Changes in serum transforming growth factor-β1 levels in chronic hepatitis C patients under antiviral therapy

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Abstract	**Background** Several cytokines including transforming growth factor (TGF)-β1 have been suggested to be involved in the pathogenesis of fibrosis in chronic hepatitis C. We examined the changes of TGF-β1 serum levels and their predictive value in patients with chronic hepatitis C under antiviral therapy.

Methods We included 84 patients with chronic hepatitis C who were treated with pegylated interferon-α and ribavirin between 2008 and 2009. Treatment was given for 24-48 weeks depending on HCV genotype. Serum TGF-β1 levels were measured by an ELISA assay at baseline, at the end of therapy (EOT), and at 6 months after the EOT. Liver fibrosis was evaluated by transient elastography.

Results Of the 84 patients, 76.2% achieved sustained virological response (SVR), 8.3% responded at the EOT but relapsed during post-therapy follow up (RR) and 15.5% had no response (NR). In all patients, mean TGF-β1 levels were 16,980 pg/mL at baseline and decreased significantly at EOT (12,041 pg/mL) and at 6 months of post-treatment follow up (13,254 pg/mL) ($P \leq 0.001$). In particular, mean TGF-β1 levels decreased significantly from baseline to EOT and to six months of post-treatment follow up in patients with SVR and numerically but not significantly in patients with RR or NR. TGF-β1 levels were not associated with the severity of liver stiffness estimated by transient elastography.

Conclusion Our data show that TGF-β1 serum levels decrease significantly at the EOT and remain decreased 6 months after the EOT mostly in chronic hepatitis C patients who achieve SVR after pegylated interferon-α and ribavirin combination treatment.

Keywords TGF-β1, HCV, elastography, antiviral treatment, liver fibrosis

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Introduction

Chronic hepatitis C virus (HCV) infection is one of the most common causes of cirrhosis comprising 40% of chronic liver disease and representing a major health problem worldwide [1]. Chronic HCV infection is also associated with increased risk for development of hepatocellular carcinoma (HCC) [2]. However, many patients with chronic hepatitis C do not exhibit progressive disease or progress so slowly that cirrhosis does not develop within their lifespan. Thus, the factors determining increasing fibrosis and progressive disease remain largely unknown [3,4]. For patients with clinically significant hepatic fibrosis there is widespread agreement that antiviral therapy is indicated because of the high risk of cirrhosis [5]. Therefore, it would be desirable to have specific predictive markers to distinguish patients potentially developing progressive liver disease from those with mild or stable disease.

Currently, therapeutic armamentarium for chronic HCV infection is rapidly changing since the classic therapeutic combination of pegylated interferon-α (Peg-IFNa) plus ribavirin gives places to specific antiviral drugs that achieve high sustained virological response (SVR) rates with excellent tolerance and safety [6]. However, the extremely high cost of the new therapies limits their widespread use in several parts of the world restricting them to patients with advanced fibrosis or cirrhosis or other specific subgroups [6]. Therefore, many patients with chronic hepatitis C may be still treated with the previous treatment combination. In any case, the possible effects of antiviral treatment on the liver are expected to depend mostly on the virological response and HCV eradication but not on the type of therapy.

Recently, the scientific research has focused on several cytokines which may be associated with the persistence of HCV infection and the development of fibrosis. Such a cytokine is transforming growth factor (TGF)-β1, a 25-kDa homodimeric protein encoded by closely related genes; its physiological role lies in the regulation of cell growth and differentiation and depends on the target cells either as a positive stimulator or inhibitor of cell growth. In mammals, three isoforms have been thus far identified; TGF-β1, 2 and 3 [7,8]. In the liver, TGF-β1 has been involved in hepatic fibrogenesis, regulation of liver cell growth, tumor development and induction of hepatocellular apoptosis [9,10]. TGF-β1 is produced by non-parenchymal liver cells, enhances the activity of hepatic stellate cells (HSCs) and inhibits the growth and proliferation of hepatocytes. In that way, TGF-β1 stimulates extracellular matrix and fibrous production [11,12]. Increased levels of TGF-β1 mRNA have been identified in patients with chronic liver disease with a positive correlation with levels of aminoterminal peptide of type III procollagen, a serum marker of hepatic fibrogenesis [13]. Some studies pointed out that serum levels of TGF-β1 are also increased in patients with chronic hepatitis C and are correlated with the degree of liver fibrosis while other investigators provide contradictory results [14]. Currently, there are some but not conclusive data on the changes of TGF-β1 levels after treatment with Peg-IFNa and ribavirin. Therefore, the aim of current study was to examine the changes of TGF-β1 serum levels in patients with chronic hepatitis C under antiviral therapy.

