Requirement of Activation for Hepatitis B Virus Infection

by

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Abstract

Although *in vitro* models of human hepatitis B virus replication are established, so far none could approximate infection efficiency as expected from *in vivo* observations. Susceptibility for HBV infections has only been reported for primary hepatocytes of human, chimpanzee or Tupaia belangeri and the cell line HepaRG. Here we show that the insusceptible human hepatoma cell line HepG2 can be infected, when the virus was beforehand activated by passage over whole duck liver cell cultures. That suggests an activation step to be performed by specialized liver cells.

The hepatitis B virus (HBV) is a small single stranded DNA-containing enveloped virus. Depending on its relatively strict organotropicity HBV infects mainly hepatocytes and causes severe liver diseases in humans [1]. For this reason it was so far only possible to infect *in vitro* cultures of primary human or Tupaia belangeri hepatocytes [2,3], whereas human hepatoma cell lines are usually refractory to HBV infection. We succeeded in infecting the hepatoma cell line HepG2 with HBV by 'activating' the virus in a culture of primary duck liver cells (PDLCs).

Using duck HBV (DHBV)-infected PDLCs as a model data were published indicating that eventually scavenging liver sinusoidal endothelial cells (LSECs) play the key role in the initial uptake and targeting of hepadnaviruses in the liver [4]. These data implicated that in the process of transcytosis through LSECs the viral surface of HBV may be altered as a prerequisite for the infection procedure, a step that may be limiting for the infection of hepatoma cell lines like HepG2 (Fig. 1b). Further *in vitro* experiments performed in our laboratory using preparations of embryonal PDLCs [5,6] infected with purified DHBV virions [7] revealed that at least co-staining of single LSECs could be measured using fluorescence-labeled acetylated low density lipoprotein for the identification of LSECs and anti-DHBc antibodies for the localization of the virions (not shown). Taking into account a possibly high conservation of LSEC function we predicted that the human HBV could also be found in association with LSECs in a duck liver cell culture. Indeed, immunofluorescence studies performed with rabbit antisera against HBc or the human nuclear factor of activated T cells (NFATc1; Santa Cruz) as a marker of LSECs revealed 24 h after the addition of HBV, similarly as described for DHBV-infected PDLCs [8], co-localization of HBV and NFATc1 in a PDLC culture (Fig. 1a). Cells, negative for

NFATc1 but strongly reacting with anti-HBc antiserum, are possibly Kupffer cells with their typical phagocytic activity. As a source of HBV virions, HepG2.2.15 cells, HepG2 cells stably transfected with cloned HBV-DNA, were utilized for the production of infectious HBV [9], which was concentrated 50-fold from the collected supernatants [10].

The calculation of virions was performed by DNA dot blot, using a gel-purified full-length *Eco*RI fragment of cloned HBV DNA labeled with [α-^{32}P] dCTP as a probe [6]. When we measured the amount of virions after the passage through a PDLC culture, designated as HBV*, against the initial amount of HBV, we found a reduction of about 60 %, supposedly lost by unspecific adsorption and phagocytosis (Fig. 1c). In order to test the infectivity of HBV* for HepG2 cells after passage through primary duck LSECs, a time course kinetic for the release of virions after infection was performed (Fig. 2a). It revealed a steady increase of the amount of viral DNA (again measured by dot blot) in the supernatants of HepG2 cells, comparable to the cell growth-dependent increase of viral output in the stably transfected hepatoma cell line HepG2.2.15. Otherwise, no viral DNA could be detected in the supernatants when untreated HBV was used directly for infection. To study viral DNA replication in infected HepG2 cells in more details we performed a Southern blot using the same radioactively labeled probe as described above. Besides the full-length relaxed circular DNA single-stranded replicative intermediates of HBV DNA were also detected (Fig. 2b). In this study we could find out three further details: first, an incubation time of HBV with LSECs of 24 h instead of 4 h did not lead to an increase of infectivity; second, transfer of HBV-incubated activator cells instead of activated virus was also able to initiate HBV-infection; third, to employ another cell type, primary duck kidney cells [8], as activator cells would not support subsequent infection of

