SOCS3 and IRS-1 gene expression differs between genotype 1 and genotype 2 hepatitis C virus-infected HepG2 cells

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Abstract

Background: The poor response to antiviral treatment of hepatitis C virus (HCV)-infected patients with genotype 1b has been associated with a higher prevalence of metabolic syndrome. However, the molecular link between these clinical entities is not clear. The goal of this study was to clarify the role of genotype 1b and 2 in the genetic expression of suppressor of cytokine signaling 3 (SOCS3) and insulin receptor substrate 1 (IRS-1).

Methods: We infected human hepatocellular carcinoma cell line (HepG2) cells with human HCV genotype 1b or 2 and measured the gene and protein expression of SOCS3 at various times. We also evaluated impairment in the insulin pathway by analysis of IRS-1 and phospho-AKT. For the control, we used HepG2 cell cultures treated with non-infectious serum. We also demonstrated the occurrence of HCV infection by the detection of both positive and negative strands in the cells and culture medium. To test infection of the HepG2 cells, we performed quantitative real-time polymerase chain reaction (qRT-PCR) of viral load at different time points. We analyzed the viral genotype in the pellet and supernatant.

Results: At each time point, we found positive and negative strands in the infected cells, while in the medium we found positive, but no negative strands. We also detected the presence of the correct genotype in the medium. Two weeks following infection when the viral load was higher, we tested genotype 1b and 2 infected cells. SOCS3 gene expression was significantly higher in genotype 1b-infected cells (median 2.56; mean 2.82±0.59) compared with genotype 2 (median 1.34; mean 1.46±0.31) (p = 0.04) and significantly lower in genotype 1b-infected cells (median 1.09; mean 1.02±0.11, p = 0.02).

Conclusions: Up-regulation of the SOCS3 gene might be one of the mechanisms governing non-response to therapy and expression of insulin resistance mediated via a direct mechanism at this level of genotype 1b HCV.


Keywords: hepatitis C virus; insulin receptor substrate 1 (IRS-1); suppressor of cytokine signaling 3 (SOCS3).

Introduction

Therapy with peginterferon α and ribavirin is the standard of care for hepatitis C virus (HCV)-related chronic hepatitis (1, 2). This treatment results in a high percentage of sustained response and depends primarily on the type of infection; 90% in genotype 2 viral infection and 50% in genotype 1 viral infection (3). We previously identified a link between the response to antiviral treatment of HCV-related liver disease and a variety of genes, some of which are up-regulated and others down-regulated (4). Recently, we found that the suppressor of cytokine signaling 3 (SOCS3) gene, which affects the insulin pathway, was over-expressed in non-responding patients with genotype 1b HCV compared with patients with genotype 2 HCV. Also, the clinical expression of insulin resistance was significantly higher in patients with genotype 1 (5). Alterations in the insulin receptor substrate 1 (IRS-1) may induce insulin resistance via defects in intracellular transduction signaling (6).

SOCS3 expression has been found to be increased in liver tissue from patients with chronic genotype 1 HCV infection who did not respond to treatment (7). Huang et al. (8) confirmed this finding in cell cultures and chimpanzees. They postulated that HCV affects SOCS3 expression by stimulating cytokines, such as TNF-α and IL-10. Subsequently, we showed that up-regulation of SOCS3 in non-responding patients is...
related to a peculiar polymorphism of the gene, namely, –4874 A/G (9).

The goal of this study was to verify the in vitro effect of HCV on SOCS3 overexpression in order to determine whether the level of SOCS3 expression differs between infection with HCV genotype 1 and 2, and whether this difference might interfere with the protein concentrations of IRS-1.

