RESEARCH ARTICLE

Serum cytokine levels as putative prognostic markers in the progression of chronic HCV hepatitis to cirrhosis

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ABSTRACT. Hepatitis C virus (HCV) infection can present as an acute manifestation, and can lead to severe complications such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). It represents a global health problem because there is no vaccine currently available. Cytokines play an important role in viral clearance, infection control, inflammation, regeneration and fibrosis, and also are implicated in the pathological processes occurring in the liver during viral infection. Immunological markers of chronic HCV hepatitis progression as compared to cirrhosis and HCC would be extremely useful, particularly for distinguishing between the molecules produced during HCV-induced chronic inflammation and those secreted during cirrhosis and HCC. In this work, we evaluated the serum levels of several cytokines, chemokines and growth factors in 30 patients affected by chronic HCV (HC), 30 patients affected by HCV-related cirrhosis (LC) and 20 healthy, control subjects. We used a multiplex biometric ELISA-based immunoassay in order to identify molecules that might be useful for monitoring the progression of HCV to liver cirrhosis and, possibly, to cancer. Our results show that some pro-inflammatory molecules are significantly up-regulated, and play a role as immunological markers in the intermediate steps towards liver cancer, and that hepatocyte growth factor (HGF) is a specific marker of liver cirrhosis. Finally, these data will be used to define a cytokinome profile, which might prove useful for studies involving the transition of chronic inflammation to neoplastic processes.

Keywords: cytokines, chemokines, growth factors, HCV, cirrhosis, cancer

Hepatitis C virus (HCV) is a small, enveloped, positive-sense, single-stranded RNA virus that belongs to the *Hepacivirus* genus, within the *Flaviviridae* family. It is very efficient at evading the host immune response using mechanisms such as escape mutation selection, specific T cell anergy induction or resistance to the effects of alpha-interferon [1]. HCV infection can present with acute manifestations, and may lead to complications such as chronic hepatitis, cirrhosis, hepatocellular carcinoma (HCC), and extrahepatic manifestations. In contrast to HBV, the HCV genome does not enter the cell nucleus; it infects human liver without killing the infected cells. In fact, the virus triggers in the infected subjects, an immune-mediated inflammatory response (hepatitis) that either clears the infection or slowly destroys the liver [2, 3]. In particular, hepatitis C virions show a rapid turn-over (with a half-life of about 3 h), and up to 1012 complete viruses are produced per day, on average, in an infected person [4]. About 80% of newly infected patients develop chronic infection; an estimated 10% to 20% will develop cirrhosis, and 1% to 5% progress to end-stage liver cancer (HCC) over a period of 20 to 30 years. Moreover, the development of significant hepatic fibrosis (stage 4 fibrosis, according to the Ishak index, or cirrhosis), dramatically increases the incidence of HCC [5, 6].

HCV infection and associated liver inflammatory diseases represent a major global health problem, affecting about 3% of the world population [7]. In particular, cirrhosis and HCC jointly, are the primary indication for liver transplantation in the United States and Europe [8]. The currently recommended treatment for chronic HCV infection and cirrhosis is the combination of pegylated interferon alpha and ribavirin. Unfortunately, this treatment is effective in only about 55% of cases and is burdened by side effects. A preventative vaccine for HCV infection is not yet available [9]. Cytokines, chemokines and growth factors that are secreted by immune system

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cells and other cell types, play an important role in viral clearance, infection control, inflammation, regeneration and fibrosis. However, these molecules are also implicated in both viral persistence and in the liver damage seen during chronic HCV infection [10]. In recent years, there has been a great interest in the search for possible immunological markers of progression of chronic HCV to cirrhosis and HCC. However, few data have been reported in the literature. It is well known that persistent inflammatory conditions can induce cancer formation; cytokines and chemokines are involved in cancer-related inflammation and might thus represent a target for innovative diagnostic and therapeutic strategies [11].

In our recent paper, we evaluated the serum levels of numerous cytokines, chemokines and growth factors in patients affected by HCC with chronic HCV hepatitis and liver cirrhosis. The data obtained showed that some pro-inflammatory molecules were significantly up-regulated in our patients compared to healthy control subjects, and highlighted the complexity of the cytokine network. Moreover, these results also demonstrate the need to distinguish between cytokines, chemokines and growth factors induced by chronic HCV infection, and those secreted during HCC [12].

