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HBsAg blocks TYPE I IFN induced up-regulation of A3G through inhibition of STAT3

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ABSTRACT

Interferon (IFN) is a regularly utilized therapeutic for the treatment of chronic hepatitis B and appears to induce superior HBeAg seroconversion comparing nucleos/tide analogs. However, the mechanisms underlying IFN inhibition of HBV replication, as well as poor responses to IFN are unclear. Apobec3G has been reported to be involved in regulating HBV replication. In this study, we investigated Apobec3G expression and regulatory pathways during HBV infection. We show that over-expression of A3G leads to inhibition of HBV replication. We also show that IFN induces a significant increase in A3G protein expression, which is associated with STAT3 activation. We further show that A3G expression in HBV patients is lower compared to non-infected controls, possibly by HBsAg which inhibits IFN induced A3G up-regulation in a dose dependent manner. This process is likely mediated through inhibition of STAT3-Ser727 phosphorylation. The results presented in this study indicate that STAT3 plays an important role in IFN-induced A3G production, and HBsAg may correlated with poor response to IFN treatment.

1. Introduction

Hepatitis B virus (HBV) is a major cause of acute and chronic viral hepatitis worldwide [1]. World Health Organization (WHO) estimates that more than 2 billion people worldwide have been infected with HBV. Indeed, around 240 million people are chronically infected. Three quarters of the world's population live in areas with endemic HBV infection and every year, there are over 4 million acute presentations of HBV infection, despite HBV vaccination. Nearly 1 million people a year die from HBV infection related complications including severe chronic hepatitis, cirrhosis or primary liver cancer. Interferon (IFN) therapy is an approved therapeutic treatment of chronic hepatitis B. However, severe side effects associated with treatment, as well as the poor response to IFN therapy result in restrained clinical applications. There is a need to understand interaction between IFN and HBV replication to improve IFN treatment.

HBV contains a 3.2 kb, partially double-stranded, relaxed-circular DNA genome. HBV replicates through reverse transcription of an RNA intermediate, the pregenomic RNA (pgRNA) [2]. The HBV DNA genome contains four overlapping open reading frames, which encode core, envelope (surface), polymerase and X proteins. HBV covalently closed circular DNA (cccDNA), which resides in the nuclei of infected hepatocytes and functions as a transcription template, plays a key role in the viral lifecycle and is required for the persistence of infection [3].

Apobec3 family members, including A3A-H, are involved in innate defense against viral infection and they edit cytidines and convert them to uridines through deamination of single-stranded DNA (ssDNA) [4,5]. One of their members, Apobec3G (apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G, here after referred to as A3G), is a restriction factor that was reported to strongly inhibit HIV and HBV replication by inducing G-to-A hypermutation in viral DNA [6–8]. In addition, A3G may also inhibit HBV lifecycle by interacting with HBV core protein [9]. In order to evade A3G antiviral function, the virus adapts and inhibits antiviral gene expression. Viral infection factor (Vif), an accessory protein of HIV-1, can interact with A3G and form an ubiquitin ligase complex with Cullin5 (CUL5), Elongin B/C (ELOB/C) and CBFb that ubiquitinate and degrade A3G in infected cells [10–13]. Indeed, similar interactions occur in HBV infection. Although IFN induced abundant A3G expression [14]; A3G expression is also inhibited following HBV infection [15], which may lead to poor response to IFN treatment. However, the mechanism by which inhibition of

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A3G expression occurs due to HBV is still poorly understood.

STAT1 is thought to play a critical factor in ISGs(Interferon stimulated genes) production in response to IFN stimulation [16]. Indeed, it is interesting to note that the induction of A3G by IFN is STAT1 independent [17]. Few studies investigated transcriptional factors responsible for A3G induction by IFNα.

In the present study, we investigated factors involved in regulating A3G expression under IFN stimulation. We show that A3G induction by IFN is STAT3 dependent, and that A3G expression in HBV infected patients is reduced. We also demonstrate that HBsAg may result in the reduction in A3G expression.

2. Materials and methods

2.1. Cell culture, plasmids and reagents

Human embryonic kidney cell line HEK293T, HepG2.2.15 and HepG2 cells were maintained in DMEM (Invitrogen Life Technologies) medium containing 10% inactivated fetal bovine serum, penicillin (100 IU/mL) and streptomycin (100 mg/mL) at 37 °C with 5% CO_2. Antibodies against Tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, sc-53646). Antibodies against p-STAT3 (Y705 and S727), p-STAT1 (Y705), STAT3, and STAT1 were provided by Cell Signaling Technology (Danvers, MA). Primary antibodies anti-HA (Invitrogen, 715500), The antibody against GAPDH was supplied by Proteintech (Wuhan, China).

