Gene expression in obese patients with non-alcoholic steatohepatitis

A. Cayón, J. Crespo, A. R. Guerra and F. Pons-Romero

Gastroenterology and Hepatology Unit. “Marqués de Valdecilla” University Hospital. Santander, Spain

RESUMEN

La fisiopatología de la enfermedad hepática por depósito de grasa sólo se conoce de forma parcial. En este trabajo hemos analizado la expresión génica intrahepática de citocinas, quimioquinas, receptores celulares, factores de crecimiento, transductores de señales intracelulares y proteínas de comunicación extracelular en el tejido hepático de sujetos obesos con y sin steatohepatitis no alcohólica, en un intento de determinar un perfil de expresión génica asociado a las formas severas de la steatohepatitis no alcohólica (EHNA).

Se analizó un grupo de 38 pacientes obesos con un IMC > 35, que fueron sometidos a cirugía bariátrica. La expresión génica intrahepática se determinó en el tejido hepático dividiendo a los pacientes en tres grupos: a) pacientes obesos sin datos histológicos sugestivos de EHNA (n= 12); b) pacientes con EHNA sin fibrosis (n = 13); y c) pacientes con EHNA y fibrosis (n = 13). Se consideró que existía una sobreexpresión génica cuando la diferencia en la expresión era, al menos, de dos veces respecto al grupo control. Los resultados se confirmaron mediante PCR en tiempo real. Se detectó una expresión diferencial de 14 genes (10 sobreexpresados y 4 infraexpresados). Los genes sobreexpresados incluyeron prohibitina, TNF, TNF RI (p55), MCSF, R2-TRAIL, TGF-b1, CTGF, FGF, VEGF, BIGH3 y ObRb. La expresión de los genes inhibición de factores de crecimiento-1, inhibición de factores de crecimiento-2, interleuquina-2 y tyrosine-receptor fue menor que en el grupo control.

En conclusión:
1. Los pacientes obesos con EHNA sin fibrosis muestran una sobreexpresión de genes proinflamatorios y proapoptóticos. En los pacientes con EHNA y fibrosis, se observa, además, una sobreexpresión de genes profibrogénicos, incluyendo el gen del receptor de la leptina.
2. La expresión de prohibitina en los pacientes con EHNA, tanto con fibrosis como sin fibrosis, fue superior que en los controles, lo que sugiere una disfunción mitocondrial en los pacientes con EHNA.

Palabras clave: Estatohepatitis no alcohólica. Enfermedad hepática por depósito de grasa. Obesidad mórbida.

ABSTRACT

Although the molecular basis for the pathophysiology of non-alcoholic steatohepatitis (NASH) is poorly understood, we evaluate the hepatic gene expression of cytokines, chemokines, cell receptors, growth factors, intracellular transducers and extracellular communication proteins in liver tissue of obese patients (with and without NASH), and we determine the specific intrahepatic gene expression profiles associated with histological severe NASH.

Thirty-eight obese patients with BMI > 35 were analyzed, who underwent bariatric surgery. Biopsy specimen samples were snap-frozen in liquid nitrogen. Hepatic gene expression was determined in liver biopsy specimens from 3 groups: a) obese patients without NASH (n = 12); b) patients with NASH without fibrosis (n = 13); and c) patients with NASH and fibrosis (n = 13). Genes were considered to be expressed differentially in NASH only if there was a greater than 2-fold difference in abundance of mRNA when compared with each of the control group. These results were confirmed by real-time PCR. Fourteen genes were differentially expressed (10 overexpressed and 4 underexpressed) in patients with NASH. Genes that were significantly overexpressed included prohibitin, TNF, TNF RI (p55), MCSF, R2-TRAIL, b1-CCTGF, FGF, VEGF, and BIGH3OB. Inhibition of growth factor-1, inhibition of growth factor-2, interleukin-2 and tyrosine-receptor were underexpressed in NASH patients.

In conclusion:
1. The obese patients with NASH without fibrosis show an overexpression of proinflammatory and proapoptotic genes. Also, the NASH patients with fibrosis show an overexpression of fibrogenic genes, including the leptin receptor Ob-Rb.
2. The up-regulated gene expression of prohibitin suggests mitochondrial dysfunction in NASH patients.

