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ORIGINAL ARTICLE

Is interleukin-8 an additional to histopathological changes diagnostic marker in HCV-infected patients with cryoglobulinemia?

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Abstract
Purpose The aim of this study was to analyze histopathological changes in the liver and serum inflammatory cytokine level in hepatitis C virus (HCV)-infected patients with and without cryoglobulinemia.
Methods The study group consisted of 34 patients with chronic hepatitis C, confirmed by serological and virological markers. Ten out of 34 patients had cryoglobulinemia. The control group consisted of 21 healthy persons. Liver biopsy specimens of HCV-infected patients were evaluated by light microscopy using the grade and the stage according to Batts and Ludwig classification. The quantitative measurements of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor in sera were performed by flow cytometry.
Results The mean age of HCV-infected patients with cryoglobulinemia was higher than age of HCV-infected patients without cryoglobulinemia. Microscopic examination of liver biopsy specimens revealed necroinflammatory activity slightly more prominent in patients with cryoglobulinemia. The most prominent inflammatory changes connected with abundant lymphoid aggregates in most of the examined portal tracts and piecemeal necroses were diagnosed in patients with several extrahepatic manifestations, such as cutaneous manifestations, nephrotic syndrome, polyneuropathy, and arthropathy. Liver fibrosis was similar in patients with and without cryoglobulinemia.
Conclusions The serum levels of all proinflammatory cytokines, especially IL-8, were significantly higher in the patients with cryoglobulinemia in comparison with the patients without cryoglobulinemia and healthy persons. All microscopic features did not correlate with the level of any investigated proinflammatory cytokines.

Keywords HCV infection · Cryoglobulinemia · Liver biopsy · Serum cytokine level · IL-8

Introduction

Hepatitis C virus (HCV) infection is one of the most common viral infections of the liver, with a global prevalence of 3% (about 170 million people), leading usually to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). The proportion of acute and chronic disease in HCV infection is estimated as 15 versus 85% [1]. Postinflammatory cirrhosis leads to HCC in 1–6% per year. The clinical course of HCV infection is dependent on individual differences, such as genetic factors, acquired immunity, age, gender, and duration of infection. Because of its hepatotropic and lymphotropic features, replication in hepatocytes, lymphocytes, and macrophages, HCV can induce systemic disease with extrahepatic manifestation.

Chronic HCV infection can be connected with cryoglobulinemia, characterized by the presence of circulating

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immunoglobulins precipitating at low temperature, which may show rheumatoid factor activity. Cryoglobulinemia develops in 10–50% HCV-infected patients [2, 3]. HCV-infected patients with cryoglobulinemia frequently present extrahepatic manifestations, such as vasculitis and arthralgia, fatigue, thrombocytopenic purpura, glomerulonephritis, polyneuropathy, corneal ulcer, porphyria cutanea tarda, and necrotizing cutaneous vasculitis [4].

The key role in defence mechanisms against viral infections is maintained by T lymphocytes and their secretory response and production and release of cytokines. Cytokines produced by T helper cells mediate a broad spectrum of interactions and influence the inflammatory reaction of the liver and other organs [5].

The aim of this study was to analyze histopathological changes in the liver and serum inflammatory cytokines level in HCV-infected patients with and without cryoglobulinemia.

Materials and methods

Patients

The study group consisted of 34 patients (20 females, 14 males) with chronic hepatitis C, evaluated by liver biopsy and confirmed by serological (anti-HCV antibodies positive) and virological markers (HCV-RNA positive by PCR). Patients were diagnosed and treated in the Department of Hepatology and Acquired Immunodeficiencies, Warsaw Medical University. All patients underwent percutaneous liver biopsy. In parallel with the liver biopsy, the serum cytokine level was examined.

We divided all patients into two groups. Group 1 consisted of 24 patients without cryoglobulinemia (12 females and 12 males, mean age 44.5 years). Group 2 consisted of ten patients with cryoglobulinemia (eight females and two males, mean age 51.6 years). Group 1 consisted of consecutive patients in contrast to group 2 which was selected on the basis of the presence of cryoglobulinemia.

All patients with cryoglobulinemia demonstrated extrahepatic manifestations: cutaneous manifestations (six patients), nephrotic syndrome (five patients), polyneuropathy (four patients), and arthropathy (two patients). Six patients had more than one extrahepatic manifestation (Table 1). All patients included in this study did not receive interferon (IFN)/ribavirin therapy 1 year prior to the liver biopsy and cytokine profile examination.

Group 3, the control group, consisted of 21 healthy persons (16 females and 5 males, mean age 44.5 years) without HCV infection. They were employees of the National Institute of Public Health, routinely tested for anti-HCV antibodies presence.