hepatoma cell lines. In a next experiment we replaced every 24 h the culture medium of HBV infected HepG2 cells for the calculation of the amount of the viral surface proteins with a commercially available anti-HBs-coated micro particle enzyme immunoassay (Abbott AXSYM system, Germany) as a measure of virus production per day (Fig. 2c). Seven days after infection the cells were disrupted to reveal HBV proteins after electrophoretical separation over a 5 to 20 % polyacrylamide gradient by Western blots with rabbit antisera raised against the surface proteins (HBs) or the core protein (HBc) of HBV as described [6]. Only in HBV*-infected cultures the synthesis of HBV core and surface proteins could be demonstrated by immunoblotting (Fig. 2d). The alteration HBV virions acquire during passage through PDLC cultures would supposedly concern envelope protein integrity or reversible modification. We therefore compared their appearance in immunoblotting. The general glycosylation pattern of the viral envelope proteins of HBV and HBV* was unchanged. Yet, we found the amount of the unglycosylated small surface protein of HBV to be significantly reduced; whereas the glycosylated as well as the unglycosylated middle and large surface proteins did not show particular alterations (Fig. 1d).

We conclude that hepatoma cell lines like HepG2 are susceptible to infection with HBV after a yet to define activation step for HBV virions that takes place in whole liver cell cultures and may even play a significant role in physiological HBV infections. The main suspect for HBV activation would be LSECs as they constitute a permeable barrier between the blood stream and the liver parenchyma and thus necessarily come into close contact with trespassing particles [11]. The absence of morphological features comparable to the liver architecture in cell cultures or defined conditions for the maintenance of

specialized cell types may be the major player that hampers *in vitro* hepadnaviral infections. Another possibility to influence infectivity of HBV virions would only be secretion of relevant molecules by a cell type in the culture. The establishment of pure LSEC cultures or LSEC cell lines and close pursuance of viral particles within the cells will permit to throw light on their participation in the proposed viral activation.

