

Increased Concentrations of Tumor Necrosis Factor and Interleukin-6 Contribute to the Hemostatic Abnormalities in Advanced Liver Disease

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Abstract

Abnormal cytokine levels have been described in patients with chronic liver disease, but studies correlating cytokine homeostasis with abnormalities in coagulation and fibrinolysis are lacking. In order to establish a link between cytokines and the hemostatic changes the following parameters were determined in 44 patients with cirrhosis (alcoholic = 15, postnecrotic = 22, others = 7): TNF- α , IL-6, thrombin-antithrombin (TAT) complexes, prothrombin fragment 1 + 2 (F1 + 2) and t-PA by using enzyme-linked immunosorbent assays, and PAI-1, plasminogen and α_2 -antiplasmin (α_2 -AP) by using chromogenic substrates. All patients were at stages B and C of Child's classification when entering the study. Mean cytokine concentrations were significantly higher in cirrhotic patients as compared to age- and sex-matched controls ($p < 0.009$). There was a significant increase of TAT ($p < 0.02$) and F1 + 2 ($p < 0.001$) in the patients groups, suggesting a grade of intravascular coagulation. A hyperfibrinolytic state as demonstrated by an increase of t-PA and decrease of plasminogen and α_2 -AP was also observed ($p < 0.001$). We could define a subgroup of patients with cytokine values higher than 20 pg/ml. Interestingly, in this group there was a significant increase of TAT ($p < 0.04$) and t-PA ($p < 0.02$) levels and a decrease of plasminogen and α_2 -AP ($p < 0.02$) as compared to values observed in patients with cytokines lower than 20 pg/ml. We conclude that high levels of TNF- α and IL-6 may contribute to hyperfibrinolysis and intravascular coagulation in patients with liver cirrhosis, as assessed by the increase of TAT and t-PA levels and the reduction of plasminogen and α_2 -AP.

Key Words

Liver disease
Tumor necrosis factor
Interleukin-6
Thrombin-antithrombin complexes
Prothrombin fragment 1 + 2
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Introduction

Because the liver is the primary site of synthesis of most of the identified coagulation factors and inhibitors of coagulation and serves as a key site for clearance of activated and degraded factors from the circulation, complex and often variable derangements of hemostasis may occur in patients with liver disease. Impaired hemostatic function results from reduced synthesis of clotting factors and normally occurring inhibitors of coagulation, synthesis of abnormal clotting proteins, vitamin K deficiency, disseminated intravascular coagulation (DIC), enhanced fibrinolytic activity and quantitative as well as qualitative platelet defects [1-3].

Hepatic synthesis of plasma proteins is subjected to the regulatory role of cytokines [4]. Tumor necrosis factor (TNF) and interleukin-6 (IL-6), also known as B-cell stimulatory factor 2, belong to the endogenous mediators of the complex reaction of the host to injury or infection. In addition to their pathophysiological role as mediators of the immune and inflammatory responses they are involved in the pathogenesis of a variety of chronic disease states when overexpressed *in vivo* [5-8]. The prevailing hypothesis is that at high local concentration or when released into the systemic circulation, these peptide regulatory molecules have the potential to exert harmful effects.

Several reports indicate that a cytokine excess can lead to tissue damage and fibrosis and thus the interest in the role of cytokines in normal liver function and in liver disease has increased [4, 9, 10]. However, studies correlating cytokine homeostasis with abnormalities in coagulation and fibrinolysis are lacking.

We, therefore, sought to correlate circulating concentrations of TNF and IL-6 to clinical and hemostatic parameters in patients with advanced liver disease.

Patients and Methods

Patients

Forty-four patients (30 men and 14 women) with chronic liver disease and 30 age- and sex-matched, nonalcoholic, healthy control subjects were studied. Patients (mean age 51 years, range 24-74) had grade B (n = 21) or C (n = 23) cirrhosis in Child's classification according to well-established clinical and analytical criteria: ascites and/or encephalopathy, high bilirubin and low albumin levels, and prolonged prothrombin time. In all cases diagnosis was confirmed by histology. Final diagnosis was alcoholic cirrhosis in 15 patients, postnecrotic cirrhosis in 22 and cirrhosis due to different liver diseases in 7 patients (biliary cirrhosis = 3, Wilson's disease = 2, hemochromatosis = 1 and α_1 -antitrypsin deficiency = 1).

