Increased production of IL-1α and TNF-α in lipopolysaccharide-stimulated blood from obese patients with non-alcoholic fatty liver disease

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Abstract

Enhanced pro-inflammatory cytokine production is considered a pathogenic factor in non-alcoholic fatty liver disease (NAFLD). Peripheral blood production of interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) was studied in relation to the severity of histological changes of the liver in obese NAFLD patients. Basal levels in serum and production of IL-1α and TNF-α in peripheral blood cell cultures after stimulation with lipopolysaccharide (enzyme-linked immunoabsorbent assays) were measured in 11 patients with steatosis and 15 with steatohepatitis, who underwent gastrectomy with a gastro-jejunal anastomosis in roux and Y, and in 9 controls who underwent anti-reflux surgery. Production of IL-1α and TNF-α was 122 and 67% higher in patients with steatosis than control values, respectively. In patients with steatohepatitis, IL-1α production was 300 and 80% higher and that of TNF-α 110 and 26% higher, as compared with controls and steatosis patients, respectively. Production of IL-1α was positively correlated with that of TNF-α (r = 0.78, p < 0.0001). IL-1α and TNF-α production were both positively correlated with the degree of steatosis (r = 0.68, p < 0.001 and r = 0.74, p < 0.0001) and steatohepatitis (r = 0.77 and r = 0.75, p < 0.0001) at liver biopsy, and with the homeostasis model assessment index (r = 0.73, p < 0.0001 and r = 0.63, p < 0.01), respectively. Basal serum IL-1α and TNF-α levels were comparable in the three groups studied. It is concluded that elevated production of IL-1α and TNF-α by in vitro stimulated whole blood cell cultures occurs in NAFLD obese patients, which might play a pathophysiological role upon inflammatory leukocyte infiltration of the liver.

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition with a wide spectrum of liver damage including simple steatosis, which may have the best prognosis in spite of having the potential to progress to steatohepatitis, with or without fibrosis and cirrhosis [1]. Frequently associated with obesity, non-insulin-dependent diabetes mellitus, and hyperlipidemia, NAFLD is related to insulin resistance and oxidative stress as critical pathogenic factors [1–3]. In this respect, hepatic fat accumulation in NAFLD may be determined by (i) an increased peripheral lipolytic activity secondary to insulin resistance, with higher fatty acid (FA) and glycerol fluxes to the liver [4], and (ii) depletion of hepatic long-chain polyunsaturated FA (LCPUFA), particularly of the n-3 series, probably caused by defective desaturation and elongation of PUFA and

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increased peroxidation of LCPUFA due to oxidative stress [5]. The latter condition may direct FA away from oxidation and secretion and towards triacylglycerol storage, considering that LCPUFA suppresses lipogenic gene expression and induces that of genes related to lipid oxidation and export [6,7].

In the setting of obesity associated with insulin resistance or diabetes, a sustained higher oxidative stress status may promote progression of liver damage from steatosis to steatohepatitis [8,9], either (i) by inducing severe oxidative damage to biomolecules with loss of their functions and impairment of cell viability [7], and/or (ii) by activating redox-sensitive transcription factors such as NF-κB and AP-1 [10,11]. The latter mechanism may involve the expression and release of pro-inflammatory cytokines such as interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α), chemokines, adhesion molecules, and/or fibrogenic mediators by Kupffer cells and other resident cells of the hepatic sinusoid [10], as well as by the recruitment and activation of inflammatory leukocytes [1,3].

Considering that hepatocyte sensitization to IL-1α and TNF-α mediated cytotoxicity is a critical factor under prolonged oxidative stress conditions, the aim of this study was to test the hypothesis that enhanced production of IL-1α and TNF-α by peripheral blood leukocytes, mainly monocyte/macrophage series, occurs in obese NAFLD patients, as a contributory factor in the development of non-alcoholic steatohepatitis (NASH) upon inflammatory cell recruitment in the liver. For this purpose, production of IL-1α and TNF-α was assessed in whole blood cultures upon stimulation with lipopolysaccharide (LPS) [12], and the results obtained were analyzed in relation to the clinical and histological features of NAFLD. In this assay, LPS mainly stimulates the monocyte/macrophage system to release IL-1, TNF-α, and other cytokines [12,13], to a higher extent than that found in peripheral blood mononuclear cell cultures, probably due to the maintenance of the blood microenvironment and avoidance of the extraction associated with modification of cell ratios and activation [12].