**Materials and methods**

**Viral inoculation and sample collection**

Human hepatocellular carcinoma cell line (HepG2) culture and infection were performed as described previously (10–12). For infection of serum, 50 μL of patient serum was diluted in 1.5 mL of medium and added to cells plated in 35 mm dishes. Following an overnight incubation, the cells were rinsed 6–10 times with phosphate buffered saline (PBS) and 2 mL of fresh growth medium was added. The final wash was collected for HCV testing (shown as time 0 in the Figures). Cells were grown for 48 h to semi-confluence in complete medium, washed twice with fetal bovine serum (FBS)-free medium, then inoculated with serum samples from four patients infected with HCV genotype 1b, four patients with HCV genotype 2 [real-time polymerase chain reaction (RT-PCR) and antibody positive], and four healthy subjects who served as negative controls (500 μL serum and 500 μL FBS-free Dulbecco’s modified Eagle medium [DMEM]/3×10⁶ cells). The HCV genotypes were characterized as genotype 1b and genotype 2 as previously reported (5, 13). The viral load in sera was quantified using RT-PCR, and the average copy number was 1.9×10⁵ copies/L. After 90 min, DMEM (GIBCO-Invitrogen, Milan, Italy) containing 10% FBS (Celsio, Milan, Italy) was brought to a final volume of 8 mL, including the volume of human serum used for infection as reported above. Cells were maintained overnight at 37°C in 5% CO₂. The following day, adherent cells were washed three times with culture medium to remove any remaining serum, and cell growth was continued for 4 weeks in complete medium containing 10% FBS, with a change of medium every 3 days. Cells and supernatant were collected on day 3, and 1, 2, 3 and 4 weeks after inoculation with HCV. The cellular level of viral infection was quantified at the various time points with RT-PCR amplification of sense and antisense strands. Genotyping analysis was also performed at these same points and genotypes were tested on the cells and culture medium.

**Extraction and quantification of HCV-RNA**

HCV-RNA was isolated from HepG2 cells using the guanidinium isothiocyanate method with TRIzol reagent (Invitrogen, Milan, Italy) (14), and from 200 μL of culture medium and 200 μL of serum using High Pure Viral RNA Kit (Roche Diagnostics, Milan, Italy).

Extracted RNA was amplified and quantitated using sequence-specific hybridization probes with the Reaction Mix LightCycler RNA Master HybProbe (Roche Diagnostics, Milan, Italy) with the LightCycler system (Roche Diagnostics, Milan, Italy). The amplification primers are located in the highly conserved 5′ untranslated (UTR) region of the HCV genome, which identifies an amplicon of 244 bp. Their sequences are: sense 5′-CTC gCA AgC ACC CTA TCA ggC gAgT-3′ and antisense 5′-gCA gAA AgC gTC TAg CCA Tgg CgT-3′ (TIB MOLBIOL, Berlin, Germany).

The sequences of hybridization probes are: the donor probe 5′-gCA gCC TCC Agg ACC CCC C-3′ labeled with 5,6-carboxyfluorescein attached to 3′-O-ribose. The adjacent acceptor probe 5′-CCC ggg AgA g CC ATA gTg gTC Tg-3′ is labeled with LightCycler Red 640 attached to the 5′ terminus (both from TIB MOLBIOL, Berlin, Germany). Following hybridization to the template DNA, the two probes are in close proximity resulting in fluorescence resonance energy transfer (FRET) between the two fluorophores. The emitted fluorescence of the LightCycler Red 640 is then measured with the LightCycler Instrument. The RT-PCR reaction was performed in a final volume of 20 μL, with 8 μL of RNA. The thermal profile was 30 min at 61°C, 95°C for 30 s, followed by 45 cycles of 1 s at 95°C, 10 s at 56°C, and 1 s and 13 s at 72°C. An external standard curve to quantify the HCV genomes present in the sample was constructed using a control target 5′UTR fragment cloned from patients infected with HCV (15).

The assay targets the 5′-UTR region, and the primers are also used in the Amplicro system. The detection limit of the assay was 10 IU/mL, corresponding to ~25 copies/mL (according to the WHO, 1 IU corresponds to 2–5 genome-copies, depending on the HCV-RNA method).