Key words: Non-alcoholic steatohepatitis. Non-alcoholic fatty liver disease. Morbid obesity.
INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) covers a broad spectrum of liver pathologies, including simple steatosis and steatosis with inflammation, frequently associated with fibrosis and known as non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis (1-4). The dual-impact theory is currently accepted in the pathogenesis of NASH. Steatosis, a consequence of insulin resistance in most patients, constitutes the first impact. Subsequently, liperoxidation and secondary oxidative stress are able to induce various pathways involved in the secretion of proapoptotic, proinflammatory, and profibrogenic cytokines (5-8). Multiple systems are involved in the pathology of NASH, including those related to insulin resistance, mitochondrial activity, and oxidative stress, and those involved in the development of the inflammatory and fibrogenic response (9-14).

Previous work from our group demonstrated the involvement of TNF-α, TGFβ1, adiponectin, and leptin in the pathogenesis of NASH. In short, we found an increased expression of TNF-α mRNA in the liver and adipose tissue of obese patients with NASH, and an increased expression of TGFβ1 in patients with NASH and fibrosis (15,16). On the other hand, we reported a dramatic decrease in the expression of adiponectin and adiponectin receptor 2 in the liver tissue of patients with NASH, as compared with a control group consisting of subjects with hepatic steatosis (17). Finally, we observed that leptin receptor overexpression is linked to hepatic fibrosis (unpublished results). Nonetheless, it must be pointed out that multiple other genes, potentially involved in the pathogenesis of this disease, still need to be classified (18,19).

The purpose of our study was to examine, by cDNA microarray analysis, the intrahepatic gene expression of several cytokines, cytokine receptors, chemokines, growth factors and transducers of intracellular and intercellular signaling involved in the fibrosis and inflammation of obese patients with NASH. Subsequently, we sought to identify a differential expression pattern of the different genes associated with NASH patients, based on the presence of steatosis, inflammation, and fibrosis.

MATERIAL AND METHODS

Patients

Thirty-eight obese patients who underwent bariatric surgery at Institute of Digestive Diseases, “Marqués de Valdecilla” University Hospital (Santander, Spain), from September 1999 to January 2002, were considered for inclusion in this study with a mean body mass index (BMI) of 46.8 ± 7.8 kg/m². Patients were interviewed and physical data were obtained, as well as a blood sample for laboratory testing and an available liver tissue specimen. Exclusionary criteria included the following:

1. Daily alcohol intake > 30 g in men or > 20 g in women. Alcohol history was confirmed by physicians and family members who were in close contact with the patient.
2. Use of drugs known to produce liver injury including corticosteroids, amiodarone, tamoxifen or tetracycline.
3. Absence of other relevant liver diseases. All patients were negative for hepatitis B surface antigen and antibody for hepatitis C. No patients had anti-mitochondrial antibody or significantly elevated titers of anti-nuclear, anti-smooth muscle actin or anti-liver-kidney microsome antibody.

Laboratory evaluation

Laboratory studies included glucose, creatinine, cholesterol, triglyceride, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT), albumin, and total protein levels with a standard clinical automatic analyzer; hepatitis B surface antigen and antibody to hepatitis C virus; autoimmune serology; studies of iron metabolism, ceruloplasmin, and 1-antitrypsin levels.

Histologic assessment

All patients underwent biliopancreatic diversion and we collected intraoperative liver biopsies by tru-cut from all patients. Moreover, an informed written consent was obtained from all subjects before the study, according to the guidelines in Helsinki Declaration II. One third of the sample was immediately frozen in liquid nitrogen and stored at -80 °C until the extraction of RNA, and the remainder was used for histological study. A single pathologist (MM), blinded to clinical and laboratory data, assessed liver histology. Biopsies were processed routinely, cut at 4 μm in thickness, and stained with hematoxylin-eosin. Tissue specimens were fixed with 10% neutral formalin and embedded in paraffin. The grading of histological lesions in NASH patients was based on a systematic approach as described previously, following the scheme proposed by Brunt et al. (20,21).

