Assessment of pro-inflammatory cytokines in sera of patients with hepatitis C virus infection before and after anti-viral therapy

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Abstract

Introduction: A number of cytokines have been implicated in hepatitis C virus (HCV)-related liver disease. This study aimed to assess the serum levels of pro-inflammatory cytokines in patients with HCV infection before (naïve) and after successful treatment (sustained responders) with Pegylated interferon and ribavirin.

Methodology: The present study included 19 naïve HCV patients and 8 sustained responders. Additionally, 20 healthy individuals were included as a control group. The serum levels of the pro-inflammatory cytokines interleukin-8 (IL-8), IL-6, IL-10, IL-1β, and IL-12p70 were measured using flow cytometry.

Results: The serum IL-8 levels were significantly higher in the naïve group (21.5±10.7 pg/mL; p = 0.02) than in the control group (14.1±1.7 pg/mL) and the sustained responder group (10.4±6.2 pg/mL; p = 0.002). The serum IL-6 levels were significantly higher in the naïve group (7.3±2.06 pg/mL; p = 0.02) than in the control group (5.9±1.01 pg/mL) whereas IL-6 in sustained responder group (6.4±1.5 pg/mL) was no different than naïve HCV patients or the controls. The serum IL-10 levels were significantly higher in the naïve group (4.42±0.64 pg/mL) than in the control group (3.6±0.34 pg/mL; p =0.0002) and not the sustained responder group (4.1±0.86 pg/mL). Moreover, the serum IL-12p70 levels were higher in the sustained responder group (3.43±0.84 pg/mL; p =0.05) than in the control group (2.76±0.83 pg/mL). There were no differences in the serum IL-1β levels among the groups.

Conclusion: Successful anti-viral therapy against HCV was associated with significant reductions in the serum IL-8 levels and skewing of the pretreatment Th2 dominant immune response to the Th1 response.

Key words: Pro-inflammatory cytokines; hepatitis C virus; anti-viral therapy


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Introduction

Hepatitis C virus (HCV) infection is associated with significant morbidity and mortality owing to progression of a high percentage (85%) of HCV-infected patients to chronic hepatitis, which might lead to the development of liver cirrhosis or hepatocellular carcinoma [1,2]. The clinical course of HCV infection is highly variable and the mechanisms involved in the pathogenesis of HCV infection remain unclear. A previous study reported that both innate and adaptive immune responses might play critical roles in the pathogenesis of HCV infection primarily because of the non-cytopathic nature of the virus [3]. Among the several immunological responses, cytokine production is considered the key factor influencing the clinical outcome of HCV infection, particularly in determining the chronicity of the infection [4,5].

Based on the pattern of cytokine production, helper T lymphocytes have been classified into Th1 and Th2 subsets [6]. Interleukin-2 (IL-2) and interferon-gamma (IFN-γ) predominantly released by Th1 lymphocytes promote cell mediated immunity and have been linked with the clearance of HCV infection [3]. However, IL-4, IL-10, and IL-13 released by Th2 lymphocytes promote humoral immunity and are associated with not only chronicity, but also progression of HCV infection [7,8]. These findings indicate that an imbalance between Th1 and Th2 lymphocytes in terms of cytokine production is critical in determining the outcome of HCV infection [9,10]. Despite the presence of convincing evidence defining the roles of cytokines produced by Th1 and Th2 lymphocytes in HCV infection, controversy exists as lack of Th2...
predominance has been reported in patients with chronic HCV infection [11].

Chronic inflammatory response associated with HCV infection is believed to be the main cause of liver injury [12]. The role of pro-inflammatory cytokines, besides interferons, in HCV infection is poorly understood. Pro-inflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) have been shown to be released by macrophages subsequent to their activation from antigen-Toll like receptor interactions [13]. A significant increase in the number of macrophages in liver tissues of patients with chronic HCV infection might therefore be critical for the maintenance of chronic inflammation in HCV infection [14]. The present study aimed to assess the serum levels of pro-inflammatory cytokines both before and after therapeutic intervention in patients with HCV infection.

