Inflammatory cytokines and S-100b protein in patients with hepatitis C infection and cryoglobulinemia

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Manuscript submitted 15th March, 2007

Manuscript accepted 27th May, 2007

Clin Invest Med 2007; 30 (5): E167-E176.

Abstract

Objective: To investigate a predictive role for the protein S-100b and serum circulating levels of Th1/Th2 cytokines in patients with chronic hepatitis C virus (HCV) infection with and without mixed cryoglobulinemia (MC).

Methods: Sixty chronically HCV-infected patients were divided into two groups: 30 with and 30 without MC. Patients with MC presented detectable mixed cryoglobulins and clinical weakness, purpura and arthralgias. HCV-RNA and genotype, serum levels of cryoglobulins, principal hepatic indexes and levels of IL-6, IL-18 and S-100b protein were evaluated. Twenty uninfected healthy subjects were a control group to evaluate serum levels of S-100b protein.

Results: IL-6 and IL-18 serum levels were higher in the MC+ group than the MC- group (8.7 ± 4.5 pg/mL *versus* 4.6 ± 2.3 pg/mL $P < 0.0001$ and 743.5 ± 128.2 pg/mL *versus* 578.5 ± 296.5 pg/mL $P < 0.001$ respectively). S-100b serum levels were higher in HCV+ with MC (0.23 ± 0.07 microg/L) respect to HCV+ patients without MC (0.17 ± 0.05 microg/L, $P < 0.0001$) and were statistically higher than in

the control group (0.08 ± 0.03 microg/L, $P < 0.0001$ and $P < 0.0001$, respectively). A positive correlation was shown between serum levels of S-100b protein and levels of cryoglobulins in the group of HCV+ patients MC+ ($r = 0.72$ and $P < 0.0001$).

Conclusion: HCV patients with MC have a worse inflammatory condition than those without MC. Moreover, S-100b protein seems to be a sensitive marker of endothelial and tissue damage in chronic HCV hepatitis with cryoglobulinemia.

Keywords: Cytokines, S-100b protein, hepatitis, HCV, cryoglobulinemia

Chronic hepatitis C-virus (HCV) infection is associated with several extrahepatic manifestations, the most important of which is mixed cryoglobulinemia (MC).^{1,2} MC is a chronic systemic disease, characterized by a symptom triad of weakness, purpura and arthralgias (Meltzer and Franklin disease). Histology of

MC is characterized by vasculitic lesions involving medium and small-sized blood vessels of the skin and internal organs (kidneys, nervous system, etc.) secondary to deposition of circulating immune complexes and complement. About 30% of HCV-infected patients develop circulating cryoglobulins.³⁻⁶ This may depend on several factors such as duration of chronic infection, virus characteristics (some authors assert that HCV genotype 2a is more frequently associated with MC), or host genetic factors (HLA system, activity of lymphocytes and natural killer cells). The majority of HCV-infected patients with circulating cryoglobulins are either asymptomatic or have non-specific findings.⁷⁻¹⁰ The triad of purpura, asthenia and arthralgia is evident at the onset of the disease in a variable percentage of cases.¹¹ MC prevails in advanced age (50-60 yr) and in women. The term cryoglobulinemia refers to the presence in the serum of one or more proteins, named cryoglobulins, that reversibly precipitate below 37°C and redissolve on re-warming and consist of a mixture of monoclonal or polyclonal IgM that have antiglobulin activity and bind to polyclonal IgG. According to the clonal composition of immunoglobulins in the cryoprecipitate (cryoglobulins). Cryoglobulinemia is usually classified into three subgroups: type I, composed of a single monoclonal immunoglobulin (monoclonal IgG or IgM); type II, composed of two immunoglobulins, one monoclonal (usually IgM) and other polyclonal (usually IgG); type III, characterized by polyclonal IgG and IgM. Type I cryoglobulinemia is also named monoclonal cryoimmunoglobulinemia; it is almost invariably associated with hematological disorders and it is frequently asymptomatic. Type II and type III are defined as mixed cryoglobulinemia (MC) and they may be secondary to numerous infections or immunological disorders. When isolated, MC may represent a distinct disease, the so-called “essential” MC. Given the striking association with HCV infection (>90%), the term “essential” is now referred to a minority of MC patients (<10%).⁶ Cryoglobulinemic vasculitis, predominantly involving the smaller vessels, is observed in less than 10% of patients.⁶ The most frequently affected organs are the skin (purpura, leg ulcers), nerves (peripheral neuropathy) and kidneys

(glomerulonephritis). Cryoglobulins are quantified either as the cryocrit or as the protein concentration.