All patients were enrolled in the study between the fifth and tenth days of hospitalization. At the time of enrollment, no patient had evidence of detectable alcohol levels or infection. Patients with malignant neoplasm were excluded from the study.

Methods

Blood Collection. Blood samples were obtained between 8 and 10 a.m. in the fasting state and drawn into vacuum blood collection tubes (Becton Dickinson, Meylan, France). Venous blood was anticoagulated with 3.8% trisodium citrate (9:1 v/v). Platelet-poor plasma was obtained by centrifugation at 2,000 g during 15 min at 4°C. Aliquots of plasma were stored at -70°C until used. For t-PA activity blood was collected on Stabilyte tubes (Biopool, Sweden) for acidification purposes. Serum was prepared from blood that had been allowed to clot at room temperature for 30 min and immediately analyzed or stored at -70°C in aliquots.

Laboratory Methods. Thrombin-antithrombin (TAT) complexes were determined by an ELISA assay (Enzygnost TAT, Behringwerke AG, Marburg, Germany) as described by Pelzer et al. [11]. Prothrombin fragment 1 + 2 (F1 + 2) was assayed by an ELISA assay (Enzygnost F1 + 2, Behringwerke) [12].

Quantitative determination of cytokines was performed on stored samples from all patients and controls using ELISA kits with specific monoclonal antibodies (Coaliza IL-6 and Coaliza TNF- α , Kabi Diagnostica, Sweden).

t-PA activity, PAI-1 activity, plasminogen, α_2 -antiplasmin and endotoxin were assayed with chromogenic substrates using commercially available kits (Coatest t-PA, Coatest PAI-1, Coatest Plasminogen, Coatest α_2 -Antiplasmin, and Coatest Endotoxin; Kabi Diagnostica, Sweden).

Table 1. Laboratory values of patients group (mean values \pm SD)

Parameter	Patients (n = 44)	Reference ranges
Total bilirubin, g/dl	4.0 \pm 1.3	0.3–1.1
AST, IU	56.2 \pm 28.5	6.1–28.6
Albumin, g/dl	3.0 \pm 1.6	3.2–5.0
Prothrombin index, %	55.7 \pm 16.2	70–120

t-PA antigen was measured with a commercially available ELISA kit based on monoclonal antibodies (TintElize t-PA, Biopool, Sweden).

Serum albumin, total protein, bilirubin, ALT, AST and prothrombin time were analyzed with standard laboratory assays.

Statistical Analysis

Results are reported as mean \pm SD. Statistical comparison of mean values between normal controls and cirrhotic patients was performed by the Student's *t* test for a normal distribution. Correlations were calculated by Spearman regression analysis. The Mann-Whitney test was used for comparisons within patients according to the cytokine levels. $p < 0.05$ was considered significant.

Results

Forty-four patients with advanced liver disease were included in the study. Abnormal laboratory tests at the time of entering the study are shown in table 1.

Results showed a significant increase of cytokine concentrations, both IL-6 (50.6 \pm 29.4 pg/ml) ($p < 0.003$) and TNF- α (30.1 \pm 11.9 pg/ml) ($p < 0.009$) in the group of patients, whereas they were practically undetectable (< 5 pg/ml) in samples from healthy controls (fig. 1). A good correlation ($r = 0.92$, $p < 0.0001$) between TNF- α and IL-6 was observed. However, no significant differences in the cytokine concentrations in relation to the etiology of the liver disease (alcoholic or hepatitis) nor to the grade in Child's classification could be demonstrated (not shown).

Table 2 shows the mean \pm SD values of the different hemostatic parameters analyzed in the studied groups. We found a significant increase of F1 + 2 ($p < 0.001$) and TAT complexes ($p < 0.02$) in patients as compared to controls. Fibrinolysis parameters showed a significant increase of t-PA activity ($p < 0.004$) and antigen ($p < 0.001$) and a marked reduction of plasminogen and α_2 -antiplasmin ($p < 0.0001$) in the group of patients as compared to controls, with no differences for PAI-1 activity, suggesting that a hyperfibrinolytic state is commonly present in these patients.

