Relationship between plasma levels of retinol and tocopherol and disease severity in patients with liver cirrhosis

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Summary. In a previous study we found that plasma levels of retinol and tocopherol tend to decrease for increasing degrees of liver cirrhosis (LC) (J Lab Clin Med 1991;118: 176). Aiming to verify whether retinol and tocopherol could be employed as markers of LC severity, we measured them in 141 consecutive cirrhotics and 48 healthy controls. Statistically significant differences in plasma retinol levels were found between controls (2.37 ± 0.94 μmol/l), Child-Pugh (CP) A (1.50 ± 0.90 μmol/l, n = 60), CPB (0.84 ± 0.42 μmol/l, n = 51) and CPC patients (0.56 ± 0.38 μmol/l, n = 30; $p < 0.01$ for CPA vs CPB, CPA vs CPC, CPA vs controls, CPB vs controls, CPC vs controls and $p < 0.05$ for CPB vs CPC). Patients with hepatocarcinoma (HCC) superimposed on LC showed lower levels of retinol as compared to those without HCC (0.38 ± 0.07, n = 34 vs 0.83 ± 0.08 μmol/l, n = 107, $p < 0.05$). While a decreasing trend was observed also for tocopherol, between-group differences were not always significant. Moreover, tocopherol levels were similar in cirrhotics with and without HCC. Age, sex and body mass index did not influence the relationship between retinol or tocopherol levels and LC degree (p = ns, ANCOVA). Our study suggests that retinol has the potential to be employed as an index of disease severity in LC patients.

Riassunto. In uno studio precedente abbiamo osservato che i livelli plasmatici di retinolo e tocoferolo tendono a decrescere per gradi crescenti di severità della cirrosi epatica (LC) (J Lab Clin Med 1991;118: 176). Volendo verificare se i livelli plasmatici di retinolo e tocoferolo possono essere impiegati come indicatori del grado di severità della LC, abbiamo misurato retinolemia e tocoferolemia in 141 cirrotici consecutivi e 48 controlli sani. Differenze statisticamente significative nei livelli plasmatici di retinolo sono state riscontrate tra i controlli (2.37 ± 0.94 μmol/l), i pazienti in classe A di Child-Pugh (CP) (1.50 ± 0.90 μmol/l, n = 60), quelli in classe B (0.84 ± 0.42 μmol/l, n = 51; CPB) e quelli in classe C (0.56 ± 0.38 μmol/l, n = 30; CPC; $p < 0.01$ per CPA vs CPB, CPA vs CPC, CPA vs controlli, CPB vs controlli, CPC vs controlli e $p < 0.05$ per CPB vs CPC). I pazienti con epatocarcinoma (HCC) secondario a LC presentavano valori di retinolo inferiori rispetto a quelli senza HCC (0.38 ± 0.07, n = 34 vs 0.83 ± 0.08 μmol/l, n = 107, $p < 0.05$). Un trend discendente veniva osservato anche per il tocoferolo ma le differenze inter-gruppo non erano sempre statisticamente significative. Inoltre, i livelli di tocoferolo erano simili nei pazienti con e senza HCC. Età, sesso e indice di massa corporea non influenzavano la relazione tra retinolo o tocoferolo e grado di LC (p = ns, ANCOVA). Il nostro studio suggerisce che il retinolo plasmatico ha il potenziale per essere utilizzato come indicatore di severità della cirrosi epatica.

Running title: retinol and tocopherol in liver cirrhosis

Keywords: retinol, tocopherol, liver cirrhosis, hepatocarcinoma

Introduction

Plasma retinol and tocopherol levels are characteristically decreased in patients with liver cirrhosis (LC) (Kanematsu et al., 1989; Rocchi et al., 1991; Bell et al., 1992; Ward et al., 1992; Clot et al., 1994; Janczewska et al., 1995). Retinol has a major role in cell proliferation and tocopherol acts as a free-radical scavenger *in vitro* (Basu et al., 1996a; Basu et al., 1996b). Cell proliferation is a constant feature of LC and the production of reactive oxygen species (ROS) plays a major role in the patho-

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genesis of chronic liver disease (Pietrangelo, 1996). The decreased levels of retinol and tocopherol commonly seen in LC patients may be the result of their intervention in these processes.

