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Original Contribution

Immune responses against oxidative stress-derived antigens are associated with increased circulating tumor necrosis factor-α in heavy drinkers

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ABSTRACT

Growing evidence indicates that pro-inflammatory cytokines play a key role in alcoholic liver disease (ALD). This study investigates whether immune response toward oxidative stress-derived antigens could be involved in promoting cytokine production in alcohol abusers.

Cytokine profile and circulating IgG against human serum albumin modified by malondialdehyde (MDA-HSA) and against oxidized cardiolipin (Ox-CL) were evaluated in 59 heavy drinkers (HD) with (n=30) or without (n=29) ALD and 34 healthy controls.

IgG against MDA-HSA and Ox-CL were significantly higher in HD with ALD than in HD without liver injury or healthy controls. The elevation of these antibodies was associated with higher circulating levels of IL-2 (p=0.005) and TNF-α (p=0.001), but not of IL-6 or IL-8. The prevalence of abnormal TNF-α was 5-fold higher in HD with oxidative stress-induced IgG than in those without. HD with the combined elevation of both TNF-α and oxidative stress-induced IgG had 11-fold (OR 10.7; 95%CI 1.2-97.2; p=0.023) greater risk of advanced ALD than those with high TNF-α, but no immune responses. Moreover, the combined elevation of TNF-α and lipid peroxidation-derived IgG was an independent predictor of ALD in HD.

We propose that immune responses towards oxidative stress-derived antigen promote TNF-α production and contribute to liver damage in alcohol abusers.

Introduction

Alcoholic liver disease (ALD) is a common consequence of long-term alcohol abuse and a major cause of morbidity and mortality worldwide. It encompasses a broad spectrum of morphological features ranging from minimal injury to advanced liver damage, including hepatitis and cirrhosis [1]. The mechanisms involved in alcohol-related liver injury have, however, remained poorly understood.

Recently, experimental and clinical studies have underscored the role of inflammation in the progression of ALD [2–4]. Serum concentrations of pro-inflammatory cytokines/chemokines, including TNF-α, IL-6, IL-8 and MCP-1, are increased in alcohol-fed rats as well as in patients with ALD [4]. Plasma TNF-α levels also correlate with disease mortality [4]. Moreover, hepatic necro-inflammation caused by long-term alcohol feeding is attenuated by Kupffer cell inactivation with gadolinium chloride as well as in mice knockout for TNF-α receptor-1 [2,4]. Although an increased translocation of gut-derived endotoxins to the portal circulation has been shown to stimulate the Kupffer cell secretion of pro-inflammatory cytokines in alcohol-fed animals [3], the activation of Kupffer cells by endotoxins might not be the only factor responsible for maintaining an inflammatory milieu in the liver. Alcoholic liver disease is often associated with the development allo- and auto-immune reactions against a variety of antigens [5–7]. Inflammatory infiltrates containing both $CD8^+$ and $CD4^+$ T-lymphocytes have been found to occur in the portal and peri-portal areas of about 40% of patients with advanced ALD correlating with the degree of intralobular inflammation, piecemeal necrosis and septal fibrosis [8,9]. Earlier studies have shown that the covalent interaction of acetaldehyde, the first metabolite of ethanol, with liver proteins induces specific immune responses in alcohol fed rodents [10,11]. Consistently, increased levels of IgA and IgG antibodies against protein adducts with acetaldehyde are detectable in the serum of ALD patients [12–14]. Oxidative stress induced by alcohol also cause modifications of hepatic proteins, which trigger immune reactions [15,16]. In particular, elevated titres of IgGs towards adducts originating from the reaction between liver proteins and lipid peroxidation products (malondialdehyde, 4-hydroxy-nonenal, arachidonyl-hydroperoxides) are frequent in patients with alcoholic hepatitis and/or cirrhosis, but rare in heavy drinkers with fatty liver only [17,18]. Moreover, the condensation of MDA and acetaldehyde generates malonildialdehyde-acetaldehyde adducts (MAA) [19], that

Abbrevations: ALD, Alcoholic Liver Disease; ELISA, Enzyme-linked immunosorbent assay; HD, Heavy drinkers; HSA, Human serum albumin; MDA, Malondialdehyde; MDA-HSA, Malondialdehyde-modified human serum albumin; Ox-CL, Oxidized cardiolipin; PBS, Phosphate buffered saline; TNF-α, Tumor necrosis factor-α.