Patients and methods

Our study group consisted of 84 patients with confirmed chronic HCV infection evaluated at our outpatient liver clinics over a period of 12 months. Inclusion criteria were: 1) confirmed chronic HCV infection (positive anti-HCV antibodies for at least 6 months and detectable serum HCV-RNA); 2) no history of previous HCV treatment (naïve patients); and 3) completion of a course with Peg-IFNa and ribavirin. Patients were excluded if they: 1) were co-infected with human immunodeficiency virus (HIV) or other hepatitis viruses (HBV, HDV); 2) had HCC or any other malignancy;

3) were active users of illicit drugs; and 4) were alcohol abusers.

Blood samples were collected at baseline, at the end of therapy (EOT) and 6 months after the EOT. A full blood count, biochemical evaluation including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (T.Bil), albumin, coagulation tests [prothrombin time (PT) and international normalized ratio (INR), activated partial thromboplastin time (APTT)] were performed in each patient on the same day using commercially available assays. Samples were stored at -70°C and analysis of TGB-β1 in pg/mL was performed retrospectively on the same day using an ELISA method according to the manufacturer's instructions (Invitrogen Corporation, Camarillo, CA). HCV-RNA was determined by quantitative polymerase chain reaction (PCR) and measured in international units (IU/mL) while HCV genotype was defined by INNOLIPA.

Hepatic fibrosis was assessed by transient elastography (TE) (Fibroscan® Echosens Paris) according to current guidelines [15,16]. Liver stiffness measurements by TE were expressed in kilopascals (kPa) and were evaluated in relation to interquartile range (IQR) and to the success rate of measurements. IQR less than 30% of mean liver stiffness and success rate more than 60% in more than 10 validated measurements were indicative of a successful measurement [15]. Based on previous studies, cut-off values of <6 KPa were considered normal, 6-8 KPa were considered within the gray range, 8-12.5 KPa F3 fibrosis, and a cut-off of >12.5 KPa was considered as F4 fibrosis [17].

All patients were treated with Peg-IFNa in combination with ribavirin. Patients infected with genotypes 1 and 4 were treated for 48 weeks, whereas patients infected with genotypes 2 and 3 were treated for 24 weeks. For patients who stopped prematurely treatment due to side effects or no response (NR), the EOT was considered as the last day of treatment. These patients were re-evaluated 6 months later and blood samples were collected for analysis. We considered as sustained responders (SVR) those patients with undetectable HCV-RNA (<50 IU/mL) at the EOT and at six-month follow up, whereas as non-responders (NR) those patients with detectable HCV-RNA at the EOT; patients with HCV-RNA undetectable at the EOT but detectable at six-months of post-treatment follow up were considered as relapsers (RR) [18].

Statistical analysis

Results were subjected to routine statistical analysis using SPSS v. 20 (SPSS Inc, an IBM Company, Chicago, IL). Quantitative variables were expressed as mean values [standard deviation (SD)]. Comparisons of quantitative variables between two groups were performed using t-test, while comparisons of quantitative variables among more than two groups were performed using one way analysis of variance (ANOVA). Comparisons of the changes over time of quantitative variables within the same group were performed using paired t-test. A P value less than 0.05 was considered to be statistically significant.

Results

The mean age of patients was 46 (SD: 15) years and 52% of them were male. Baseline characteristics are presented in Table 1. Thirty seven of the patients had comorbidities including hypertension (n=11, 30%), diabetes mellitus II (n=3, 8%) or other (n=9, 24%). Genotype 1 was detected in 24 (28.6%), genotype 2 in 7 (8.3%), genotype 3 in 35 (41.7%) and genotype 4 in 17 patients (20.2%), while genotype could not be determined in 1 patient (1.2%).

Route of transmission of HCV infection was unknown in 46 (55%) patients. Of the remaining 38, 26 patients (68%) were intravenous drug users, 10 (26%) had a history of blood transfusions and 2 (5%) had a tattoo performed with unsterilized tools.