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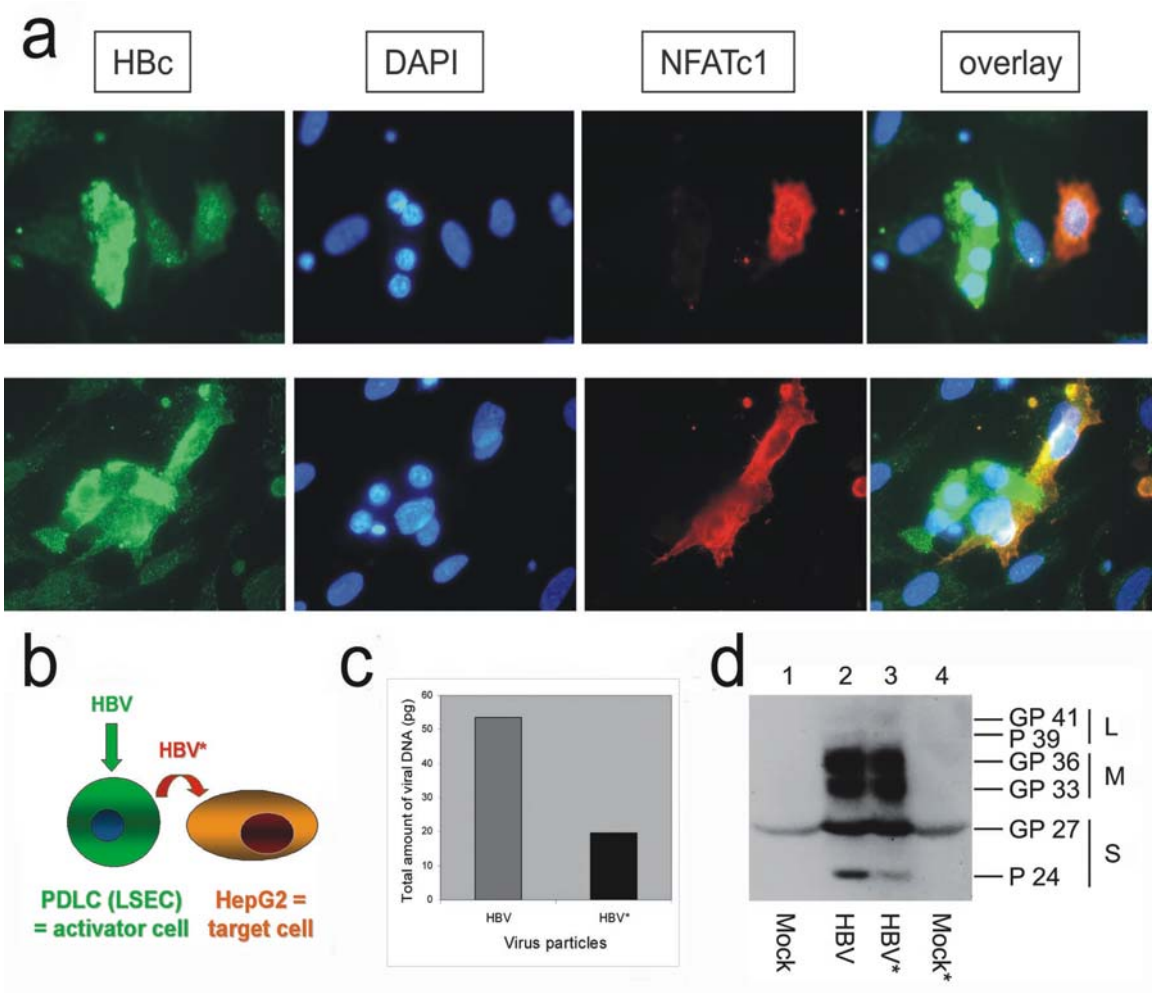
Figure Legends

Fig. 1. Activation of HBV by PDLCs for infection of human hepatoma cells. (a) Co-immunofluorescence staining of PDLCs after incubation with HBV using anti-HBc and anti-NFATc1 antisera. (b) Model to demonstrate the requirement for activation of HBV by PDLCs before the infection of target cells like HepG2. (c) Analysis of HBV DNA before and after the addition to PDLCs. HBV was added to PDLCs and removed again after an incubation time of 4 h. Calculation of the viral DNAs before and after incubation with PDLCs revealed a loss of HBV virions of about 60 %; the presented dot blot data shows the mean of 3 measurements. (d) The same amount of virus particles was used to investigate changes of the viral envelope proteins before and after incubation with PDLCs; GP, glycoprotein, and P, protein, indicate the molecular weight in kD of the HBV large (L), middle (M), and small surface proteins (S).

Fig. 2. Study of viral DNA replication and protein synthesis in HepG2 cells. (a) Dot blot kinetic of viral DNA in the supernatants over 7 days following infection of HepG2 cells with HBV* and HBV or seeding of HepG2.2.15 cells. (b) Infectivity of HBV* for HepG2 cells; prior incubation of HBV with PDLCs was performed for 4 or 24 h (lanes 2 and 3). Alternatively HBV-treated PDLCs or primary duck kidney cells were transferred into the HepG2 culture (lanes 5 and 6); lane 1, HepG2.2.15 cells; RC, relaxed circular, and SS,

single-stranded, DNAs of HBV; M, viral marker DNA. (c) Kinetic study of HBs for one week; here, day 1 represents viral input. All data points represent the mean of 3 calculations. (d) Analysis of the production of viral surface (left) or core (21.5 kD) proteins (right) by Western blot one week after infection with HBV* or HBV; mock*- and mock-treated inputs refer to the supernatants of HepG2 cells similarly passaged over PDLCs or not; P, PDLCs after removal of HBV*.



