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NS5A protein of HCV enhances HBV replication and resistance to interferon response

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Abstract

HCV and HBV are the major causes of chronic liver diseases worldwide. Patients with both viruse's co-infection tend to develop severer liver diseases and are at high risk of liver-related death. NS5A protein of HCV plays key roles in HCV replication and inhibition of host immune responses. In this study, we described the establishment of HepG2-derived cell line that stably expresses NS5A protein and the application of a cellular system for HBV replication based on a recombinant adenovirus carrying HBV genome. Our results demonstrated that NS5A enhances the expression of S and E proteins of HBV, as well as the synthesis of viral DNA. Moreover, we showed that NS5A assists HBV to escape interferon responses. These data suggested that NS5A of HCV may employ multiple strategies contributing to the enhancement of HBV replication and interferon resistance during the co-infection of HCV and HBV.

Keywords: HBV; HCV; Co-infection; Interferon; HBV replication; Viral gene expression

Acute and chronic infections of HCV and HBV are leading causes of hepatic inflammation, necrosis, and hepatocellular carcinoma (HCC) worldwide [1–9]. Approximately 3–18% of patients with chronic HBV infection are also infected with HCV [7,15]. Clinical reports showed that patients diagnosed with HCV and HBV co-infection had increased risk of liver-related death compared to individuals infected with HCV or HBV with the mortality rates of 3.2%, 5.3%, and 7.1% for HBV infection, HCV infection, and HBV/HCV co-infection, respectively [8]. Patients with HCV and HBV co-infection face a higher mortality risk from continued drug use than from the infection [8,9].

Interferons, including IFN-α and IFN-β, regulate diverse biological processes including antiviral activities, cellular growth and differentiation, and immune functions [10–13]. Double-stranded RNA-activated protein kinase (PKR) stimulated by extracellular stresses including presence of dsRNA, virus infection, and expression of extrinsic genes plays key roles in antiviral and antiproliferative responses [17–19]. IFN-α is the most common medicine for patients suffering from HBV or HCV infection [14,25]. It is 25–50% effective in patients with chronic hepatitis B [15], and 20% effective in patients with chronic hepatitis C [16], and barely effective in individuals with HBV and HCV co-infection [7].

The non-structural protein 5A (NS5A) of HCV binds to the dimerization domain of PKR and abolishes its catalytic activity in host cells [20–27]. NS5A serves as an inhibitor of IFN-α-induced antiviral activity and thus, plays a major role in resistance to IFN treatment in patients with hepatitis C [20–35]. Influenza virus expressing the NS1 protein, a non-structural protein that binds to and inactivates PKR, is always co-infected with other microbes by depressing the induction of IFN and other immune factors [31–36]. The VAI RNA of adenovirus, as a mature bio-product, magnifies the expression of other extrinsic genes [37].

In this study, we attempted to address these clinical issues by establishing HepG2-derived cell line expressing

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NS5A and by applying a cellular assay for determining HBV replication based on a recombinant adenovirus carrying HBV genome. The roles of NS5A of HCV in the expression of HBs and HBe proteins of HBV and in the production of core-associated DNA of HBV were determined. In addition, the effects of IFN-α on the expression of HBs and HBe, and the production of viral DNA were also investigated. Our results suggested that NS5A may employ multiple strategies contributing to the enhancement of HBV replication and its resistance to interferon during HBV and HCV co-infection. These results have provided insights into the mechanism involved in the co-infection with HBV and HCV.

Materials and methods

Cell cultures and transfection. Hepatocellular carcinoma cell line HepG2 (from CCTCC) was maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (50 μg/ml) in 5% CO_2 at 37 °C. HepG2 was transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction at 80% confluence. Twenty-four hours post-transfection, cells were split at a 20:1 dilute and cultured in the selected medium containing 400 μg/ml of G418 (Merck). The cell culture medium was changed every 3–4 days. After 3–4 weeks of selection, G418-resistant colonies were isolated.

