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Association between Plasma Interleukin-18 Levels and Liver Injury in Chronic Hepatitis C Virus Infection and Non-Alcoholic Fatty Liver Disease

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Abstract. There is significant upregulation of interleukin-18 (IL-18) expression in viral infectious diseases and in some chronic hepatic diseases, especially (i) hepatitis C virus (HCV) infection, (ii) HCV infection with persistently normal ALT levels (PNAL), and (iii) non-alcoholic fatty liver disease (NAFLD). The aim of this study was a better understanding of the implications of plasma IL-18 levels in the above-mentioned liver diseases. Thirty-four patients with HCV infection, 13 with NAFLD, and 10 controls were enrolled. The HCV-RNA and HCV-genotypes and the serum or plasma levels of IL-18, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GT), alkaline phosphatase, total cholesterol, triglycerides, α_1-fetoprotein, and ferritin were evaluated. Patients with HCV showed higher levels of IL-18 than the NAFLD patients ($p < 0.01$) and the controls ($p < 0.005$). Patients with NAFLD showed higher values of body mass index and liver disease parameters, compared to HCV-infected subjects or controls. These data confirm previous reports of enhanced expression of IL-18 in patients with HCV and NAFLD, compared to healthy subjects, and suggest that IL-18 is important as a marker of liver diseases.

Keywords: interleukin-18, HCV-infection, viral hepatitis, non-alcoholic fatty liver disease

Introduction

Hepatitis C virus (HCV) infection induces chronic hepatitis with fluctuating serum aminotransferase (ALT) levels and detectable HCV-RNA [1-3], identifying chronic active HCV hepatitis (CAH-C). About 20% of chronically infected patients develop cirrhosis with enhanced risk of hepato-cellular carcinoma [4-5], because of the difficulty in eradicating HCV [6].

Approximately 30% of patients with HCV have persistently normal ALT level (PNAL) HCV infection [7-8], characterized by (i) presence of anti-HCV antibody, (ii) positive assay for HCV-RNA by RT-PCR, and (iii) normal transaminases in at least 3 evaluations at intervals of 2 mo, for a period of 6 mo [9-10]. These patients tend to have lower degrees of inflammation and fibrosis in liver biopsies [11-14]. Previous studies suggested that these patients have slower disease progression [15-16]. It is now recognized that not all HCV-infected patients with normal serum ALT levels have mild liver disease with slower progression. In fact, up to 14-20% of these patients have advanced fibrosis or cirrhosis on liver biopsy and the degree of liver injury may not differ from matched controls with elevated serum ALT levels [17-19]. Other studies provide evidence that patients with HCV infection

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and PNAL have progressive liver disease that can be identified in liver biopsies [20].

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent cause of chronic liver disease in developed countries [21]. It is currently defined as fat accumulation in liver that exceeds 5% to 10% by weight, and is practically estimated as the percent of fat-laden hepatocytes observed by light microscopy [22]. No proven treatment is currently available for patients with NAFLD. It is unclear why steatosis is stable in some patients, whereas in others it progresses to fibrosis and cirrhosis [23].

Poynard et al [3] studied the natural history of liver fibrosis in HCV patients and identified the factors associated with its progression, suggesting that host factors, rather than virologic factors, play key roles. Helper T-cell subsets are polarized by some cytokines and are believed to govern, at least in part, the immune response to viral infection [24-25]. Helper T-cells fall into subsets depending on the production of either interferon-γ (IFN-γ) or interleukin 4 (IL-4), in combination with other cytokines. Helper T-cell subsets and the cytokines they produce are believed to play a pivotal role in the defense against HCV [26].

In fact, viral infections and some chronic injuries are known to suppress the immune system. In this context, interleukin-18 (IL-18), or interferon-γ-inducing factor, is a 18-kD cytokine synthesized by Kupffer cells and by activated macrophages with proinflammatory activity at a very early step in the immune response. IL-18 generates cell-mediated immune responses via activation of Th1 T-helper cell sub-populations primarly through the induction of interferon-γ [27,28]. There are few data on IL-18 in relation to infectious diseases, but it plays a prominent role in chronic HCV infection [29].

In chronic hepatitis C virus infection, a significant upregulation of IL-18 occurs in the inflammatory infiltrate, suggesting a role of this cytokine in the chronic cellular immune response to hepatocytes in the course of the disease. In particular, it has been shown that the administration of IFN-γ exerts an anti-inflammatory action in vivo by induction of IL-18 binding protein and late suppression of IL-18 [30-31].

