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Harmful effect of adipose tissue on liver lesions in patients with alcoholic liver disease☆

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Background & Aims: Adipose tissue is an important source of cytokines. Excess weight is an independent risk factor for steatosis, acute alcoholic hepatitis (AAH), and cirrhosis in patients with alcoholic liver disease (ALD). In this study, we investigated the role of adipose tissue in human ALD.

Patients and methods: Fifty patients with ALD underwent liver and abdominal subcutaneous adipose tissue biopsies and supplied blood samples for the investigation of cytokine gene expression and secretion, as well as liver histology.

Results: The levels of TNF-α and IL-10 in adipose tissue were higher in patients with AAH. IL-10 level in adipose tissue was also correlated with fibrosis score. TNF-α gene expression in adipose tissue was correlated with Maddrey score, blood C-reactive protein (CRP) concentration and liver IL-6 concentration. IL-6 production levels in the liver were higher in patients with AAH and correlated with AAH score, liver histological lesions, liver TNF-α concentration, Maddrey score, and blood CRP concentration. Plasma concentrations of soluble forms of TNF-receptor were correlated with inflammatory lesions in the liver, Maddrey score and fibrosis score.

Conclusion: In patients with ALD, inflammation occurs not only in the liver, but also in the adipose tissue. Adipose tissue inflammation is correlated with the severity of pathological features in the liver. Our findings may account for the harmful interactions between body mass index, AAH, fibrosis, and cirrhosis in alcoholic patients.

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Introduction

Alcoholic liver disease (ALD) is defined histologically as a combination of steatosis, hepatocyte injury and necrosis, inflammatory infiltration, and fibrosis. The severity, extent and relative contributions of these four basic features vary between cases. Almost all cases of ALD display some degree of fibrosis. We have previously identified excess weight as an independent risk factor for steatosis, acute alcoholic hepatitis (AAH), and cirrhosis in patients with ALD [1]. We have also shown that body mass index (BMI) is an independent risk factor for fibrosis in non-obese alcoholic patients [2]. We also recently demonstrated that high blood pressure, apolipoprotein A-1 concentration and BMI are predictive of steatosis [3]. These results suggest that adipose tissue is involved in the pathogenesis of human ALD.

Cytokines are mediators of the inflammatory response exerting multiple actions in an autocrine, paracrine, and endocrine manner. Immune cells, including macrophages and hepatocytes, were initially identified as the main source of cytokines [4]. There is little doubt that cytokines play a major role in the development of chronic alcoholic liver disease [5–7]. Pro-inflammatory cytokines, such as TNF (tumor necrosis factor)-α and IL (interleukin)-6, are directly involved in the pathogenesis of alcoholic hepatitis [8]. Ethanol directly induces the secretion of IL-8 by hepatocytes [9], but the main route of cytokine secretion is thought to involve endotoxins and reactive oxygen species activating Kupffer cells by binding to Toll-like receptors [10]. The cytokines released by these cells initiate the cascade of events that ultimately leads to the development of hepatocyte necrosis,

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Abbreviations: ALD, alcoholic liver disease; AAH, acute alcoholic hepatitis; BMI, body mass index; PMN, polymorphonuclear neutrophil; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; IL-10, interleukin-10.

the recruitment of neutrophils and immune cells and liver fibrosis. The anti-inflammatory system is simultaneously upregulated, to control the inflammatory response and to limit tissue damage. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of pro-inflammatory cytokines by T helper type 1 T cells, monocytes/macrophages, and neutrophils. IL-10 is synthesized by several cell types within the liver, including Kupffer cells and hepatocytes. Endogenous IL-10 has been shown to modulate the inflammatory response and to limit hepatotoxicity in several models of liver injury, such as alcoholic hepatitis [11,12].

Adipocytes have recently been identified as an important source of cytokines, including TNF-α, IL-6, IL-8, IL-10, transforming growth factor (TGF)-β, nerve growth factor, and adipokines. The secretion of cytokines by adipose tissue plays an important role in non-alcoholic fatty liver disease (NAFLD) (reviewed in [13]). However, despite the harmful effects of excess weight in patients with ALD, the role of adipose tissue as a source of pro- and anti-inflammatory cytokines in non-obese patients with ALD has never been studied.

We tested the hypothesis that adipose tissue plays a role in human ALD as a potent source of pro- and anti-inflammatory cytokines. We compared cytokine gene expression in adipose tissue and liver, between patients with and without AAH. We then considered the correlation between the levels of cytokines and individual pathologic features of ALD in non-obese patients. We also assessed the correlations between cytokine gene expression and the severity of liver injury, as assessed by Maddrey score [14] and between cytokine gene expression and systemic inflammation, as assessed by C-reactive protein (CRP) concentration.

Patients and methods

Patients

The patients included in this prospective study were admitted to the Hepatogastroenterology Department of Antoine Béclère University Hospital, Clamart, France, due to alcoholism and alcoholic liver disease. Patients were eligible for inclusion if they had high serum aminotransferase levels, had drunk at least 50 g of alcohol per day over the previous year and had not stopped drinking before admission, had no detectable hepatitis B surface antigen or antibodies against hepatitis C virus in their serum, gave informed consent and had no contraindications (including blood coagulation defects or ascites) for intercostal liver and adipose tissue biopsies. The exclusion criteria were gastrointestinal bleeding, bacterial infection, hepatocellular or other carcinoma, acute pancreatitis, severe associated disease, the presence of anti-HIV antibodies, dyslipidemia, and diabetes mellitus.