Using a multiplex biometric ELISA-based immunoassay, we also evaluated the serum levels of certain cytokines, chemokines and growth factors in patients with chronic HCV and HCV-related cirrhosis, in order to identify molecules which might prove to be useful markers of the progression of HCV to liver cirrhosis and HCC, and to identify a cytokinome profile specific for the transition of chronic inflammation to cancer.

DONORS AND METHODS

Patients

In this study, we enrolled thirty patients (15 women, 15 men) with chronic HCV hepatitis (HC), thirty patients (16 women, 14 men) with HCV-related cirrhosis (LC), and 20 healthy control subjects (11 women, 9 men). This was based upon our interest in studying the progression from chronic liver damage to cirrhosis according to the degree of liver fibrosis. The clinical characteristics of these patients are listed in *table 1*. All patients had transaminase values (AST and ALT) higher than the control range, as evaluated in healthy donors. Moreover, patients with liver cirrhosis presented higher birilubin and lower albumin values and platelets counts (PLT) with respect to the control range. All patients had normal alpha-fetoprotein (AFP) values, and did not present cancer. The stage of fibrosis was assessed for the HC patients according to the Ishak index [13]. In particular, F2 corresponding to fibrosis of the majority of portal tracts, F3 to fibrosis of the majority of portal tracts with occasional port-portal septa, and F4 to fibrosis of the majority of portal tracts with port-portal and port-central septa.

The serum concentrations of certain cytokines, chemokines and growth factors were evaluated in all patients and healthy donors.

BioPlex assay

Blood samples were collected from a peripheral vein and kept on ice. Serum was collected by centrifugation (3,000 rpm for 10 min at 4°C), aliquoted, and stored at - 80°C until analyzed. A multiplex biometric ELISA-based immunoassay, containing dyed microspheres conjugated with a monoclonal antibody specific for a target protein was used according to the manufacturer's instructions (Bioplex, Bio-Rad Lab., Inc., Hercules, CA, USA). Soluble molecules were measured using two commercially available kits: *i)* 12-Plex panel: IL-1β, IL-1ra, IL-2R, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17, CXCL10, CCL3; *ii)* 21-Plex panel: IL-1α, IL-2R, IL-3, IL-12p40, IL-16, IL-18, CCL27, CXCL1, CXCL9, CXCL12, HGF, IFN-α2, LIF, MCP-3, M-CSF, MIF, β-NGF, SCF, SCGF-β, TNF-β, TRAIL.

Each experiment was performed in duplicate, using the same procedure described in our recent paper [12]. Serum levels of all proteins were determined using a Bio-Plex array reader (Luminex, Austin, TX) that quantitates multiplex immunoassays in a 96-well format with very small fluid volumes. The analyte concentration was calculated

Table 1

Clinical characteristics of patients with chronic inflammation (HC) and liver cirrhosis (LC). We report the number of patients to which the parameters refer. For clinical data, the mean value and the related control range, evaluated in healthy donors, are shown

	HC	LC	Control range
Age	63.86	67.96	60.92
Gender	15M-15F	14M-16F	9M-11F
AST (IU/L)	70.69	80.54	5-40
ALT (IU/L)	120.90	71.96	7-56
Tot bilirubin (mg/dL)	0.91	1.70	0.20-1.30
Albumin (g/dL)	4.11	2.61	3.5-5
PLT (mL)	187413.79	113875	150,000-400,000
HCV – PCR RNA	Positive	Positive	
HCV genotype	1: 18; 2: 12	1: 22; 2: 8	
Ishak index	F2: 8; F3: 14; F4: 8		
AFP (ng/mL)	< 10	< 2 0	
Child-Pügh [28, 29]		A:12; B:10;C:8	

Table 2
P-values obtained for all significant molecules in chronic inflammation (HC) and liver cirrhosis (LC) patients, with respect to healthy control subjects using the nonparametric Mann-Whitney U test

	HC *vs* controls	LC *vs* controls
IL-1α	0.0196*	0.0077**
IL-1β	< 0.0001***	< 0.0001***
IL-2R	0.0355*	0.0053**
IL-6	0.0032**	0.0024**
IL-8	0.0004***	0.0001***
CXCL1	0.0076**	0.0034**
CXCL9	0.0004***	0.0002***
CXCL10	0.0015**	0.0003***
CXCL12	0.0364*	0.0443*
MIF	0.04*	0.0033**
β-NGF	0.0008***	0.0002***
HGF		0.0028**

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

using a standard curve, with software provided by the manufacturer (Bio-Plex Manager Software).