2. Materials and Methods

2.1. Patients and laboratory investigations

Twenty-six NAFLD patients (average body mass index (BMI) of 42.9 ± 0.9 kg/m², age range of 21–65 years), who underwent therapeutic gastrectomy with a gastro-jejunal anastomosis in roux and Y as a therapy for obesity, were included in this study. Nine patients (BMI of 24.3 ± 0.4 kg/m², aged between 24 and 60 years) who underwent anti-reflux surgery were also studied as control group. The protocol was explained in detail to the subjects, who then gave their written informed consent to participate in the study before any procedure was undertaken. Nutritional and alcohol consumption histories with anthropometric measurements were obtained. Laboratory tests included serum liver enzymes (aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (γ-GT)) and bilirubin. Exclusion criteria included positive hepatitis B or C serology, positive antibodies (antinuclear, anti-mitochondrial, and anti-smooth muscle antibodies), smoking habits or non-smokers <1 year cessation, and consumption of more than 40 g of ethanol per week. Insulin resistance was calculated from the fasting insulin and glucose values by homeostasis model assessment (HOMA) of insulin resistance analysis (fasting insulin (µunits/ml) × fasting glucose (mmol/l)/22.5) [14].

The patients were subjected to a diet of 25 kcal/kg body weight (where 1 kcal = 4.184 kJ), with 30% of the energy given as lipids and 15% as proteins, for at least 2 days prior to surgery, and liver biopsies for histological diagnoses were taken during surgery. The samples were fixed in 10% formalin, paraffin embedded, and sections were stained with hematoxylin/eosin and Van Gieson’s stain. Sections of each liver were observed in a blinded manner and evaluated for histological alterations by means of previously defined codes [15,16]. Patients were grouped according to the findings of their liver histology as normal (controls; Fig. 1A), the presence of macrovesicular steatosis alone (steatosis group; Fig. 1B), or steatosis and lobular inflammation with hepatocyte ballooning with or without fibrosis (steatohepatitis group; Fig. 1C). The pathology score was defined as the sum of the steatosis and inflammation scores, each graded as absent (0), mild (1), moderate (2), and severe (3). The Ethics Committee of the University of Chile Clinical Hospital approved the study protocol, according to Helsinki criteria.

2.2. Basal levels of cytokines in serum and whole blood stimulation assay for cytokine production

Venous blood was collected in apyrogenic EDTA-tubes and samples were divided into aliquots for either direct measurement of basal serum IL-1α and TNF-α levels or subjected to dilution with 1/10 RPMI-1640 (Gibco, Rockville, MD, USA) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and distributed in 2-ml wells [12], for LPS-stimulated cytokine production assay. Aliquots from these cultures were taken before (zero time) and after LPS addition (100 ng/ml endotoxin from S. enteritisidis from Sigma, St. Louis, MO, USA) and wells were incubated at 37 °C with 5% CO₂ for 24 h [12]. The contents of the wells were collected and centrifuged at 900 × g for 10 min, and the supernatants were stored at −20 °C. IL-1α and TNF-α concentrations were measured in the serum samples, as well as in the culture supernatants, with specific enzyme-linked immunosorbent assays (Pharmingen, San Diego, CA, USA) according to the manufacturer’s specifications. Samples containing high cytokine levels were repeated after dilution to ensure assay results within the range of the calibration curve. Results were expressed in pg/ml. Data from culture samples at time zero were subtracted from those found at 24 h.