Semi-quantitative RT-PCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Total RNA extract was treated with DNase I (Promega) at 37 °C for 30 min. 1.0 μg of the total RNA was used as template in reverse-transcription reaction by MLV-RT (Promega) with an oligo-dT(18) at 42 °C for 60 min. MLV-RT was inactivated at 75 °C for 15 min. NS5A gene was amplified with the following reagents: 20 pmol forward primer (5′-GGAGGATGAGAGGGAAGTAT-3′), 20 pmol reverse primer (5′-TAGACCAAGACCCGTCGCTG-3′), 1 U Taq DNA polymerase (MBI). The PCR was performed in a Thermal Cycler 480 (Perkin-Elmer) under the following cycling conditions: heat activation of the polymerase for 5 min at 95 °C, followed by 32 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s; with a final extension at 72 °C for 10 min. To amplify the 447 bp of partial β-actin fragment, β-actin-specific forward (5′-TGAAGTGTGACGTGGACATCCG-3′) and reverse (5′-GCTGTCACC TTCACCGTTCCAG-3′) primers were used. PCR was performed by 18 cycles with the same conditions described above. The amplified products were electrophoresed on a 1.5% agarose gel.

Western blot. Cells were collected from 6-well plates, washed with cold phosphate-buffered saline (PBS), and then resuspended with PBS containing 0.1% Triton X-100, 0.01% EDTA, and 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma). The lysates were centrifuged at 12,000 rpm for 10 min after sonication. The supernatants were mixed with 4× loading buffer followed by boiling for 5 min. The concentration of protein was measured with a Bio-Rad Protein Assay kit II (Bio-Rad Laboratories). 50 μg of solubilized lysate proteins was separated on 10% SDS–PAGE gels and then transferred to Hybond-C nitrocellulose membrane (Amersham-Pharmacia). After blocking with 5% non-fat dried milk in PBS/Tween buffer, the blots were incubated with monoclonal antibodies anti-Flag (Sigma) for 2 h at room temperature and were subsequently incubated for 40 min with goat anti-mouse secondary antibody (Santa Cruz). Then, the membranes were incubated with enhanced chemiluminescence solution (Pierce) for 5 min and exposed to X-ray film (Kodak).

Virus infection. Cells were cultured at a density of 1×10^6 cells/well in 6-well plates. Prior to virus infection, cells were treated with or without recombinant human IFN-α (500 U/ml) for 24 h and then challenged with 5 m.o.i. of Ad-HBV per dish using the procedures described previously [38].

Assay for HBV protein expression. Forty-eight hours after infection, the level of HBs and HBe proteins in cell culture media was determined by enzyme-linked immunosorbent assay (ELISA) using HBV S antigen and HBV E antigen diagnostic kit (Shanghai KeHua Biotech Co. Ltd.), respectively.

Analysis of HBV DNA by real-time PCR. To detect the effect of NS5A on HBV replication with or without IFN-α, intracellular core-associated DNA of HBV was extracted as described previously [8]. Briefly, cells were lysed and centrifuged at 25 °C, and then magnesium chloride was added to the supernatant. DNA not protected by HBV core was digested with deoxyribonuclease (DNase I). After inactivating the DNase I, cell lysate was treated with proteinase-K and extracted with phenol/chloroform. Core-associated HBV DNA was recovered by ethanol precipitation and quantified by real-time PCR as described by the manufacturer (PG Biotech, Shenzhen, China). The HBV DNA in the supernatants was also quantified following the procedure provided by the manufacturer (PG Biotech, Shenzhen, China). Primers used in RT-PCR were: P1, 5′-ATC CTGCTGCTATGCCTCATCTT-3′ and P2, 5′-ACAGTGGGGAAAG CCCTACGAA-3′. The probe was 5′-TGGCTAGTTTACTAGTGCCA TTTTG-3′. PCR was carried out and analyzed by a PE Gene Amp 7700 (Perkin–Elmer).

Results

HepG2-derived cell line expressing NS5A protein was established

HepG2 cells were transfected with pCMV-tag2B-NS5A to establish cell lines that stably express the NS5A protein of HCV. Four transformants (HepG2/NS5A-1, HepG2/NS5A-2, HepG2/NS5A-3, and HepG2/NS5A-4) were obtained and further characterized. Results from semi-quantitative RT-PCR using NS5A specific primers showed that the NS5A mRNA was detected in HepG2/NS5A-1, HepG2/NS5A-3, and HepG2/NS5A-4, but not in HepG2/NS5A-2, while β-actin mRNA was detected in all cell lines (Fig. 1A). Similar results were obtained from Western blot analysis, which showed that NS5A protein was expressed in HepG2/NS5A-1, HepG2/NS5A-3, and HepG2/NS5A-4, but not in HepG2/NS5A-2, and β-actin protein was observed in all four cell lines (Fig. 1B). These results indicated that NS5A was stably expressed in HepG2/NS5A-1, HepG2/NS5A-3, and HepG2/NS5A-4. Since both NS5A mRNA and its protein were expressed at the highest levels in HepG2/NS5A-4, we used it as NS5A expressing cell line in this study.