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stimulate immune reactions in ALD patients [20]. Patients with advanced ALD also exhibit $CD4^+$ T-lymphocyte responses towards malondialdehyde-derived antigens, suggesting that oxidative mechanisms are involved in promoting both humoral and cellular immune responses [21]. Oxidative stress may also favour auto-immune reactions because 60-80% of patients with advanced ALD have high titres of circulating anti-phospholipid auto-antibodies directed specifically against oxidized phospholipids, namely oxidized cardiolipin (oxCL) [22]. A possible role of such immune mechanisms in modulating inflammatory processes associated with alcohol hepatotoxicity has been suggested by recent experiments using intragastric alcohol-fed rats with low circulating endotoxins [23]. In these animals the amounts of lipid peroxidation adduct in the liver are associated with hepatic TNF-α mRNA expression and the severity of hepatic inflammation, while the supplementation with antioxidants reduces both immune and inflammatory responses [23]. These observations prompted us to investigate the possible role of immune reactions triggered by oxidative stress in sustaining the hepatic production of pro-inflammatory cytokines/chemokines.

Experimental procedures

Patients and control subjects

We studied 59 male alcoholic patients (mean age 48±11 yr) who referred to specialized liver clinic or detoxification at Central Hospital Seinäjoki and Tampere, Finland. All the patients showed a well-documented history of ethanol consumption exceeding 80 g/day either continuously or during repeated episodes of binge drinking. Among the subjects investigated 30 had biopsy-proven liver disease, the liver histology ranging from mild fibrosis and fatty change to cirrhosis with a wide distribution of morphological abnormalities related to alcoholic hepatitis, as assessed according to previously established combined morphological index (CMI) of liver disease severity [24]. The remaining 29 were heavy drinkers, who had been admitted for detoxification, but were devoid of clinical and laboratory evidence of significant liver disease. Because of ethical considerations, these patients were not biopsied. The documentation of ethanol intake was based on detailed personal interviews using a time-line follow-back technique. The mean duration of abstinence prior to sampling was 2±2 days. All patients were negative for hepatitis B virus antigen or hepatitis C serology. The reference population consisted of 34 apparently healthy male volunteers (mean age 49±12 yr) who were either abstainers (n=17) or moderate drinkers (n=17) with mean daily ethanol consumption of about 20 g (range 1–40 g). All serum samples were stored at −70°C until analysis. All participants of the study gave their informed consent and the study was carried out according to the provisions of the Declaration of Helsinki. The main clinical and laboratory characteristics of the study population are summarized in Table 1.

Preparation of the antigens

Human serum albumin modified by malondialdehyde (MDA-HSA) was prepared by reacting for 2 h at 37°C 2 mg/ml HSA with 100 mmol/L MDA obtained by the acid hydrolysis of MDA-bis-dimethylacetal, as previously reported [25]. Cardiolipin (88 μg/mL in PBS) oxidation was induced by free radicals originating from the thermal decomposition of 1 mmol/L 2,2″-azo-bis-(2-amidinopropane) hydrochloride (Polyscience Inc., Warrington, PA) as previously described [25]. Oxidized cardiolipin was extracted in 5 mL of chloroform, dried under nitrogen, and resuspended in ethanol (50 μg/mL final concentration).

