THE IMPACT OF HCV KNOCKDOWN ON STAT3 AND ADI1 EXPRESSION IN HUMAN CON1 CELLS

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ABSTRACT
Hepatitis C virus (HCV) is one of the leading causes of chronic liver disease besides, it is the main causes of liver-related morbidity and mortality. There are many host genes modulate viral infection and are an underappreciated target for antiviral therapy. From the important host genes playing role in HCV infection are STAT3 and ADI1 genes. In this study, siRNA targeting the 5'HCV (siHCV) was designed using siVIRUS software. This siRNA Human Con1 cells (Huh-7 expressing HCV con1 I377/NS3-3’ replicon). Cells were transfected with the siHCV siRNAs and the all star negative control at 10nM concentrations. RNA was isolated using Qiazol buffer (Qiagen, USA) according to manufacturer instruction. RNA was subsequently cleaned up using RNAeasy mini Kit (Qiagen, USA). HCV, STAT3, and ADI1 copy numbers were quantified using QuantiFast Sybergreen RT-PCR. The copy numbers were normalized to the house keeping beta actin gen. Con1 cell transfection with 10nM siHCV significantly reduced the viral replication as compared to negative control treatment. Consequently, HCV knockdown resulted in significant reduction in both STAT3 and ADI1 gene expression as compared to negative control treatment. The results from this study revealed the strong relation between STAT3 and ADI1 host genes and HCV replication. Consequently, they could be used as potential targets in the fight against HCV infection.

INTRODUCTION
Hepatitis C virus (HCV) is one of the leading causes of chronic liver disease, affecting more than 170 million individuals worldwide. It is one of the main causes of liver-related morbidity and mortality. The virus establishes a persistent liver infection, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinomas. HCV belongs to the genus Hepacivirus of the family Flaviviridae. HCV is a single-stranded, positive-sense RNA virus with a genome of approximately 9500 nucleotide. Host genes...
modulate viral infection and are an underappreciated target for antiviral therapy. From the important host genes playing role in HCV infection are STAT3 and ADI1 genes. Transducer and activator of transcription (STAT-3) is an oncogenic transcription factor that is activated upon tyrosine phosphorylation in response to extracellular signals, such as cytokines and growth factors[3]. Studies from animal models have shown that STAT3 plays an important role in cell proliferation, survival, and transformation in the liver [4]; however, the role of STAT3 in human liver disease is less clear. STAT proteins play an important role in the regulation of inflammatory responses by Antigen presenting cells (APCs). In monocytes, STAT3 is a particularly critical transcription factor in limiting excessive inflammatory responses. It is commonly activated via canonical JAK-STAT signaling where a cytokine or extracellular factor binds its cognate receptor, resulting in the auto-phosphorylation of JAK and subsequent tyrosine phosphorylation of STAT3. In addition to STAT-3 activation by tyrosine phosphorylation, Ser727 phosphorylation mediated by mitogen-activated protein kinases (MAPKs) contributes to its maximal transcriptional activity[1]. Knockout of STAT3 in macrophages dysregulates inflammatory responses and leads to severe colitis. In addition, macrophages and dendritic cells within the tumor microenvironment constitutively phosphorylate STAT3 and suppress the generation of potent anti-tumor T cell responses. Importantly, treatment with pharmacologic STAT3 inhibitors reverses the suppression. Recently, the activation of STAT3 has been shown to be responsible for inducing genes involved in the differentiation of myeloid-derived suppressor cells [5].

Human aci-reductonedioxygenase 1 (ADI1) is a member of the Cupin superfamily. It was found to bind to and inhibit the activities of membrane-type 1 matrix metalloproteinase protein which interact with the tight junction protein, claudin-1. Previously, a variant protein, named submergence-induced protein-like factor (Sip-L), consisting of ADI1 amino acids 64-179. It was reported that over-expression of human ADI1 in 293 cells (293-ADI1 cells) also supported HCV infection and replication. Using serum-derived HCV as an infectious source, enhanced cell uptake of HCV to a Northern blot detectable level was found in 293 cells over-expressing both CD81 and ADI1 (293-ADI1-CD81 cells)[6].

MATERIAL AND METHODS

Si RNA design

Si RNA targeting the 5’HCV (siHCV) was designed using siVIRUS software [7]. This siRNA was following three of guidelines of siRNA design [8-10]. The designed siRNA was blasted against the reference human mRNA database for excluding siRNAs with possible partial
alignments to human mRNA transcripts. The selected siRNA sequence was as follow: sense, gggggcgucuagcaggcguagu, antisense, acuacgggauagagccccc.