RNA isolation

Total RNA was extracted from frozen liver tissue with the acid guanidinium phenol chloroform method using TRIzol® reagent (GIBCO BRL, Gaithersburg, Maryland, USA) according to the manufacturer’s instructions; this protocol is based on the method described by Chomezynski and Sacchi (22). RNA concentration was assessed by absorbance spectroscopy, and RNA integrity was confirmed by non-denaturing agarose gel electrophoresis.
Macroarray gene expression

Gene expression was studied in fifteen patients using macroarrays. Patients were divided into 3 groups: 13 NASH patients with liver fibrosis, 13 NASH patients without fibrosis, and 12 control patients without NASH. In order to determine the relative intrahepatic gene expression profiles in the 3 study groups, we analyzed the relative abundance of mRNA using high-density oligonucleotide macroarrays containing probes for 268 genes (Atlas™ human cytokine array, Clontech). Total RNA (40 µg, 1 µg/µl) was incubated at 37 °C for 30 min with Dnase I, RNAase free (Roche, Germany). The samples were: Phenol, chloroform extracted twice, then precipitated and resuspended in 15 µl of RNAase-free water in order to completely remove RNAses. Each macroarray hybridization was performed using 5 µg of total liver RNA pooled from five patients. Five micrograms of RNA were then reverse transcribed using CDS primer mix (Clontech) in the presence of 32P dATPs (3,000 Ci/mmol, 10 µCi/µl, Amersham), thus allowing the labeling of the probe. Probes were then purified with a PCR purification kit: Atlas Nucleospin® Extraction Kit (Clontech), according to the manufacturer’s instructions.

Samples of cDNA were hybridized in the recommended buffer to macroarrays (BD Express Hyb, Clontech) overnight with continuous agitation at 68 °C. The samples were stained and washed according to the manufacturer’s protocol on a wash solution 1 (2 XSSC, 1% SDS) and wash solution 2 (0.1 XSSC, 0.5% SDS) at 68 °C.

The signal was detected by exposing the membrane to X-ray film at -70 °C for two and five days. The luminescence intensities of hybridized cDNA probes were analyzed using Bio-Rad Quantity One™ software (Bio-Rad Laboratories, California, USA). Identification of genes that are expressed differentially was accomplished using Microsoft Excel. In order to determine the specific intrahepatic gene expression profiles associated with histologically progressive NASH, analyses were performed in two steps. Initially, the levels of expression in each array were normalized using several highly expressed housekeeping genes, including genes coding for ubiquitin, GAPDH, HLA class I histocompatibility Ag C-4 subunit (HLA C-4), β-actin, tubulin alpha 1, 60S ribosomal protein L13A, and 40S ribosomal protein S9. Finally, the percentage change in gene expression was calculated as ([intensity in subjects with NASH/intensity in subjects without NASH] [intensity in NASH subjects with fibrosis/intensity in subjects without NASH]). Genes were considered to be expressed differentially (over- or underexpressed) in NASH only if there was a greater than 2-fold difference in the abundance of mRNA when each group was compared to the control group.

Real time PCR

Real-time reverse transcription polymerase chain reaction (RT-PCR) was used for the validation of differentially expressed genes identified by DNA arrays. Double stranded cDNA was made as previously described (9). The probes and primer sets used for Taqman real time PCR (QPCR) of prohibitin, TNFRSF10B, ILF-1, ILF2, VEGF, FG, CTGF, CSF1, TGF and r18S were obtained from Applied Biosystems® (Foster City, California, USA): Prohibitin (Hs00855044), TNFRSF10B (Hs00187196), ILF-1 (Hs00153126), ILF2 (Hs001712554), VEGF (Hs00173626), FG (Hs00265254), CTGF (Hs00170014), MCSF (Hs00174164), TGFβ1 (Hs00165908) and r18S (Hs). TaqMan assays were performed in a total volume of 20 µl containing final concentrations of Taqman universal PCR master mix (1X) and gene expression assay mix (1X). A no-template control (where water replaced cDNA) was included in every run. The expression of the measured genes was normalized for 18S ribosomal RNA expression. PCR reactions were performed in duplicates in MicroAmp 96-well reaction plates (Applied Biosystem®), and the amplification was carried out in the ABIPrism 7000 Sequence Detector System (Applied Biosystem®). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 s at 95 °C, followed by 1 min at 60 °C. Results were analyzed using the ABI Prism 7000 SDS software.