Of the 84 patients, based on HCV-RNA measurements, 64 patients (76%) achieved SVR, 7 patients (8.5%) responded at the EOT but relapsed during post-therapy follow up (RR) and 13 patients (15.5%) had NR. Nineteen (79.2%) patients with genotype 1 responded at the EOT and 15 (62.5%) achieved SVR at a six-month follow up. All 7 patients (100%) with genotype 2 achieved SVR, 31 (88.6%) patients with genotype 3 responded at the EOT and 30 (85.7%) achieved SVR and 14 (82.4%) patients with genotype 4 responded at the EOT and 12 (70.6%) achieved SVR. It should be noted that 14 patients showed adverse reactions to treatment. The most common adverse events were cytopenias (neutropenia and/or anemia) in 6 (7%), rash in 3 (4%), thyroiditis in 3 (4%) and depression in 2 (2%). Twelve patients (14%) stopped therapy prematurely due to adverse events (4 with genotype 1, 4 with genotype 3 and 4 with genotype 4). Mean (SD) baseline HCV-RNA levels were 2.9×10^6 (8.6×10^6) IU/mL, while liver stiffness at baseline had a mean (SD) level of 8.27 (6.06) kPa (Table 1).

Mean (SD) TFG-β1 levels were 16,980 (11,643) pg/mL at baseline, 12,041 (8,972) pg/mL at the EOT and 13,254 (6,607) pg/mL at 6 months of follow up. Compared to baseline levels, TGF-β1 decreased at the EOT [mean difference: -3,829 pg/mL, 95% confidence interval (CI): -2,314 to -5,344; P<0.001 by paired *t*-test] and at 6 months of post-treatment follow up (mean difference -3327 pg/mL, 95% CI: -946 to -5,708; P=0.001 by paired *t*-test). In particular, mean (SD) TGF-β1 levels decreased significantly at the EOT and six months of post-treatment follow up compared to baseline only in patients with SVR (17,166 vs. 12327 pg/mL, P<0.0001 and 17,166 vs. 13,555 pg/mL, P=0.007, respectively, by paired *t*-test) (Fig. 1) (Table 2). On the other hand, a numerical but not statistically significant decrease was usually observed in mean TGF-β1 levels from baseline to the EOT or to 6 months of post-treatment follow up in patients with RR (11,487 vs. 12,241 pg/mL, P=0.814 and 11,487 vs. 9,648 pg/mL, P=0.344, respectively) or NR (19,394 vs. 10,357 pg/mL, P=0.065 and 19394 vs. 11,812 pg/mL, P=0.797, respectively] or RR and NR together [16,319 (12,928) vs. 11,090 (7,322) pg/mL, P=0.227 and 16319 (12,928) vs. 11,382 (3031) pg/mL, P=0.695, respectively] (Fig. 1) (Table 2). No significant changes in mean (SD) TGF-β1 levels were observed from the EOT to 6 months

Table 1 Baseline characteristics of 88 chronic hepatitis C patients treated with pegylated interferon-α and ribavirin therapy

Sex (male/female)	44/40
Age (years)	46 (15)
Origin (Greek, Egyptian, other)	65/10/9
Height (cm)	170 (9)
Weight (kg)	72 (15)
Comorbidities (yes/no)	30/54
White blood cells (/mm^3)	6,674 (1,960)
Hemoglobin (g/dL)	14.05 (1.38)
Platelets (/mm^3)	227,880 (71,518)
AST (IU/mL)	65 (80)
ALT (IU/mL)	115 (199)
Albumin (g/dL)	4.46 (0.38)
Bilirubin (mg/mL)	0.65 (0.26)
HCV RNA (IU/mL)	2.9×10^6 (8.6×10^6)
Liver stiffness (kPa)	8.27 (6.06)
TGF-β1 (pg/mL)	169,780 (11,644)

All quantitative variables are expressed as mean (standard deviation) values; all quantitative variables had normal distribution except for ALT, AST and HCV RNA

