ORIGINAL ARTICLE

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Propagation of Hepatitis B Virus in a Rat Hepatoma Cell Line Stably Transfected with Human Annexin-V

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Background and Aims: **Hepatitis B virus (HBV) displays a distinct hepatotropism and a narrow host range *in vivo*. However, very little is known about the interaction of HBV with its host cells, mainly because of difficulties in the development of suitable tissue culture system. We present here confirmatory evidence of a putative role of annexin-V in HBV infection.**

Methods: **HBV from both human sera and from culture supernatants from HepG2 2.15 cells were used to infect FTO9.1 cells (a rat hepatoma cell line transfected with a construct containing human annexin-V). Cells and culture supernatants were assayed at various times post-infection by immunofluorescent microscopy (HBcAg staining in nucleus), and by HBV cccDNA-specific PCR. Supernatants from these initially infected cells were then used to infect fresh FTO9.1 cells with a similar outcome to primary infection.**

Results: **Core and surface gene PCRs were positive on days 2, 5 and following transfer experiments. cccDNA-specific PCR confirmed internalisation of the virus into the nucleus. HBcAg fluorescence showed nuclear staining on days 2, 5 and following transfer experiments. Addition of recombinant annexin-V and DMSO to the cell culture medium resulted in a greater efficiency of infection. Later washes were negative for HBV-DNA, ruling out contamination of the cells by external HBV particles.**

Conclusions: **This cell line does appear to be useful in the study of the early stages of HBV infection, but requires further evaluation.**

Keywords: **HBV, In Vitro, FTO 9.1 Cell Line, Human Annexin-V**

Introduction

Hepatitis B virus (HBV) displays a distinct hepatotropism and a narrow host range in vivo [1, 2]. Several studies using transfection and/or transgenic technology have suggested that the species barrier of HBV infection and replication may be located at the early step of viral adsorption [3, 4]. However, information is incomplete concerning the interaction of HBV with its host cells, mainly because of difficulties in the development of a suitable tissue culture system. HBV infection in *in vitro* systems involves chromosomal replication of HBV-DNA but does not include the early events of infection. Transformed cell lines have been used for short-lived, single cycle culture of HBV after infection; however, the cells are thought to lose some of their liver specific functions, leading to loss of susceptibility to HBV.

During the last decade, the studies on the putative receptors for HBV have revealed controversial and confusing results. It has been suggested that the HBV pre-S1 domain, in

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particular amino acid residues 21-47, contains the most important attachment site to liver cells [5, 6]. Several receptors on the liver cell membrane have been proposed. Asialoglycoprotein receptor (ASGPR), a transmembrane molecule specifically expressed on the hepatocellular membrane [7], human interleukin-6 [8], and transferrin [9], are all proposed to contain recognition sites for the HBV pre-S1 domain. Furthermore, in an attempt to identify cell surface receptor(s) for hepadnaviruses, several proteins which have binding sites for the HBV pre-S region have been proposed, including glycoproteins of 170-180 kd [10, 11], 120 kd [12], 80 kd [13] and 44 kd [14].

Among studies performed for identification of receptor(s) in the initiation of infection by HBV, the role of annexin-V has been supported by several observations [15, 16]. Annexin-V, previously referred to as endonexin II, is a member of the family of Ca^{2+} dependent phospholipid-binding proteins comprising 1% of total protein in some cells, and may function in membrane fusion and exocytosis [17]. Annexins have also been found to be involved in the initial step of cytomegalovirus and of influenza virus [4], and hepatitis delta virus infections [18]; it is present on human hepatocyte plasma membranes and appears to bind to small HBsAg [4, 8]. The receptor-ligand relationship between hA-V and small HBsAg has been studied by using antibodies against hA-V which inhibit the binding of HBsAg to intact human hepatocytes [6]. Rat annexin-V does not bind to HBsAg, although it has amino acid homology of more than 90% as compared with hA-V [4], suggesting the existence of a "receptor-ligand" relationship between hA-V and HBsAg.

In the present study, we confirm this involvement of hA-V in HBV infection and replication. Transfection of a non-susceptible rat hepatoma cell line, FTO2B (which is not infectable by HBV) with a construct containing the hA-V gene (FTO9.1), under selection by a neomycin-resistance gene, resulted in hA-V-expressing cells that are susceptible to HBV infection. We extend this earlier observation [4] to include multiple passage of the virus and to show enhancement of the infection with additional annexin-V added to the system. Because formation of cccDNA only occurs after virus entry, a method that enables differentiation between the repaired and unrepaired genome can be used in vitro infection to determine whether HBV originating from an inoculums has only bound to a cell or has been successfully taken up. We used the method described by Kock *et al.* [19] to amplify viral cccDNA.