**Cell culture propagation**

Human Con1 cells (Huh-7 expressing HCV con1 I377/NS3-3’ replicon) (kind gift from Dr Charles Rice, USA) were used in siRNA transfection experiments. Cells were maintained in the proper conditions. The cells were cultured in DMEM medium supplemented with 100 IU/ml penicillin G sodium, 100 IU/ml streptomycin sulfate, 1% L-glutamine, 1% G418 and 10 % fetal bovine serum (FBS) at 37 ºC in a humidified incubator with 5 % CO2. The cells was be harvested after trypsinization (0.025 % trypsin and 0.02 % EDTA) and washed twice with Dulbecco's phosphate-buffered saline (DPBS). When the cell density reached approximately 80 %, cells were split for further culture. Experiments were conducted when the cells were in the logarithmic growth phase.

**siRNA transfection**

Cells were seeded in 24 well plate at a seeding density of 30,000 cells / well. Next day, cells were transfected with the siHCVsiRNAs and the all star negative control siRNA (Qiagen, USA) at 10nM concentrations. Hiperfect transfection reagent (Qiagen) was used for transfection. Breiefly, siRNA was diluted using serum free media and then 3ul of transfection reagent was added and incubated for 15 mins to form the transfection complex, after that this complex was added to the cells. All Stars negative control siRNA (Qiagen) was used as control. The media was changed 24 hours after transfection and the cells were incubated with complete media for another 72 hours.

**RNA isolation, clean up and Quantitative real time RT-PCR**

RNA was isolated using Qiazol buffer (Qiagen, USA) according to manufacturer instruction. RNA was subsequently cleaned up using RNAeasy miniKit (Qiagen, USA). HCV, STAT3, and ADI1 copy numbers were quantified using QuantiFastSybergreen RT-PCR. The copy numbers were normalized to the house keeping beta actin gene. 5’UTR primer sequence was as follow: Forward, 5’aactactgtcttcacgcagaa 3’; reverse, 5’ tgcctcgttgctaggtgata3’. The sequence for human Beta-actin was Forward, cctctggtgcattgctcct; Reverse, ggacagtatggctcttc.

Primers for STAT3 and ADI1 were provided by Qiagen, (USA). The RT and subsequent PCR cycling conditions were as follow, 50C for 10 mins, 95C for 5 mins, 60C for 30sec, then 95 for 15 seconds, the number of cycles were 40 cycles.
RESULTS
As demonstrated in fig (1), Con1 cell transfection with 10nM siHCV significantly reduced the viral replication as compared to negative control treatment. Consequently, HCV knockdown resulted in significant reduction in both STAT3 and ADI1 gene expression as compared to negative control treatment (fig 2,3).

Fig (1): HCV knockdown using siHCV in Con1 cells.
U, untreated Con1 cells; siNC, cells treated with all star negative control siRNA; siHCV, cells treated with siHCV siRNA. Data are represented as mean±SEM, *Significant difference as compared to negative control treatment, P<0.05.

Fig (2): The effect of HCV knockdown on STAT3 gene expression in Con1 cells.
U, untreated Con1 cells; siNC, cells treated with all star negative control siRNA; siHCV, cells treated with siHCVsiRNA. Data are represented as mean±SEM, *Significant different as compared to negative control treatment, P<0.05.
Fig (3): The effect of HCV knockdown on ADI1 Gene expression in Con1 cells.

U, untreated Con1 cells; siNC, cells treated with all star negative control siRNA; siHCV, cells treated with siHCVsiRNA. Data are represented as mean±SEM. *Significant different as compared to negative control treatment, P<0.05.

DISCUSSION

In the current study, HCV knockdown resulted in significant reduction in STAT3 and ADI1 gene expression in Con1 cells. These findings shade lights on the importance of these two genes in HCV replication. The exact signal HCV gene expression triggers in these intracellular events needs further extensive studies to elucidate the effects on host genes. Tacke et al., [5] suggested that HCV core-induced STAT3 activation plays a critical role in the alteration of inflammatory responses by APCs which leads to impaired anti-viral T cell responses during HCV infection. Once STAT-3 is activated in HCV-infected cells, it may regulate gene expression of survival factors to ensure an antiapoptotic environment in the cells, a situation favorable for oncogenesis[11]. STAT-3 exerts its growth-deregulating activity by activating the expression of cellular genes that are involved in cell cycle progression, such as fos, cyclin D1, myc, and pim-1, and by activating antiapoptotic proteins, such as Bcl-2 and Bcl-XL (3,11, 12, 13). The role of ADI1 gene expression in supporting viral replication showed many controversies. A variant protein, named submergence-induced protein-like factor (Sip-L), consisting of ADI1 amino acids 64-179, was found to support hepatitis C virus (HCV) infection and replication in 293 cells. However, Cheng et al., [6] found that transfection of HCV replicon RNA by electroporation into naïve 293 and 293-ADI1 cells revealed no difference in replication efficiency. Using the infectious J6/JFH chimera as an infectious source, the infectivity was compared between 293-ADI1-CD81 and Huh-7.5 cells, more infection foci were formed in the 293-ADI1-CD81 cells in the first round of infection. The results from this study support the strong relation between STAT3 and
ADI1 host genes and HCV replication. And hence, they could be used as potential targets in the fight against HCV infection.

REFERENCES