Statistical analysis

Data were expressed as mean ± SEM. Differences between groups were calculated using Student’s t-test for independent samples. Statistical significance was inferred at a two-tailed p value of less than 0.05. Correlation coefficients were calculated with Pearson’s method. SPSS for Windows (version 11.0) was used for the statistical analysis.

RESULTS

Clinical and histological characteristics

Of all 38 patients included in the study, 13 had NASH with fibrosis, 13 had NASH without fibrosis, and 12 had a histologically normal liver or low-grade steatosis, and were considered as controls. All patients in all three groups were similar in terms of biomedical characteristics. Table I shows the data on demographic, clinical, and laboratory characteristics.

Degree of steatosis, degree of inflammation, and fibrosis stage were studied in all subjects. Histological characteristics are shown in table II.

Macroarray gene expression

This study allowed us to detect overexpressed and inhibited genes in all patients with NASH in comparison with the control group. This analysis allowed us to detect overexpressed and inhibited genes in all patients with NASH in comparison with the control group.
Table I. Characteristics of the study groups included

<table>
<thead>
<tr>
<th></th>
<th>NASH with fibrosis</th>
<th>NASH without fibrosis</th>
<th>No NASH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>12/1</td>
<td>10/3</td>
<td>10/2</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>39</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>Body mass index (± SD)</td>
<td>45 ± 5</td>
<td>46 ± 6</td>
<td>51 ± 10</td>
</tr>
<tr>
<td>AST (U/l ± SD)</td>
<td>29 ± 9</td>
<td>30 ± 8</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>ALT (U/l ± SD)</td>
<td>31 ± 13</td>
<td>29 ± 27</td>
<td>24 ± 7</td>
</tr>
<tr>
<td>GGT (U/l ± SD)</td>
<td>24 ± 8</td>
<td>63 ± 18</td>
<td>29 ± 9</td>
</tr>
<tr>
<td>Cholesterol (mg/dl ± SD)</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Glucose (mg/dl ± SD)</td>
<td>101 ± 36</td>
<td>118 ± 74</td>
<td>116 ± 84</td>
</tr>
<tr>
<td>Triglycerides (mg/dl ± SD)</td>
<td>96 ± 43</td>
<td>120 ± 58</td>
<td>84 ± 23</td>
</tr>
</tbody>
</table>

Table II. Histological characteristics of patients included

<table>
<thead>
<tr>
<th></th>
<th>Degree of steatosis</th>
<th>Degree of inflammation</th>
<th>Stage of fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.6 (0-1)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NASH without fibrosis</td>
<td>1.4 (1-2)</td>
<td>1.6 (1-2)</td>
<td>0</td>
</tr>
<tr>
<td>NASH with fibrosis</td>
<td>2 (1-3)</td>
<td>1.4 (1-2)</td>
<td>2.6 (2-3)</td>
</tr>
</tbody>
</table>