**Methodology**

**Study groups**

This study was performed in the department of Pathology, King Khalid University Hospital (KKUH), Riyadh between November 2012 and December 2013. A total of 27 patients infected with HCV attending the hepatology clinic at KKUH were enrolled in the study. Of these, 19 patients (13 female and 6 male patients; mean age, 49 ± 12.8 years) were included before the initiation of anti-viral therapy ( naïve group) and 8 patients (3 female and 5 male patients; mean age, 47 ± 8 years) were included after treatment with anti-viral therapy (sustained responder group). Majority of the patients were lost to follow-up, and therefore, the sustained responder group included only 2 patients from the naïve group. For comparison, 20 healthy individuals (all males; mean age, 32 ± 10.6 years) were included in the study (control group). The control group was screened for evidence of current or past infection of human immunodeficiency virus (HIV), human T-lymphotropic virus, and hepatitis B and C viruses, and all the participants tested negative. The sustained responder group had been treated with a combination of Pegylated interferon (PegIFN) and ribavirin for one year duration and had undetectable levels of HCV for the last six months. The naïve group on the other hand had a high mean viral load of 1.35x10^6±1.72x10^6 IU/mL (Table 1). This study was approved by the Institutional Review Board of the College of Medicine, and all patients signed an informed consent form.

**Assessment of hepatitis C viral load**

The hepatitis C viral load was measured from serum samples using the COBASTaqMan analyzer and the COBAS AmpliPrep/COBAS TaqMan HCV Quantities test, version 2.0 (Roche Diagnostics, Germany).

**Measurement of pro-inflammatory cytokines**

The levels of IL-8, IL-6, IL-10, IL-1, and IL-12 were measured in serum samples from patients and controls using the cytometric bead array (CBA) human inflammatory cytokine kit (BD Biosciences, San Jose, USA) in accordance with the manufacturer’s instructions. This assay is designed for the simultaneous detection of several cytokines in a single sample with flow cytometry using microbeads conjugated with specific antibodies. Briefly, the human cytokine standard (20× bulk) was prepared by mixing 2 mL of assay diluent and lyophilized cytokine standard, and incubating the mixture for 15 minutes. Two-fold serial

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**Table 1. Characteristics of patients and controls included in the study.**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Number / Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>20</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>20/0</td>
</tr>
<tr>
<td>Age (mean ± S.D) [range]</td>
<td>31.6±10.56 [19-51] years</td>
</tr>
<tr>
<td><strong>Naïve-HCV infected patients</strong></td>
<td></td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>6/13</td>
</tr>
<tr>
<td>Age (mean ± S.D) [range]</td>
<td>49.75±12.76 [23-71] years</td>
</tr>
<tr>
<td>Mean viral load ± S.D [range]</td>
<td>1.3x10⁶±1.7x10⁶ [200 - 681,986] IU/mL</td>
</tr>
<tr>
<td>HCV genotype (# of cases)</td>
<td>type 1(4), type 1a(1), type 2(1), type 4(12), ND(1)</td>
</tr>
<tr>
<td>Mean ALT ± S.D</td>
<td>84.10±75.17 U/L</td>
</tr>
<tr>
<td>Mean AST ± S.D</td>
<td>54.42±39.87 U/L</td>
</tr>
<tr>
<td>Mean ALP ± S.D</td>
<td>146.26±44.10 U/L</td>
</tr>
<tr>
<td><strong>Sustained responders HCV</strong></td>
<td>8</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>5/3</td>
</tr>
<tr>
<td>Age (mean ± S.D) [range]</td>
<td>47.25±8.05 [32-58] years</td>
</tr>
<tr>
<td>Mean viral load ± S.D</td>
<td>HCV not detected</td>
</tr>
</tbody>
</table>

HCV = Hepatitis C virus, Naïve patients = Pretreatment group, Sustained responders = successfully treated group. ALT = Alanine transaminase, AST = Aspartate Transaminase, ALP = Alkaline phosphatase, ND = not determine, IU/mL= International unit/milliliter.
dilutions of the standard were prepared, and 50 μL of each standard and serum sample were transferred into fluorescence activated cell sorting (FACS) tubes. Mixed human cytokine capture beads were prepared by mixing 10 μL of each type of cytokine capture bead for each sample. Mixed capture beads (50 μL) were added to both the standard and sample tubes, and the tubes were incubated for 90 minutes at room temperature. The contents of the tubes were washed, and 50 μL of human cytokine PE-detection reagent was added. The tubes were then incubated for 90 minutes at room temperature, washed, and re-suspended in 300 μL of wash buffer before being analyzed using the BD FACS Caliber analyzer (BD Biosciences). The results were interpreted using FCAP software (BD Biosciences) to determine the cytokine levels in pg/mL.