While the pathogenesis of chronic HCV infection has not been clearly defined, many researchers believe that cytokines play an important role in both immunoregulation and immune impairment. The aim of this study was to clarify the significance of IL-18 in regard to the degree of inflammation in viral liver disease and NAFLD by comparing serum IL-18 levels with various markers of liver disease.

Materials and Methods

Thirty-four caucasian patients with chronic hepatitis C-virus (HCV) infection and 13 subjects with non-alcoholic fatty liver disease (NAFLD) were recruited at the time of first examination at the Infectious Diseases Division of the G. D'Annunzio University of Chieti. In addition, 10 non-obese, uninfected healthy subjects, matched for ethnicity and age, were included as a control group. All subjects gave written informed consent; the study was approved by the Medical Ethics Committee of our Medical School.

All patients underwent an extensive medical and laboratory evaluation, including a liver ultrasound scan and biopsy, the results of which were used to assign the patients into 2 groups on the basis of the presence of chronic hepatitis (1st group: HCV) or steatosis (2nd group: NAFLD). The liver biopsies were at least 15 mm long. Slides were routinely stained with H&E. Liver biopsies were read by a single liver pathologist who was unaware of each patient's clinical and laboratory data. Biopsies from patients with chronic hepatitis C were graded by hepatitis activity index scores, according to Knodell et al [32]. All of the patients were afflicted with mild to moderate degrees of chronic hepatitis C infection. Diagnosis of non-alcoholic steatohepatitis was defined histologically by a combination of macrovescicular steatosis and lobular inflammation, plus either balloooning of hepatocytes or abnormal fibrosis [23].

The HCV group was then divided into subgroups of subjects with chronic active HCV hepatitis (CAH-C) and subjects with persistently normal ALT level (PNAL) HCV infection. Finally, 14 patients with CAH-C were classified as genotype 1 HCV-infected (n = 9) or genotype non-1 HCV-infected (n = 5). The diagnosis of HCV infection was defined by the usual biochemical and histological data and by detection of anti-HCV antibodies (Abbot Axsym HCV-3 and Ortho HCV 3.0 ELISA), plus HCV RNA in serum by polymerase chain reaction (PCR) (Amplicor method, Roche Molecular Diagnostics, Milan, Italy), with a detection limit <600 HCV RNA copies/ml of plasma. HCV genotyping was performed by PCR and patients were screened for viral 1 or non-1 genotypes. The entire study population was negative for other forms of viral hepatitis and human immunodeficiency virus (HIV) infection. Other conditions known to cause liver dysfunction were excluded on the basis of clinical evaluation.

The diagnosis of NAFLD was established according to the following criteria: (i) persistently elevated serum aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) levels; (ii) weekly ethanol consumption of less than 140 g; (iii) liver biopsy consistent with the diagnosis of

NAFLD; and (iv) exclusion of other liver diseases [25]. No patients were taking steatogenic or antiviral drugs and they showed no clinical signs of advanced liver disease; the plasma prothrombin time, serum albumin, and serum total bilirubin levels were within normal limits.

Body mass index was calculated as weight (kg) divided by the square of height (m^2). Fasting blood samples were drawn to measure serum levels of aspartate aminotransferases (AST), alanine aminotransferases (ALT), γ-glutamyltranspeptidase (γ-GT), alkaline phosphatase, total cholesterol, and triglycerides by (automated enzymatic methods (Ortho Clinical Diagnostics, Rochester, NY, USA), and serum α_1-fetoprotein and ferritin (LIAISON AFP and ferritin assay kits, DiaSorin, Vercelli, Italy). Plasma IL-18 levels were assayed by an enzyme-linked immunosorbent assay (IL-18 ELISA, R & D Systems, Minneapolis, MN, USA). The minimum detection limit for IL-18, estimated by serial diluition, was 12.5 pg/ml.

Data are reported as means ± SD. Statistical significance was assessed by Student's t-test for unpaired data. Linear regression analysis was performed to evaluate the correlations between plasma IL-18 and serum AST, ALT, γ–GT, and alkaline phosphatase levels. P values <0.05 were considered statistically significant.

