Plasma concentrations of zinc, copper, interleukin-6 and interferon-γ, and plasma dipeptidyl peptidase IV activity in chronic hepatitis C

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Abstract. Copper and zinc are essential trace elements which play an important role in various biological processes. Along with various cytokines, glucocorticoids, glucagon and insulin, the acute-phase protein metallothionein is involved in the regulation of immune cell functions. Metallothionein is the central protein regulating zinc concentration. Zinc deficiency is often found in patients with chronic liver disease, chronic kidney disease and diabetes mellitus, and in those with acute infectious diseases. In contrast, copper deficiency is rarely reported. In order to determine whether there is a correlation between zinc and/or copper and selected immunological parameters in patients with chronic liver disease, we investigated plasma levels of zinc and copper, concentrations of interleukin-6 (IL-6) and interferon-γ (IFN-γ), and the enzymatic activity of dipeptidyl peptidase IV (DP IV, CD26) in patients with chronic hepatitis C and in healthy control subjects. Whereas zinc plasma levels did not differ between patients and control subjects, copper concentrations revealed gender-specific differences. The mean copper concentration was higher in female patients with chronic hepatitis C and in female controls compared with the respective male groups. The immunological parameters of IFN-γ concentration and DP IV activity showed similar levels in the patient and control groups. Of note, the mean IL-6 plasma concentrations were significantly elevated in patients with chronic hepatitis C compared with healthy control subjects. In summary, there was no correlation between either zinc or copper concentrations and the immunological parameters measured (IL-6 and IFN-γ levels and DP IV activity) in patients with chronic hepatitis C and in healthy control subjects.

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Introduction

Zinc and copper are essential trace elements that play an important role in numerous biological processes in humans, animals and plants. Plasma and tissue levels of these trace elements are influenced by stress (1-2), trauma (3) and infection (4-5). Under these conditions, zinc plasma concentrations are normally reduced and copper levels are elevated (6). Similar observations have been reported in the liver tissue of patients with hepatocellular carcinoma (7).

During inflammation, tissue injury and stress, the pro-inflammatory cytokines interleukin (IL)-1 and -6, and tumor necrosis factor α (TNF-α) are mainly produced by activated macrophages. These mediators in turn induce the synthesis of acute-phase proteins, such as coeruloplasmin and metallothionein, in the liver. Metallothionein is a metal-binding protein that protects against oxidative damage and oxygen metabolites resulting from inflammation and tissue damage (8).

The main zinc metabolism occurs in the liver hepatocytes. There are two storage pools: slow and fast zinc. Metallothionein is the central protein regulating zinc concentration. The regulation of metallothionein synthesis depends not only on cytokine levels, but is also regulated by insulin, glucagon and glucocorticoids. A strong interaction between metallothionein, zinc and copper exists as the two trace elements compete to bind to metallothionein (8).

In addition to the regulation of zinc resorption, distribution and cellular accumulation by metallothionein, zinc uptake itself leads to increased metallothionein synthesis. With the help of tracer studies, it has been revealed that a complete zinc exchange takes two days (8). In patients with zinc deficiency, reduced zinc concentrations in the liver are one of the causes of impaired hepatocyte regeneration.

The pathogenesis of liver cell injury in chronic hepatitis C infection is poorly understood, and the exact mechanism whereby hepatitis C virus (HCV) establishes itself and persists remains unclear. Previous studies have demonstrated that T-cell immunoregulatory cytokines contribute to liver damage. Escape mutations by HCV itself, insufficient T-cell response and weak humoral immune responses have been proposed as the main culprits for the chronicity of HCV infection (9-12).

Recently, Yuasa *et al* (13) demonstrated that zinc may play an important role as a negative regulator of HCV replication in genome-length HCV RNA-replicating cells. Thus, zinc supplementation appears to offer a novel approach for future strategies in the treatment of intractable chronic hepatitis C.

Activation of the cellular immune response is implicated in the eradication of HCV infection. It has been shown that the outcome of HCV infection depends on the Th1/Th2 cytokine balance. Th1 cytokines (IL-2 and IFN-γ) stimulate phagocyte-mediated immune reactions, whereas Th2 cytokines (IL-4, -5 and -10) stimulate the production of IgE and eosinophil/mast-cell-mediated immune reactions, and down-regulate Th1 responses. The Th1 profile appears to be associated with a benign and favorable clinical outcome in HCV infection. A prevalent Th2 response may contribute to the chronicity of HCV infection and the severity of liver disease (14-15).