**Statistical analysis**

Data were statistically analyzed using the GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA). The non-paired t-test and Mann-Whitney U test were used to evaluate the statistical significance between the groups. A p-value < 0.05 was considered to indicate statistical significance.

**Results**

Figure 1 presents the comparison of data for serum levels of IL-8, IL-6, IL-10, IL-1, and IL-12 among the naïve, sustained responder, and control groups. Alterations in the serum levels of IL-8, IL-6, IL-10, and IL-12p70 were evident among the groups evaluated (Figure 1 A, B, C, and E). The serum IL-8 levels were significantly higher in the naïve group (21.5 ± 10.7 pg/mL) than in the control group (14.1 ± 1.7 pg/mL) and sustained responder group (10.4 ± 6.2 pg/mL) (both, p = 0.02). However, there were no differences in the serum IL-6 levels between the control group and the sustained responder group (Figure 1A). The serum IL-6 levels were significantly higher in the naïve group (7.3 ± 2.06 pg/mL) than in the control group (5.9 ± 1.01 pg/mL; p = 0.02). However, there were no differences in the serum IL-6 levels between the sustained responder group (6.4 ± 1.5 pg/mL) and the naïve or control group (Figure 1B). The serum IL-10 levels were significantly higher in the naïve group (4.42 ± 0.64 pg/mL) than in the control group (3.6 ± 0.34 pg/mL; p = 0.0002). Although IL-10 levels decreased in the sustained responder group (4.1 ± 0.86 pg/mL) the difference was not statistically significant when compared with the naïve groups. There were no differences in the serum IL-10 levels between the sustained responder group and the control group (Figure 1C). The serum IL-12p70 levels were higher in the sustained responder group (3.43 ± 0.84 pg/mL) than in the control group (2.76 ± 0.83 pg/mL; p = 0.05). However, there were no differences in the serum IL-
12p70 levels between the naïve group (3.19 ± 0.86 pg/mL) and the control or sustained responder group (Figure 1E). There were no differences in the serum IL-1β levels among the control (3.52 ± 0.71 pg/mL), naïve (4.4 ± 2.1 pg/mL), and sustained responder (3.8 ± 0.90 pg/mL) groups (Figure 1D).

Discussion

Alterations in the serum levels of IL-8, IL-6, IL-10, and IL-12p70 were observed in patients with HCV infection either before or after therapeutic intervention with a combination of PegIFN and ribavirin. IL-8 (CXCL8) is a CXC chemokine that plays a role in neutrophil trafficking towards the site of tissue injury [15]. It is produced by a variety of cells expressing toll-like receptors, particularly monocytes and macrophages [16,17]. IL-8 binds to the chemokine receptors CXCR1 and CXCR2, and both these receptors are expressed by neutrophils, monocytes, and macrophages [18,19]. The presence of elevated IL-8 levels in both the intrahepatic tissues and peripheral blood of patients with chronic liver disease suggests that increased tissue infiltration and activation of hepatic macrophages in HCV infection is mediated by the IL-8-CXCR1 interaction [20]. Moreover, high IL-8 levels in patients with HCV infection have been implicated in disease progression, leading to chronicity of the disease [21]. The elevated serum IL-8 levels detected in the present study among naïve HCV patients are consistent with the levels reported in a previous study [21]. The reduction of the serum IL-8 levels following therapeutic intervention among sustained responders indicates that alterations in the serum IL-8 levels may be associated with disease activity. Therefore, the evaluation of serum IL-8 levels in patients with HCV infection may serve as a useful tool to monitor not only disease activity, but also the efficacy of therapeutic intervention.