2.3. Statistical analysis

Results are presented as means ± SEM for the number of patients indicated. The sources of variation for multiple
comparisons were assessed by one-way ANOVA, followed by Bonferroni’s multiple comparison test, as post-hoc test. To analyze the association between different variables, the Spearman rank order correlation coefficient was used. All statistical analyses were computed using GraphPad Prism version 2.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Clinical and biochemical characteristics of NAFLD patients and control subjects

Patients’ characteristics are shown in Table 1. NAFLD patients were predominantly female and significantly more obese than control subjects, as revealed by their BMI being 75 and 79% higher over control values for steatosis and steatohepatitis, respectively. Fasting blood glucose levels were comparable in the studied groups, but fasting insulin was higher over control values in both groups of NAFLD patients. Insulin resistance, assessed by the HOMA analysis, was increased both in the steatosis and steatohepatitis groups, the latter being significantly higher than that of the steatosis group \((p < 0.05)\). Liver function tests, including serum AST, ALT, \(\gamma\)-GT, and bilirubin levels, were within normal ranges. Average steatosis and pathology scores were \(0.2 \pm 0.1 \) (\(n = 9\)) in controls and \(1.2 \pm 0.2 \) (\(n = 11\)) in the steatosis group, whereas patients with steatohepatitis (\(n = 15\)) exhibited a steatosis score of \(2.2 \pm 0.2\) and a pathology score of \(3.7 \pm 0.4\), determined as described in Section 2.

3.2. Basal IL-1\(\alpha\) and TNF-\(\alpha\) levels in serum and production in LPS-stimulated whole blood cell cultures

Basal serum levels of IL-1\(\alpha\) (controls, \(26.9 \pm 9.2\) pg/ml (\(n = 9\)); steatosis, \(16.6 \pm 3.2\) (\(n = 11\)); steatohepatitis, \(37.7 \pm 15.4\) (15)) and TNF-\(\alpha\) (controls, \(3.6 \pm 0.6\) pg/ml (\(n = 9\)); steatosis, \(3.3 \pm 0.7\) (\(n = 11\)); steatohepatitis, \(4.1 \pm 0.5\) (\(n = 15\)) in control subjects and patients with steatosis or steatohepatitis were comparable.

![Fig. 1. Hematoxylin/eosin-stained liver sections from a control patient (A) and from NAFLD patients with steatosis (B) or steatohepatitis (C). Magnification, 70×.](image)

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls ((n = 9))</th>
<th>NAFLD patients with steatosis ((n = 11))</th>
<th>NAFLD patients with steatohepatitis ((n = 15))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>(40 \pm 4)</td>
<td>(33 \pm 3)</td>
<td>(42 \pm 3)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>2/7</td>
<td>0/11</td>
<td>3/12</td>
</tr>
<tr>
<td>BMI (&lt;25 kg/m(^2))</td>
<td>(24 \pm 0.4)</td>
<td>(42 \pm 1^a)</td>
<td>(43 \pm 1^a)</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>(90 \pm 2)</td>
<td>(87 \pm 3)</td>
<td>(98 \pm 10)</td>
</tr>
<tr>
<td>(&lt;110 mg/dl)</td>
<td>(10 \pm 1)</td>
<td>(23 \pm 2^a)</td>
<td>(31 \pm 4^a)</td>
</tr>
<tr>
<td>Fasting insulin (4-25 (\mu)g/ml)</td>
<td>(28 \pm 7)</td>
<td>(44 \pm 8)</td>
<td>(50 \pm 10)</td>
</tr>
<tr>
<td>HOMA index</td>
<td>(2.2 \pm 0.1)</td>
<td>(4.9 \pm 0.4^b)</td>
<td>(7.5 \pm 0.8^c)</td>
</tr>
<tr>
<td>AST (&lt;40 IU/l)</td>
<td>(27 \pm 4)</td>
<td>(20 \pm 1)</td>
<td>(30 \pm 4)</td>
</tr>
<tr>
<td>ALT (&lt;50 IU/l)</td>
<td>(44 \pm 8)</td>
<td>(26 \pm 2)</td>
<td>(40 \pm 4)</td>
</tr>
<tr>
<td>(\gamma)-GT (&lt;78 units/l)</td>
<td>(28 \pm 7)</td>
<td>(44 \pm 8)</td>
<td>(50 \pm 10)</td>
</tr>
<tr>
<td>Bilirubin (&lt;1.1 mg/dl)</td>
<td>(0.63 \pm 0.08)</td>
<td>(0.48 \pm 0.06)</td>
<td>(0.48 \pm 0.05)</td>
</tr>
</tbody>
</table>