Materials and Methods

Cell Culture and Infection

FTO 9.1 cells (Innogenetics, Ghent, Belgium) were grown at 37°C, 5% CO_2 in Hams F12: Dulbecco's modified Eagle's medium (1:1 v/v) containing 10% foetal bovine serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Life Technologies, Paisley, UK), and 600 µg/ml G418. Cells were passaged every 3-4 days. Cos-7 cells were grown in DMEM with 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin (the FTO-2B cell line, i.e. rat hepatoma without annexin-V, was not available for study, so we used COS-7 cells as a non-permissive cell control. HepG2 2.15 cells were grown under category III containment. These are stably transfected with a full length HBV genome and infectious virus is released into the culture medium. For a cccDNA positive control, DNA was extracted with phenol/chloroform from paraffin-embedded HBV positive liver tissue. Using a standard technique, HBV-DNA was also extracted from HBeAg positive sera for C and S gene PCR controls.

For infection, cells were plated at 3×10^5 cells in 24 well plates. Infection was carried out for 16 hrs with HepG2 2.15 supernatant containing ~5×10^6 genome equivalents (geq)/ml, diluted in FCS to give a multiplicity of infection (MOI) of 100. Similarly, HBV-positive sera, obtained from an HBeAg positive patient, were diluted in normal sera to the appropriate MOI. At the time of infection, either 2% DMSO or rAV (10 µg/ml) was added to the media. For all experiments Cos-7 cells were used as a negative control under the same culture conditions. Cells were infected overnight at 37°C, then washed thoroughly (7 times) with PBS. 200 µl of the last wash was extracted and tested for HBV-DNA to confirm that the positivity of FTO cells seen at day 5 was not as a result of amplification of DNA from virions present in the original inoculum. For passaging of the virus, supernatant of infected cells from day 5 was carefully removed to prevent contamination with cells, and then spun at 14000 rpm. Then 500 µl of this supernatant was added to the fresh cells, and incubated over night at 37°C. This was again performed using supernatants of first passage infected cells (termed second passage and for a third passage).

DNA Extraction and cccDNA-Specific PCR

HBV-DNA was extracted and C and S gene PCR were carried out, as described previously [19]. For cccDNA amplification (to confirm internalisation

of the virus into the cells), the PCR protocol and specific primers were used as described by Kock *et al.* [19]: HBV 2537+ (CCT CTG CCG ATC CAT ACT GCG GAA C) and HBV 470- (CTG CGA GGC GAG GGA GTT CTT CTT C) to give the product that amplified over the gap and/or nick of cccDNA, so confirming the presence of active, natural replication.

Immunofluorescence

Cells were fixed with 0.4% sucrose and 1% formaldehyde in PBS. After permeabilization with 0.5% Triton-X100, rabbit anti-core polyclonal IgG (Zymed, San Francisco, USA) and FITC conjugated goat anti-rabbit IgG (Sigma, Poole, UK) were used as primary and secondary antibodies, respectively. Cells were examined under a Nikon Microphot-SA microscope with 75× magnification.

Results

Cell Passage Number and Infection

Our preliminary experiments showed that an early passage of FTO 9.1 cell line (passage number 8) was more infectable than a later passage number, i.e., ≥20. This may be due to gradual loss of the plasmid. As a result of this, we used the cells only at passage number 18 or less (results not shown).

HBcAg Staining

Figure 1 shows the expression of HBcAg on days 1 and 5 days after initial infection. HBcAg staining can be clearly seen in the nuclei of the cells on day five of initial infection (Figure 1, primary), and day five of the first and second passage (Figure 1, P1 and P2). This experiment has been repeated multiple times with consistent results.

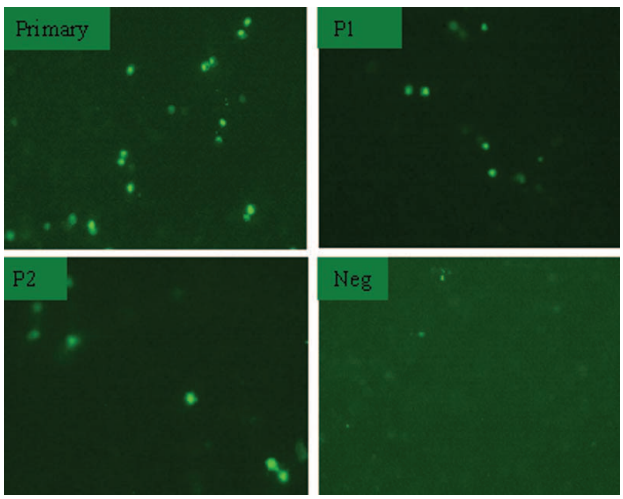

Figure 1. FTO 9.1 cells infected with HepG2 2.15 conditioned media (Primary: infection day 5; P1: day 5, 1st passage; P2: day 5, 2nd passage; Neg: mock-infected cells).