Table III. Differentially expressed genes in histologically progressive NASH

<table>
<thead>
<tr>
<th>Gene</th>
<th>NASH vs. control</th>
<th>NASH with fibrosis vs. control</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prohibitin</td>
<td>+3.0</td>
<td>+3.0</td>
<td>Mitochondrial</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+2.7</td>
<td>+2.8</td>
<td>Inflammation</td>
</tr>
<tr>
<td>TNF R1</td>
<td>+2.0</td>
<td>+2.2</td>
<td>Inflammation</td>
</tr>
<tr>
<td>M-CSF</td>
<td>+3.5</td>
<td>+3.8</td>
<td>Inflammation</td>
</tr>
<tr>
<td>TRAIL-R2</td>
<td>+2.1</td>
<td>+2.3</td>
<td>Apoptosis</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>+3.2</td>
<td>+3.2</td>
<td>Fibrotic</td>
</tr>
<tr>
<td>CTGF</td>
<td>+2.8</td>
<td>+2.8</td>
<td>Fibrotic</td>
</tr>
<tr>
<td>FGF</td>
<td>+2.5</td>
<td>+2.5</td>
<td>Fibrotic</td>
</tr>
<tr>
<td>VEGF</td>
<td>+2.7</td>
<td>+2.7</td>
<td>Fibrotic</td>
</tr>
<tr>
<td>IGF1</td>
<td>-2.3</td>
<td>-2.5</td>
<td>Insulin resistance</td>
</tr>
<tr>
<td>IGF2</td>
<td>-2.6</td>
<td>-2.0</td>
<td>Insulin resistance</td>
</tr>
</tbody>
</table>

TNF-R1: Tumor necrosis growth factor receptor I; M-CSF: Macrophage colony-stimulating factor (+3.5); TRAIL-R2: TNF-related apoptosis-inducing ligand receptor 2; CTGF: Connective tissue growth factor; FGF: Fibroblast growth factor; IGF: Insulin-like growth factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor.

The + indicates overexpressed in NASH and the - indicates underexpressed in NASH.

with the control group, which consisted of obese subjects without NASH (Table III).

When compared to healthy patients without NASH, NASH patients with inflammation and without fibrosis showed 5 overexpressed genes and 2 inhibited genes. These overexpressed genes were: a) three genes involved in the inflammatory response: Tumor necrosis growth factor alfa (TNF-α, +2.7), tumor necrosis growth factor receptor I (TNFRI, +2), and macrophage colony-stimulating factor (M-CSF, +3.5); b) one gene involved in the apoptotic response: TNF-related apoptosis-inducing ligand receptor 2 (TRAIL-R2, +2.1); and c) one gene involved in the mitochondrial function: Prohibitin (PHB, +3). Genes inhibited in these patients with regard to obese patients without liver damage are involved in insulin resistance: Insulin-like growth factor I (IGFI, -2.3) and insulin-like growth factor II (IGFII, -2.6).

When compared with the control group without NASH, NASH patients with fibrosis show six overexpressed genes and one inhibited gene. The overexpressed genes are: a) one gene involved in the inflammatory response: TNF-α (+2.8); b) four genes involved in the fibrotic response: Transforming growth factor β1 (TGFβ1, +3.2), fibroblast growth factor (FGF, +2.5), vascular endothelial growth factor (VEGF, +2.7) and connective tissue growth factor (CTGF, +2.8); and c) one gene involved in the mitochondrial function: PHB, +3. The inhibited gene was IGFII (-2.5), related to insulin resistance.

Real time PCR

Validation of gene expression data was performed by quantitative real time PCR (QPCR) and cytokine results were normalized to the internal standard: the 18S ribosomal gene. The overexpression of TRAIL-R2, M-CSF and prohibitin, as well as the inhibition of IGFII and IGFII was confirmed by QPCR in NASH patients with fibrosis compared with patients without NASH: TRAIL-R2 (3.1 ± 2 vs. 1.6 ± 1.4; p < 0.04), M-CSF (1.6 ± 1.2 vs. 0.8 ± 0.4; p < 0.008), prohibitin (1.3 ± 1.1 vs. 0.8 ± 0.4; p < 0.05), IGFII (0.6 ± 0.5 vs. 1.1 ± 0.4; p < 0.03), IGFII (0.6 ± 0.8 vs. 1.2 ± 1.4; p < 0.02). The increased hepatic gene expression of TNF-α mRNA and TNFRI mRNA in patients with NASH compared with obese patients without NASH was previously revealed by semiquantitative PCR (9).