In a previous study we found lower levels of retinol and tocopherol in cirrhotics (n = 53) with alcoholic, viral and mixed (alcoholic + viral) causes of cirrhosis as compared to healthy controls (n = 32) (Rocchi et al., 1991). An interesting finding of this study was that plasma levels of retinol and tocopherol tended to decrease for increasing degrees of LC severity, as determined by the Child-Pugh classification. However, a larger study sample was needed to test this hypothesis more accurately. Moreover, we were interested to establish whether levels of retinol and tocopherol differ between cirrhotics with hepatocarcinoma (HCC) and without HCC.

The present study aimed therefore to answer two questions: 1) are plasma levels of retinol and tocopherol different in patients with different degrees of cirrhosis ?, and, 2) are these levels different in cirrhotics with HCC as compared to those without HCC ?

Materials and methods

Subjects

One hundred and forty-one patients with biopsy-proven diagnosis of LC were consecutively enrolled into the study at the Medicine II of Modena University. Etiology of LC was viral in 70% of cases, alcoholic in 10% and mixed in the remaining 20%. Forty-eight healthy subjects recruited from the medical staff of the Clinic were used as controls. The study protocol had been approved by the Ethical Committee at Modena University and all subjects gave their informed consent. The following exclusion criteria were applied to cirrhotics: 1) etiology of cirrhosis other than viral, alcoholic or mixed; 2) presence of chronic organ disease other than LC; 3) presence of malabsorption, as detected by chemico-physical analysis of feces; 4) alcohol consumption not suspended from at least 3 months; 5) recent (< 3 months) hemorrhagic complications; 6) absence of significant cholestasis as defined by bilirubin levels > 51.3 μmol/l. All patients and controls were on a free diet at home and the patients consumed a standard diet during their hospital stay.

Clinical evaluation

The criteria of Child-Pugh were used to classify patients in 3 progressively worsening stages of cirrhosis: A (CPA), B (CPB), C (CPC) (Pugh et al., 1973). This classification, which is based on the values of serum albumin and bilirubin, prothrombin time, degree of ascites and degree of encephalopathy, is widely employed as an indicator of LC severity. Body weight (Wt) and height (Ht) were measured following the Anthropometric Standardization Reference Manual (Lohman et al., 1988). Body mass index (BMI) was calculated as Wt (kg)/Ht^2 (m^2) (Garrow et al., 1985).

Retinol and tocopherol

Fasting plasma samples were stored at -80° C. They were added to a double volume of ethanol containing retinol acetate and tocopherol acetate as internal standards and to 3 vol N-heptane containing butylated hydroxytoluene (0.5 g/l). They were extracted, evaporated under nitrogen and resuspended in the mobile phase (acetonitrile:methyl alcohol, 50:50, v/v). The chromatographic procedure, employing a Waters 501 HPLC apparatus connected to a programmable multi-wavelength detector (model 490, Waters Associates, Mildford, Massachusetts, USA) was developed in our laboratory and is described in detail elsewhere (Rocchi et al., 1991). Analyses of retinol, retinol acetate (the first internal standard), tocopherol and tocopherol acetate (the second internal standard) were performed in a single run by scanning the sample at wavelengths of 325 and 295 nm for retinol and tocopherol respectively. All samples were analyzed within 1 week from their collection. With our method, the within-week coefficients of variation (CV) for repeated measurements of retinol and tocopherol are 1.2 and 1.5% respectively (Rocchi et al., 1991).