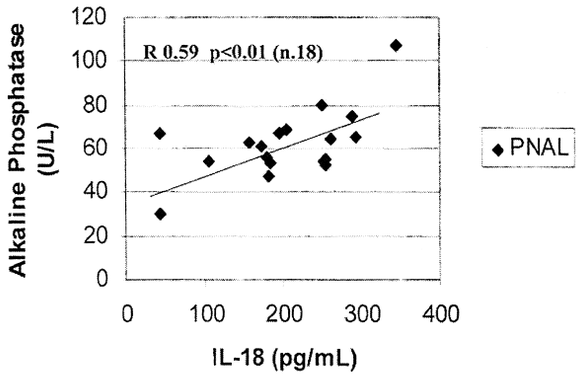

Fig. 1. Pearson's correlation coefficient (r = 0.59, p <0.01) for plasma interleukin-18 levels and serum alkaline phosphatase activities in 18 cases of chronic active hepatitis infection with persistently normal serum ALT level (PNAL).

Table 1. Basic characteristics and analytical results in patients with chronic hepatitis C virus infection (HCV, n = 34), in patients with non-alcoholic fatty liver disease (NAFLD, n = 13), and in healthy control subjects (n = 10) (means ± SD).

Parameters	HCV group	NAFLD group	Control group
gender (M/F)	20/14	9/4	7/3
age (yr)	54.6 ± 15.5	49.7 ± 17.6	46.8 ± 7.5
body mass index (BMI, kg/m^2)	25.6 ± 4.5	29.3 ± 5.1*	23.4 ± 3.5§§
serum alanine aminotransferase (ALT, U/L)	63.2 ± 48.1	73.5 ± 62.3***	47.3 ± 10.7
serum aspartate aminotransferase (AST, U/L)	58.7 ± 47.3	47.1 ± 29.5	45.8 ± 13.2
serum γ-glutamyltranspeptidase (γ-GT, U/L)	47.3 ± 37.5	44.5 ± 28.2	33.2 ± 25.3
serum alkaline phosphatase (U/L)	77.2 ± 31.7	206.8 ± 72.2***	75.2 ± 23.5§§§
serum α_1-fetoprotein (ng/dl)	12.0 ± 19.9	2.5 ± 0.9	3.47 ± 0.5§§
serum ferritin (μg/L)	166.9 ± 160.1	245.1 ± 177.5	48.2 ± 50.7† §§§
serum total cholesterol (mg/dl)	173.4 ± 31.5	244.9 ± 42.5***	170.45 ± 37.8§§§
serum triglycerides (mg/dl)	136.6 ± 36.3	174.2 ± 69.9	141.0 ± 40.5
blood platelet count (10^9/L)	164.0 ± 59.0	188.1 ± 27.9	230.0 ± 75.0†† §
plasma interleukin-18 (IL-18, pg/ml)	369.5 ± 249.3	175.4 ± 78.4**	113.2 ± 86.4†††

HCV subjects versus NAFLD subjects: ***p <0.001; **p <0.01; *p <0.02
Control subjects versus HCV subjects: †††p <0.005; ††p <0.01; †p <0.05
Control subjects versus NAFLD subjects: §§§p <0.005; §§p <0.01; §p<0.05

Results

Patients with HCV infection and patients with NAFLD showed higher plasma IL-18 levels than the control group ($p < 0.005$). Notably, IL-18 was higher in patients with HCV (370 ± 249 pg/ml) than in patients with NAFLD (175 ± 78 pg/ml) ($p < 0.01$) (Table 1).

No statistically significant differences were found between the HCV group and the NAFLD group in respect to age, AST, γ-GT, α_1-fetoprotein, ferritin, triglycerides, or platelet count. Patients with NAFLD showed higher values of BMI ($p < 0.02$), ALT ($p < 0.001$), alkaline phosphatase ($p < 0.001$), and total cholesterol ($p < 0.001$) than the HCV group. They also had higher values of BMI, alkaline phosphatase, α_1-fetoprotein, ferritin, total cholesterol, and platelet count when compared to the controls (Table 1).