**PCR on HCV genomic RNA strands**

Extracted RNA was analyzed using a strand-specific reverse transcriptase (RT)-nested PCR assay with Tth DNA Polymerase for positive and negative strands of HCV-RNA. In the

![Figure 1](image-url)
Viral load in HepG2 cells performed with qRT-PCR. Monitoring of infection in HepG2 cells treated with HCV infected sera of both genotype 1b and genotype 2. Viral levels were monitored periodically showing fluctuations in viral load ranging from 80 copies/mL to $6.7 \times 10^6$ copies/mL.

Figure 3 Genotyping of HepG2 cells and culture medium with Inno Lipa 2.0 Kit. RNA extracted from HepG2 cells and culture medium was used for genotyping. Using reverse primer P1 in the RT step, cDNA copies were generated from viral RNA. The cDNA was amplified using PCR with outer primers in the first round and inner primers in the second round contained in the HCV Genotype Amplification kit (LIPA – SIEMENS, Bayswater VIC, Australia). DNA products were amplified and biotinylated in the Versant HCV genotype assay (LIPA – SIEMENS, Australia) (18).
RNA isolation, RT-PCR and quantitative real-time (qRT)-PCR analysis of SOCS3, Mx-A and IRS-1

Total cellular RNA was extracted from the cultured cells using the guanidinium thiocyanate method with the TRIzol reagent (Invitrogen, Milan, Italy) (19). All RT reactions were performed using the iScript™ cDNA synthesis kit (BioRad, Milan, Italy), according to the manufacturer’s instructions. qRT-PCR was performed using the SYBR Green PCR Master Mix with the Applied Biosystem Model 7900HT Sequence Detection System. The primers were designed by the Primer Express 2.1 program (Applied Biosystems, Branchberg, NJ, USA). Primer sequences are as follows: SOCS3, forward 5’-CTTTCTGATCCGCGACAGCT-3’, reverse 5’-TGGTCCCAGACTGGGTCTTG-3’; Mx-A, forward 5’-GGAGAGATCTTTCAGCACCTG-3’, reverse 5’-TGGATGATCAAAATGGGATGTGG-3’; IRS-1, forward 5’-GCCAGAGACGGGTCGCTAGCT-3’, reverse 5’-AGGATTTGCTGAGGTCTTATGTTAGGT-3’. All PCRs were performed in duplicate. β-Actin (forward 5’-CGTGCTGTCGACCGAGG-3’, reverse 5’-GAAGTTGCTCAAA-CATGATCTGGGT-3’) was used as internal control.

Western blotting

Harvested cells were washed three times with ice-cold PBS and homogenized with ice-cold buffer for extracting the cytosolic fraction at 4°C (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 1 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol and a complete protease inhibitor cocktail (Roche Diagnostics, Milan, Italy)). Protein content was measured with the Bradford assay (BioRad, Milan, Italy). Using the method of Laemmli (20), 30 μg of cytosolic lysate was loaded onto two different
Table 1  SOCS3 gene expression analysis in all experimental points.

<table>
<thead>
<tr>
<th>Collection points</th>
<th>SOCS3 mRNA levels [mean (SE); median]</th>
<th>p-Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p-Value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative Genotype 2 Genotype 1b</td>
<td></td>
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</tr>
<tr>
<td>3 days</td>
<td>1.08 (0.27); 1.28</td>
<td>0.73 (0.06); 0.73</td>
<td>N.S.</td>
</tr>
<tr>
<td>1 week</td>
<td>1.08 (0.24); 0.96</td>
<td>1.66 (0.40); 2.03</td>
<td>N.S.</td>
</tr>
<tr>
<td>2 weeks</td>
<td>1.02 (0.11); 1.09</td>
<td>2.82 (0.59); 2.56</td>
<td>0.04</td>
</tr>
<tr>
<td>3 weeks</td>
<td>1.00 (0.05); 0.98</td>
<td>0.93 (0.12); 0.99</td>
<td>N.S.</td>
</tr>
<tr>
<td>4 weeks</td>
<td>1.14 (0.37); 0.89</td>
<td>1.10 (0.12); 1.07</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Comparison between cell lines infected by HCV genotype 2 vs. genotype 1b; <sup>b</sup>comparison between cell lines infected by HCV genotype 1b vs. those treated with negative sera Mann-Whitney test. SOCS3, suppression of cytokine signaling 3; SE, standard error; N.S., not significant.