In patients with liver cirrhosis and hepatocellular carcinoma, the serum concentration of metallothionein is reduced compared to that of patients with chronic hepatitis and healthy control subjects. In contrast, serum copper levels are significantly elevated in patients with cirrhosis and hepatocellular carcinoma compared with patients with chronic hepatitis and healthy control subjects. Zinc concentration is reduced in all three patient groups compared with control subjects (16).

Dipeptidyl peptidase IV (DP IV, CD26) is a widely expressed transmembrane ectoenzyme that is also present as a soluble form in biological fluids. It has been implicated in T-cell activation, hepatocyte-extracellular matrix interactions and fibroblast proliferation. Elevated levels of soluble DP IV activity have been described in patients with chronic HCV infection (17-19). Moreover, serum DP IV activity has been proposed as an indicator of HCV-induced liver injury (18).

The present study aimed to evaluate concentrations of zinc and copper, as well as those of IL-6 and IFN-γ, and plasma DP IV activity in patients with chronic HCV infection and in control subjects who showed no sign of liver disease or infection. In particular, in our investigation of a possible correlation between the levels of trace elements and immunological parameters, differences in measurements between the genders were taken into account.

Patients and methods

Patients. Fifty patients with native chronic HCV infection were included. The diagnosis of chronic hepatitis C was based on patient history, physical examination and laboratory findings. In 37 cases, the diagnosis was confirmed by liver biopsy and histological examination. All patients were positive for HCV antibodies and HCV-RNA. The mean ages of females (n=32) and males (n=18) were 57 and 48 years, respectively. Four of the female patients (12.5%) had used oral contraceptives.

As controls, 30 HCV-antibody-negative subjects were included in the study. These subjects had neither a history of liver or infectious disease, nor clinical or laboratory signs of it. The mean ages of females (n=23) and males (n=7) were 47 and 45 years, respectively. Eight of the female control subjects (28.7%) had used oral contraceptives. Patient and control subject characteristics are listed in Table I. Patients and control subjects gave their informed consent to the study. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki, 1985.

Zinc and copper measurements. Venous blood was collected in the morning on an empty stomach. Plasma concentrations of zinc and copper were measured using atomic absorption spectroscopy (spectrometer 2100 for zinc and 4100 ZL for copper; both from Perkin Elmer, USA. Reference values were 55-150 μg/dl for zinc and 74-122 μg/dl for copper.

Cytokine measurements. The IL-6 and IFN-γ concentrations of the plasma samples were determined using commercially available sandwich enzyme-linked immunosorbent assays (high-sensitivity Quantikine HS human IL-6 ELISA and Quantikine human IFN-γ ELISA; R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions. The sensitivity of the IL-6 ELISA was <0.11 pg/ml and that of the IFN-γ ELISA <8 pg/ml. The cut-off values of IL-6 and IFN-γ were <20 pg/ml.

Measurements of dipeptidyl peptidase IV enzymatic activity. DP IV enzymatic activity was determined in a microplate assay using 1.6 mM Gly-Pro-4-nitroanilide (Gly-Pro-pNA, Bachem, Heidelberg, Germany) and 10 μl plasma in the reaction mixture (20). The cut-off value of DP IV activity was <450 pkat/ml.

Statistics. For statistical analyses, the Excel (Microsoft, Redmond, USA) and Statview 5.0 (SAS Institute Inc., Cary, USA) software programs were used. Data were compared using linear regression analysis. Correlations were calculated using the Spearman rank correlation test. Correlations ≤-0.7 or ≥0.7 were considered significant.

Descriptive statistics are shown as the means ± standard error of the mean (SEM) or as a range (min-max), and P-values are based on the exact permutational distribution of the test statistics rather than being a normal approximation. $P<0.05$ was considered to be statistically significant. Since the underlying investigations were of an exploratory nature, any kind of α adjustment was omitted.

Results

Serum zinc concentrations in patients with chronic HCV infection and those in the control group showed no significant differences between men and women, and ranged within normal values (Table II). An analysis of individual patients and control subjects revealed that: a) in 9 patients with chronic HCV and in 4 healthy subjects, a slight zinc deficiency was found (<71 μg/dl); b) out of 50 patients who were partially followed-up for 9 years, 18 had a zinc deficiency requiring zinc substitution; and c) 4 out of 6 patients with liver cirrhosis exhibited hypozincemia.