HCV is involved in the pathogenesis of the majority (> 90%) of essential MC.¹²⁻¹⁴ Between 40 and 56% of HCV-infected patients have MC.¹⁵⁻¹⁷ The behaviour of MC is closely linked to the natural history of HCV chronic infection but the MC is also the result of concomitant genetic and/or environmental factors which remain largely unknown. HCV infection may exert a chronic stimulus on the immune system. The interaction between the HCV envelope protein E2 and the CD81 receptor on B cells¹⁸⁻²¹ may lower the activation threshold of these cells, thus facilitating the production of various autoantibodies, including IgM rheumatoid factor (RF). Transformation from polyclonal B cell proliferation (type III MC) to oligo/monoclonal B cell proliferation (type II MC) and then to overt malignant lymphoma is a multistep process that probably requires multiple genetic aberrations. The high prevalence of Bcl-2 rearrangement in patients with HCV-related MC is consistent with this hypothesis. T(14;18) translocation of the Bcl-2 oncogene results in overexpression of the gene, which may prolong the lifespan of B lymphocytes, favoring the emergence of a dominant clone capable of producing monoclonal IgM- κ RF.²

Several studies suggest that the immune response, especially the pro-inflammatory cytokines play an important role in liver injury induced by viral factor, particularly IL-6, a pleiotropic Th2 cytokine traditionally considered an activator of acute phase responses and a lymphocyte stimulatory factor²² and it plays a role in immune responses that may lead to viral clearance.²³ IL-18 is a potent proinflammatory Th1 cytokine, correlated with metabolic and viral hepatitis diseases that has pathophysiological roles in several inflammatory conditions. It is produced mainly by monocytes/macrophages but it is expressed in a wide variety of cells. Therefore, the functions of IL-18 *in vivo* are very heterogeneous and complicated. IL-18 enhances Th1 immune responses, but it can also stimulate Th2 immune responses.²⁴ The Th1/Th2 cytokine balance seems to play a pivotal role also in extrahepatic im-

munological complications of HCV infection such as MC.²⁵

Recently, the extracellular functions of S100 proteins such as the ability to enhance neuritic outgrowth and the involvement in inflammation, have attracted more attention.²⁶⁻²⁸ S-100 proteins are small dimeric extracellular and cytosolic proteins that modulate the activity of other target proteins in a calcium-dependent manner and regulate intracellular processes such as cell growth and motility, cell cycle regulation, transcription and differentiation.²⁹⁻³³ S-100 is a calcium-binding protein with a molecular weight equivalent to 21000 daltons synthesized in astroglial cells in all parts of the Central Nervous System (CNS). It is present in the body in different subchains formed by several α and β sub-unit combinations of which the β form (96%) predominates in the brain. S-100b protein is present in the cytosol of glial and Schwann cells, and also in adipocytes and chondrocytes, although in very low concentrations in the latter two. The role of protein S-100b is not fully understood. It is suggested that it has intracellular and extracellular neurotropic, as well as neurotoxic function. At nanomolar levels, S-100b stimulates neuritic outgrowth and enhances survival of neurons. However, at micromolar levels it stimulates the expression of inflammatory cytokines and induces apoptosis. Moreover, it has been demonstrated that S-100b protein is located around the cell nucleus of endothelial cells and that it is translocated in the cytoplasm and cell membrane upon different stimulation.²⁸ Recently, serum S-100b protein has been proved to be an attractive surrogate marker of primary severe brain injury and secondary insults. It can be measured in the arterial and venous serum; it is not affected by haemolysis and remains stable for several hours without the need for immediate analysis.