Statistical analysis

Statistical analysis was performed on a MacOS computer using the Statview 5.0 software package (SAS Institute, Cary, NC, USA). All variables of interest were normally distributed and their between-group variances were homogeneous, as detected by the Kolmogorov-Smirnov-Lilliefors and Levene tests respectively. Between-group comparisons were performed by ANOVA using the Games-Howell test for post-hoc analyses. (The employed sample size ensured a power of 1.0 and 0.97 for between-group comparisons of retinol and tocopherol respectively, with p set to 0.05). To control for the confounding effect of sex, age and BMI, general linear models (GLMs) were employed in which retinol or tocopherol were entered as the dependent variable and disease status (0: control, 1: cirrhotic), disease degree (0: control; 1: CPA cirrhotic, 2: CPB cirrhotic, 3: CPC cirrhotic) or HCC status (0: cirrhotic without HCC; 1: cirrhotic with HCC) as the independent variable; confounding variables (sex, age and BMI) were then entered as covariates or factors in the GLMs in order to test their interaction with the independent variable.

Results

The characteristics of the study subjects are given in Table 1.

Preliminary analyses showed that etiology did not influence plasma levels of retinol and tocopherol (data not shown) so that it was not considered in further analyses.

Effects of sex, age and BMI

The majority of the enrolled patients were male, reflecting in part the higher prevalence of LC in male subjects. To control for the possibly confounding effect of sex, we entered it as a factor in the GLMs. Age was significantly higher in CPC cirrhotics than controls ($p < 0.05$, ANOVA; Table 1). Thus, we entered it as a covariate in the GLMs. Wt, Ht and BMI were similar in cirrhotics but Wt and BMI were lower in cirrhotics than controls ($p < 0.05$, ANOVA; Table 1). Since BMI takes into account the effects of both Wt and Ht and is a better indicator of body composition than Wt (Garrow et al., 1985), it was entered as a covariate in the GLMs to control for the effects of body composition. The statistical significance of the between-group differences in BMI and the interaction of this latter with the independent variable did not change when cirrhotics with ascites were removed from the dataset employed to generate the GLMs (data not shown).

Effect of LC

Retinol was significantly lower in cirrhotics (n = 141) than controls (1.08 ± 0.77 vs 2.37

Table 1

Anthropometric characteristics and plasma levels of retinol and tocopherol in controls and cirrhotics (mean ± SD). Abbreviations: CPA = Child-Pugh class A; CPB Child-Pugh class B; CPC = Child-Pugh class C; Wt = body weight; Ht = body height; BMI = body mass index.

	Controls	CPA	CPB	CPC
n	48	60	51	30
Sex (m:f; %)	77:23	79:21	85:15	90:10
Age (yr)	53 ± 15^{a}	$59 \pm 10^{a,b}$	$60 \pm 9^{a,b}$	63 ± 8^{b}
Wt (kg)	80.1 ± 12.3^{a}	72.1 ± 10.1^{b}	70.0 ± 14.4^{b}	70.9 ± 9.8^{b}
Ht (cm)	170.8 ± 9.3^{a}	165.2 ± 7.0^{b}	$166.0 \pm 9.4^{a,b}$	$165.8 \pm 7.3^{a,b}$
BMI (kg/m^2)	23.5 ± 3.6^{a}	21.8 ± 2.6^{b}	$21.0 \pm 3.7^{a,b}$	21.4 ± 3.3 a,^{b}
Retinol (μmol/l)	2.37 ± 0.94^{e}	1.50 ± 0.90^{f}	0.84 ± 0.42^{g}	0.56 ± 0.38^{h}
Tocopherol (μmol/l)	21 ± 5^{a}	18 ± 6^{b}	18 ± 5^{b}	15 ± 5^{c}

[a,b,c,d] Values not sharing the same superscript are significantly different at the $p < 0.05$ level; [e, f, g, h] Values not sharing the same superscript are significantly different: $p < 0.01$ for CPA vs CPB, CPA vs CPC, CPA vs controls, CPB vs controls, CPC vs control; $p < 0.05$ for CPB vs CPC.