Figure 5  Levels of the SOCS3 protein in HepG2 2 weeks after HCV infection.

(A) Western blot of SOCS3 and β-actin in HepG2 cells inoculated with serum from 3 HCV genotype 1b- and 3 HCV genotype 2-infected patients; 3 healthy, HCV-negative subjects served as negative control. (B) The protein level was significantly higher in cells infected with HCV genotype 1b vs. cells infected with HCV genotype 2 (Mann-Whitney test; *p < 0.04) and non-infected cells (Mann-Whitney test; **p = 0.03). Data are presented as median and mean ± SEM. OD, optical density.
healthy control sera, at day 3, and 1, 3 and 4 weeks following inoculation with HCV (Table 1). Two weeks following HCV infection, SOCS3 mRNA levels were significantly higher in cells infected with HCV genotype 1b (median 2.56; mean 2.82 ± 0.59) vs. cells infected with genotype 2 (median 1.34; mean 1.46 ± 0.31, p = 0.04) and vs. cells that were not infected (median 1.09; mean 1.02 ± 0.11, p = 0.02) (Figure 4A and B). Genotype 2 cells did not differ from control cells. There was an inverse relationship between SOCS3 gene expression and the expression of Mx-A (Figure 4C), which is consistent with previous data (9). In particular, Mx-A was down-regulated significantly in HepG2 cells infected with HCV genotype 1b (median 0.76; mean 0.77 ± 0.05) compared with cells infected with genotype 2 (median 1.04; mean 1.04 ± 0.01, p = 0.02) and cells that were not infected (median 1.03; mean 1.01 ± 0.06, p = 0.04). Western blot analysis confirmed that SOCS3 protein levels were significantly higher in cells infected with genotype 1b (median 16.68; mean 16.67 ± 0.01) with respect to genotype 2 (median 16.48; mean 16.48 ± 0.02, p = 0.04) and control cells (median 16.19; mean 16.26 ± 0.13, p = 0.03) (Figure 5A and B).

**IRS-1 gene expression and protein levels in HepG2 cells infected in vitro**

We analyzed IRS-1 gene expression and IRS-1 protein levels in genotype 1b- and 2-infected cells at 3 days and 2 weeks following infection with HCV. SOCS3 expression peaked 2 weeks following infection with genotype 1-infected HepG2 cells (Figure 1). IRS-1 expression, measured with qRT-PCR, did not differ between genotype 2- and genotype 1b-infected cells (Figure 6A). Conversely, when measured with Western blotting, the IRS-1 protein level was significantly lower in genotype 1b-infected cells (median 15.45; mean 15.47 ± 0.03) than in cells infected with HCV genotype 2 (median 16.60; mean 16.59 ± 0.01, p = 0.02) (Figure 6B and C). Of note, there was no difference in protein levels between HepG2 cells infected by HCV genotype 2 and cells that were not infected (p = 0.15).

**AKT protein levels in HepG2 cells infected in vitro**

We analyzed the protein level of AKT and its phosphorylation state (pAKT) by Western blotting (Figure 7A) using the same samples that had been analyzed...
Figure 7  AKT and phospho-AKT (Ser473) protein levels in HepG2 infected cells. (A) Levels of the AKT and phospho-AKT proteins in HepG2 2 weeks after HCV infection. (B) The pAKT/AKT ratio was significantly lower in genotype 1b-infected cells vs. cells not infected with genotype 1b (genotype 2-infected and not infected) cells (Student t-test, *p < 0.03). The y-axis shows the optical density (OD) of the phospho-AKT expression compared to AKT protein. Data are presented as median and mean ± SEM.

for IRS1 and SOCS3 expression. We observed a reduction of pAKT/AKT ratio in genotype 1b-infected cells when compared to genotype 2-infected cells (25.9%) and cells that were not infected (21.9%). A statistically significant difference was observed when comparing the pAKT/AKT ratio in genotype 1b-infected cells (0.19 ± 0.034) and cells not infected with genotype 1b (genotype 2-infected and not infected) cells (0.253 ± 0.004, p = 0.03) (Figure 7B).