The plasma viral load in subjects with HCV infection was 546 ± 352 x 10^4 copies/ml. In regard to the 2 subgroups of HCV patients, the CAH-C group showed higher values of IL-18 ($p < 0.001$), ALT ($p < 0.001$), AST ($p < 0.001$), γ-GT ($p < 0.001$), ferritin ($p < 0.001$), alkaline phosphatase ($p < 0.01$) and α_1-fetoprotein ($p < 0.01$), compared to the PNAL group (Table 2). In the HCV and CAH-C groups, linear regression analysis showed that plasma IL-18 was positively correlated with serum ALT ($p < 0.001$ and < 0.05, respectively), AST ($p < 0.001$ and < 0.01, respectively), γ-GT ($p < 0.001$ and < 0.02, respectively), and alkaline phosphatase ($p < 0.001$ and < 0.005, respectively) (Table 3). In the PNAL group, plasma IL-18 was positively correlated only with serum alkaline phosphatase ($p < 0.01$) (Fig. 1).

When the 14 subjects with CAH-C were classified in 2 groups based on their viral genotypes, plasma IL-18 levels were significantly higher in patients with viral genotype 1 (678 ± 299 pg/ml) than in patients with viral genotype non-1 (358 ± 175 pg/ml) ($p < 0.05$). Plasma IL-18 levels were positively correlated with serum ALT, AST, and γ-GT activities in patients with viral genotype 1 (Table 4).

Discussion

In this study we measured plasma levels of cytokine IL-18 in HCV-positive (CAH-C and PNAL) patients and NAFLD patients to obtain information about the roles of IL-18 in liver inflammation and immune response.

Several cytokines mediate hepatic processes including inflammation, apoptosis, necrosis, cholestasis, and fibrosis, but paradoxically, they are also involved in regeneration of liver tissue after injury. Evidence suggests that the immune response, and expecially the pro-inflammatory cytokines, plays an important role in liver injury induced by viral

Table 2. Analytical results (means ± SD) in patients with chronic active HCV hepatitis (CAH-C, n = 16) and in patients with HCV infection with persistently normal ALT levels (PNAL, n = 18).

Parameters	CAH-C group	PNAL group
serum ALT (U/L)	92.6 ± 57.0	37.1 ± 10.0***
serum AST (U/L)	94.0 ± 48.4	27.3 ± 7.0***
serum γ-GT (U/L)	73.4 ± 39.7	23.6 ± 10.8***
serum alkaline phosphatase (U/L)	95.9 ± 36.5	62.2 ± 15.8**
serum α_1-fetoprotein (ng/dl)	20.3 ± 23.4	2,3 ± 0.7**
serum ferritin (µg/L)	256.7 ± 187.1	78.3 ± 66.6***
plasma IL-18 (pg/ml)	556.3 ± 242.4	203.4 ± 82.3***

CAH-C subjects versus PNAL subjects: ***$p < 0.001$; **$p < 0.01$

Table 3. Pearson's correlation coefficients (r) of plasma IL-18 levels versus serum enzyme activities in the total group of patients with hepatitis C virus infection (n = 34) and in the subgroup of patients with chronic active HCV hepatitis (CAH-C, n = 16).

Parameter	TOTAL HCV patients (corr. coef. (r) vs plasma IL-18)	CAH-C patient (corr. coef. (r) vs plasma IL-18)
serum ALT (U/L)	0.68 ($p < 0.001$)	0.50 ($p < 0.05$)
serum AST(U/L)	0.78 ($p < 0.001$)	0.57 ($p < 0.01$)
serum γ-GT (U/L)	0.72 ($p < 0.001$)	0.52 ($p < 0.02$)
serum alkaline phosphatase (U/L)	0.74 ($p < 0.001$)	0.63 ($p < 0.005$)

HCV: hepatitis C virus
CAH-C: chronic active HCV hepatitis
PNAL: hepatitis C virus infection with persistently normal serum ALT levels
ALT: alanine aminotransferase
AST: aspartate aminotransferase; γ-GT: gamma-glutamyltranspeptidase

and dysmetabolic factors. Indeed, Th-1 type cytokines, such as IL-18, are crucial in enhancing cell-mediated immunity and in protecting against a number of viruses, including HCV [1].

Our data confirm previous reports of elevated plasma levels of IL-18 in HCV+ patients and in NAFLD patients, compared to healthy subjects. McGuinness et al [29], for instance, documented that IL-18 mRNA expression is significantly upregulated in HCV-associated chronic hepatitis [29].

Viral infections are known to suppress the immune system; induction of IL-18 binding protein and inhibition of IL-18 by endogenous IFN-α may contribute to the immunosuppressive state during viral infections [30].

