Leptin Receptor Isoforms mRNA Expression in Peripheral Blood Mononuclear Cells from Patients with Chronic Viral Hepatitis

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There is accumulating evidence that leptin has a pleiotropic role in hematopoiesis, immune response, fibrogenesis, and hepatocarcinogenesis. We investigated the expression of leptin and leptin receptor (OB-R) at the protein level by flow cytometry and also quantified by real-time reverse transcriptase–polymerase chain reaction (RT-PCR) the two major leptin receptor isoforms (*OB-Rl, OB-Rs*) in peripheral blood mononuclear cells (PBMCs) of patients with hepatitis B (HBV; $n = 31$), hepatitis C (HCV; $n = 34$), and nonviral liver disease ($n = 25$), and healthy controls ($n = 36$), as well as in liver tissues of HBV ($n = 8$), HCV ($n = 7$), and healthy individuals ($n = 6$). Serum leptin levels were measured in all participants ($N = 126$). We observed significantly lower *OB-Rl* and *OB-Rs* mRNA levels in PBMCs of HBV and HCV patients compared with healthy controls and nonviral liver disease patients ($P < 0.05$). Flow cytometry analysis confirmed the real-time RT-PCR results. Expression of leptin and *OB-Rl* was significantly increased in viral hepatitis liver tissues compared with healthy tissues ($P < 0.01$). *OB-Rl* mRNA levels were not associated with hepatitis patients' clinical status (inactive, chronic hepatitis, or cirrhosis). We also found decreased serum leptin in HBV and HCV patients compared with healthy individuals and the nonviral liver disease group. Leptin was expressed in 3 of 34 HCV (8.8%) and 19 of 25 (76%) nonviral liver disease patients. Moreover, expression of *OB-Rl* and *OB-Rs* were associated when all individuals were grouped together ($r = 0.78$, $P < 0.001$). In conclusion, our findings may suggest the involvement of the leptin system in the immunopathology of chronic viral hepatitis. Exp Biol Med 231:1653–1663, 2006

Key words: leptin; leptin receptors; hepatitis B virus; hepatitis C virus; cirrhosis

Introduction

Leptin, the 16-kDa nonglycosylated protein product of the *OB* gene, is an essential hormone/cytokine that is secreted from adipocytes and links nutritional status with neuroendocrine and immune functions (1–4). As a hormone, leptin regulates appetite and energy homeostasis at the hypothalamic level by activating specific neuroendocrine pathways (2, 5, 6). As a cytokine, leptin affects thymic homeostasis and, similar to other proinflammatory cytokines, modulates the onset of immune responses (1, 7, 8). In humans, there are four isoforms of leptin receptor, generated by alternative splicing, which are membrane-spanning glycoproteins with cytoplasmic domains of varying length (9). The longest isoform (OB-Rl) is responsible for fully functional leptin signaling, while the shorter isoforms (OB-Rs) have truncated cytoplasmic domains with reduced signal transduction capabilities (10–12).

Leptin receptors are expressed in a variety of peripheral tissues including the brain, liver, pancreas, placenta, lung, skeletal muscle, heart, hematopoietic cells, and peripheral blood mononuclear cells (PBMCs) (9, 13). It has been observed that leptin-deficient (*ob/ob*) and leptin receptor–deficient (*db/db*) mice present a complex syndrome that consists of abnormal reproductive function, hormonal imbalances, immune dysfunction, and reduced levels of peripheral T and B cells, suggesting a role for leptin in

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lymphopoiesis (14). Human leptin deficiency caused by a missense mutation in the *OB* gene can also lead to immune system dysfunction as evidenced by decreased T helper cell 1 (Th1) and increased Th2 responses (15). Leptin can also induce proliferation, differentiation, and functional activation of hematopoietic cells and increase the secretion of proinflammatory mediators. More specifically, leptin promotes the Th1 immune responses by increasing γ-interferon (IFN-γ), tumor necrosis factor-α (TNF-α), and interleukin-2 (IL-2) secretion by Th1 or naive T cells. Leptin promotes activation and secretion of proinflammatory factors such as leukotriene B4, cyclooxygenase-2 (Cox-2), and nitric oxide (1, 14, 16–21) in monocytes and macrophages.

Leptin has also been associated with chronic inflammatory diseases, such as pelvic endometriosis, chronic pulmonary inflammation, inflammatory nephritis, Behcet's syndrome, Graves' disease, rheumatoid arthritis, and nonalcoholic or viral hepatitis (1). It has been suggested that activation of T cells and macrophages is one of the initial events during viral hepatitis infection (22). Activated T cells are either directly cytotoxic to hepatocytes or release proinflammatory cytokines, which mediate hepatocyte damage in various animal models (23, 24). The T cell–modulating activity of leptin, the observation that OB-Rl is expressed on CD4 and CD8 positive cells, and the presence of OB-Rl and OB-Rs in hepatic sinusoidal cells suggest that leptin may be directly involved in the pathophysiological mechanisms that take place during viral hepatitis (25, 26).

To further assess the role of leptin and its receptors in chronic viral hepatitis infection, we measured serum leptin levels and investigated, for the first time to our knowledge, expression of leptin receptor isoforms in PBMCs of chronically-infected hepatitis B virus (HBV) and hepatitis C virus (HCV) patients and in patients with nonviral liver disease. We measured mRNA levels of leptin receptor isoforms using quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) and protein expression using flow cytometry. We found that leptin receptor mRNA and protein expression is significantly decreased in PBMCs of HBV and HCV patients compared with healthy controls and patients with nonviral liver disease. We also observed a notable increase of leptin receptor mRNA in the livers of HBV and HCV patients compared to healthy controls, suggesting a possible involvement of the leptin system in the immunopathogenesis of HBV and HCV.

