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Interferon alpha Induced Intrahepatic pSTAT1 Inversely Correlate with Serum HCV RNA Levels in Chronic HCV Infection

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Abstract

Background—The Jak-STAT signaling of hepatitis C virus (HCV) infected hepatocyte is critical for the antiviral action of endogenously produced interferon (IFN) as well as exogenously administered interferon alpha (IFN- α). The activation of cellular Jak-STAT signaling by IFN- α results in the phosphorylation and nuclear translocation of pSTAT1 and pSTAT2 proteins to induce antiviral gene transcription. Clinical studies show that chronic HCV patients with high viral load show poor response to interferon alpha and ribavirin combination therapy.

Aim—We seek to determine whether the IFN- α induced activation of pSTAT1 and pSTAT2 in hepatocytes isolated from liver biopsy of patients chronically infected with hepatitis C virus could be related to the viral load.

Method—Hepatocytes were isolated from liver biopsies of 18 chronic HCV patients using the collagen digestion method. Induction of pSTAT1 protein in the isolated hepatocyte was measured after IFN- α treatment. The fold change in the levels of pStat1 in the cell lysates due to IFN-treatment was measured by Western blot analysis followed by densitometry analysis.

Results—Results of our study indicate that IFN- α induced pSTAT1 levels vary in chronically infected hepatocytes from chronic HCV patients. Semi-quantitative analysis of the pSTAT1 bands revealed a median induction of 7.4- fold in non-infected primary hepatocytes and 2.3-fold in chronic hepatitis C patients ($p < 0.001$). Total STAT1 levels were not significantly different between treated and untreated primary hepatocytes. We also found a significantly inverse correlation between the intrahepatic pSTAT1 inductions with the serum HCV RNA levels.

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Conflict of interest statement

The authors declare no conflict of interest.

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Conclusion—We have developed an antibody based Western blot detection method to measure intrahepatic pStat1 and pStat2 levels to assess the cellular response to exogenous IFN- α . Our results indicate that pStat1 activation is a good indicator to assess the level of HCV replication in chronic HCV patients.

Keywords

Chronic HCV infection; Liver biopsy; Jak-Stat Signaling; Interferon- α ; pStat1; pStat2

Introduction

Hepatitis C virus (HCV) infection is a worldwide public health problem (1–3). Most of the individuals infected with hepatitis C virus develop chronic liver disease that often progress to liver cirrhosis and hepatocellular carcinomas (4,5). The current standard of care for the treatment of chronic HCV infection includes the combination of pegylated IFN- α , ribavirin and one of the protease inhibitors. The overall sustained virological response of this combination therapy has improved significantly (6,7). The response rate of triple therapy has not been satisfactory among patients who are nonresponders to PEG-IFN and ribavirin (8,9). The mechanisms underlying the resistance to IFN-treatment are not understood. Several viral and host related factors are associated with negative treatment responses such as high viral load of more than 600,000 IU/ml, virus genotype, age, sex, race, obesity, presence of insulin resistance, presence of liver fibrosis, pre-activation of endogenous interferon system in the liver, and the IL-28B genotype (10,11). In addition to these clinical parameters, the interaction of viral and host cellular protein also modulates the treatment response (12).

Interferon alpha binds to type I IFN-receptor (IFNAR1 and IFNAR2) which activates the receptor associated Jak-kinase leading to the phosphorylation of Stat1 and Stat2. The phosphorylated Stat1 and Stat2 proteins along with IRF9 translocate to the nucleus where they bind to the promoter element of interferon inducible genes to initiate antiviral gene transcription (13,14). There are number reports suggesting that HCV can modulate the cellular Jak-Stat signaling by number of mechanisms (15–20). The importance of cellular Jak-Stat signaling in the interferon alpha antiviral response has been confirmed in our laboratory using stable sub-genomic HCV replicon cell lines as well as infectious HCV cell culture system. We showed that HCV replicon cell culture system with a defective Jak-Stat signaling due to expression of truncated interferon alpha chain 1 of the type I IFN receptor is resistant to interferon alpha. Over expression of wild type IFNAR1 restored the IFN-alpha sensitivity and antiviral response. (21). We also showed that interferon alpha resistance mechanisms of HCV infected cell culture is also related to the defective Jak-Stat signaling due to the selective degradation of IFNAR1 chain of the type I IFN-receptor (22). Results of these published reports have indicated the Jak-Stat signaling pathway of infected cell is critical for HCV antiviral response to exogenous added or endogenously produced interferon. The significance of these cell culture results needs further validation using infected human hepatocytes.