ALT, alanine aminotranferase; AST, aspartate aminotransferase; TGF-β1, transforming growth factor-β1
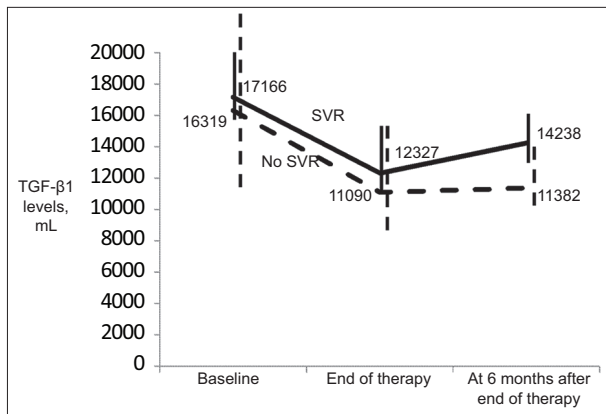
Table 2 Changes of mean levels of transforming growth factor (TGF)-β1 (pg/mL) before (at entry), at the end and at 6 months after the end of pegylated interferon-α and ribavirin therapy in patients with chronic hepatitis C in relation to their virological responses

	Mean (SD) TFG-β1 levels		
	Sustained responders	Relapsers	Non-responders
Before therapy	17,166 (11,360)	11,487 (4,047)	19,394 (15,734)
End of therapy	12,327 (9,447)	12,241 (10,542)	10,357 (4,789)
At 6 months after end of therapy	13,555 (6,985)	9,648 (1,499)	11,812 (3,277)

of post-treatment follow up in patients with or without SVR [12,327 (9,447) vs. 13,555 (6,985) pg/mL, P=0.557 or 11,090 (7,322) vs. 11,382 (3,031) pg/mL, P=0.118, respectively, by paired *t*-test].

Univariate analysis was performed to search possible associations between demographic and laboratory tests with response to antiviral treatment. The analysis showed that patients with than without SVR were significantly younger [mean (SD) age: 44 (15) vs. 55 (13) years, P=0.015] and had significantly higher white cell blood counts (WBC) at baseline [mean (SD) WBC: 6,926 (1,984)/mm^3 vs. 5,317 (1,440)/mm^3, P=0.002].

Patients were stratified using baseline cut-off stiffness values of less than 7.5 kPa and greater or equal to 7.5 kPa; statistically significant association with TGF-β1 values was not found

Figure 1 Serum levels of transforming growth factor (TGF)-β1 (pg/mL) before (at baseline), at the end of therapy, and at 6 months after the end of pegylated interferon-α and ribavirin therapy in patients with chronic hepatitis C in relation to their sustained virological response (SVR). Compared to baseline, TGF-β1 levels were significantly lower at end and 6 months after end of therapy in sustained responders (P<0.001 and P=0.001), but the change did not reach statistical significance in non-sustained responders (P=0.227 and P=0.695)

between the two groups. A second stratification with baseline cut-off stiffness values less than 6.5, between 6.5 and 9.5 and greater than 9.5 was performed; again, we could not find any statistically significant association with TGF- β1 values among the three groups.

Discussion

In this study, we included patients with chronic hepatitis C of all genotypes who attended our liver clinics and we measured serum TGF-β1 levels before and after treatment with Peg-IFNa and ribavirin. We found that TGF-β1 levels decreased substantially at the EOT and this difference was more pronounced and statistically significant only in patients who achieved SVR. Mean TGF-β1 values slightly increased in patients with SVR during the 6-month period of follow up compared to values at the EOT but they never reached the pre-treatment levels and the difference remained statistically significant compared to baseline. Mean TGF-β1 values had numerical declines from baseline to EOT and 6 months after EOT in patients without SVR, but the changes did not reach statistical significance. Given that the number of patients without SVR was smaller than the number of patients who achieved SVR, a type II error for this association cannot be excluded.

We did not estimate the tissue expression of TGF-β1, as we used non-invasive methods (TE) for the estimation of liver fibrosis. Given that TGF-β1 is a pleiomorphic cytokine acting mostly locally at the site of inflammation [19], its serum level is a crude estimation of liver immunopathogenesis of HCV infection and treatment induced changes. However, it has previously shown that plasma levels of TGF-β1 are significantly correlated with TGF-β1 content in liver tissue indicating that plasma levels may correspond reliably to tissue expression [20].