In contrast, significant gender-specific differences were observed for plasma copper concentrations. These were significantly higher in the female vs. male control groups ($P=0.0068$) as well as in the female vs. male patient groups ($P=0.0098$).

As seen in Table II, the highest copper levels were found in the female control group (159±7.72 μg/dl) followed by

Table I. Clinical data of patients (n=50) and healthy control subjects with no sign of liver disease or infection (n=30).

Patients with chronic HCV infection		
Women (n=32)	Mean age 57.25 years	
Men (n=18)	Mean age 47.56 years	
Stage of chronic HCV infection		(No.)
Liver cirrhosis		6
Chronic hepatitis without fibrosis		17
Chronic hepatitis with fibrosis stage 1-3 (Desmet and Scheuer)		20
No liver histology		7
Additional complaints		(No.)
Overlap syndrome (chronic hepatitis plus primary biliary cirrhosis)		2
Diabetes mellitus		5
Hypothyroidism		3
Hemophilia		3
Rheumatism		3
Prostate cancer		1
Lichen ruber		1
Laboratory data		Normal range
ALAT (μmol/sl)	0.86±0.49	0.17-0.58
ASAT (μmol/sl)	0.83±0.38	0.17-0.58
GGT (μmol/sl)	1.07±0.86	0.58-1.10
Control subjects		
Women (n=24)	Mean age 47.00 years	
Men (n=6)	Mean age 45.33 years	
Diseases present in controls		(No.)
No disease		15
Hypothyroidism, subclinical		4
Diabetes mellitus		4
Chronic gastritis		4
Colitis ulcerosa		1
Morbus Crohn		1
Rheumatism		1

Table II. Plasma concentrations of zinc and copper in patients with chronic hepatitis C and in healthy control subjects with no sign of liver disease or infection.[a]

Chronic hepatitis C	Female	Male
Zinc		
Mean ± SEM	84±2.65	87±2.83
Range (min-max)	46-112	65-108
Copper		
Mean ± SEM	147±6.02	123±5.89
Range (min-max)	94-255	95-179
Zinc:copper ratio	0.57	0.71
Healthy control subjects	**Female**	**Male**
Zinc		
Mean ± SEM	88±2.71	89±4.16
Range (min-max)	58-105	72-101
Copper		
Mean ± SEM	159±7.72	105±6.06
Range (min-max)	91-227	83-123
Zinc:copper ratio	0.55	0.85

[a]Data are presented as the means ± SEM (μg/dl), range of concentration (μg/dl) and zinc:copper concentration ratio. No significant differences were found between plasma concentrations in the patient and control groups (zinc, P=0.305; copper, P=0.092. Reference values are 55-150 μg/dl for zinc and 74-122 μg/dl for copper.

the female patient group (147±6.02 μg/dl). Conversely, in the male patient group, the mean values for copper were elevated as compared to the male control group (123±5.89 vs. 105±6.06 μg/dl). Notably, mean copper levels in the female control and patient groups were higher than the established reference values of 74-122 μg/dl. The mean copper concentration for the male patients with chronic HCV infection was only slightly higher than the normal value (122 μg/dl). In 9/32 female patients with chronic hepatitis C and in 12/23 female control subjects, copper levels >160 μg/dl were found. The highest copper concentrations, of 225 μg/dl, were measured in a female patient with chronic hepatitis C. Of 18 male patients, 4 showed copper levels >140 μg/dl. In the male control group, the highest copper levels measured were 123 μg/dl (Table II).

Taken together, these results suggest that plasma copper concentrations show a gender dependency. This observation was confirmed by calculating the zinc:copper ratio (Table II). There was no difference in the ratio between the female patients and control subjects (0.57 vs. 0.55). In contrast, in the male patient group the ratio was clearly lower than in the male control group (0.71 vs. 0.85).

We further assessed the plasma concentrations of the cytokines IL-6 and IFN-γ, as well as DP IV activity (Fig. 1). IL-6 plasma concentrations were significantly higher in female and male patients with chronic hepatitis C ($p<0.05$; mean 5.5±1.76 and 6.4±3.22 pg/ml, respectively) as compared to the control subjects (female subgroup, mean 1.4±0.29 pg/ml; male subgroup, mean 2.5±1.51 pg/ml) (Fig. 1A).