The overexpression of VEGF, CTGF, TGF beta1, and prohibitin, as well as the inhibition of ILGI, were confirmed by QPCR in patients with NASH and without fibrosis when compared with patients without NASH: VEGF (1.8 ± 2.3 vs. 0.7 ± 1.2; p < 0.05), CTGF (2.2 ± 1.2 vs. 1.1 ± 0.6; p < 0.011), TGF-β1 (2.66 ± 1.32 vs. 1.3 ± 0.9; p < 0.002), PHB (1.8 ± 1.4 vs. 0.9 ± 0.6; p < 0.014), ILFI (0.4 ± 0.4 vs. 0.9 ± 0.5; p < 0.01). FGF showed similar expression patterns in all patients with NASH and fibrosis, NASH without fibrosis and patients without NASH (1.2 ± 0.6 vs. 1.2 ± 0.7; p = ns). The overexpression of TNF-α mRNA in patients with NASH and fibrosis when compared with patients without NASH was previously revealed by semiquantitative PCR (10).

DISCUSSION

Macourray gene expression analyses were performed with the purpose of identifying the expression pattern of
certain genes playing a role in the inflammation and fibrosis of NASH patients. We compared the gene expression of 268 cytokine genes, cytokine receptors, transducers of intracellular and intercellular signaling and growth factors in patients with NASH (with and without fibrosis) and in obese patients without liver damage. Only a few studies with a small number of patients. In the course of this study, we found that all obese patients with NASH, when compared with obese subjects without NASH (control group), showed a marked overexpression of the genes coding for the mitochondrial protein prohibitin, and for the inflammatory cytokine TNF-α. Prohibitin is a chaperon involved in the assembly of the mitochondrial respiratory chain (23-26). The inhibition of this protein was previously described in S-adenosylmethionine deficient rats, which develop NASH (24). On the other hand, it was observed that abnormal expression of this protein in obese patients constitutes a risk factor for the development of steatohepatitis (27).

Nonetheless, other authors reported overexpression of prohibitin in primary liver tumors in response to metabolic stress (28). Our results suggest that altered expression of prohibitin may damage the functional and native organization of the respiratory chain and thus compromise the functionality of the mitochondria. In this sense, Solis-Herruzo revealed that mitochondrial dysfunction plays a crucial role in the pathogenesis of NASH (14). Besides, Sanyal showed that peripheral insulin resistance promotes beta oxidation of fatty acids and proved that oxidative stress is present both in steatosis and in steatohepatitis, whereas NASH is only associated with defects in the mitochondrial structure (14,29). TNF-α overexpression was already determined by semiquantitative PCR analysis. Moreover, this increase in TNF-α mRNA was higher in patients with greater inflammatory infiltrate.

Obese patients with NASH presenting a higher degree of inflammation in liver biopsies show a marked overexpression of the genes coding for TNFRI, MCSF and TRAIL-R2 when compared with the control group (subjects with simple liver steatosis). The overexpression of TNFRI, as well as TNF-alpha, was determined by semiquantitative PCR analysis. MCSF is a cytokine which enhances the number of macrophages and consequently, inflammation (30,31). Serum MCSF levels were already shown to increase with necrosis and liver inflammation in patients with acute hepatitis and in patients with cirrhosis (32). An increase in IL-1β, TNF-α and IL-6 correlated with MCSF and liver inflammation was also observed in total peripheral blood (33). Our results reveal an increased expression of mRNA in both cytokines MCSF and TNF-α in the liver, suggesting the activation of the cytokine cascade, which results in the proliferation of macrophages in vivo in the liver. The other gene overexpressed in NASH patients with a higher degree of inflammation with respect to controls was TRAIL-R2. The TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily, which induces apoptosis in a variety of cancer cell types with little or no effect on normal cells (34). In the past, other authors utilized a model of adenovirus-mediated hepatitis to demonstrate that apoptosis in infected hepatocytes is mediated by TRAIL-R2 and TRAIL (35). Our study reveals an overexpression of TRAIL-R2 in NASH patients, suggesting that the TRAIL system may be an important apoptosis mediator in these patients.