Table 4. Pearson's correlation coefficients (r) of plasma IL-18 levels versus serum enzyme activities in patients with viral genotype 1 of chronic active hepatitis C (CAH-C, n = 9).

Parameter	corr. coef (r) vs plasma IL-18 levels
serum ALT (U/L)	0.72 ($p < 0.02$)
serum AST (U/L)	0.70 ($p < 0.02$)
serum γ-GT (U/L)	0.70 ($p < 0.02$)

ALT: alanine aminotransferase
AST: aspartate aminotransferase
γ-GT: gamma-glutamyltranspeptidase

There are indications that HCV+ patients present a Th1 type immune response, with over-production of IFN-γ. [29,33-35]. In contrast, Fan et al [36] documented a Th2 type immune response during CAH-C infection.

As IL-18 promotes the differentiation of naive T cells into Th1 cells, it may also have a negative role in the immunopathogenesis of chronic hepatitis C [30]. The different levels of IL-18 in separate compartments of the body should be dynamically evaluated in order to clarify Th1/Th2 balance in HCV infection [1]. We found strong positive correlations between plasma IL-18 levels and indices of inflammation and necrosis in patients with HCV, CAH-C, and PNAL.

In regard to an association between IL-18 and HCV-RNA, our in vivo results documented moderately higher levels of plasma IL-18 in virus genotype 1 compared to genotype non-1, in spite of the small number of patients. In contrast to these findings, possibly due to the small number of our subjects, Schvoerer et al [1] found in vitro that IL-18 levels were lower in genotype 1 HCV-infected patients than in healthy donors, while comparable IL-18 production was observed in genotype non-1 HCV-infected patients and controls. In vivo, plasma IL-18 levels tended to be lower for genotype 1 HCV-infected patients than for controls, although not significantly [1]. Lastly, Schvoerer et al [1] suggested that IL-18 levels and viral genotype

should be examined together during the progression of HCV infection in order to better clarify their possible involvement in the pathogenesis. Genotype 1 HCV-infected patients are supposed to be exposed to a more severe liver disease [1,37]. Moreover, low peripheral blood production of IL-18 in genotype 1 HCV-infected patients could be sufficient to favor HCV persistence. Thus, the relationships among genotype 1 HCV, plasma IL-18 levels, and the progression of HCV disease need further study.

As for NAFLD, it represents a potential evolution towards non-alcoholic steatohepatitis (NASH), which is a new syndrome that usually occurs in obese patients with a liver biopsy consistent with alcoholic steatohepatitis, but without a history of alcohol abuse. As documented by our NAFLD patients, who had a body mass index higher than either the HCV-infected patients or control subjects, obesity is a major risk factor for these syndromes. The magnitude of the problem is impressive, because as many as 20-25% of the general population is believed to be affected by NAFLD, and one-fourth to one-fifth of these may have NASH [38].

The etiology of NASH and NAFLD remains difficult to explain, but most investigators agree that a critical baseline of steatosis requires a second strong stimulus capable of inducing inflammation, fibrosis, or necrosis to develop NASH. The interaction of cytokines with oxidative stress [39-41] and lipid peroxidation has been postulated to play a key role in the induction of steatohepatitis [42,43]. Kugelmas et al [43] documented that plasma levels of IL-18 did not decrease with therapy in patients with NASH and suggested that the metabolic defect, which permits overproduction of cytokines in response to lipopolysaccharide (LPS) stimulation, was not corrected by short-term modest weight loss, with or without antioxidant therapy.

Our NAFLD patients showed, although not significantly, higher plasma levels of IL-18 than the controls, suggesting that IL-18 might have an important role in the prediction of worsening NAFLD. Moreover, we documented, for the first time, increased plasma levels of IL-18 in 2 HCV-subgroups (CAH-C and PNAL), with the highest IL-18 levels in CAH-C. Thus, most HCV-infected patients with persistently normal serum ALT activities will have mild disease in terms of degree of activity and fibrosis.

In conclusion, we showed that, in HCV (CAH-C and PNAL) and NAFLD patients, elevated plasma levels of IL-18 may have an important role as a marker of both inflammation and hepatic-biliary injury progression. In addition, we observed positive correlations between plasma IL-18 levels and cholestatic indices. Although the plasma IL-18 level is envisioned as an important marker of global liver injury, little is known about plasma IL-18 responses in HCV infection and hence further studies are needed.

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