We selected a subgroup of 7 patients (15.9%) who presented higher levels of cytokines (> 20 pg/ml of both TNF- α and IL-6) and compared the results of the different hemostatic parameters with those obtained in the remaining patients. Table 3 shows the mean \pm SD values of hemostatic parameters in relation to the cytokine concentrations. There was a significant increase of TAT complexes ($p < 0.04$) and t-PA antigen ($p < 0.02$) and a reduction of plasminogen ($p < 0.009$) and α_2 -antiplasmin ($p < 0.001$) in patients with increased cytokines as compared to values observed in patients with levels of cytokines < 20 pg/ml. No differences between groups were observed in the levels of F1 + 2 and functional t-PA and PAI-1.

No correlation between cytokine concentration and parameters of liver injury (AST and serum bilirubin) or hepatic synthesis function (prothrombin time and serum con-

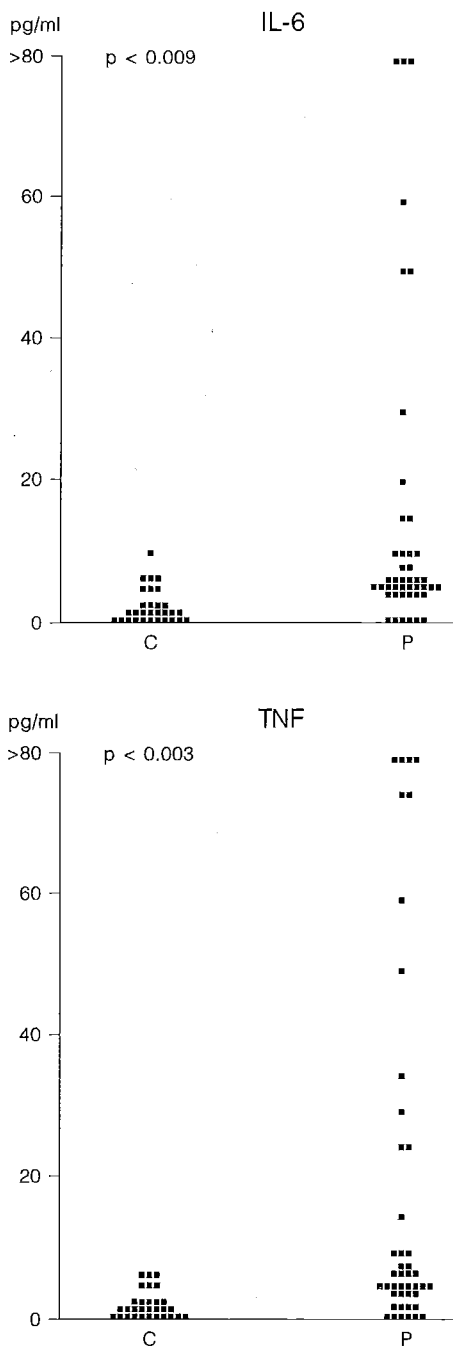


Fig. 1. Individual values of IL-6 (top) and TNF- α (bottom) in patients with advanced liver disease (P) and controls (C).

centrations of albumin) could be demonstrated (not shown).

The mean plasma endotoxin concentration in patients was 69.5 ± 8.6 pg/ml (not detectable in controls, the detection limit of the assay being 10 pg/ml). No correlation between endotoxin and plasma concentrations of IL-6 and TNF was found.

Discussion

This study shows that a significant increase of TNF- α and IL-6 is present in a proportion of patients with advanced liver disease which may contribute to the hemostasis abnormalities. The observed increase was not related to the etiology of the process, as has been reported previously [13].

The liver appears to be an important source of cytokine production as well as the main clearance organ of circulating cytokines [13–15]. Some authors have also found a significant increase of TNF, IL-6 and other cytokines in chronic hepatitis, liver cirrhosis and in fulminant hepatic failure [16–23].

Other investigators have demonstrated increased cytokine secretion by endothelial cells [24, 25] and LPS-stimulated monocytes isolated from patients with both alcoholic and nonalcoholic liver disease, suggesting that these cells may be the source of circulating cytokines [26, 27].

The increase in plasma levels probably reflects higher production, but decreased clearance might also be involved. Although endotoxin and possibly other abnormal components present in serum of patients with liver cirrhosis could induce cytokine expression [28], we could not demonstrate a relationship between cytokine concentrations and peripheral endotoxemia, which agrees with a recent report by Khoruts et al. [23].

