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Hepatitis B virus and hepatitis C virus interaction in Huh-7 cells☆

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***Background/Aims*: Co-infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) increases the risk of development and the severity of chronic liver disease. Although dominant and suppressive effects of each virus over the other have been reported *in vivo*, *in vitro* studies of HBV/HCV co-infection have been limited to analysis of the effects of over-expression of HCV proteins on HBV replication.**

***Methods*: We have re-examined HBV/HCV interactions in Huh-7 cells following co-infection with cell culture-propagated HCV (HCVcc; genotype 2a) and a recombinant adenovirus vector capable of delivering a replication-competent HBV genome (AdHBV; genotype A).**

***Results*: While intracellular HCV RNA levels were significantly increased when cells were pre-infected with AdHBV, HCV replication and virion secretion were not altered by simultaneous infection with AdHBV or AdHBV superinfection of HCV-infected cells. Likewise intracellular and secreted HBV DNA levels and HBV promoter activities were either unchanged or modestly increased by HCVcc infection. Despite this, HCV E2 and HBsAg proteins colocalized extensively in co-infected cells suggesting shared stages in viral egress.**

***Conclusions*: These studies indicate that there is little direct interaction of HBV and HCV in co-infected hepatocytes and imply that indirect effects of host-viral interactions dictate viral dominance in HBV/HCV co-infected individuals.**

***Keywords*: HBV; HCV; Co-infection; Superinfection; Hepatocytes; Pathogenesis**

1. Introduction

Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are the major causes of serious liver disease worldwide. Furthermore, co-infection with both HBV and HCV is not uncommon and is frequently associated with increased risk of development of cirrhosis and hepatocellular carcinoma, compared with infection with either virus alone [1–4]. In this respect occult HBV infection, in particular, is often associated with accelerated progression of liver disease in HCV infected individuals [5]. While clinical studies have indicated that HCV dominates over HBV in co-infected individuals

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Abbreviations: HCV, hepatitis C virus; HBV, hepatitis B virus; GFP, green fluorescent protein; HBsAg, hepatitis B virus surface antigen; HBcAg, hepatitis B virus core antigen; ER, endoplasmic reticulum; HCVcc, cell-culture propagated HCV; HCVpp, HCV pseudoparticles.

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[6–9], it has also been reported that active replication of HBV can suppress the replication of HCV [10–12].

Despite evidence to suggest that both HBV and HCV can replicate in the same hepatocyte *in vivo* [13], there is little information regarding interaction(s) between the two viruses at the molecular level that may explain observations of viral dominance in the clinic. However *in vitro* studies have demonstrated that over-expression of HCV core or NS5A proteins suppresses HBV gene expression and replication [14–17], with the latter inhibiting HBV replication via its ability to activate phosphatidylinositol-3-OH kinase (PI-3K)/Akt signalling. However, the above observations should be interpreted with caution as HCV protein localization [18], and functional effects may differ when examined in the context of a complete virus lifecycle. It is therefore, crucial to study the interaction of HBV and HCV in the context of both replicating viruses to better understand pathogenic mechanisms associated with co-infection. Here we report that HBV and HCV can replicate concurrently and that replication and particle secretion of one virus are, for the most part, unaltered by co-infection with the other virus. This suggests that clinical observations of unidirectional or reciprocal suppression of viral replication in co-infected individuals may be largely attributable to host-derived factors and/or the host immune response, not a direct interaction between the two viruses.

2. Materials and methods

2.1. Plasmids

The plasmid pJFH1 [19] was the generous gift of T. Wakita (Tokyo, Japan). The plasmid pBB45HBV1.3 [20] consists of a 1.3-unit length HBV genome (genotype D, subtype *ayw*) contained within pBlueBac4.5 (Invitrogen; Carlsbad, CA). HBV promoter/enhancer regions were cloned into the luciferase reporter plasmid pGL3-basic (Promega; Madison, WI) and confirmed by automated sequencing (see Sup. Table 1). Plasmid pRL-HL (kindly provided by S.M. Lemon, Galveston, USA) is a bicistronic reporter construct that dictates translation of *Renilla* luciferase from a 5′ cap structure and *Firefly* luciferase from the genotype 1b HCV IRES element [21].

2.2. Cell culture

A Huh-7 cell line that supports HCVcc (JFH1) and HBV replication and the genomic HCV replicon cell line, NNeo/C-5B[RG] [22] (kindly provided by S.M. Lemon), were cultured as described [23].