Data analysis and statistics

Data were analysed using the Bio-Plex Manager software version 3.0 (Bio-Rad Lab.). The nonparametric Mann-Whitney U test was used to evaluate differences between cytokine, chemokine and growth factor ratios from HC and LC patients, and healthy controls. One asterisk (*) indicates values where $p < 0.05$, two asterisks (**) values where $p < 0.01$, and three asterisks (***) values where $p < 0.001$. Student's T-test was used to compare the serum levels of these proteins evaluated in HC and LC patients. The correlations between the cytokine levels and clinical data were determined using the Pearson correlation coefficient. Values of $p < 0.05$ were considered to be statistically significant. The statistical program Prism 4 (GraphPad Software, San Diego, CA, USA) was used.

RESULTS AND DISCUSSION

Comparison between patients with chronic inflammation or liver cirrhosis, with respect to healthy donors

In *table 2*, we report the different levels of the pro-inflammatory cytokines, chemokines and growth factors that were present in the serum of patients with chronic inflammation (HC) or liver cirrhosis (LC), with respect to healthy controls; data that were not statistically significant were not reported. Greater amounts of IL-1α, IL-1β, IL-2R, IL-6, IL-8, CXCL1, CXCL9, CXCL10, CXCL12, MIF, and β-NGF were secreted by both HC and LC patients. The significantly increased serum levels of IL-1β, IL-2R, IL-6, IL-8 found in our patients with LC are in agreement with data recently reported elsewhere [14]. In particular, all four chemokines (CXCL1, CXCL9, CXCL10, CXCL12), which orchestrate the immune response to viruses including hepatitis C virus, are crucial for viral elimination. Their persistent expression in chronic HCV infection can actually drive tissue damage and inflammation [15]. In particular, CXCL9 and CXCL10, two related chemokines, are increased in both