Table 2. Hemostatic parameters in patients and controls (mean values \pm SD)

Parameter	Patients (n = 44)	Controls (n = 30)	p
TAT, $\mu\text{g/l}$	8.8 \pm 7.1	3.1 \pm 2.5	<0.02
F1 + 2, nmol/l	1.8 \pm 1.1	1.0 \pm 0.2	<0.001
t-PA activity, U/ml	7.9 \pm 6.8	2.0 \pm 1.2	<0.004
t-PA Ag, ng/ml	18.3 \pm 10.9	5.3 \pm 2.6	<0.001
PAI-1, U/ml	4.8 \pm 4.1	4.9 \pm 3.6	NS
Plasminogen, %	41.6 \pm 16.3	97.3 \pm 11.5	<0.0001
α_2 -Antiplasmin, %	52.2 \pm 26.1	93.3 \pm 11.3	<0.0001

Table 3. Mean \pm SD values of hemostatic parameters in relation to the cytokine concentrations

Parameter	Cytokine levels <20 pg/ml	Cytokine levels >20 pg/ml	p
TAT, $\mu\text{g/l}$	7.5 \pm 6.9	16.0 \pm 9.2	<0.04
F1 + 2, nmol/l	1.8 \pm 1.2	1.5 \pm 0.7	NS
t-PA activity, U/ml	6.4 \pm 4.4	15.2 \pm 11.8	NS
t-PA antigen, ng/ml	17.8 \pm 10.5	27.4 \pm 8.9	\leq 0.02
PAI-1, U/ml	4.2 \pm 3.9	8.0 \pm 6.9	NS
Plasminogen, %	44.9 \pm 12.8	28.2 \pm 16.7	<0.009
α_2 -Antiplasmin, %	58.6 \pm 21.5	19.8 \pm 15.4	<0.001

Cytokines values were not different according to the type of cirrhosis nor with the Child's B and C stage. These results are in agreement with data from Kakumu et al. [13] who found that local IL-6 correlates with disease activity regardless of its etiology. Khoruts et al. [23] also detected normal cytokine values in alcoholic patients without chronic liver disease.

Significant changes in parameters of coagulation and fibrinolysis were observed in the group of patients. We found an increase of F1 + 2 and TAT suggesting specific enzymatic activity of factor X upon prothrombin and thrombin generation [29–34]. Other authors suggest, however, that evidence implicating DIC is indirect in liver cirrhosis and the increased levels of prethrombotic markers would only reflect a decreased clearance [35, 36].

Fibrinolysis parameters showed the typical pattern of liver failure with a significant increase of t-PA activity and antigen and decrease of plasminogen and α_2 -antiplasmin in patients as compared to controls, suggesting that extensive alterations of the fibrinolytic system are commonly present in chronic liver disease [34, 37–39]. The lack of differences in PAI-1 activity between patients and controls could be related to the increased t-PA concentrations since other authors have found elevated concentrations of PAI-1 antigen [38].

We could define a subgroup of patients with higher plasma concentrations of both TNF- α and IL-6 and analyzed different hemostatic and fibrinolysis parameters according to the cytokine levels. Interestingly, we observed that in patients with increased cytokine concentrations there was a significant increase of TAT complexes and t-PA antigen

and reduction of plasminogen and α_2 -antiplasmin as compared to patients with lower cytokine values. However, no differences in F1 + 2 levels were observed between both groups. This could indicate higher thrombin generation and hyperfibrinolysis in patients with abnormal cytokine values although the possibility of decreased clearance of TAT cannot be ruled out.

It can be argued that the observed changes on hemostasis are exclusively related to liver damage and do not necessarily mean that cytokines are responsible for the abnormalities. It appears, however, that the high levels of these inflammatory mediators could contribute to hypercoagulability, assessed by increased TAT levels, as well as to hyperfibrinolysis, as determined by high t-PA and low α_2 -antiplasmin in patients with liver cirrhosis. Similar effects on coagulation and fibrinolysis

have also been found by infusion of cytokines in normal and pathological situations [40–44].

In conclusion, besides their possible role as mediators of the pathophysiological events leading to tissue damage and fibrosis, the increased endogenous production of cytokines (TNF, IL-6) may contribute to the observed hemostatic changes leading to DIC and pathological fibrinolysis in patients with chronic liver disease. Furthermore, it is in the group of patients with higher cytokine levels that this correlation is stronger, regardless of the hepatic function. Further studies of cytokine actions may lead to a better understanding of their role in the pathogenesis of liver disease and delineate their possible contribution to the altered hemostasis in these patients.

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