malignant transformation into HCC even in the absence of continued oncogenic stimuli [36,39–42]. It has also been proposed that malignant transformation induced by viruses may involve a ‘hit-and-run’ phenomenon, whereby exposure to viral genes and/or products are sufficient to initiate malignant transformation, but persistence of the malignant phenotype will occur despite the absence of continued expression of viral genes or viral replication [44,45]. The frequent absence of HBx protein and hepadnaviral DNA from HCC tumour and peri-tumour tissue [46,47] suggests a similar mechanism may also exist for HBV-associated hepatocarcinogenesis.

Currently available nucleos(t)ide agents for HBV appear only partially effective in reducing the risk of developing HCC, especially in patients with cirrhosis of the liver [25,48]. In contrast to nucleos(t)ide analogues, interferon-based treatment has been shown to reduce the long-term risk of progression to cirrhosis and HCC development [49], although the mechanism by which interferon is able to achieve this reduction is unknown. However, two key signal transduction pathways that interact directly with IRS-1 protein are PI3K/Akt and MAP kinase pathways [50], and the over-expression of IRS-1 may account for the increased activation of Akt and ERK seen here in HBV-infected cells. IRS-1 protein is an intracellular substrate for the interferon-α receptor [43]. In addition, interferon-α, which is known to have antiviral activity against HBV, activates STAT1, inhibits cyclin D3 and cdc25A expression and induces apoptosis of virally infected cells [51]. Activation of STAT1 is believed to have an oncoprotective effect in solid tumours [52]. With these effects in mind, we are currently investigating the concomitant effects of interferon-α on reversing HBV-induced changes in PI3K-Akt, MAP kinase pathways and cell cycle dysregulation.

Although the cell culture system used here is unable to replicate the in vivo situation, it nonetheless serves as a useful model to investigate the early events in HBV infection that may initiate tumour development. The range of continuous cell lines that may be considered suitable for studies of HBV-associated hepatocarcinogenesis is limited and primary hepatocytes are also not readily available. Although we performed our experiments using a hepatoma cell line (Huh7), we have previously shown that the pattern of changes in MAPK and Akt signalling and cell cycle regulatory proteins is remarkably similar to those that were observed in HBV-infected primary marmoset hepatocytes [18]. Also, the replication capacity of HBV and antiviral activity of lamivudine against HBV has previously been established in Huh7 cells [53]. These findings encouraged our belief that Huh7 cells, rather than the poorly differentiated hepatoblastoma cell line HepG2, are a suitable cell line for studies investigating mechanisms of HBV-associated hepatocarcinogenesis. HepG2 cells are known to over-express P53 and IRS-1 with constitutive activation of the MAPK and PI(3)K/Akt pathways [43,54]. Other cell lines we considered were also not appropriate because of the presence of integrated HBV genome in the HepG2.215 and Hep3B cell line and the HeLa cell origin of Chang cells. Our system serves as a useful in vitro model to elucidate more precisely the mechanistic details of the effects of HBV on p21<sup>ras/raf</sup> and the Akt effector pathways (in particular, cross-talk with the cytokine transduction) and to determine whether or not emerging chemotherapeutic drugs that target the PI3K/Akt may be effective in reversing HBV-induced dysregulatory signals.

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**Disclosure statement**

The authors declare no conflicts of interest.

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