Values represent means ± SEM for the number of subjects indicated in parentheses. Significant differences between the groups (one-way ANOVA and Bonferroni’s test): \(^a p < 0.05\) versus control; \(^b p < 0.05\) versus control and steatosis; \(^c p < 0.05\) versus control and steatohepatitis. HOMA is calculated as (fasting insulin (\(\mu\)units/ml) \times\) fasting glucose (mmol/l)/22.5. Abbreviations: BMI, body mass index; HOMA, homeostasis model assessment; AST, aspartate aminotransferase; ALT, alanine aminotransferase; \(\gamma\)-GT, \(\gamma\)-glutamyltransferase; IU, international units.
LPS-stimulated peripheral blood production of IL-1α was found to be 122% higher in patients with steatosis and 300% higher in those with steatohepatitis compared with controls, being steatohepatitis group values 80% higher than those in the steatosis group \((p < 0.05)\) (Fig. 2A). In turn, stimulated whole blood production of TNF-α in the steatosis group was 67% higher than control values \((p < 0.05)\) and that in patients with steatohepatitis was 110 and 26% higher than control and steatosis groups \((p < 0.05)\), respectively (Fig. 2B). Furthermore, there was a positive and significant correlation between LPS-stimulated whole blood IL-1α and TNF-α production among the three studied groups \((r = 0.78, p < 0.0001)\) (Fig. 2C).

### 3.3. Association of IL-1α and TNF-α production with histological variables and insulin resistance

The analysis of the association of IL-1α production in LPS-stimulated whole blood cell cultures from NAFLD patients and that of controls showed positive and significant correlations with the respective degree of steatosis (Fig. 3A, upper panel), the pathology score indicating the grade of steatosis and inflammation (Fig. 3A, middle panel), and with the HOMA index (Fig. 3A, lower panel). Similar results were observed for TNF-α production and either the steatosis and the pathology scores or the HOMA index (Fig. 3B). Within the group of obese NAFLD patients, insulin resistance was significantly correlated with IL-1α \((r = 0.50; p < 0.005; n = 26)\) and TNF-α \((r = 0.36; p < 0.05; n = 26)\) production, whereas no significant correlations were found between BMI (BMI range of 33–51 kg/m²) with IL-1α \((r = 0.17; p = 0.39; n = 26)\) or TNF-α \((r = 0.04; p = 0.85; n = 26)\).

### 4. Discussion

Cytokines are low molecular weight proteins playing a major role in the regulation of immune responses under both normal and pathological conditions. The data presented here show that stimulated monocytes/macrophages system from obese NAFLD patients overproduced IL-1α and TNF-α, pointing to a metabolic disturbance allowing the occurrence of an enhanced cytokine production in response to LPS by these cells. The above response is characterized (i) by being significantly greater in NASH patients than in those with steatosis alone (80 and 26% higher for IL-1α and TNF-α, respectively), and (ii) by a positive correlation between the production of IL-1α and TNF-α among the studied patients, as well as between the production of cytokine and the respective histology scores, namely, the degree of steatosis and the grade of steatosis plus inflammation. These observations suggest that the overproduction of IL-1α and TNF-α observed in vitro could play a role in the progression of liver damage from an uncomplicated steatosis to a more severe grade of liver damage in NASH patients with inflammatory infiltrate. Thus, hepatocyte sensitization to injury may be accomplished by exacerbation of the toxic effects that share IL-1α and TNF-α, including (i) the synthesis of chemokines and cellular adhesion molecules that further amplifies the inflammatory response \([10,17]\) and/or (ii) the inhibition of the mitochondrial electron-transport chain leading to ATP depletion and further generation of reactive O₂ species \([1,18]\), which correlates with the higher liver oxidative stress status observed in steatohepatitis over steatosis \([7]\). Under these pro-oxidant conditions, an enhancement in the capacity of immune cells to produce IL-1α and TNF-α may involve...
the activation of redox-sensitive transcription factors controlling their expression, namely, NF-κB and AP-1 [10,19,20]. However, further studies are needed to elucidate this proposal.