Discussion

To evaluate if the HCV genotype affects the molecular expression of SOCS3, we reproduced genotype 1 and 2 virus infection in HepG2 cells. We used HepG2 cells rather than a lymphoblastoid cell line because their biosynthetic pathway is similar to that of hepatocytes (22), and because overexpression of SOCS1 and SOCS3 in human hepatoma HepG2 cells suppresses IFN-α-induced STAT activation and gene expression of the antiviral proteins 2′,5′ OAS (2′-5′-oligoadenylate synthetase and MxA (21). In addition, lymphoblastoid lines immortalized by Epstein-Barr virus interferes with HCV infection. Finally, propagation of HCV in HepG2 cells has been well validated (10, 23). We inoculated HepG2 cells with either genotype 1 or genotype 2 according to Lázaro et al. who reported that serum infected cells still showed high virus titer up to 2 months in both the cell pellet and the culture medium (12). In our system, cells showed fluctuating virus levels up to 30 days and greater (data not shown), in a cyclic pattern with peaks at 4, 15 and 24 days. This may reflect the effect of host responses to the virus as reported by others (12, 24, 25). Moreover, the analysis of genotypes by reproducing the genotype specific infection in both cell culture and culture medium also confirms that our system is able to support viral replication.

After 2 weeks of viral exposure, SOCS3 expression was significantly higher in cells infected with genotype 1b vs. cells infected with genotype 2, and also vs. non-infected cells. There was no difference between genotype 2-infected and non-infected cells. Of interest was the “mirror effect” during the peak of viremia, SOCS3, IRS-1 and AKT in genotype 1-infected cells. Namely, the HCV-RNA peak was associated with a significant increase in SOCS3 and a significant decrease in the intracellular levels of IRS-1 and pAKT. This observation is in agreement with the finding that SOCS3-related IRS-1 degradation occurs via the ubiquitin system (22, 23). Moreover, the reduction in AKT phosphorylation confirms the altered intracellular insulin metabolic pathway, and supports the idea that genotype 1b HCV might somehow interfere at this level. The inverse regulation between SOCS3 and IRS-1 suggests that genotype 1b HCV affects the expression of SOCS3 and IRS-1. These results are in agreement with our previous report of enhanced SOCS3 in non-responding patients with genotype 1 HCV infection.
(5), and supports the concept that virus-related SOCS3 gene up-regulation underlies the non-response to therapy and the clinical expression of insulin resistance in genotype 1-infected patients.

Pazienza et al. (23) reported significantly greater insulin resistance in genotype 1 patients, and found that the mechanism underlying IRS-1 down-regulation was not related to SOCS3. Our data do not support this observation, and instead support the finding that the HCV down-regulates IRS-1 and 2 by up-regulating SOCS3 (26). Moreover, our finding supports the concept that the IFN-α antagonistic activity of HCV core protein involves SOCS3 induction (27). This is consistent with the association between lack of antiviral therapy and obesity and hepatic expression of SOCS3 in genotype 1 patients (6). However, insulin resistance is a predictor of sustained viral response in patients who respond to treatment (genotype 2 and 3) (28). The apparent discrepancy between this observation and our data could be that non-responding patients infected with “easy” genotypes might have a SOCS3 polymorphism that results in higher levels of SOCS3. This fact, together with jak/stat suppression, might interfere with the insulin intracellular pathway. Further studies are needed to address this hypothesis.

In conclusion, we demonstrate that genotype 1b HCV plays a crucial direct role in up-regulation of the SOCS3 gene, conditioning the response to antiviral therapy and expression of insulin resistance. This observation challenges the concept that the response to therapy is governed by insulin resistance. Further studies on the biological mechanism underlying the interaction between HCV and SOCS3 might lead to new combinations of therapeutic strategies that are effective in patients who do not respond to traditional therapy.

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