Table 1 Demographic data, histological activity, clinical parameters and cytokines level of HCV-infected patients (group 1 and group 2) and healthy persons (group 3)

Group number and description	*N*	Age mean (±SD)	Sex (F/M)	Transaminases level U/l mean (±SD)		Histological activity mean (range)		Extrahepatic manifestations	Cytokine level pg/ml mean (±SD)					
				ALT	AST	G	S		IL-12p70	TNF	IL-1β	IL-6	IL-8	IL-10
1 patients without cryoglobulinemia	24	44.5 (12.2)	12/12	137 (116)	115 (118)	2 (0–4)	2 (0–4)	Not observed	1.2 (1.42)	1.81 (1.72)	1.16 (0.97)	2.57 (3.10)	7.14 (4.74)	1.81 (1.14)
2 patients with cryoglobulinemia	10	51.6 (12.9)	8/2	106 (88)	97 (83)	2.7 (2–4)[a]	2.0 (0–3)[b]	Cutaneous manifestations [6] Nephrotic syndrome [5] Polyneuropathy [4] Arthropathy [2]	3.4 (5.49)	4.7 (7.5)	2.68 (2.54)	7.07 (5.79)	24.47 (22.62)	4.65 (5.29)
3 healthy persons	21	44.5 (12.3)	16/5	n.d.	n.d.	n.d.	n.d.	Not observed	0.06 (0.26)	0.88 (0.62)	0.29 (0.47)	0.89 (0.62)	1.98 (0.96)	0.41 (0.6)

n.d. not done

[a] G0/G1/G2/G3/G4 = 0/0/5/3/2

[b] S0/S1/S2/S3/S4 = 1/1/6/2/0

Cryoglobulin evaluation

Serum specimens were maintained at 37°C, allowed to clot for 90 min, centrifuged at 37°C, and stored at 4°C for 4 days. Samples with visible, reversible protein precipitation after 4–6 days incubation at 4°C were considered as positive.

Liver biopsy—microscopic examination

Liver biopsy specimens from 34 HCV-infected patients were fixed in 4% neutral buffered formalin and embedded in paraffin. Sections displaying at least ten portal tracts were routinely stained by hematoxylin–eosin, Periodic Acid-Schiff method, with and without diastase digestion, Gomori silver impregnation, Azan, or Masson Trichrome. Histological interpretation was performed using internationally accepted criteria and reviewed retrospectively by two independent pathologists [6]. All histological features were finally scored using the five degree scale for the grade (inflammatory activity) and stage (fibrosis) of the disease. The grade was assessed as: 0—portal inflammation only, without piecemeal necrosis, without lobular inflammation and necrosis; 1—minimal portal inflammation; minimal, patchy piecemeal necrosis, occasional spotty necrosis; 2—mild portal inflammation; mild piecemeal necrosis involving some or all portal tracts, little hepatocellular damage; 3—moderate portal inflammation: moderate piecemeal necrosis involving all portal tracts, moderate lobular inflammation with noticeable hepatocellular change; and 4—severe portal inflammation; severe piecemeal necrosis with bridging necrosis, severe lobular inflammation with prominent diffuse hepatocellular damage. The stage was assessed as: 0—no fibrosis, normal connective tissue; 1—portal fibrosis with fibrous portal expansion; 2—periportal fibrosis, periportal or rare portal-portal septa; 3—septal fibrosis, fibrous septa with architectural distortion; and 4—cirrhosis.

Cytokine evaluation

Cytokines were determined in sera taken altogether from 55 persons (34 patients and 21 healthy persons as controls). The quantitative measurement of IL-1β, IL-6, IL-8**,** IL-10, IL-12p70, and tumor necrosis factor (TNF) was performed by flow cytometry with Cytometric Bead Array technique (CBA Human Inflammatory Cytokines Kit, Becton–Dickinson, USA). CBA technique combines the principles of the sandwich immunoassay with the capability of flow cytometry and allows for simultaneous determination of six cytokines concentration in a single sample. The analysis of Becton Dickinson Cytometric Bead Array (BD CBA) data was performed using Fcape software which allows to optimize in reading analyzed concentrations from ten-point standard curves.

Statistical analysis

Comparison of cytokine levels between investigated groups was done using Kruskal–Wallis test. If significant result was obtained, each pair of groups was compared using Mann–Whitney test with Bonferroni correction. Non-parametric tests were used when non-normal distribution of cytokines was observed. ROC curve was used to describe prediction of cryoglobulinemia by cytokine profile in HCV-infected patients. Cochran–Armitage trend test was used to compare G and S among groups 1 and 2. Analysis was performed using SPSS 18.0 statistical program.