± 0.94 μmol/l, $p < 0.0001$, ANOVA). Moreover, differences between controls, CPA, CPB and CPC patients were statistically significant (Table 1). With retinol as the dependent variable in the GLM, the interactions of disease status and disease degree with sex, age and BMI were not significant (p = ns).

Tocopherol was significantly lower in cirrhotics than controls (17±6 vs 21±5 μmol/l, $p < 0.0005$, ANOVA). While a decreasing trend was observed for tocopherol among controls, CPA, CPB and CPC patients, differences between CPA and CPB patients were not statistically significant (Table 1). As in our previous study (Rocchi et al., 1991), standardization of plasma tocopherol on total serum lipids did not modify this trend and its statistical significance (data not shown). With tocopherol as the dependent variable in the GLM, the interactions of disease status and disease degree with sex, age and BMI were not significant (p = ns).

Effect of HCC

Thirty-four (24%) of the 141 patients had HCC superimposed on LC (as diagnosed by liver biopsy). Retinol levels were significantly lower in cirrhotics with HCC than those without HCC (0.38 ± 0.07 vs 0.83 ± 0.08 μmol/l, $p < 0.05$, ANOVA) but tocopherol levels were similar (18 ± 6 vs 17 ± 5 μmol/l, p = ns, ANOVA). The interactions of sex, age and BMI with HCC status were not significant for both retinol and tocopherol (p = ns). Also the interaction of disease degree with HCC status proved to be not significant for retinol. While this finding is interesting since it suggest that HCC *per se* may be associated to modifications of plasma retinol, it should be taken with caution due to the relatively low number of patients with HCC that were (randomly) recruited into this study.

Discussion

The cirrhotics recruited for this study were on a free diet and had similar values of Wt and BMI. Within the limits allowed by anthropometric assessment, it appears therefore that they had a similar body composition. They were also abstaining from alcohol from at least 3 months and had no significant cholestasis, conditions which may cause retinol and tocopherol deficiency independently of LC (Basu et al., 1996b; Basu et al., 1996a). Moreover, age, sex and BMI had no effect on the relationship between retinol, tocopherol and LC severity.

In our patients, we found a strong relationship between plasma retinol and LC severity (Table 1). Moreover, patients with HCC had decreased plasma levels of this vitamin as compared to those without HCC. That plasma retinol levels tend to modify with liver disease is not surprising if one considers that 90% of body retinol is stored in the liver (Basu et al., 1996a). However, the pathophysiological explanation of this finding may not be a simple one. While it is tempting to speculate that the relationship between plasma retinol, LC and HCC may reflect an increasing damage of the liver, it should in fact be considered that tissue levels of this and other vitamins are frequently uncorrelated with their plasma levels, especially in HCC patients (Rocchi et al., 1997). Quite interestingly, however, liver transplantation restores plasma retinol to normal levels, thus reinforcing the possibility that plasma retinol can be used as a marker of the severity of liver disease (Janczewska et al., 1995).

A less strong but nonetheless interesting relationship was observed between plasma tocopherol and LC severity (Table 1). However, tocopherol levels were similar in patients with HCC and without HCC. In animal models, vitamin E protects against ROS-generating hepatotoxins although supplementation studies in cirrhotics have shown no substantial benefit on the progression of the underlying disease (Pascoe et al., 1987; de la Maza et al., 1995; Sokol, 1996). Contrarily to retinol, tocopherol never accumulates in large amounts in the liver and this could partly explain why it is less sensitive to the severity of liver cirrhosis as compared to retinol.

In summary, our study shows that both retinol and tocopherol tend to decrease for increasing levels of liver cirrhosis. However, retinol is clearly a better marker of disease severity than

tocopherol. Longitudinal studies of cirrhotics are needed to ascertain the full clinical relevance of this finding.

Acknowledgements: partially supported by MURST (Ministero Università Ricerca Scientifica e Tecnologica) grants.

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