Many investigators believe that cytokines play an important role in both immunoregulation and immune impairment.³⁴⁻³⁵ The Th1/Th2 cytokine balance is likely important in determining the rate of HCV infection chronicity and HCV-induced liver injury.³⁶ The circumstances that predispose HCV-infected patients to developing MC remain unclear. The aim of this

study was to evaluate the pro-inflammatory cytokine production (IL-6 and IL-18) in the serum of HCV-positive patients with cryoglobulinemia, respect to HCV-positive patients without cryoglobulinemia and to explain the implications of IL-18 and IL-6 and the degree of inflammation in viral liver disease in HCV+ patients with cryoglobulinemia. Moreover, considering that the evolution of MC determines central and peripheral nervous system alteration and endothelial damage, we evaluated the predictive role of the S-100b protein serum level in patients affected with HCV+ with MC compared with controls.

Materials and Methods

All subjects gave written informed consent and the study was approved by the Medical Ethics Committee of the "G. D'Annunzio" University Medical School.

Patients

Sixty Caucasian patients with chronic Hepatitis C-Virus (HCV) infection were recruited on their first examination at the Infectious Diseases Clinic of the G. d'Annunzio University of Chieti. Twenty uninfected healthy subjects, selected for ethnicity, sex and age, were included as a control group to evaluate serum levels of S-100b protein.

Patients diagnosed with MC presented a detectable circulating mixed cryoglobulins and a systemic disease clinically characterized by the triad: weakness, purpura and arthralgias. They were divided on the basis of the presence of HCV infection with MC (14 male and 16 female, aged 57.3 ± 13.0 yr) and HCV infection without MC (22 males and 8 females, aged 55.5 ± 16.2 years).

All had histologically proven chronic active liver disease, and positive HCV antibodies and RNA. They were negative for hepatitis B surface antigen and human immunodeficiency virus antibodies. MC-positive patients had serum cryoglobulins $> 0.05\text{g/l}$, on at least two occasions. MC-negative patients never had detectable cryoglobulins.

Virological and immunological serum markers

The HCV-RNA in serum was determined by Polymerase Chain Reaction (PCR) (Amplicor method - Roche Mol. Diagn., Milan, Italy), with detection limit ≥ 600 HCV-RNA UI/mL plasma, was valuated also HCV genotype.

Cryoglobulins were isolated from the patients' sera, purified, and characterized by immunoblotting at 37 °C, as previously described. In this study, patients were considered to have a significant amount of serum cryoglobulins if they had a minimum level of 0.05 g/l on two occasions. According to the classification of Brouet et al.³ all patients had either type II or type III MC.

Clinic and biochemical characteristics

Blood samples were taken to measure serum levels of aspartate aminotransferases (AST), alanine aminotransferases (ALT), gamma-glutamyltranspeptidase (γ -GT), alkaline phosphatase, levels of fasting glucose (glucose-oxidase method), total cholesterol and triglycerides (automated enzymatic method - Ortho-Clinical Diagnostics, Rochester, NY, USA), α_1 -fetoprotein (LIAISON AFP, DiaSorin, Vercelli, Italy) and ferritin (LIAISON Ferritin, DiaSorin, Vercelli, Italy).

IL-6, IL-18 and S-100b protein

Blood was taken at the time of liver biopsy in sterile heparinized tubes, transported on ice to the laboratory, centrifuged at 6 °C and the serum was kept frozen at -70 °C until assayed. Cytokine levels were measured once in all patients. The cytokine IL-6 was evaluated by Cytometric Bead Array Assay (Human Th1/Th2 Cytokine kit, BD Biosciences, San Diego, CA). For this assay, soluble cytokines are captured on microparticles and then measured using a fluorescence-based detection system and flow cytometry analysis as previously described. A series of 10 dilutions from cytokine standards were run in each assay for the genera-

tion of standard curves. Samples were analyzed in a FACSCalibur flow cytometer using the BD CBA Analysis Software. For each study subject, the mean value for each cytokine was used for statistical analysis.

IL-18 plasmatic levels were calculated by an enzyme-linked immunosorbent assay (IL-18 ELISA - R & D Systems, Minneapolis, MN, USA). The minimum detection limit estimated by serial dilution was 12.5 pg/mL since the mean +2 SD of the 6.25 pg/mL was lower than the mean -2 SD of the 12.5 pg/mL.