The primary aim of this study is to measure the Jak-Stat signaling in human hepatocytes that are chronically infected with HCV. We have developed a method to measure the

intracellular pStat1 and pStat2 levels by Western blot using hepatocytes isolated from liver biopsies. Our results show that a low level of pStat1 is detectable in most of the chronically infected human hepatocytes. There are some differences in the induction of pStat1 level in the infected human hepatocytes as compared to uninfected primary human hepatocytes. The IFN- α induced pStat1 activation varies among chronic HCV patients. We found that the activation of Jak-Stat signaling measured by the induction of pStat1 in an infected human hepatocyte is inversely correlated with serum HCV RNA titer.

Materials and Methods

Patients

The study was performed after receiving IRB approval from the office of the Senior Vice President of Tulane University Health Sciences Center, New Orleans, LA. This study was performed using 18 chronic hepatitis C patients attending the Tulane Liver Clinic. All the patients were informed about the purpose of this study. A serum antibody to HCV was detected by an enzyme linked immunosorbent assay (ELISA) and serum HCV RNA levels were quantified by real-time RT-PCR assay.

Interferon alpha sensitivity of hepatocytes isolated from liver biopsy

Liver biopsy specimens were obtained from the operating room using a sterile container containing DMEM with 10% fetal bovine serum. Hepatocytes were isolated from liver biopsy using a standard protocol described earlier (23,24). Briefly, liver tissue was transferred to a 1.5 ml sterile ependroff tube in DMEM with 10% FBS and then digested with 0.04% collagenase P (Roche) for 15-minutes at 37°C. After digestion cells were passed through a 70- μ m nylon cell strainer (BD Falcon, Bedford, MA) to obtain a single cell suspension. Hepatocytes were enriched by low speed centrifugation (50Xg) for 3 minutes. The viability of hepatocytes was determined by trypan blue exclusion assay (25). The total number of hepatocytes isolated from a liver biopsy was divided into two parts one half was treated with interferon- α (1000 IU/ml) for 30 minutes and other half left untreated.

Western blotting for pStat1 and pStat2

Hepatocytes were lysed in ice-cold RIPA lysis buffer (21). Total protein in the lysate was quantified using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Approximately 10 μ g of protein from each sample was mixed in 4X SDS-loading buffer. Proteins were separated by NuPAGE 12% gel and then transferred onto a nitrocellulose membrane (GE healthcare, Buckinghamshire, UK). The membrane was blocked with 5% fat-free milk powder in 50mM TBS pH 7.6 with 0.1% Tween-20 (TBS-Tw20) at room temperature for 1 hour. The membrane was washed three times and incubated overnight at 4°C with either mouse monoclonal antibody to pStat1 or pStat2 at 1:500 dilutions (Cell Signaling, MA) or a mouse monoclonal anti-Beta-actin at 1:1000 dilutions in TBS-Tween 20 containing 5% fat-free milk powder. After this step, the membrane was washed three times with TBS-Tween 20 and reacted for one hour with the secondary antibody (anti-mouse supplied in the ECL kit) that was conjugated with horseradish peroxidase (HRP) at a dilution of 1:2000. The bound antibodies were detected using the ECL Plus Western blotting detection system (GE

healthcare, Buckinghamshire, UK) and the chemiluminiscent signals were detected using high performance chemiluminescence film (GE healthcare, Buckinghamshire, UK).

Immunolocalization of pStat1 in primary human hepatocytes: Hepatocytes were washed in PBS then incubated with PBS containing 0.05% saponin for two minutes then dipped in 100% ice cold methanol for 5 minutes. Then the slides were blocked with 1 ml of blocking reagent (Background Sniper, Biocare Medical, BS966) for 10 minutes at room temperature. The slides were then incubated with a primary mouse monoclonal antibody pStat1 at 1:50 dilutions (Cell Signaling) at room temperature for 1 hour, followed by a secondary reagent mouse probe (MACH 4 mouse probe, UP534) for 10 minutes, then tertiary reagent HRP polymer (Mach 4 HRP polymer, Biocare Medical, MRH534) for 20 minutes. At the final step, the slides were counterstained with hematoxylin.

Statistical analysis

Statistical analysis was performed using Student's t-test, x2 test and Fisher's test. Any p value <0.05 was considered significant.