Measurements of the antibody titres

Polystyrene microwell plates for ELISA (Nunc-Immuno Maxi-Sorb; Nunc, S/A, Roskilde, Denmark) were coated for 16 hours at 4°C with 0.05 mg/mL of either modified or native HSA solubilized in 0.1 mol/L bicarbonate buffer, pH 9.6. After incubation, the solutions were removed and replaced by 0.3 mL of coating buffer containing 3% (w/v) bovine serum albumin in PBS, pH 7.4. The plates were further incubated for 1 hour at 37°C to block non-specific binding sites. The coated wells were washed 3 times with PBS containing 0.25% (v/v) Triton X-100. The sera of the patients were diluted 1:50 with the coating buffer and added in duplicate as aliquots of 0.20 mL to the appropriate wells and incubated 1 hour at 37°C. After washing the plates 3 times with PBS 0.25% Triton X-100, peroxidase-linked goat antihuman IgG (dilution 1:6000) (Dako S.P.A., Milano, Italy) were added and incubated for 1 hour at 37°C. The antibody binding was revealed by the addition of 0.15 mL of a reaction mixture containing 0.4 mg/mL of 1-phenylendiamine, 0.4 μL/mL hydrogen peroxide (30%), 5.1 mg/mL citric acid, and 6.1 mg/mL anhydrous Na_2HPO_4 at pH 5.0. After 15 minutes, the reaction was stopped by adding 50 μL 2 N H_2SO_4, and absorbance was measured at 490 nm using a Bio-Rad microplate reader (Bio-Rad Laboratories Inc., Hercules, CA). The results were expressed by subtracting the background reactivity with unmodified HSA.

For the determination of antibody towards oxidized cardiolipin, ELISA plates (Nunc-Immuno Poly-Sorb Nunc, S/A, Roskilde, Denmark) were coated by adding 30 μL of the oxidized cardiolipin ethanol solution to each well, and the solvent was evaporated under vacuum. The same amount of ethanol was added to reference wells. After 2 washes with PBS (0.3 mL), non-specific binding sites were blocked by 1 hour incubation at 37°C with 1% (w/v) solution of polyethylenglycol compound in PBS, pH 7.4. The coated wells were then washed 3 times with PBS. Patients and controls sera (1:50 dilution in PBS) were incubated for 1 hour at 37°C, and the plates were washed 3 times with PBS. IgG binding was revealed as described above. The results were expressed by subtracting the background reactivity in the wells treated with ethanol alone. The threshold values of the IgG reactivity towards the different oxidative stress-related antigens were calculated at the 95th percentile from the group of healthy subjects.

Cytokine measurement

The concentrations of interleukins (IL-2, IL-6, IL-8 and TNF-α) in serum were determined using Quantikine high sensitivity ELISA kits according to the instructions of the manufacturer (R&D Systems, Abington Science Park, UK). Assay cut-offs were based on ROC analyses and reference limits given by the assay manufacturer.

Other biochemical determinations

Serum aminoterminal propeptide of type III procollagen (PIIINP) was measured by a radioimmunological procedure (Orion Diagnostica, Espoo, Finland). Serum hyaluronic acid was measured using an enzyme-linked

Table 1
Clinical and biochemical characteristics of the study subjects

	Controls	Heavy drinkers without ALD	Heavy drinkers with ALD
Number of observations (n)	34	29	30
Age (Years)	48.8±12.2	45.2±11.4	50.8±9.5
Aspartate aminotransferase (U/L)	33.8±26.6	38.7±18.4[a]	62.2±54.3[c]
Gamma glutamyltranspeptidase (U/L)	34.4±24.3	88.8±79.6[b]	280.7±360.3[d]
Bilirubin (μmol/L)	14.8±6.1	13.9±8.7	17.9±15.0
Albumin (g/L)	42.6±3.4	45.3±3.9[a]	40.9±5.5[ac]
IL-2 (U/L)	1.3±2.2	1.2±4.4	7.1±9.9[bc]
IL-6 (pg/mL)	0.8±1.9	4.3±7.5	8.5±8.6[bc]
IL-8 (U/L)	33.7±33.8	44.5±42.9	75.4±60.8[bc]
TNF-α (U/L)	3.7±5.1	11.0±13.8	40.8±24.4[cd]
Hyaluronic acid (ng/mL)	39.6±27.4	36.4±34.0	198.7±170.2[d]
PIIINP (ng/mL)	3.8±1.2	3.2±1.4	10.2±5.3[d]

The values are expressed as means±S.D. Statistical significance: [a] $p<0.05$ or [b] $p<0.005$ vs abstainers or moderate drinkers; [c]$p<0.005$ or [d]$p<0.0005$ vs heavy drinkers without ALD or controls.