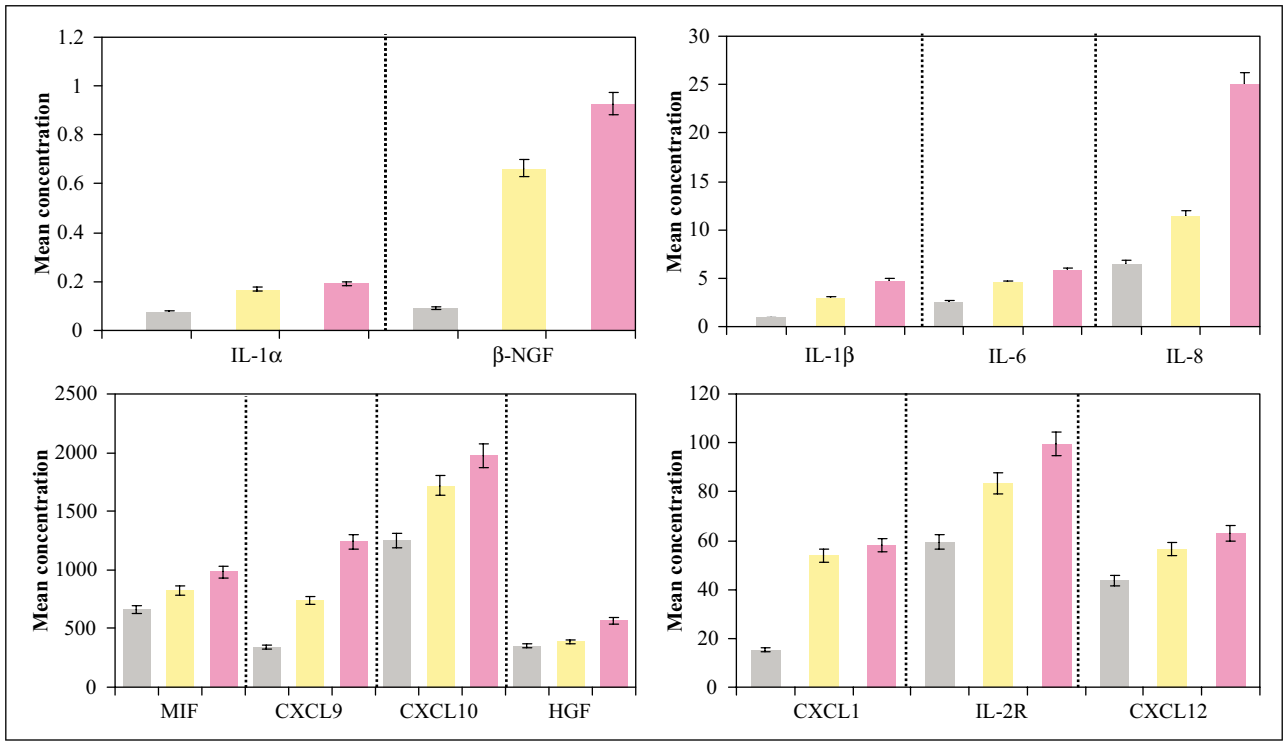

Figure 1
Mean concentrations of significant pro-inflammatory cytokines, chemokines and growth factors in healthy control subjects (light grey), in chronic inflammation (yellow), and liver cirrhosis patients (pink).

HC and LC patients, and are indicated as markers of liver inflammation status and HCC progression [10, 16]. CXCL12 is reported to be involved in advanced liver diseases, being associated with the HCV virus [17]. Also in the literature, it is reported that MIF correlates with inflammatory changes only in human chronic hepatitis B infection, and is involved in liver injury [18, 19], whereas β-NGF has been detected in diseased liver tissues and is involved in LC leading to HCC [20].

As shown in *table 2*, in the chronic inflammation and LC patients, the same molecules were increased, the only exception being that hepatocyte growth factor (HGF) was significantly up-regulated only in those patients with LC and not in those with chronic inflammation. HGF is a multifunctional growth factor that regulates growth and cell motility. It exerts mitogenic effects on hepatocytes and epithelial cells, and plays diverse roles in organ development, tissue regeneration, and tumor progression [21]. Moreover, it is implicated, along with IL-6, IL-8 and IL-1, in the hepatic stellate cell-activation pathway. However, numerous reports have examined the relationship between HGF and either the facilitation or suppression of HCC, and have suggested that this growth factor could be used as an index of cellular growth and of the development of HCC in LC patients [22-24]. In fact, it is very interesting that levels of this molecule were significantly different in LC patients, as compared to both healthy controls and patients with chronic inflammation, and that its concentration in HCC patients was higher than in patients with LC. This suggests that the levels of HGF might have increased during the progression of chronic inflammation to LC and cancer. We therefore suggest that HGF might represent the degree of the carcinogenic state in the liver of chronic inflammation and LC patients, and could possibly be used for predicting the progression to HCC in patients with chronic HCV-related liver disease, in accordance with papers already published [22-24].

Comparison between patients with chronic inflammation and LC

Since IL-1α, IL-1β, IL-2R, IL-6, IL-8, CXCL1, CXCL9, CXCL10, CXCL12, MIF, and β-NGF were increased in both HC and LC patients as compared to healthy control subjects, we compared their mean concentrations using Student's T-test. As shown in *figure 1*, the concentrations of all these proteins were higher (with $p < 0.05$) in patients with LC than in those with chronic inflammation. In particular, IL-8, CXCL9 and β-NGF showed different levels of expression between HC and LC patients, their concentrations being higher in patients with LC.Our