Results

Interferon alpha induced Jak-Stat signaling in Primary Human Hepatocytes

Interferon alpha binds to type I IFN cell surface receptors (IFNAR1 and IFNAR2) result in the activation of Jak1 and Tyk2 kinases. The activated kinases phosphorylate Stat1 and Stat2 proteins. The phosphorylated Stat1 and Stat2 along with IRF9 form complex which then translocate to the nucleus to initiate antiviral gene transcription (Fig. 1). The activity of cellular Jak-Stat signaling has been linked to interferon alpha and ribavirin treatment response of chronic HCV infection. However most studies have been carried out using Huh-7 based cell culture system. We first examined whether the cellular Jak-Stat signaling can be assessed in a primary human hepatocytes after interferon alpha stimulation. For this purpose, commercially available uninfected primary human hepatocytes were treated with IFN-alpha (1000 IU/ml) for 30- minutes and then phosphorylation of Stat1 and Stat2 was examined by Western blot analysis. Interferon alpha stimulated phosphorylation of Stat1 and Stat2 that can be reliably measured by Western blotting (Fig. 2A). Phosphorylated Stat1 protein either homodimerize or heterodimerize with Stat2 that results its nuclear translocation to initiate antiviral gene transcription. The nuclear translocation of pStat1 in primary hepatocytes culture was examined by fluorescence microscopy (Fig. 2B). Immunohistochemical analysis showed a very strong pStat1 staining in the nucleus of most of the primary human hepatocytes (Fig. 2C). These results suggest that the cellular Jak-Stat signaling of primary human hepatocytes can be reliably assayed using antibody based staining of pStat1 or pStat2.

Interferon alpha sensitivity of HCV infected human hepatocytes isolated from liver biopsy

Cell culture studies indicate that the cellular Jak-Stat signaling and pStat1 levels are significantly reduced in Huh-7 cells infected with cell culture grown HCV particles (22). The Jak-Stat signaling of infected hepatocytes isolated from chronic HCV patients has not been investigated to validate these results. We summarized the method that was developed

to isolate small amount of viable hepatocytes from liver biopsies and measure the activity of cellular Jak-Stat signaling in vitro (Fig. 3A). Hepatocytes isolated by this method are viable and 90% pure does not have any contaminating red blood or blood lymphocytes (Fig. 3B). The interferon alpha stimulated pStat1 and pStat2 levels can be measured by Western blot analysis (Fig. 4A). Low level of pStat1 activation was detected in most of HCV infected human hepatocytes without interferon alpha treatment. We are able show that interferon alpha induced pStat1 levels vary among the HCV infected human hepatocytes isolated from liver biopsies. A semiquantitative analysis of the phosphorylated pStat1 band in primary hepatocytes was approximately 7.4- fold induction as compared to HCV infected hepatocytes with an average of 2.3 fold. In contrast pre-activation of pStat2 was not observed in any of the HCV infected human hepatocytes without interferon alpha treatment. Interferon alpha induced pStat2 activation was lower in chronically HCV infected liver as compared to uninfected primary human hepatocytes. Variation in the interferon alpha induced pStat2 activation was observed in HCV infected human hepatocytes.

Interferon alpha induced intrahepatic pStat1 levels inversely correlate with viral load

Since the cellular Jak-Stat signaling is critical to the host cellular response to infection to exogenous and endogenous interferon. We selected to correlate the intrahepatic induction of pStat1 detection by Western blot method with viral load using a group of chronic HCV patients. Induction of pStat1 level of each patient was measured by Western blot analysis with or without interferon alpha treatment. The fold induction of pStat1 due to interferon alpha treatment was determined by measuring the intensity of Western blot band before and after treatment. The fold induction values were compared with serum HCV RNA titers. The correlations of serum HCV RNA level with the fold induction of pStat1 in all chronic HCV patients found to be inversely correlated and the results are statistically significant (Fig. 4B). The differences are not due to inadequate amount of protein available in the lysate used in the analysis since all the samples were normalized to beta-actin levels.

Discussion

IFN- α binds to the cell surface receptor that activates cellular Jak-Stat signaling and phosphorylation of Stat1 and Stat2 proteins leading to antiviral gene transcription resulting in the inhibition of HCV replication. Using replicon as well as infectious cell culture model we found that the activity of cellular Jak-Stat signaling is critical for responding to interferon alpha treatment. Chronic HCV patients with high baseline serum HCV RNA levels have been shown to be one of the risk factors for response to interferon and ribavirin even in combination with new FDA approved protease inhibitors (26). The contribution of cellular Jak-Stat signaling in the antiviral treatment of interferon-alpha in infected human hepatocytes was investigated. We developed an antibody-based test that can determine the cellular response to exogenous IFN- α by examining the pStat1 and pStat2 levels by Western blot analysis. This test can be done within a couple of hours by using hepatocytes isolated from liver biopsy. Initially, we focused on testing the IFN- α induced Stat1 and Stat2 phosphorylation as measure of the activity of cellular Jak-Stat signaling to understand its role in HCV replication. This is because Stat activation relates to the biological response to endogenous as well as exogenous IFN- α (IFN-alpha), IFN- γ (IFN-gamma) and IFN- λ (IFN-

lambda) (27). In humans, the defect in IFN-gamma-Stat1 axis have been linked with increased susceptibility to mycobacterial infection (28,29). In addition, HCV viral proteins have been shown to block the Stat1 phosphorylation, nuclear translocation, nuclear export, directly degrade Stat1 protein that resulted in a defective Jak-Stat signaling of infected hepatocytes.