NS5A enhances HBV protein expression and viral replication

To evaluate the effects of NS5A protein on HBV gene expression and viral replication, HepG2 and HepG2/NS5A were infected with recombinant adenovirus (Ad-HBV) carrying HBV genome. Forty-eight hours post-infection, the levels of S protein (HBs) and E protein (HBe) of HBV in the supernatant of cell cultures were measured by ELISA. Results showed that the level of HBs in HepG2/NS5A was 2.6-fold higher than that in HepG2 (Fig. 2A), while the level of HBe was increased by 5.2-fold in HepG2/NS5A comparing to that in HepG2 (Fig. 2B).

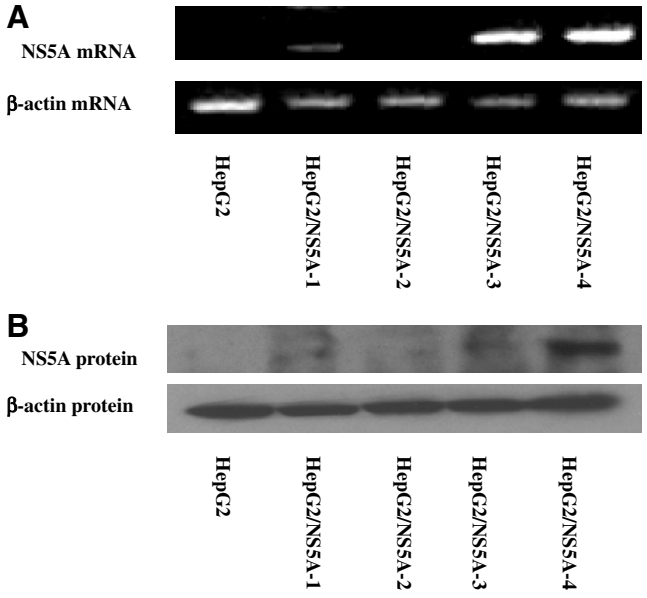

Fig. 1. Establishment of HepG2-derived cell lines that stably express NS5A. HepG2 cells were transfected with pCMV-tag2B-NS5A carrying NS5A gene to establish HepG2 cell lines stably expressing NS5A protein. Four transformants (HepG2/NS5A-1 to HepG2/NS5A-4) were characterized by semi-quantitative RT-PCR and Western blot analysis. (A) RT-PCR analysis: 1.0 μg of total RNA isolated from HepG2 cells and its derivatives was used as template in RT-PCR using primers specific to NS5A or to β-actin. (B) Western blot analysis: whole-cell extracts were prepared from HepG2 cells and its derivatives and equal amounts of extracts were resolved by SDS–PAGE. Proteins were detected by Western blot analysis using antibody to NS5A or to β-actin.

These results demonstrated that the expression of HBs and HBe proteins of HBV was stimulated by NS5A protein of HCV.

The above results also implied that NS5A of HCV has a stimulatory effect on HBV replication. To confirm such effect, the levels of intracellular core-associated DNA of HBV were determined by real-time PCR. Results showed that the level of HBV DNA was increased by 14.2-fold in HepG2/NS5A comparing to that in HepG2 (Fig. 2C). These results clearly indicated that NS5A of HCV can enhance HBV replication.