LIAISON Sangtec 100 is a two-site immunoluminometric assay (sandwich principle) based on paramagnetic particles coated with two monoclonal antibodies and a monoclonal tracer antibody labelled with an isoluminol derivative. The S-100b concentration was determined by the induced chemiluminescence reaction. The serum detectable limit considered for S-100b was <0.15 microg/L.

Statistics

Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was assessed by Student's *t* test for unpaired data and χ^2 test for frequency variables. Spearman's correlation coefficient between serum levels of S-100b protein and the cryoglobulin levels was computed. A *P* value ≤ 0.05 was required.

Results

The main characteristics of 60 patients are summarized in Table 1 (sex, age, duration disease, hepatic index, viral load and genotype). The characteristics of 30 patients HCV+ with MC were as follows: the mean cryoglobulin level was 1.4 ± 0.8 g/l. The mean duration of HCV infection since age of diagnosis was 7.4 ± 4.4 yr for MC patients and 3.8 ± 3.3 yr for patients without MC ($P < 0.0001$). The mean ALT serum level was 124.5 ± 48.9 U/L in MC patients and did not differ from those HCV patients without MC, as well as AST, γ -GT and alkaline phosphatase. Mean virologic data were similar in both groups, genotype (genotype 1 in 60% of MC patients vs 56.6% in MC negative

TABLE 1. Parameters and clinical laboratory measurements (mean $\pm$ SD) in the study populations.

Parameter (and units)	HCV-infected patients with MC (n=30)	HCV-infected patients without MC (n=30)
Sex: M/F	7/8	2 3/4
Age (yr)	57.3 $\pm$ 13.0	55.5 $\pm$ 16.2
Disease duration (yr)	7.4 $\pm$ 4.4	3.83 $\pm$ 3.3 ***
Viral load (log 10 ⁴ UI/mL)	24.9 $\pm$ 30.9	32.9 $\pm$ 33.2
Genotype		
1 (%)	18 (60)	17 (56.6)
2 (%)	6 (20)	12 (40)
3 (%)	4 (13.3)	0
4 (%)	2 (6.6)	1 (3.3)
Cryoglobulin levels (mcg/L)	1.46 $\pm$ 0.8	—
Serum AST activity (U/L)	143.8 $\pm$ 122.0	153.8 $\pm$ 103.6
Serum ALT activity (U/L)	124.5 $\pm$ 48.9	127.6 $\pm$ 59.5
Serum γ -GT activity (U/L)	62.4 $\pm$ 59.7	77.7 $\pm$ 70.9
Serum Alkaline Phosphatase (U/L)	95.2 $\pm$ 37.9	103.0 $\pm$ 51.2
Serum α_1 -fetoprotein (ng/dL)	14.1 $\pm$ 17.5	9.0 $\pm$ 13.0
Serum ferritin (mcg/L)	429.5 $\pm$ 326.8	376.7 $\pm$ 261.2
Serum cholesterol (mg/dL)	146.9 $\pm$ 23.7	158.6 $\pm$ 42.6
Serum triglycerides (mg/dL)	104.5 $\pm$ 32.0	120.4 $\pm$ 38.3
Serum fasting glucose (mg/dL)	92.6 $\pm$ 18.0	87.1 $\pm$ 20.2
Blood platelet count (10 ³ cells/L)	142 $\pm$ 75	156 $\pm$ 62

HCV: Hepatitis C Virus

MC: Mixed cryoglobulinemia

AST: aspartate aminotransferase; ALT: alanine aminotransferase;

 γ -GT: gamma-glutamyltranspeptidaseHCV MC+ subjects vs HCV MC- subjects:*** $P < 0.0001$;

patients) and HCV viremia were positive in all patients. Liver biopsy specimens often showed signs of chronic active hepatitis, but there were no significant differences in the distribution of necroinflammatory lesions or fibrosis between the groups.

The two inflammatory cytokines showed that IL-6 was higher in the MC+ group than the MC- group (8.7 ± 4.5 pg/mL versus 4.6 ± 2.3 pg/mL $P < 0.0001$), moreover the IL-18 serum levels were higher in patients with MC+ (743.5 ± 128.2 pg/mL) than in those with MC- (578.5 ± 296.5 pg/mL) ($P < 0.001$) (Fig. 1).