In the present study, the serum IL-6 levels were higher in naïve HCV patients than in controls; however, the levels were not different between sustained responders and controls. Elevated serum IL-6 levels among patients with HCV infection have been shown to be associated with the severity of the disease [22]. A direct relationship between increased serum IL-6 levels and HCV infection was recently demonstrated in animal studies, where immunization of mice with recombinant HCV core protein resulted in the production of a high amount of IL-6 [23]. Therefore, it is possible that the reduction in the serum IL-6 levels following successful anti-viral treatment in the present study was associated with the clearance of infection. Among patients co-infected with HCV and HIV, high IL-6 and IL-9 levels have been reported to be strong predictors of treatment failure with interferon-alpha (IFN-α) and ribavirin [24]. The validity of high IL-6 and IL-9 levels as predictors of treatment failure in patients co-infected with HCV and HIV appears to be questionable, as HIV is known to be associated with increased apoptosis of CD4+ T helper cells [25]. It is therefore possible that the degree of HIV-induced immune deficiency contributes to treatment failure.

Pretreatment serum IL-10 levels were high in patients with HCV infection, and following successful treatment with anti-viral therapy, the IL-10 levels declined with a concomitant increase in the serum IL-12p70 levels among sustained responders. IL-10 is a member of the Th2 group of cytokines, while IL-12 is a member of the Th1 group of cytokines [26]. High serum IL-10 levels in the pretreated HCV patients suggested Th2 polarization of the immune response in HCV infected patients that changed to a Th1 immune response following successful anti-viral therapy, which was indicated by an increase in the IL-12 levels and reduction in the IL-10 levels. A Th2-skewed immune response in patients with HCV infection has been reported in previous studies [7,8], and serum IL-10 levels are believed to be elevated in patients with HCV infection owing to high production of this cytokine by monocytes [27]. The elevation of serum IL-12 levels following PegIFN-α2A/ribavirin treatment has been linked with sustained virological response, whereas high serum IL-10 levels are believed to indicate a predisposition to late virological relapse [28]. These observations suggest that a favorable therapeutic outcome following PegIFN/ribavirin treatment is associated with a change in the pretreatment Th2 cytokine immune response to the Th1 response.

There have been concerns over the low-level persistence of HCV RNA in peripheral blood mononuclear cells (PBMCs) after successful treatment of chronic hepatitis C. HCV RNA can be detected 52–66 months after sustained virological response following treatment with PegIFN-α2A/ribavirin, along with high levels of transcripts for IL-6, IL-8, IL-12, TNF-α, and macrophage inflammatory protein 1β [29]. HCV RNA appears to be passively adsorbed by PBMCs [30], leading to up-regulation of cytokine transcripts because of persistent antigen stimulation. IL-6, a cytokine of the innate immune response, promotes T cell proliferation and B cell differentiation and survival [31], whereas IL-8, a neutrophil specific cytokine, is believed to be involved in macrophage infiltration and activation [20]. It is therefore possible that both the cytokines participate in not only the innate, but also the
adaptive immune response against HCV. However, increased serum IL-12 levels observed in the present study following anti-viral therapy suggest not only Th1 polarization of the immune response, but also enhancement of anti-viral and cytotoxic activities [32].

The present study had some limitations. The study included a small number of patients and controls in each group. Moreover, majority of the naïve patients were lost to follow-up; therefore, the sustained responder group included only 2 of the 19 patients from the naïve group. A large-scale follow-up study is needed to further evaluate the pre-therapeutic and post-therapeutic alterations in serum cytokine levels in patients with HCV infection.

Conclusion

Successful anti-viral therapy against HCV was associated with significant reductions in the serum IL-8 levels and change of the pretreatment Th2 dominant immune response to the Th1 response.

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Authors’ contributions

A. Alhethel designed the study, conceived the idea, performed experimental work, data analysis and contributed in manuscript preparation. A. Albarrag and Z. Shakoor contributed in study design, data analysis, preparation and revision of the manuscript. K. Alswat, A. Abdo, and W. Alhamoudi contributed in data collection, data analysis and manuscript preparation.

References


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