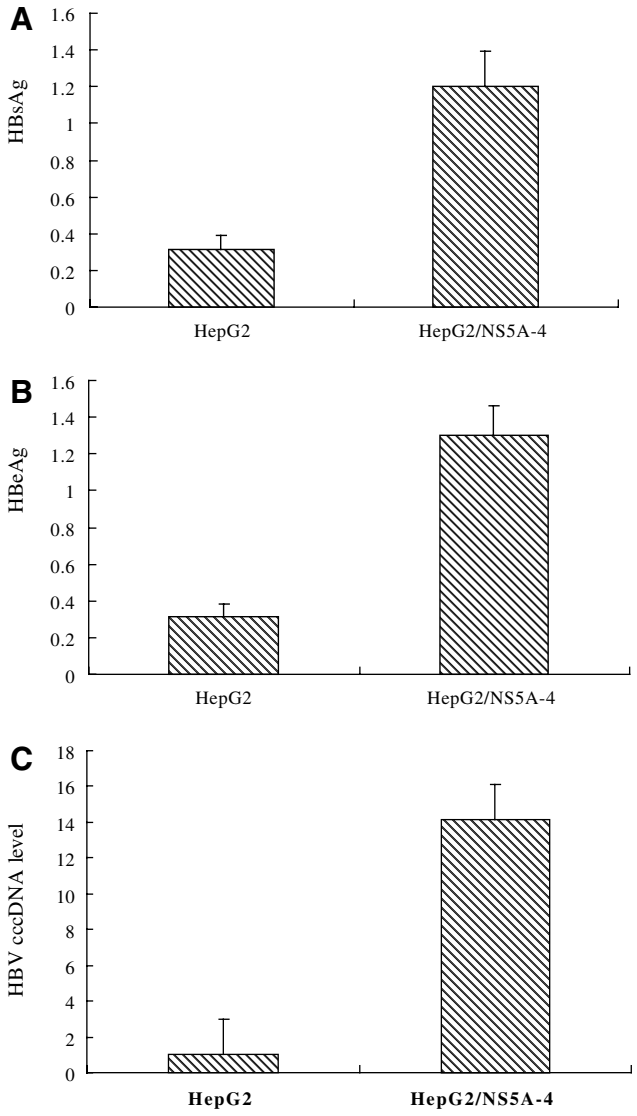

Fig. 2. Determination of the roles of NS5A in HBV gene expression and viral DNA replication. HepG2 and HepG2/NS5A were infected with Ad-HBV at 5 m.o.i and harvested 48 h post-infection. The expression of S protein (A) and E protein (B) of HBV in the supernatant of cell culture was measured by ELISA using diagnostic kits for HBV. The production of core-associated DNA (C) of HBV was determined by real-time PCR. Results represent means of three independent experiments, with derived standard errors shown.

NS5A eliminates response of HBV to IFN-α treatment

To evaluate the effects of IFN-α on the expression of S and E proteins of HBV in the presence of NS5A protein of HCV, HepG2 and HepG2/NS5A were treated with or without IFN-α and then infected with Ad-HBV. Results from ELISA analysis showed that the level of S protein was reduced by 2-fold in HepG2 treated with IFN-α comparing to that without treatment (Fig. 3A), while the level of S protein remained relatively constant in HepG2/NS5A in the presence and absence of IFN-α (Fig. 3B). Similar results were observed when the E protein of HBV was analyzed, which showed that the level of HBe was reduced by 2-fold in HepG2 cells with the treatment of IFN-α. However, IFN-α had no effect on the expression of HBe protein in HepG2/NS5A, where NS5A was present (Fig. 3B). These results suggested that NS5A inhibits the stimulatory effects of IFN-α on HBV protein expression.

The production of core-associated DNA of HBV in the presence or absence of IFN-α was also determined. Results from real-time PCR analyses showed that the level of HBV DNA in HepG2 was significantly changed from 1.80E + 06 (in the absence of IFN-α) to <1.00E + 03 (in the presence of IFN-α) (Table 1). However, the levels of HBV DNA in HepG2/NS5A were relatively unchanged (2.66E + 07 vs. 1.37E + 07) with or without IFN-α treatment (Table 1).