Moreover, patients with NASH and fibrosis show, with respect to controls, an overexpression of 4 genes involved in hepatic fibrosis: TGFβ1, CTGF, FGF and VEGF. An increased production of TGF beta1 was previously detected in fibrosis in other liver diseases, including HCV-related chronic hepatitis, alcoholic liver disease and autoimmune hepatitis (36-38). In this sense, we recently found overexpression of TGF beta1 in the hepatic tissue of NASH patients with fibrosis by real time PCR analysis, correlating with the expression of leptin receptor, suggesting TGF beta1 activation caused by the action of leptin (15), and thus promoting fibrosis in NASH patients. CTGF is a protein implicated in the regulation of cell proliferation, differentiation and embryogenesis (39,40). It was also reported to be involved in the pathogenesis of multiple diseases characterized by tissue fibrosis (41,42). Further studies demonstrated a CTGF overexpression in the liver in the course of NASH, both in human liver biopsies and in experimental models of obesity and diabetes (43). Further in vitro studies showed that glucose and insulin induce a significant increase in CTGF mRNA and the protein by hepatic stellate cells (HSC) and that CTGF upregulates several components of the extracellular matrix (44). It has been suggested that the continuous presence of TNF-α and TGFβ1 may be necessary to stimulate matrix protein synthesis and to maintain hepatic stellate cells (HSC) activated when liver damage has occurred (45,46). Besides, both cytokines are able to stimulate CTGF expression in hepatic stellate cells and upregulate the synthesis of the protease inhibitor in activated stellate cells, which reduces matrix degradation, contributing to liver fibrosis (47,48).

Overexpression of these three genes (TNF-α, TGFβ1 and CTGF) in NASH patients with fibrosis suggests that their simultaneous overexpression explains the progression of fibrosis in these subjects. In contrast, liver disease does not progress if the expression of these cytokines is not simultaneous. The proliferation of stellate cells is also stimulated by other cytokines, including thrombin, FGF and VEGF (49). Macroarray gene expression analysis and QPCR showed an overexpression of VEGF, whereas the overexpression of FGF was only confirmed by macroarrays but not by real time PCR.

Experimental models proved that FGF is overexpressed in concanavalin A-induced liver fibrosis, as well as TGFβ1 (50); however, the role played by these two genes (VEGF and FGF) in the development of NASH was not confirmed by previous studies and thus, their implication in the pathogenesis of this disease should be
further analyzed. Inhibited genes in NASH patients with or without fibrosis with respect to patients without liver damage are insulin-related growth factors: IGF1 and IGFBP-1. These growth factors are anabolic hormones, markers of nutritional status and liver function, whose bioavailability is reduced in liver cirrhosis (52-54). Low levels of IGF1 and high levels of IGFBP-1 (IGF-1 binding protein) were previously described in association with cirrhosis and insulin resistance (55,56). Insulin is the major regulator of hepatic IGFBP-1 expression, in response to nutritional status. The effects of increased insulin resistance and liver function, whose expression of IGFBP-1 are due to a decreased intracellular availability of free IGF1 and the consequential insulin resistance (57). Sreekumar first reported overexpression of IGFBP-1 in NASH patients without cirrhosis with respect to patients without liver damage (58). In this sense, we found inhibition of IGF1 in patients with NASH and without cirrhosis. These results suggest that steatohepatitis is associated with alterations in IGF1 expression and other mediators of insulin action.

To sum up, our results reveal the implication of genes involved in proinflammatory, proapoptotic and profibrogenic pathways as well as in insulin resistance in the progression of NASH. When compared with controls, obese patients with NASH show activation of inflammatory and apoptotic cytokines and inhibition of genes involved in insulin resistance. Patients with NASH and fibrosis also show, with respect to controls, activation of fibrotic cytokines. Besides, the overexpression of the prohibitin gene suggests the mitochondrial dysfunction in all patients with NASH with and without fibrosis. The results of our experiments provide insight into gene expression, not transcription or post-transcriptional regulation. Further studies are required to determine the mechanism and the physiologic significance of these findings.

ACKNOWLEDGMENTS

This work was supported by the I + D Program (SAF 2001/0876), by a grant from the Fondo de Investigaciones Sanitarias de la Seguridad Social (PI 020858) and by Red temática de investigación cooperativa: Estudio de los mecanismos patogénicos de las hepatitis víricas y estetohepatitis: implicaciones en la terapia farmacológica (G03/015).

REFERENCES