Results

The difference between age of HCV-infected patients ($n = 34$) versus the control group ($n = 21$) was not statistically significant (Student's t test; $p = 0.664$). The mean age of HCV-infected patients with cryoglobulinemia (group 2) was higher (51.6 years) than the age of HCV-infected patients without cryoglobulinemia (group 1) (44.5 years) and healthy persons (group 3) (44.5 years). The demographic data, histological activity, clinical parameters, and cytokines level of HCV-infected patients and control group are shown in the Table 1.

Microscopic examination of liver biopsy revealed necroinflammatory activity, described as the grade of the disease, more prominent in group 2 (mean 2.7) than in group 1 (mean 2.0). Piecemeal necrosis, lobular inflammation, portal lymph follicle formations, and vasculitis were observed in all cases. The most prominent inflammatory changes connected with abundant lymphoid aggregates in all portal tracts and piecemeal necrosis were diagnosed in patients with extrahepatic manifestations, such as cutaneous manifestations and arthropathy. For statistical purposes, we assumed grades 3 and 4 as high inflammation and grades 0, 1, and 2 as low inflammation.

Liver fibrosis, described as the stage of the disease was identical in group 1 and group 2 (mean 2.0). However, we have found complete cirrhosis in two patients of group 1. Similar to the grade, for statistical purposes we considered stages 3 and 4 as high fibrosis, and stages 1 and 2 as low fibrosis.

Levels of cytokines were presented as median, 1st and 3rd quartiles, minimum and maximum values (Table 2). Statistically significant differences between group 3 (control) versus group 1, and group 3 versus group 2 were observed for each cytokine. Statistically significant differences between group 1 and group 2 were observed for TNF, IL-6, IL-8, and IL-10. ROC curve analysis showed that usefulness of IL-6, IL-8, and IL10 can be classified as "good" (as area under curve (AUC) was greater than 0.8);

Table 2 Comparison of cytokine levels in sera of HCV-infected patients (group 1 and group 2) and healthy persons (group 3)

Group	IL-12p70	TNF	IL-1beta	IL-6	IL-8	IL-10
1 Patients without cryoglobulinemia						
N	24	24	24	24	24	24
Median	0	1.48	1.32	1.645	5.435	1.59
Q1	0	1.088	0	1.343	3.52	1.31
Q3	1.45	1.96	1.735	2.6	11.263	2.01
Min	0	0	0	0	2.07	0
Max	5.2	6.83	3.26	16.1	20.62	5.01
2 Patients with cryoglobulinemia						
N	10	10	10	10	10	10
Median	1.255	2.125	1.71	4.315	18.31	2.995
Q1	0.84	1.57	1.343	2.263	11.728	1.98
Q3	3.86	3.873	3.035	12.583	28.783	4.555
Min	0	1.37	0.95	1.62	3.64	1.47
Max	18.1	24.26	9.27	16.52	83.59	19.18
3 Healthy patients						
N	21	21	21	21	21	21
Median	0	1.02	0	1.04	2.07	0
Q1	0	0	0	0	1.445	0
Q3	0	1.225	0.95	1.255	2.84	1.075
Min	0	0	0	0	0	0
Max	1.19	1.93	1.06	2.06	3.64	1.57
$P_{\text{Kruskal–Wallis}}$	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Group 1–Group 3	*	*	*	*	*	*
Group 2–Group 3	*	*	*	*	*	*
Group 1–Group 2		*		*	*	*

* Statistically significant differences

(Fig. 1; Table 3). However, IL-8 appeared to be the best marker from the other two cytokines for cryoglobulinemia. The AUC for IL-1beta and TNF was found statistically significant >0.5.

To eliminate impact of gender and age of patients on the obtained results, multivariate models for each cytokine with gender and age of patients were run. In none of the models, gender and age were statistically significant. Moreover, area under the ROC curve built on the basis of predicted probabilities from multivariate model was almost the same as such for ROC curve for cytokine only.

The serum levels of cytokines were compared with histological changes in the liver (grade and stage). The comparison of cytokine levels in groups with high inflammation and low inflammation did not show any significant differences (Mann–Whitney test; $p > 0.05$). Cochran–Armitage trend test was used to compare G and S among groups 1 and 2. The obtained p values were 0.34 and 0.14, respectively, so the distribution of G and S was similar in both study groups.