We documented that S-100b serum levels were higher in HCV+ with MC (0.23 ± 0.07 microg/L) respect to HCV+ patients without MC (0.17 ± 0.05 microg/L, $P < 0.0001$). Moreover, these levels of S-100b protein were statistically higher than in the control group (0.08 ± 0.03 microg/L, $P < 0.0001$ and $P < 0.0001$ respectively) (Fig. 2).

Finally we show a positive correlation between serum levels of S-100b protein and levels of cryoglobulins in the group of HCV+ patients with MC ($r = 0.72$ and $P < 0.0001$) (Fig. 3).

FIGURE 1. Serum levels of IL-6 and IL-18 in the 30 patients HCV+ with MC (columns with stippled pattern), and in the 30 patients HCV+ without MC.

Discussion

Our study on inflammatory cytokines demonstrates that patients with HCV-MC have a worse inflammatory condition than those with chronic HCV hepatitis without MC. In fact, IL-6 and IL-18 are higher in patients with MC. This data can be explained by the fact that the inflammatory state, expressed by these cytokines, is connected to hepatic viral infection and also to the presence and the action of cryoglobulins. Such cryoglobulins may cause an inflammatory damage either by a direct and injurious event on the tissue or through a secondary role that helps the damaging viral action both in the blood circulation and especially in the liver.

No correlation was found between cytokine levels, cryoglobulins and histological findings in HCV-MC patients but liver biopsy was performed once in all patients. So, liver tissue samples taken could not be representative of real liver damage.

Moreover, we documented that S-100b serum levels were higher in HCV+ with MC respect to HCV+ patients without MC. We have seen that the S-100b protein results were higher in HCV patients than in healthy controls. This may be explained by a possible involvement in inflammation of this protein associated with viral infection. The damage indicated by this protein is associated in any case with the HCV infection, with or without cryoglobulinemia. Finally, we have shown a positive correlation between serum levels of S-100b protein and levels of cryoglobulins in the group of HCV+ patients with MC. This indicates that the S-100b protein may be a marker of endothelial and neuronal damage caused by cryoglobulinemia.

Experimental evidence suggests that the immune response, especially the pro-inflammatory cytokines, plays an important role in viral induced liver injury. Our previous study demonstrated that in the course of chronic HCV hepatitis the increase of pro-inflammatory cytokines is more present. In fact, we showed high levels of IL-6 and IL-18 in patients with

FIGURE 2. Serum levels of S-100b protein in the 30 patients HCV+ with MC (columns with stippled pattern), in the 30 patients HCV+ without MC (columns with diagonal pattern) and in the 20 control subjects (columns with squared pattern).

chronic hepatitis related to HCV and HBV and between these IL-18 and IL-6 levels were higher in HCV-infected patients than in HBV. Moreover, patients with HCV-related hepatitis showed a trend towards the Th1 type response, with an increase of IFN- γ .³⁶ It is now common knowledge that the two pro-inflammatory cytokines IL-6 and IL-18 are an optimal index of hepatic damage.²⁵

The relation between cryoglobulinemia and HCV infection shows new insights in the interpretation of the link between viral infection, autoimmune phenomena and lymphoproliferative disorders evolution. The virus chronically stimulates B-cell polyclonal proliferation from which a monoclonal population may emerge. The Th1/Th2 cytokine balance seems to play a pivotal role in extrahepatic immunological complications of HCV infection such as MC.²⁵⁻²⁷ The role that the cryoglobulins plays in liver damage and in the progression of the disease in patients with chronic HCV hepatitis and cryoglobulinemia com-

FIGURE 3. Spearman's rank correlation between the serum levels of S-100b protein and cryoglobulin concentration.

pared to HCV+ without cryoglobulins has not been explained yet. Some authors showed that the cryoglobulin level does not generally correlate with the severity/activity of the disease^{2,6}; while others suggested that HCV-MC patients are at higher risk of developing cirrhosis than HCV-cryoglobulin negative patients.³⁵ Enhanced production of type-1 cytokines, such as IL-18, may account for a more severe course of liver disease.³⁵ On the other hand, patients with HCV-related autoimmune disorders showed a predominant Th2 pattern and a higher frequency of cryoglobulinemia. Increased secretion of IL-6, via stimulation of TLR2 by HCV core protein, may play a role in the pathogenesis of hepatitis C-associated MC³⁷. Moreover, a previous molecular study of HCV-MC vasculitis provides strong evidence that pro-inflammatory cytokines and oxidative stress-derived molecules have a role in the pathogenesis of HCV-MC vasculitis neuropathy.