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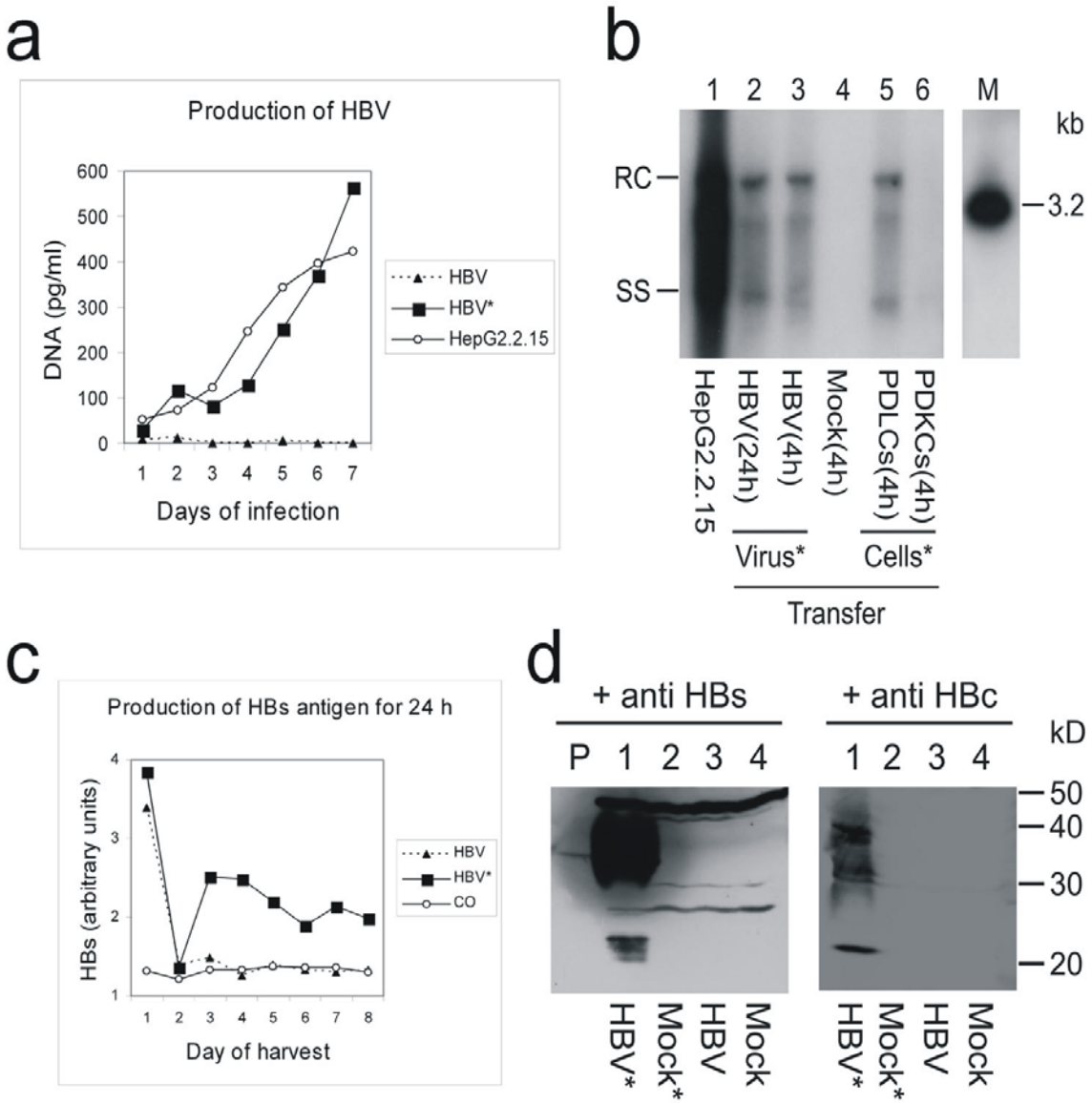

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