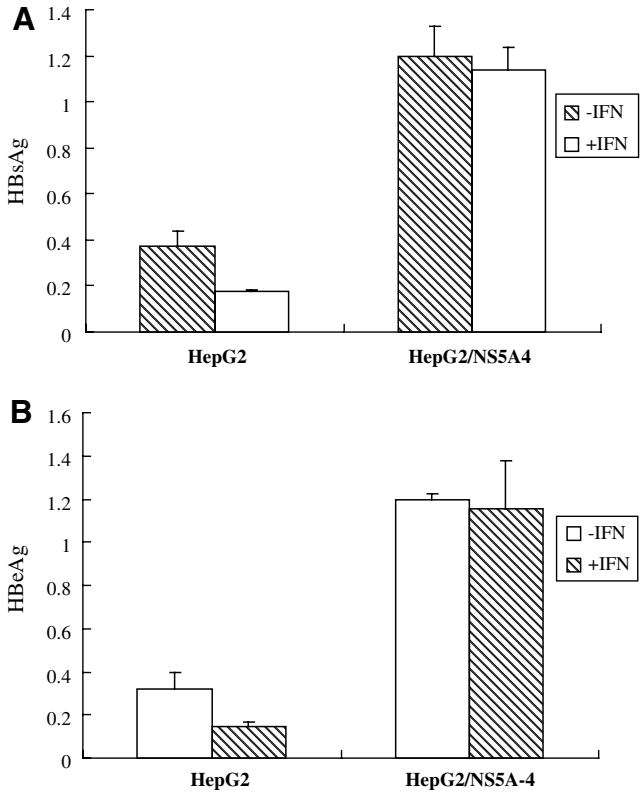

Fig. 3. Analysis of the effect of IFN-α on HBV gene expression regulated by NS5A. HepG2 and HepG2/NS5A were treated with IFN-α at 500 U/ml for 24 h, infected with Ad-HBV at 5 m.o.i., and then harvested 48 h post-infection. The expression of S protein (A) and E protein (B) of HBV in the supernatant of treated cells was measured by ELISA using HBV diagnostic kits. Results represent means of three independent experiments, with derived standard errors shown.

These results demonstrated that NS5A eliminated HBV response to IFN-α treatment in terms of viral replication.

Discussion

Epidemiological evidences revealed that patients infected with HBV or HCV, and co-infected with both viruses were facing higher risk of developing liver-related diseases than normal persons and co-infection of both viruses brought increased risk of liver-related death to patients [1–9]. In this study, we discovered that NS5A displayed stimulatory effects on the expression of S and E proteins of HBV and the synthesis of viral DNA. Thus, we provided the first evidence that NS5A protein of HCV enhances HBV replication, suggesting NS5A may be involved in the persistent HBV infection. We speculate that the effect of NS5A on HBV replication might be due to the anti-PKR activity or transcriptional activity of NS5A, but the detailed molecular mechanism involved in HBV replication regulated by NS5A remained to be determined.

INF-α has benefits for patients with chronic hepatitis B with elevated ALT and viral DNA levels, and causes a durable response in patients who lack resistance to the therapy [39]. However, IFN-α treatment for hepatitis C often led to sustained virological response (SVR) 6 months following completion of the treatment [40]. For the co-infection with both HCV and HBV, the most representative clinical problem was "serologically silent" HBV infection, which was correlated with impaired response to IFN treatment. It has shown that 4 out of 14 patients, who were chronically infected with HCV and with "serologically silent" HBV infection and treated with IFN-α, had normal ALT levels at the end of therapy, but all had relapsed within 6 month post-treatment [41,42].

NS5A is responsible for impairing IFN-responsiveness in patients [43,44]. Biochemical data showed that NS5A binds to and inactivates PKR, which resulted in the inhibition of both autophosphorylation of PKR and phosphorylation of exogenous substrates [18,20]. NS5A renders cells partially resistant to IFN and allowed growth of other viruses [27,29]. For these reasons, we investigated the effect of NS5A on HBV response to IFN-α treatment. We showed that HBV protein expression and viral DNA replication were significantly reduced under the treatment of IFN-α in the absence NS5A, but not in the presence of NS5A. Thus, we demonstrated that NS5A impaired the sensitivity of HBV to IFN-α response.

Patients co-infected with HCV and HBV face a higher mortality risk from continued drug use than from the infection per se [9]. Although IFNs were recognized first for their potent antiviral properties, they may also profoundly affect other vital cellular functions, including enhancement of histocompatibility antigens, pleiotropic hormone-like effects, stimulation of other inflammatory factors, and activation of a number of different effector cells [45]. It is noticeable that every drug has its harmful side effects. Under the condition of antiviral activity suppression triggered by NS5A, such harmful side effect of IFN is dominant, implying it is the main reason that patients with co-infection of HCV and HBV face a higher mortality risk from continued drug use.

Table 1
Effect of NS5A on HBV replication in response to IFN-α treatment

	HepG2	HepG2 + IFN-α	HepG2/NS5A	HepG2/NS5A + IFN-α
HBV cccDNA copies	1.88E + 06	<1.00E + 03	2.66E + 07	1.37E + 07

HepG2 and HepG2/NS5A were treated with 500 U/ml of IFN-α for 24 h, infected with recombinant adenovirus Ad-HBV at 5 m.o.i., and then harvested 48 h post-infection. The production of intracellular core-associated DNA of HBV was determined by real-time PCR.

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