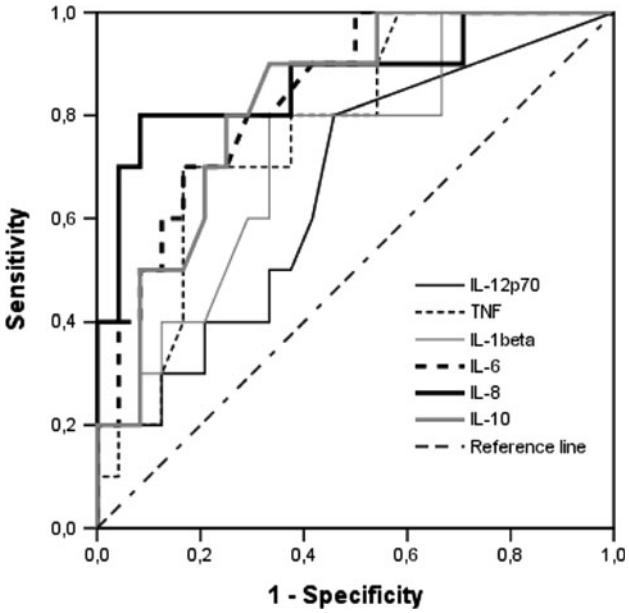

Fig. 1 ROC curve

Table 3 ROC curve analysis

	Area under curve (AUC)		*P*	Asymptotic 95% CI	
	Area	SE		Lower limit	Upper limit
IL-12p70	0.660	0.102	0.146	0.461	0.860
TNF	0.771	0.084	0.014	0.607	0.935
IL-1beta	0.729	0.092	0.038	0.548	0.910
IL-6	0.840	0.070	0.002	0.703	0.976
IL-8	0.871	0.076	0.001	0.722	1.020
IL-10	0.825	0.072	0.003	0.683	0.967

Analysis of the possibility of differentiation of cryoglobulinemia presence by means of cytokines level in HCV-infected patients

Classification of the AUC values: 0.90–1.00 = excellent; 0.80–0.90 = good; 0.70–0.80 = fair; 0.60–0.70 = poor; 0.50–0.60 = fail

The parallel analyzes made for liver fibrosis and cytokine levels gave the same result. In summary, despite the differences in the histopathological picture all microscopic features did not correlate with the level of any investigated proinflammatory cytokines.

Discussion

Cytokines are produced mostly by monocytes, macrophages, T lymphocytes, sometimes B lymphocytes. They form the signalling molecules involved in the regulation of immune response by coordinating the T and B lymphocytes response and the influx of inflammatory cells to organs. They are also produced in situ, among others, by hepatocytes and Kupffer cells in the liver.

Circulating cytokines may contribute to the diagnostic biomarker panel for a number of chronic liver diseases. They also play an important role in viral hepatitis, especially in the evolution of acute into chronic hepatitis. The role of cytokines of the Th1/Th2 balance was extensively studied. It was established that dominance of the Th1 response is a good prognostic factor of viral clearance, but the domination of the Th2 is associated with persistence of viral infection [5, 7, 8]. In our study, IL-8 has shown the most prominent serum level among other cytokines. IL-8 belongs to the seven transmembrane-spanning receptor families, which is only marginally involved in immunity, but its dominance in the extrahepatic manifestations of HCV-infected patients may suggest other mechanisms in the expansion of the disease. However, we have also observed a serum-elevated level of IL-6 and IL-10 as Th2 response and TNF as Th1 response. Stimulatory cytokines IL-1 and IL-12, as a cytotoxic lymphocyte maturation factor, secreted by macrophages, are involved in the maturation and proliferation of T cells, which could also explain their increase in the extrahepatic manifestations of HCV-infected patients.

Serum cytokine levels may be important markers of inflammation and hepatic injury in the course of hepatitis C. In a number of studies, it has been shown that the circulating cytokines are elevated in HCV-infected patients, in comparison to the control group [9–14]. However, it must be stressed that elevated serum cytokines may represent a systemic response, but do not necessarily result from increased local synthesis within the liver [9]. On the other hand, serum cytokine levels may be correlated with their production in the liver [10]. It has also been shown that IFN therapy caused a short-term increase (up to 24 h) of cytokines [15, 16]. Because the patients enrolled in this study were not under IFN/ribavirin therapy for 1 year prior to the liver biopsy and blood sample collection for cytokines examination, we did not analyze the influence of IFN administration on the cytokine level.