HBV cccDNA PCR of Cell Culture Supernatants from FTO9.1 Infected Cells

We have shown that cells were infectable by both liver tissue-derived HBV and HepG2 2.15 supernatant (Figure 2). Figure 2 shows the results of HBV cccDNA-specific PCR on cell culture supernatants from cells infected with HepG2 2.15-conditioned media. In pilot experiments, HBV-DNA from liver tissue showed a positive cccDNA band (Figure 2, lane 15); however, DNA from HBeAg-positive sera was negative, as expected as it only contains the relaxed circular form of viral DNA (Figure 2, lane 14). Results for both these positive controls using standard diagnostic C and S gene PCR were positive (results not shown).

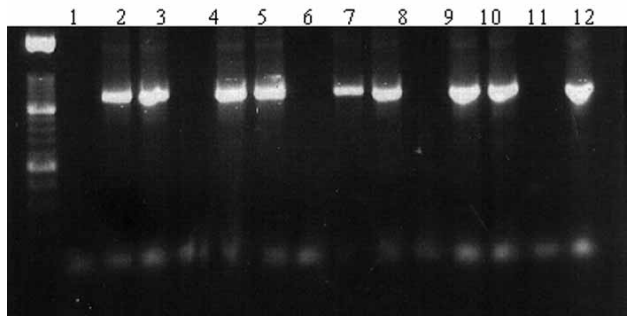

Figure 2. 1.5% ethidium bromide-stained agarose gel of cccDNA PCR products (Lane 1: M WT marker; lane 3 and 4: day 5 primary infection; lane 6 and 7: day 5, 1st passage; lane 9 and 10: day 5, 2nd passage; lane 12 and 13: day 5, 3rd passage; lane 14: HBeAg+ve serum; lane 15: HBV+ve liver DNA and lanes 2, 5, 8 and 11: mock infected cells).

Effect of Recombinant Annexin-V and DMSO on Infectivity of FTO 9.1 Cells

We added 10 µg/ml recombinant annexin-V to the cell culture medium in order to inhibit the infection. However, we noted it resulted in a more stable infection (possibly complexed with HBV). This is likely due to non-specific uptake of liver cells by annexin-V. As DMSO had been shown previously to improve infection efficiency, this was also added to the medium. In the absence of rAV, one of four wells proved positive by PCR at day 2, whereas if 10 µg/ml rAV is present, three of four wells were positive (results not shown). Cos-7 cells remained negative under all conditions.

Discussion

Several cell lines retaining some of the hepatocyte markers are available that support HBV transcription and replication upon plasmid transfection. However, even these permissive cell lines are not susceptible to true HBV infection. An inevitable conclusion is that the early steps of virus-cell attachment and entry determine the viral tropism and susceptibility. By adding chemicals (such as DMSO, PEG, etc), infectivity can be established and the viability of the cells can be extended. However, as the addition of these artificial substances does not mimic the in vitro process and may be affecting the infection mechanism, it is desirable to find another system.

Human annexin-V presents itself as a likely candidate for a species specificity determining receptor for HBV, because of the ability of a non-susceptible cell line to become susceptible following transfection with DNA for this protein [4]. The effect of rAV on infection may be explained by its competence for binding phospholipids, as HBsAg is extensively lipidated (8). However, binding, and subsequent penetration of the hepatocyte membrane by HBV via HBsAg interacting with annexin-V may not explain the hepatotropism of HBV. Clearly, since HBV virions can be produced after transfection of a rat cell line and also in transgenic mice (20-22), the species barrier for HBV is not at the level of transcription/translation of HBV specific genes. It is likely that the species barrier is present at the initial stage of entry of the virus into the host cell. Since h A-V is expressed in most tissues (23) this receptor does not account for the hepatotropism of HBV. It is possible that the tissue specificity results from downstream events and those liver specific factors are necessary for new virus production.

In our infection system, some issues need to be discussed. First FTO-h A-V cells lose expression of annexin-V over time. Second, we did not quantify HBV production. Future studies using quantitative PCR should help to elucidate the efficiency of infection these cells by HBV. Third, the Cos-7 is primate-derived, and thus closer cell line to human, originate from Green Monkey kidney and not from hepatocytes. Fourth, as shown in Cos-7 cells (negative control), the presence of A-V alone in supernatant was not sufficient to establish the infection. However, the use of A-V may lead either to development of other models of HBV infection or to extend it to other cell species, e.g., mouse, human etc.

These results should now allow the study of various aspects of the viral life cycle that could only be studied with the use of, e.g. primary hepatocytes, which are difficult to obtain and have limited growth characteristics, in term of retaining their hepatocyte specific functions. This cell line can therefore be considered both susceptible and permissive to HBV replication. The potential development of an in vitro culture system for HBV replication provided an opportunity to investigate molecular and cellular events in cloned cells.

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