A specific questionnaire was used to obtain information about alcohol consumption [15]. The patients' families were also interviewed, when possible. Patients were asked about how long their alcoholism had lasted and their daily alcohol intake over the year before their admission to our department for alcoholism or alcoholic liver disease. The questionnaire included the following items concerning alcohol consumption: duration of alcohol abuse, daily consumption of beer, wine, aperitifs and spirits (strong liquors) and the types of aperitif ingested (aniseed-based aperitifs, whisky and others). The daily intake of each beverage was expressed in grams of pure ethanol. Total daily ethanol consumption was calculated by summing the amounts of alcohol present in each type of beverage consumed.

Body mass index (BMI) was calculated by the Quetelet formula (weight in kilograms/(height in meters)^2). Blood samples were tested to determine fasting glucose, triglyceride, and total cholesterol concentrations. All patients underwent ultrasound-guided liver biopsy within one week of admission. One specimen of liver tissue was used for histological analysis and another was immediately frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. Specimens were stained with hematoxylin-eosin-saffron, Masson's trichrome and picrosirius red. All biopsy samples were evaluated by a pathologist blind to the patient's clinical and biological data and to the duration of alcoholism and daily alcohol intake. Internationally accepted morphological criteria were used for this evaluation [16]. Acute alcoholic hepatitis (AAH) was defined as the presence of both polymorphonuclear neutrophil (PMN) infiltrate and hepatocellular necrosis. We used a scoring system for AAH combining detailed alcoholism-related features: acidophilic bodies, PMN, Mallory bodies, and ballooning. Each feature was scored from 0 to 2, giving a total score of 0–8 [17,18]. Steatosis was assessed semi-quantitatively on a five-point scale: none = 0; mild (1–5% of hepatocytes) = 1; moderate (6–32% of hepatocytes) = 2; marked (33–66%) = 3; severe (67–100%) = 4 [19]. Five fibrosis lesions were assessed semi-quantitatively: enlargement of the portal tract (no = 0, mild = 1, severe = 2), fibrous septa (no = 0, between portal tracts or between portal tract and terminal hepatic venule = 1, both = 2), perisinusoidal fibrosis (no = 0, yes = 1), perivenular fibrosis (no = 0, yes = 1), cirrhosis (no = 0, macronodular = 1, micronodular = 2). Thus, the total score for fibrosis was between 0 and 8 [18]. Samples of subcutaneous adipose tissue were removed by aspiration, under local anesthesia, from the anterior abdominal wall, below the right costal margin, on the mid-clavicular line, at the level of the umbilicus. Sixteen-gauge needles from 20 ml and standard venipuncture syringes were used to obtain the samples. Maximum traction was applied to the plunger of the syringe plunger and the syringe was repeatedly rotated and pushed back and forth in the adipose tissue while suction was maintained. A 2 ml sample from each patient was immediately frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction.

Informed consent was obtained from all participants. The study was conducted in accordance with the French law concerning medical investigations (Huriet Law) and the Helsinki declaration. The protocol was approved by the ethics committee of Bicêtre Hospital.

Six patients were excluded due to the presence of esophageal carcinoma ($n = 1$), dyslipidemia ($n = 3$) and diabetes mellitus ($n = 2$).

Cytokine production by the liver and adipose tissue

RNA extraction

Adipose and liver tissues were collected and homogenized in QIAzol lysis buffer with a Polytron. Total RNA was extracted with the RNeasy Lipid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA obtained was treated with an RNAse-free DNAse kit (Qiagen), to eliminate residual DNA. The amount of RNA was determined by measuring absorbance at 260 nm in a spectrophotometer.

Amplification

Liver RNA was amplified with the Message Amp II aRNA kit (Ambion, Austin, TX, USA).

Reverse transcription (RT) of RNA

M-MuLV RT (Invitrogen, Carslbad, CA, USA) was used to reverse transcribe 5 μg of RNA from adipose tissue with pd(T) primers (Roche, Mannheim, Germany) and 5 μg of RNA from the liver with random hexamers (Roche).

PCR

A LightCycler (Roche) was used for PCR, with the LC FastStart DNA Master SYBR Green I kit (Roche). We amplified the cDNAs for TNF-α, IL-6, and IL-10.

Several studies have suggested that PCR results may depend on the choice of housekeeping gene for normalization. We quantified the RNA used for amplification and assessed the variability of 18S, β-actin, GAPDH, and SFRS4 gene expression, by amplifying equivalent amounts of mRNA for each sample, to prevent nonspecific results due to variability in housekeeping gene expression [20,21]. Based on the results obtained, we chose to normalize TNF-α, IL-6, and IL-10 mRNA levels according to 18S mRNA levels in the liver and β-actin mRNA levels in adipose tissue, as these two genes displayed the lowest variability for amplifications of equal amounts of RNA.

Plasma cytokine levels

Levels of the cytokines IL-10 and IL-6 and of the TNF-α soluble receptors (sRp), TNFsRp55 and TNFsRp75 were determined on the day on which plasma samples were taken and liver biopsies performed. Plasma TNFsR concentrations are considered a reliable indicator of TNF-α system activation [22]. Blood (5 ml) was collected into tubes containing EDTA. Plasma was obtained by centrifugation, dispensed into aliquots within 30 min, and stored at −80 °C until testing. Cytokine concentrations were assessed by ELISA, according to the kit manufacturer's recommendations (Amersham, les Ulis, France).

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