Our study indicated that serum cytokines level (IL-1β, IL-6, IL-8**,** IL-10, IL-12p70, and TNF) was increased in the group of 34 HCV-infected patients in comparison to the 21 healthy persons. Similar results were obtained by other authors who studied cytokines IL-1β, IL-6, and TNF-α in HCV-infected patients [17]. Our analysis by ROC curve has shown particularly significant increase in the IL-8 level in ten patients with cryoglobulinemia in comparison to other HCV-infected and healthy persons. This analysis has shown that not only IL-8, but also IL-6 and IL-10 cytokines had the good predictive value for the presence of cryoglobulinemia (Fig. 1). Quite similar results were obtained while studying cytokines IL-1β, IL-6, and TNF-α which were significantly raised in HCV-infected patients with cryoglobulinemia in comparison to age- and sex-matched HCV-infected patients without cryoglobulinemia ($\leq$0.04) and to controls ($p < 0.01$).

The presence of cryoglobulins is a very characteristic feature of hepatitis C infection, which occurs in 10–50% of HCV-infected patients [2, 3]. Involvement of the HCV-infected lymphoid cells is crucial in the pathogenesis of this extrahepatic manifestation. Recent studies demonstrate that in HCV infection with cryoglobulinemia, the monoclonal profile of B cells CD5+ population is less susceptible to apoptosis because of IL-4, IL-10, and IL-12 cytokine protection, and can lead to the production of autoantibodies [18]. Falasca et al. investigated the serum levels of Th1/Th2 cytokines in patients with chronic HCV infection with and without mixed cryoglobulinemia. Serum concentrations of IL-6 and IL-18 were higher in the mixed cryoglobulinemia group than in the group without this disorder ($p < 0.001$) [17]. Enhanced Th1 cytokine production in HCV-infected patients with mixed cryoglobulinemia was also described by Zignego [19]. Soluble forms

of TNF receptors were significantly higher, especially in those with cryoglobulinemia and non-Hodgkin B lymphoma, than in normal subjects [20]. Our study showed that HCV-infected patients with cryoglobulinemia had regularly higher level of cytokines than the patients without cryoglobulinemia, which is in agreement with other studies.

Our patients with cryoglobulinemia had a slightly (but insignificantly) elevated concentration of IL-12p70 in comparison to the patients without cryoglobulinemia. It was shown that IL-12 is a powerful cytokine of the Th1 type which promotes synthesis of IFN-γ and TNF-α by T lymphocytes and natural killer (NK) cells. It is difficult to interpret why IL-12 was the only cytokine which was slightly raised in patients with cryoglobulinemia. However, it is interesting to note that when cytokines level was compared in children with chronic hepatitis C and healthy blood donors, the level of IFN-γ, IL-10, and TNF-α, but not IL-12 was increased in the first group [21]. This is similar to our findings and perhaps IL-12 acts on the other stage of chronic hepatitis C.

We have demonstrated that the serum level of IL-8 was the best marker for patients with cryoglobulinemia. IL-8 is known as a chemotactic and angiogenetic cytokine, a potential mediator of the host response to injury or inflammation. Intrahepatic expression of IL-8 and IL-2 increases with fibrosis and inflammatory activity in chronic hepatitis [22]. An elevated level of IL-8 has been demonstrated in the advanced stage of hepatitis and in non-responder patients treated with ribavirin and IFN-α [23]. In the present work, we did not observe any correlation between serum IL-8 level and histological grade and stage but the presence of cryoglobulinemia was associated with prominent vascular lymphoid invasion in the portal tracts. Studies in vitro show that upregulation of IL-8 by HCV contributes to the inhibition of antiviral action [24]. Serum IL-8 can be considered and proposed as a non-invasive and predictive marker of a response to IFN and ribavirin therapy in chronic hepatitis due to HCV [23]. The serum level of IL-8 also reflects the stage and severity of alcoholic liver disease, and may serve as a predictor of survival in patients with alcoholic hepatitis [25]. Our patients showed a mild degree of steatosis, but it did not correlate with the level of IL-8. As this cytokine plays a part in the immune dysregulation, it is also elevated in Graft versus host disease (GVHD) and is proposed in the panel of four biomarkers to confirm the diagnosis of GVHD [26]. Along this line, we also propose to consider IL-8 as a good biochemical marker of cryoglobulinemia in HCV infection.

Microscopic changes in the liver, characterized by vascular changes and lymph follicles formation, sometimes by progressive fibrosis, have been described as more prominent in patients with cryoglobulinemia [26, 27]. It is possible that cryoglobulinemia results in more rapid hepatic fibrosis, but we did not find this association in our group of ten patients.

We observed an elevated level of IL-8 correlated with cryoglobulinemia. We suggest that the serum level of IL-8 may be considered as an additional marker of cryoglobulinemia in HCV-infected patients, but taking into account the relatively small number of patients enrolled in this study, this should be confirmed.

Conflict of interest None.

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