In patients with chronic hepatitis C, the concentrations of IFN-γ ranged from 3.4 to 13.3 pg/ml (female patient subgroup, mean 6.0 pg/ml; male patient subgroup, mean 5.5 pg/ml). Compared with the patients, healthy control individuals displayed similar levels of IFN-γ, ranging from 3.0 to 8.6 pg/ml (female control subgroup, mean 4.7 pg/ml; male control subgroup, mean 5.1 pg/ml) (Fig. 1B).

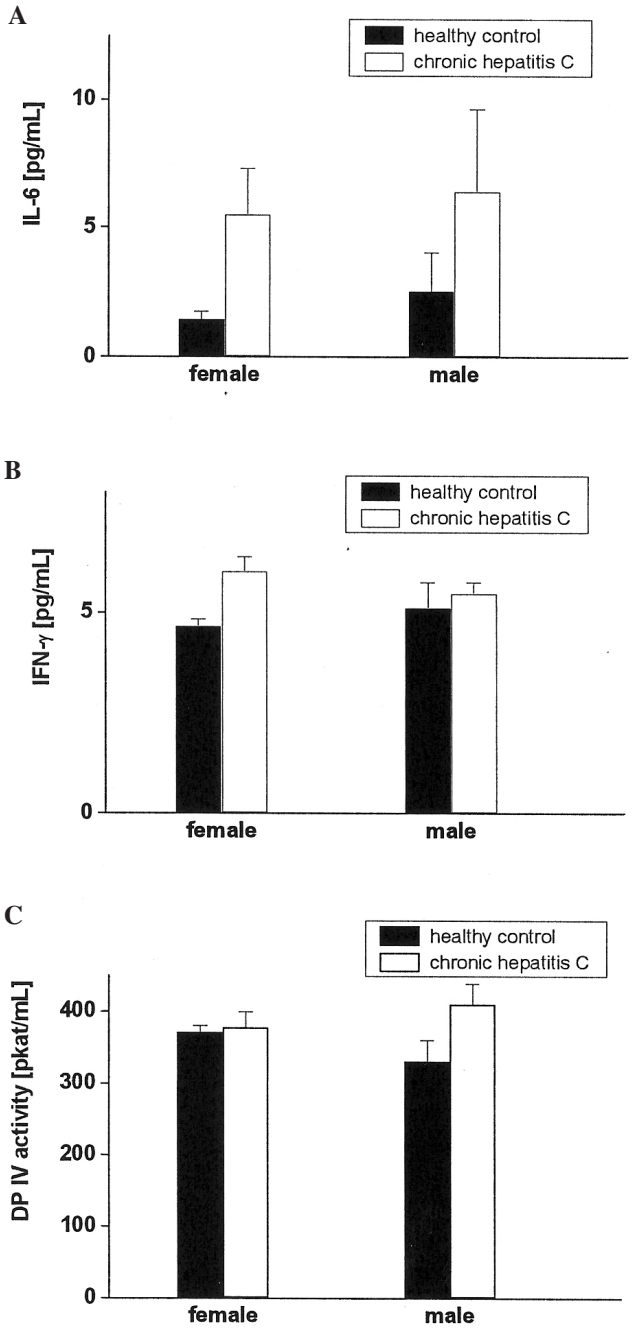

Figure 1. IL-6 and IFN-γ concentrations and DP IV enzymatic activity in the plasma of patients with chronic HCV infection and in healthy control subjects with no sign of liver disease or infection. Data are presented as the means ± SEM. Significant differences were found between (A) IL-6 plasma concentrations ($P<0.05$), but not (B) IFN-γ plasma concentrations ($P=0.135$) or (C) DP IV enzymatic activity ($p=0.538$) in the patients and controls.

There were no differences between DP IV enzymatic activity in patients with chronic hepatitis C (female subgroup, mean 377±21.94 pkat/ml; male subgroup, mean 410±28.29 pkat/ml) and healthy control individuals (female subgroup, mean 371±9.34 pkat/ml; male subgroup, mean 330±29.59 pkat/ml) (Fig. 1C).

Moreover, we found no correlations between zinc or copper plasma concentrations and IL-6 or IFN-γ levels or DP IV activity [zero, hypothesis (no correlation) for all possible positive combinations; $p<0.0001$].

Discussion

In order to investigate a possible association between immunological parameters and trace elements, we determined and compared zinc, copper, IL-6 and IFN-γ plasma concentrations and DP IV activity in patients with chronic HCV infection and healthy control individuals.