It is important to note that patients with steatohepatitis show marked insulin resistance compared to patients with only steatosis or normal livers. Considering the significant correlation established for IL-1α and TNF-α production and the HOMA index, the increased capacity of immune cells for cytokine production may contribute to an inflammatory state, leading to insulin resistance. This suggestion could be supported by the diminution in insulin-induced phosphorylation of insulin-receptor substrate-1 and in the expression of the insulin-dependent glucose transporter Glut4 afforded by TNF-α [21]. These observations and the lack of correlation between production of pro-inflammatory cytokines and BMI observed in the obese NAFLD patients studied (range of 33–51 kg/m²) support the contention that NAFLD is strongly associated with insulin resistance, which is present in approximately 98% of individuals with NAFLD [22].

Enhancement in LPS-stimulated peripheral blood IL-1α and TNF-α production in NAFDL patients occurs in the absence of significant changes in the basal serum levels of the cytokines. In this respect, earlier reports showed higher serum levels of TNF-α in NASH patients associated with either (i) increased prevalence of small intestinal bacterial overgrowth, without changes in intestinal permeability or endotoxemia [23] or (ii) hypoadiponectinemia [24]. The discrepancies observed could be explained in terms of the severity of the liver damage attained, considering that NASH patients with increased serum TNF-α exhibit more extensive liver necroinflammation [23,24] than those in the present study. NASH patients with increased serum TNF-α concentrations also showed higher levels of IL-6 and those of the chemokine IL-8, features paralleled by an overproduction of these cytokines by LPS-stimulated isolated monocytes [25]. In line with these findings, severely obese patients with NASH overexpressed TNF-α mRNA both in liver and adipose tissue, in association with higher hepatic levels of p55 TNF-α receptor mRNA, changes that were more elevated in patients with more advanced NASH [26]. Furthermore, increases in TNF-α and soluble TNF receptor 2 levels in the serum of NASH patients were found concomitantly with a diminution in those of adiponectin [24,27], with reduced hepatic expression of both adiponectin and adiponectin receptor-II [28], an adipokine having significant anti-inflammatory and metabolic effects that may prevent liver disease [29,30].

In conclusion, increased IL-1α and TNF-α production by LPS-stimulated whole blood monocytes/macrophages occurs

Fig. 3. Association between the production of interleukin-1α (IL-1α) (A) and tumor necrosis factor-α (TNF-α) (B) with the respective liver steatosis score, liver pathology score, and the HOMA index in control subjects and patients with non-alcoholic fatty liver disease showing steatosis or steatohepatitis. Both histology scores and the HOMA index were determined as described in Section 2 and data were analyzed by the Spearman correlation test.
in NAFLD patients, a response being more marked in steatohepatitis than in uncomplicated steatosis and that correlates with the respective histology scores. It is suggested that dysregulation in pro-inflammatory cytokine (Fig. 2) [23–26], adipokine [24,27,28,31], and/or chemokine [25] signaling in NAFLD associated with obesity and insulin resistance may represent key factors determining the progression from stable steatosis to NASH, in the setting of oxidative stress-mediated hepatocyte sensitization [7,9,32]. The progression of this disease seems to involve a variety of signaling molecules derived from parenchymal and non-parenchymal liver cells, adipocytes, and/or mononuclear and polymorphonuclear cells infiltrating the liver.

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