Peripheral neuropathy usually occurs in patients with chronic HCV infection and the presence of serum cryoglobulins is predictive of more severe and widespread neuropathic involvement. Two main pathogenic mechanisms have been suggested: 1) impairment of vasa nervorum microcirculation by intravascular deposits of cryoglobulins, producing ischemia

due to derangement of the microcirculation, and 2) necrotizing vasculitis induced by longstanding cryoglobulin precipitation, complement fixation, and rheumatoid factor activity. Either the vasculitis itself or the vascular occlusion causes fascicular ischemia, which ultimately results in axonal degeneration.³⁸ In HCV-infected patients without cryoglobulinemia HCV RNA has been isolated in homogenates of nerve biopsy specimens and these findings suggest a direct role of HCV in the pathogenesis of peripheral neuropathy.³⁸ Possible mechanisms include a direct nerve cytopathic effect by HCV or an immune-mediated mechanism, such as immune complex-induced changes of the epineural vessel.³⁸

An aberrant S-100b protein production has been observed in neurodegenerative disease, as Alzheimer's disease and Down's syndrome. S-100b is responsible to start up a gliotic reaction by the release of pro-inflammatory mediators, including nitric oxide (NO) and cytokines from microglia and astrocytes, which are, in turn, deleterious for neurons. Interestingly, pro-inflammatory effect of S-100b seems not be restricted to the brain.³⁹ In our preliminary study, there is growing evidence that an increase of S-100b concentrations may be used as a marker of peripheral and central cell damage since serum S-100b release has a significant correlation to damage severity.³⁹

In this study we have seen that the S-100b protein results statistically higher in HCV patients than in the healthy control group. Since S-100b protein is released passively by necrotic cells and actively by neurons and endothelial cells, its increase in all HCV+ patients may reflect a direct cytopathic role of HCV. The higher S-100b serum levels in HCV-CM patients likely indicates endothelial damage caused by the sum of direct pathogenic role of cryoglobulinemia and virus. Moreover, S-100b protein is an inflammatory protein as well as a specific receptor for advanced glycation end products (RAGE) ligand.²⁸ that induces expression of RAGE, COX-2 mRNA and other pro-inflammatory genes in monocytes in a dose-dependent manner.⁴⁰ Increasing evidence has shown RAGE, a member of the immunoglobulin superfamily of cell surface molecules that is expressed by monocytes/macrophages, endothelial cells and neurons, to be an

important part of complex interactions of the oxidative stress and pro-inflammatory responses. It has been shown that RAGE may promote cardiovascular disease and diabetic vascular and renal disease through the activation of intracellular signaling pathways that promote oxidative stress.⁴¹ Therefore, the high S-100b protein serum level in HCV+MC+ patients in addition to expressing a worse inflammatory status may be itself a promoting factor of inflammation and necrosis.

The role of cryoglobulinemia in endothelial damage, especially in manifestations linked to vasculitis, has been demonstrated previously.³⁷ The circumstances that favour the development of MC in HCV infected patients are unclear. The exact role of HCV in the pathogenesis of the disease remains still to be answered. HCV could be only the triggering factor of the MC, or it could also contribute to the self-perpetuating mechanism of the disease.

This study shows, for the first time, that the S-100b protein may indicate damage caused by HCV infection and also by cryoglobulinemia since its serum levels are strongly increased in all HCV patients and particularly in HCV-MC+ patients. Moreover, the high S-100b protein serum level in HCV+MC+ patients in addition to express a worse inflammatory status, as documented by high IL-6 and IL-18 serum levels, may be it itself a promoting factor of inflammation and necrosis. Finally, the direct correlation between cryoglobulinemia and the S-100b protein in HCV-MC patients confirms that there is a relation between endothelial damage, elevated S-100b levels and elevated cryoglobulins. In future, it would be interesting to be able to study the S-100b protein more profoundly and to examine whether progressive elevation of this protein in HCV+ patients can be used as a marker for the evolution of endothelial damage caused by cryoglobulins.

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