A

	CXCL1	IL-1α	IL-2R	MIF	CXCL9	ß-NGF	CXCL12	IL8	CXCL10	IL-1ß	IL-6	AST	ALT	PLT
CXCL1	1	0,5840	0,1032	0,1219	0,6656	0,0161	0,1688	0,7428	0,1647	0,7800	0,8332	0,1630	0,1766	0,3959
IL-1α			0,0400	0,0140	0,6653	0,4586	0,9552	0,6410	0,8311	0,6374	0,6139	0,0244	0,0205	0,7476
IL-2R				0,0002	0,0058	0,0005	0,0203	0,7297	0,8099	0,5597	0,4554	0,0001	0,0001	0,8256
MIF					0,9364	0,0189	0,2492	0,7693	0,6114	0,9228	0,7069	0,0012	0,0007	0,2942
CXCL9						0,1673	0,0003	0,6020	0,4431	0,0305	0,6275	0,9524	0,7913	0,9030
ß-NGF							0,0054	0,8974	0,3431	0,9163	0,3276	0,0170	0,0237	0,2936
CXCL12								0,1183	0,6250	0,1056	0,0122	0,2789	0,2216	0,9316
IL8									0,9185	0,0032	0,0015	0,7591	0,8088	0,5732
CXCL10										0,4945	0,7111	0,8025	0,6864	0,2954
IL-1ß											0,0301	0,8631	0,5607	0,2099
IL-6												0,4660	0,4397	0,8475
AST													0,0000	0,6579
ALT														0,5375

B

	CXCL1	IL-1α	IL-2R	MIF	CXCL9	ß-NGF	CXCL12	HGF	IL8	CXCL10	IL-6	IL-1ß	AST	ALT	PLT	Bilirubin	Albumin
CXCL1	1	0,7267	0,7382	0,8155	0,8884	0,6406	0,8072	0,0466	0,6766	0,0184	0,3533	0,2132	0,5524	0,2540	0,4220	0,6602	0,0487
IL-1α			0,0854	0,0669	0,1092	0,0049	0,3205	0,7645	0,8112	0,7491	0,8164	0,8355	0,3212	0,7929	0,6448	0,4803	0,4002
IL-2R				0,0030	0,2482	0,0010	0,3745	0,4655	0,6769	0,9569	0,3325	0,5437	0,9787	0,1704	0,9536	0,9486	0,6619
MIF					0,8507	0,0114	0,1492	0,2329	0,2016	0,9204	0,9384	0,8262	0,6730	0,0678	0,7498	0,3948	0,7117
CXCL9						0,1090	0,2487	0,2983	0,0245	0,0213	0,2623	0,1621	0,4048	0,6933	0,3732	0,3363	0,0457
ß-NGF							0,1420	0,0615	0,8675	0,7056	0,4239	0,8152	0,3635	0,5829	0,3605	0,9319	0,8650
CXCL12								0,0957	0,0520	0,5909	0,5536	0,4448	0,5042	0,5897	0,6338	0,5324	0,9622
HGF									0,1029	0,0122	0,6123	0,0157	0,4378	0,2265	0,7058	0,4270	0,0237
IL8										0,1531	0,6767	0,4790	0,3616	0,5530	0,3850	0,2470	0,3764
CXCL10											0,4475	0,0013	0,4012	0,8356	0,2830	0,0128	0,0470
IL-6												0,3036	0,5421	0,6320	0,6338	0,4725	0,8252
IL-1ß													0,4113	0,3043	0,3963	0,3550	0,3173
AST														0,0188	0,6129	0,0133	0,5341
ALT															0,8414	0,0757	0,4187
PLT																0,0742	0,1424
Bilirubin																	0,0715

Figure 2

Correlations between serum levels of significant molecules and clinical data in patients with chronic inflammation (**A**) and liver cirrhosis (**B**), using the Pearson correlation. P-values lower than 0.05 are highlighted in yellow.