We first showed that cellular Jak-Stat signaling could be activated by treatment of exogenous IFN- α that leads to phosphorylation of Stat1 and Stat2 protein using primary human hepatocytes. The phosphorylation of Stat1 and Stat2 can be reliably detected by Western blot analysis. Immunofluorescence as well as immunocytochemical staining confirmed the nuclear translocation of phosphorylated Stat1. The immunocytochemical detection of nuclear pStat1 appears to be more sensitive than the fluorescence microscopy. Using hepatocytes isolated from chronic HCV patients, we showed that low level of pStat1 is detected most of the infected hepatocytes and the level of activation varies among patients. The preactivation of Stat1 proteins in the infected hepatocytes could have been due to either intracellular virus replication or due to infected cells exposed to low level endogenously produced interferons. We found that induction of pStat1 by exogenous interferon varies among chronic HCV patients. These results are in agreement with a previous report (30). Our results show that pStat2 is more specific in terms of determining interferon alpha sensitivity as compared to pStat1 since we could not detect pre-activation of Stat2 protein in any of the patients infected with HCV.

To validate the significance of pStat1 level with HCV replication we compared the fold induction of pStat1 with the HCV RNA titer of 18-chronic HCV patients. The results show that induction of pStat1 is inversely correlated with HCV RNA levels in the serum. These results can be explained by two different ways, first it is possible that high level replication of HCV in vivo degrade the IFNAR1 leading to the suppression of the cellular Jak-Stat signaling. Second possibility is that defective Jak-Stat signaling induced due to other mechanisms could also show reduced Stat1 phosphorylation and high level HCV replication. We could not perform such comparison with pStat2 since not all patients could show Stat2 induction in their hepatocytes. In summary, we show evidence that cellular Jak-Stat signaling and phosphorylation of Stat1 and Stat2 is also altered in hepatocytes chronically infected with HCV. The low level activation of Stat1 protein shows a negative correlation with HCV viral load. This may be one of the reasons why patients with high viral load respond less to standard interferon alpha and ribavirin therapy.

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Figure 1.

Schematic diagram showing how IFN- α binding to its cell surface receptors (IFNAR1 and IFNAR2) activates Jak-Stat signaling and antiviral response against hepatitis C. IFN- α binding to the receptor phosphorylates Jak1 and Tyk2 kinases. The kinases phosphorylate Stat1 and Stat2 proteins. The Stat1 and Stat2 along with IRF9 translocate to the nucleus to initiate antiviral gene transcription that inhibit HCV replication in the infected hepatocytes.

Figure 2.

I IFN- α treatment of primary human hepatocytes activates cellular Jak-Stat signaling. A. Western blot analysis shows IFN- α induced phosphorylation of Stat1 (left panel) and Stat2 proteins (right panel). The Western blot assay was normalized with beta actin level in the protein extract. B. Shows the immunofluorescence for localizing pStat1 in the nucleus of primary hepatocytes without or without IFN- α (1000 IU/ml) treatment. Primary human hepatocytes were treated with IFN- α (1000 IU/ml) for 30 minutes at 37°C and then washed and immobilized onto a glass slide by cytospin method. Immunofluorescence staining was performed using a monoclonal antibody to pStat1 (Cell signaling) using a standard protocol. C. Shows the immunocytochemical staining of nuclear pStat1 using horseradish peroxidase (HRP) conjugated secondary antibody in uninfected primary human hepatocytes without or with IFN- α (1000 IU/ml) treatment.

Figure 3.

A. Outline of method used to measure IFN- α sensitivity of infected hepatocytes isolated from liver biopsy of chronic HCV patients. Hepatocyte suspensions were treated with IFN- α (1000 IU/ml) for 30-minutes and then hepatocytes were centrifuged and the cell pellet was lysed. Western blot analysis for pStat1 was performed by using a method described in our publication. The band intensity was measured by Computer software B. Shows light microscopy picture the hepatocytes isolated by this method.

Figure 4.

Correlation of intrahepatic pStat1 with serum HCV RNA levels of 18- chronic HCV patients. A. Western blots showing level of pStat1 and pStat2 protein in IFN-stimulated infected human hepatocytes of five chronic HCV patients. Left panel shows that preactivation of pStat1 and pStat2 in the infected hepatocytes. Right panel show IFN- α induced activation of pStat1 and pStat2. pStat2 level was not detectable in any patients without IFN-alpha addition. IFN-alpha induced pStat2 level in all patients. The levels of beta actin in the protein extracts were comparable. B: Shows an inverse correlation between the fold induction of intrahepatic pStat1 with serum HCV RNA titer in patients chronically infected with hepatitis C.