Our results are similar but not identical to those of other studies that investigated the potential changes in serum levels and/or tissue expression of TGF-β1 after interferon treatment. Flisiak *et al* [21] also showed that TGF-β1 levels after treatment with Peg-IFNa and ribavirin decreased significantly compared to baseline in a small number of patients, regardless of SVR, reaching levels observed in healthy volunteers. However, TGF-β1 levels increased significantly 24 weeks after treatment in NRs compared to patients who achieved SVR. In initial studies in the '90s using IFNa monotherapy, increased expression of TGF-β1 mRNA in liver tissue was observed with normalization after 48 weeks of IFNa administration, suggesting an important role of this cytokine in the pathogenesis of chronic hepatitis C [22,23]. Subsequent studies that estimated plasma TGF-β1 levels in patients with chronic hepatitis C who received conventional IFNa and ribavirin presented conflicting results. In one study [24] including 34 responders and 26 NRs to IFNa and ribavirin, serum TGF-β1 levels were found to decrease significantly after treatment only in the responders, while mRNA TGF-β1 expression in liver tissue decreased significantly in all patients independent of the achievement of SVR. On the other hand, while Janczewska *et al* reported that serum TGF-β1 levels decreased after treatment with IFNa and ribavirin in both responders and NRs [25]. These differences might reflect different populations, different stage of the disease or in the methodology of cytokine assessment. However, in agreement with our results, most studies have shown that IFNa administration reduces TGF-β1 plasma levels and/or TGF-β1 mRNA tissue expression in patients who achieve SVR.

Experimental studies have showed that bio-active TGF-β1 induces HSCs activation and invasion which likely contributes to liver fibrosis associated with HCV infection [26]. In our study, no association was found between TGF-β1 levels and liver fibrosis as estimated by TE at baseline or after therapy regardless of SVR. In accordance with our results, Roulot *et al* [27] reported that there was no association between TGF-β1 mRNA and either the biological and histological markers of liver fibrosis or a favorable response to IFNa therapy. Other studies have shown a correlation between plasma TGF-β1 levels and severity of liver fibrosis [28,29] or close relationship between tissue TGF-β1 mRNA expression and serum markers of liver fibrosis, suggesting an active role in the fibrotic process [30,31]. Moreover, in a recent small study including only genotype 4 patients, Gomaa *et al* [32], showed that overexpression of TGF-β1 was an independent predictor of poor outcome to IFNa based therapy. In contrary, we did not find any association between pre-treatment levels of TGF-β1 and response to therapy.

The diversity in the results of these trials is not easy to interpret. It is well recognized that advanced fibrosis is a negative predictive factor of response to IFNa treatment [18]. Profibrotic cytokines have been implicated in the impairment of IFNa signaling resulting in reduced efficacy [33]. On the other hand, there is a complex interaction between viral proteins, TGF-β1 and IFNa signaling. Recent experimental studies provide further evidence to support the hypothesis that HCV enhances hepatic derangement progression through the generation of reactive oxygen

Summary Box

What is already known:

- Chronic hepatitis C can be a progressive disease leading to accumulation of liver fibrosis and perhaps to the development of cirrhosis
- Antiviral therapy may achieve inhibition of the progression and even improvement in liver fibrosis in chronic hepatitis C patients
- Several cytokines including transforming growth factor (TGF)-β1 have been suggested to be involved in the pathogenesis of fibrosis in chronic hepatitis C

What the new findings are:

- Serum TGF-β1 levels decrease significantly after pegylated interferon-alfa and ribavirin therapy in chronic hepatitis C patients
- Serum TGF-β1 levels decrease mostly in chronic hepatitis C patients who achieve sustained virological response
- Serum TGF-β1 levels in chronic hepatitis C are not significantly associated with liver stiffness estimated by transient elastography

species and induction of TGF-β1 [34-36]. However, polymorphisms of the cytokines genes may affect the production and/or the action of TGF-β1 and despite the up-regulation provoked by HCV proteins, the final effect on the IFNa signaling is unpredictable [37,38]. Therefore, and in agreement with our results, it is not the absolute levels of TGF-β1 before treatment but possibly the changes following the administration of IFNa that reflect the ability of therapy to overcome the negative effect of TGF-β1.

A limitation of our study was that we did not measure the TGF-β1 levels in different periods during treatment to possibly define the earliest time point that TGF-β1 levels decline as a result of IFNa-based therapy. Such a study in the future could provide valuable information in support of the prognostic role of TGF-β1 levels during HCV treatment.

In conclusion, our results suggest that TGF-β1 serum levels decrease significantly at EOT and 6 months of follow up, particularly in patients achieving SVR after Peg-IFNa and ribavirin. The long-term changes of TGF-β1 serum levels after HCV therapy need to be further investigated.

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