With the exception of IL-6 concentrations, no differences were found between patients and control subjects. However, a detailed analysis of the two groups according to gender revealed gender-dependent differences for copper but not for zinc levels. The mean plasma values of copper in women were significantly higher in the patient and control groups compared with the values for men. Copper levels were higher in the female control group than in the female patient group, whereas the opposite result was seen in the male groups.

The apparent gender-dependent differences in plasma copper concentrations may be influenced by sex hormones. High copper levels, especially in the female control group, may be caused by hormonal contraception. During pregnancy, which is a hormonal situation similar to that found in contraceptive users, copper concentration increases while zinc levels decrease. It is known that the estrogen-gestagen ratio is behind these changes (21). However, in the present study, differences in copper concentration were not due to contraception only as only 12.5% of the patients and 28.7% of the control subjects had used oral hormonal contraception therapy.

Nutrition is another possible explanation for differences in copper concentration. However, in the current study one cannot expect to see clear differences in copper concentration, as the entire study sample lived in the same geographic area. Meat, especially liver, nuts and vegetables contain high concentrations of copper. Copper resorption is inhibited by high doses of vitamin C, while supplementation with ascorbic acid, cysteine and zinc can reduce copper toxicity (22). Harris *et al* (23) performed a study on 23 post-menopausal women who received an isocaloric diet containing defined copper and zinc concentrations. The authors concluded that although no antagonism exists between zinc and copper, there is interdependence between the two trace elements.

Our results, which show similar zinc and copper concentrations in patients with chronic hepatitis C and healthy control subjects, are in disagreement with those of Kalkan *et al* (5), who reported significantly reduced zinc levels and significantly elevated copper levels in patients with chronic hepatitis B and C compared with healthy control subjects. Gender-specific zinc and copper concentrations in their study were not calculated, and the differences were attributed to the effect of cytokines and other inflammatory mediators present in chronic hepatitis patients. However, this hypothesis is not supported by experimental data. Barany *et al* (24) described a positive correlation between zinc and copper in normal human blood and serum, though again no gender-specific analysis of the results was performed. Moscarella *et al* (25) reported significantly reduced serum zinc levels in patients with decompensated liver cirrhosis, while their copper and manganese concentrations remained unchanged.

Singer *et al* (26) demonstrated that plasma levels of zinc and copper were reduced in patients who had undergone orthotopic liver transplantation compared to other types of

major surgery. The low levels of these trace elements persist for up to 5 days after transplantation, despite appropriate supplementation (26). These results once more underline the physiological role of the liver in regulating the zinc and copper metabolism.

Zinc levels depend on the severity of liver disease. In patients with decompensated liver cirrhosis, zinc concentrations are reduced by up to 75%, whereas patients with chronic hepatitis B or C exhibit just moderate reduction (27). This is explained by changes in the protein and amino acid metabolism and by disturbances in intestinal resorption and hepatic zinc extraction, which are less pronounced in chronic hepatitis than in liver cirrhosis. In chronic hepatitis, the effect of proinflammatory cytokines is believed to play an important role in determining trace element concentration.

Similar to our results, Cesur *et al* (28) reported no significant differences between zinc and copper serum concentrations in patients with chronic hepatitis C and healthy control subjects. Another finding of our study is the significant increase in IL-6 concentration in patients with chronic hepatitis C. This result is consistent with previously published data and suggests that persistent infection, especially chronic hepatitis, is characterized by an elevated IL-6 concentration (29,30).

In our study, we did not observe any significant differences between IFN-γ concentrations in patients with chronic hepatitis and in control subjects. This observation is confirmed by previous studies, which reported no differences between IFN-γ concentrations in HCV-infected patients and in control subjects (31-33). Moreover, we observed no differences in DP IV activity in patients with chronic hepatitis C compared with control subjects. This result is inconsistent with previously published data showing increased DP IV activity in various liver diseases such as primary biliary cirrhosis (34), cholestasis (35) and chronic hepatitis C (18). Firneisz *et al* (18) found a strong correlation between DP IV and transaminase activity, and increased DP IV activity has been implicated in hepatocyte damage.

To the best of our knowledge, this is the first study to combine an investigation of trace elements (zinc and copper), cytokines (IL-6 and IFN-γ) and DP IV enzymic activity with special reference to gender differences. In conclusion, not excluding a ß error, we did not find a correlation between levels of the trace elements zinc and copper and immunological parameters, including IL-6 and IFN-γ levels and DP IV activity.

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