2.2. RNA extraction and quantitative PCR (Q-PCR)

Total RNA was extracted from cells using EasyPure RNA Kit (Transgen, China) and then converted to first-strand cDNA using TransScript First-Strand cDNA Synthesis SuperMix (Transgen, China). The housekeeping gene GAPDH was used as an internal control, and gene expression was quantified as previously described [18]. The primers sequences for qPCR are shown as follows: GAPDH: CGGATTTGGTCGTATTGGG, TCTCGCTCCTGGAAGATGG; HBVDNA: GAGTGTGGATTCGCACTCC, GAGGCGAGGGAGTTCTTCT; pgRNA: TCTTGCCTTACTTTTGGAAG, AGTTCTTCTTCTAGGGGACC. A3G: GCTTCTTGGACCTGGTTCCTT, AGGTGTCCCAGCAGTACTCA.

2.3. Immunoblotting

Immunoblotting was carried out as described previously [19]. Briefly, cells were collected and lysed by adding lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% NP40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT) on ice for 30 min and tapping tubes every 10 min. Protein concentration was quantified by Coomassie Plus™ protein assay Reagent (Thermo Scientific). The quantitation of immunoblotting band intensity was carried out with ChemiDoc™ XRS^+ Molecular Imager software (BioRad).

2.4. ELISA

Cells were mock transfected or transfected with A3G overexpression plasmid. After 48 h, cells were exposed to IFNα (10 ng/ml) or IFNβ (1000 U/ml) for 24 h. Supernatant was collected for analysis by ELISA (Kehua Shengwu, China).

2.5. CRISPR/Cas9 knockout

HepG2 cells were seeded in 24 well plate, 16 h later, two plasmids, one expressing Cas9 with STAT1 or STAT3 sgRNA and the other carrying a puromycin resistant gene were co-transfected into HepG2 cells using Viafect transfection reagent (Promega). At 36 h after transfection, cells were either selected by adding puromycin at a concentration of 2 μg/ml or collected for immunoblotting with specific STAT1 or STAT3 antibodies (Cell Signaling). Two days later, live cells were diluted into a 96 well plate at a density of 1 cell per well. Immunoblotting was performed again to determine the level of gene editing efficiency after the clonal expansion and DNA sequencing was performed to verify the edited genes.

2.6. Blood samples

Twenty HBV infected patients and twenty non-infected control individuals were enrolled in this study. Non-infected control individuals were chosen from our colleagues who did not have any diseases, 10 of whom were male and age ranged between 25 and 50 years old. Whole blood was withdrawn to prepare serum and PBMCs. The study protocol was approved by the IRB of Jilin University, the First Hospital.

2.7. Statistical analysis

The results were presented as mean ± SD, and differences were analyzed with Students t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Higher A3G expression was induced by type I IFN stimulation

IFN antiviral function is primarily mediated through induction of ISG expression that suppresses certain elements of the viral life cycle. In order to investigate Apobec3s expression following IFN stimulation, we examined a group of Apobec3s gene expression with quantitative PCR (qPCR) in HepG2 cells exposed to IFNα and β. Consistent with previous reports, A3G mRNA levels were significantly increased in the presence of IFNα or β. Increased mRNA levels were further supported by increased protein levels detected by Western blot (Fig. 1A, 1B).

3.2. STAT3 dependent induction of A3G up-regulation by IFN

Previously, A3G induction was reported to be STAT1 independent in response to IFNα stimulation. STAT3 has also been reported to play a role in hepatocellular carcinoma development [20]. We therefor sought to examine whether STAT3 was involved in A3G induction. After cells were treated with IFNα together with or without Stattic (STAT3 inhibitor), both STAT3(705) and STAT3(727) phosphorylation were inhibited by Stattic (Fig. 2A), and A3G was inhibited by Stattic at both mRNA and protein levels (Fig. 2B), which indicated STAT3 may participate in the regulation of A3G upon IFNα treatment. To confirm these findings, STAT1, STAT3 or both were knocked-out in 293T cells using the CRISPR/CAS9 system. A3G induction was indeed blocked in STAT3-knockout 293T and HepG2 cells (Fig. 2C and 2D). In contract, A3G remained up-regulated in the STAT1 knockout 293T cells (Fig. 2C).

3.3. A3G inhibited HBV replication

It has been shown that A3G overexpression inhibits HBV replication. In our system, A3G overexpression was induced in the transfected HepG2 cells, which resulted in a reduction of HBV DNA and pgRNA level in cells, and HBeAg and HBsAg level in the supernatant (Fig. 3A-D).