2.3. Antibodies

Pooled (5 patients) human anti-HCV antiserum was inactivated by 10-fold dilution in PBS, addition of NP-40 to 1% (v/v) and incubation at 56 °C for 30 min. Human anti-E2 monoclonal antibody (MAb) CBH-5 [24], mouse anti-NS5A MAb 9E10 [25] and rabbit anti-HCV core antiserum (RR8) [26] were generous gifts from S.K. Foung (Palo Alto, USA), C.M. Rice (NY, USA) and M. Kohara (Tokyo, Japan), respectively. Mouse MAbs against the following antigens were purchased: HBsAg (Dako [3E7]; Carpinteria, CA), HCV core (Abcam [C7-50]; Cambridge, MA), calnexin (Abcam [AF18]), Golgin-97 (Molecular Probes [CDF4]; Eugene, OR), CD81 (BD Biosciences [JS-81]; San Jose, CA) and β-actin (Sigma [AC-15]; St. Louis, MO). Rabbit anti-HBcAg (Dako), rabbit anti-SR-BI (Novus Biologicals; Littleton, CO), biotinylated sheep anti-LAMP1 (R & D Systems; Minneapolis, MN) and biotinylated goat anti-green fluorescent protein (GFP) (Rockland Immunochemicals; Gilbertsville, PA) antibodies were purchased. Horseradish peroxidise (HRP)-conjugated and AlexaFluor (488, 555 and 594)-conjugated secondary antibodies were from Pierce (Rockford, IL) and Molecular Probes, respectively. Streptavidin-conjugates of HRP and FITC were from Amersham Biosciences (Piscataway, NJ) and BD Biosciences, respectively.

2.4. Generation of virus stocks and infection

HCV RNA was transcribed from pJFH1 *in vitro* [19] and transfected into Huh-7 cells using DMRIE-C (Invitrogen). Cell culture supernatants were harvested 5 d post-transfection and secondary viral stocks were prepared by amplification on naïve Huh-7 cells. Virus titration was performed as described [27]. AdHBV, which contains a 1.5 × unit length HBV genome (genotype A, subtype *adw2*) and a CMV promoter-driven GFP reporter, and AdGFP, which lacks the HBV genome, were propagated and titrated as described [28]. Experimentally, Huh-7 cells in 12-well trays were infected with HCVcc (MOI: 0.01, 0.02 or 0.04) and/or AdHBV or AdGFP (MOI: 1) in 0.25 ml of complete media for 2 h at 37 °C/5% CO_2. Monolayers were then washed twice with PBS and returned to culture for the indicated period (24–72 h) before subsequent infection or harvesting. Murine leukaemia virus (MLV)-based vectors for the generation of retroviral pseudoparticles bearing HCV glycoproteins (H77c; genotype 1a) were kindly provided by H.E. Drummer (Melbourne, Australia) and were used to generate HCV pseudoparticle (HCVpp)-containing tissue culture fluid as described [29]. Experimentally, subconfluent Huh-7 cells in 12-well trays were infected with undiluted HCVpp for 4 h at 37 °C/5% CO_2, washed with PBS and returned to culture for 3 d before measurement of β-galactosidase activity (Promega).

2.5. Indirect immunofluorescence microscopy

HCV-infected Huh-7 cells (3 d post-infection) or NNeo/C-5B[RG] cells were grown on 0.2% gelatin-coated coverslips before transfection with pBB45HBV1.3 using FuGene6 (Roche Applied Science; Mannheim, Germany). Two days later cells were fixed with acetone/methanol (1:1) for 15 min at 4 °C, washed with PBS and incubated for 30 min in PBS containing 5% BSA. Antibody incubations were performed at room temperature for 1 h using PBS/1% BSA as antibody diluent. Coverslips were mounted using SlowFade fluorescence mounting medium (Molecular Probes). Where combinations of antibodies were used, appropriate control experiments were performed to ensure minimal non-specific binding of antibodies. Confocal microscopy was carried out using an Olympus IX70 inverted microscope linked to a Bio-Rad Radiance 2100 confocal microscope. Samples were visualized using a ×60/1.4NA water immersion lens (2–3× zoom) and images were acquired sequentially for each fluorophore and processed using Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA).

2.6. Luciferase reporter assays

Dual-luciferase assays (Promega) were performed essentially according to manufacturer's instructions. Briefly, subconfluent Huh-7 cells were co-transfected with the indicated HBV promoter/reporter plasmid and pRL-TK (Promega; at a ratio of 20:1) or transfected with pRL-HL using FuGene6 (Roche), 18–24 h prior to determination of luciferase activity.

2.7. HCV RNA quantitation

Total cellular RNA extraction, first-strand cDNA synthesis and real-time RT-PCR was performed as described [23].

2.8. HBV DNA quantitation

Intracellular HBV cores were harvested from washed cell monolayers by incubation with 0.8 ml of cold lysis buffer (0.5% NP-40, 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA) for 15 min at 4 °C. Lysates were cleared of debris by centrifugation and treated with DNase I before