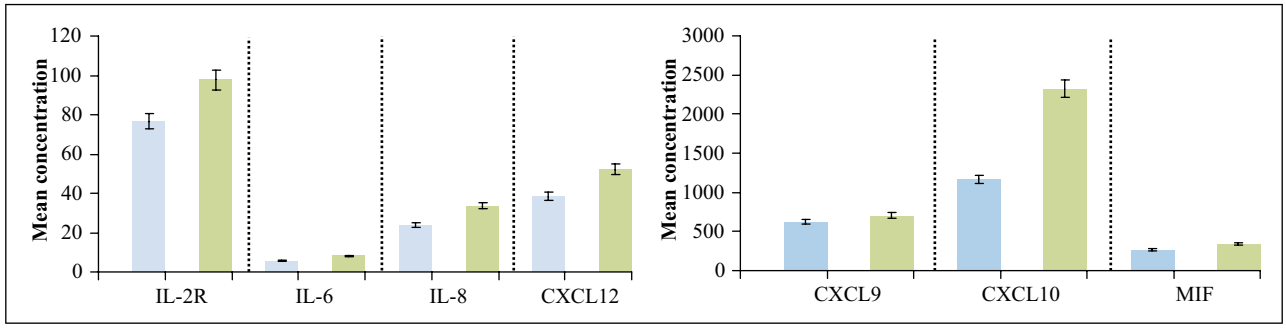

Figure 3

Mean concentrations of significant pro-inflammatory molecules in chronic inflammation patients with F2 (cyan) and F4 (green) stages of fibrosis.

results regarding these proteins are in agreement with recently reported data [14, 16, 20].

We then compared the serum levels of all cytokines, chemokines and growth factors in HC and LC patients with respect to those in HCC patients tested in our recent paper [12]. The mean concentrations of all of these molecules were higher in HCC patients than in those with LC. This suggests that the expression of these pro-inflammatory molecules tends to increase in the chronic inflammation progression that leads to LC and HCC, and thus, their evaluation could be used for prognostic studies.

We correlated the serum levels of statistically significant cytokines, chemokines and growth factors in the HC and LC patients with clinical data, using the Pearson correlation coefficient. In patients with chronic inflammation, IL-1α, IL-2R, MIF and β-NGF showed significant correlation, and a positive correlation coefficient with the transaminase values *(figure 2A)*, which were higher in these patients than in healthy controls *(table 1)*. Therefore, these proteins could be considered to be an index of immune activation. In particular, our results were in agreement with literature data reporting that IL-1 and IL-2R participate in the progression from liver injury to fibrosis [25, 26] and that β-NGF is involved in liver cancer growth and metastasis and can be used as an index of chronic infection leading to LC and HCC [27, 20]. Indeed, our work suggests, for the first time, a role for MIF in HCV-related chronic inflammation, as increased serum MIF has been reported only in HBV patients [19].

Moreover, levels of CXCL1, CXCL9, CXCL10 and HFG in patients with LC showed a significant correlation, a positive correlation coefficient between them, and a negative correlation coefficient with albumin values *(figure 2B)*, that were lower in these patients with respect to controls. Also, as HGF was the only other molecule whose levels were statistically different between HC and LC patients, our data suggest that these four proteins could be useful for diagnostic/prognostic purposes.

Comparison of patients with chronic inflammation and different stages of fibrosis

Since the stage of fibrosis in patients with chronic inflammation had been determined (Ishak index) *(table 1)*, we divided these patients into three subgroups corresponding to stages F2, F3 and F4. We compared the mean concentrations of the significant molecules in the three groups using Student's T-test. No relevant differences were observed between chronic inflammation patients with F3 and F4 grades because they corresponded to two, already well advanced stages of fibrosis. Comparing F2 and F4 patients, the concentrations of IL-2R, IL-6, IL-8, CXCL-9, CXCL-10, CXCL-12 and MIF were statistically higher (with $p < 0.05$) in patients with chronic inflammation and an F4 grade as compared to those with an F2 grade *(figure 3)*. These data are in agreement with a recent paper reporting that levels of CXCL9 and CXCL10 were significantly elevated in patients with advanced fibrosis [16]. However, these results need to be confirmed in a larger number of patients.

In conclusion, our results suggest that *i)* IL-2R, IL-6, IL-8, CXCL-9, CXCL-10, CXCL-12 and MIF could be markers of the progression of chronic hepatitis C infection to LC and *ii)* HGF, being over-expressed only in those patients with LC, could be an marker of the progression of fibrosis leading to LC.

These results, obtained using patient serum, will be confirmed using tissues and other experimental methods. Nevertheless, these data will be useful in drug-design studies for investigating molecules that are able to block the progression of fibrotic damage in patients with chronic inflammation leading to LC and then, to HCC.

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