Materials and Methods

A total of 126 human subjects were included in the study. This consisted of 65 consecutive patients with chronic HBV and HCV infections, 25 patients with nonviral liver disease, constituting the disease control group, and 36 healthy individuals. All patients were followed-up at the Outpatient Clinic of the Academic Liver Unit, University Hospital of Larissa, Larissa, Greece. Thirty-one patients had chronic HBV infection (17 male, 14 female; mean age: 51.1 years; age range: 25–72 years; mean body mass index [BMI]: 26.6 ± 4.562) and 34 patients had chronic HCV infection (13 male, 21 female; mean age: 45.7 years; age range: 18–76 years; mean BMI: 25.05 ± 3.207). Control groups consisted of 36 healthy individuals (15 male, 21 female; mean age: 47.5 years; age range: 23–65 years; mean BMI: 24.52 ± 3.343) and 25 patients with non-viral liver disease (15 with alcoholic liver disease and 10 with autoimmune liver disease; a total of 14 male, 11 female; mean age: 46.1 years; age range: 20–73 years; mean BMI: 27.1 ± 3.794). There were no age differences between the four study groups. The mean ± SD of disease duration in HBV and HCV patients was 95 ± 73.9 and 125 ± 141.57 months, respectively. The duration of disease was calculated from the age at diagnosis, as stated in other reports from our group (27–29).

Patients with chronic viral liver disease included in the study were classified into three clinical groups: (i) patients with normal levels of aminotransferases determined at 3-month intervals for at least one year before entry into the study (inactive state group; $n = 25$: 14 with HBV and 11 with HCV), (ii) patients with histologically, virologically, and biochemically proven chronic HBV and HCV (chronic hepatitis group; $n = 31$: 13 with HBV and 18 with HCV), (iii) patients with HBV or HCV–related cirrhosis (cirrhosis group; $n = 9$; four with HBV and five with HCV). None of the patients had during the follow-up and at the time of investigation any clinical, laboratory, or radiological evidence of hepatocellular carcinoma (HCC).

Diagnosis of chronic HBV infection was based on clinical, laboratory, and histological evaluations according to our previous reports (28, 29) and the recent European Association for the Study of the Liver (EASL) International Consensus Conference on Hepatitis B (30). Briefly, patients in the chronic inactive HBV state ($n = 14$) had reactivity against hepatitis B surface antigen (HBsAg; Abbott Laboratories, Wiesbaden, Germany), normal aminotransferases, and no viral replication as attested by the absence or the presence of low levels of serum HBV DNA (below 10^5 copies/ml) by PCR (cut-off: 200 copies/ml; Cobas Amplicor HBV Monitor; Roche, Branchburg, NJ). HBV patients with chronic hepatitis B ($n = 31$) met the following criteria: (i) serological evidence, using commercially available enzyme immunoassays (Abbott Laboratories), of chronic infection with HBV for at least 6 months before entry into the study, (ii) active virus replication as defined by the detection of HBV DNA ($>10^5$ copies/ml) using a commercially available PCR kit (Cobas Amplicor HBV Monitor; Roche), (iii) elevated levels of alanine aminotransferase (ALT; at least 2-fold that of the upper limit) for at least 6 months before entry into the study, (iv) histologically proven chronic HBV without any sign of cirrhosis. The diagnosis of cirrhosis due to HBV was also based on the above criteria (i), (ii), and (iii) and liver histology compatible with cirrhosis. None of the HBV patients tested positive for antibodies to HCV (anti-HCV; Murex Diagnostics, Temple

Hill, Dartford, UK) or antibodies to HIV (anti-HIV; Abbott Laboratories).

Diagnosis of chronic HCV infection was based on clinical, laboratory, and histological evaluations (27–29). Briefly, all HCV patients included in the study met the following criteria: (i) serological evidence of chronic infection with HCV as determined by the detection of anti-HCV antibodies using a third-generation enzyme immunoassay at least twice within 6 months before enrollment into the study, (ii) active virus replication as defined by detection of HCV RNA using a commercially available qualitative PCR kit (HCV Monitoring Cobas Amplicor; Roche; cut-off: 50 U/ml). None of the HCV patients were positive for HBsAg or anti-HIV.

Diagnosis of alcoholic liver disease was based on a history of alcohol consumption (more than 50 g/day ethanol) along with compatible clinical, laboratory, and liver histology. Diagnosis of autoimmune liver disease (five patients with autoimmune hepatitis and five with primary biliary cirrhosis) was based on the revised descriptive criteria for diagnosis of autoimmune hepatitis reported by the International Autoimmune Hepatitis Group (31) and, for patients with primary biliary cirrhosis, the following previously-described criteria (32): positivity for antimitochondrial antibodies (positive titer $\geq$1/40) by indirect immunofluorescence using in-house rodent tissue substrates, elevated cholestatic enzymes, and histological lesions compatible with the disease. All disease control patients tested negative for HBsAg, anti-HCV and anti-HIV.

Liver tissue specimens were collected from six healthy individuals of the control group, for whom an operation was performed for cholecystectomy (three male, three female; mean age: 43 $\pm$ 16.09 years; age range: 28–64 years), seven patients of the HCV group (three male, four female; mean age: 44.14 $\pm$ 16.94 years; age range: 28–69 years), and eight patients of the HBV group (four male, four female; mean age: 47 $\pm$ 14.89 years; age range 25–61 years). None of the patients with chronic viral hepatitis had any clinical, laboratory, or radiological evidence of HCC during follow-up at the time of investigation.

None of the patients received antiviral treatment (α-interferon alone or in combination with either ribavirin or lamivudine) for at least 24 months before entry into the study. All healthy individuals had normal ALT values (26.5 $\pm$ 5.3 U/liter), tested negative for HBsAg, anti-HCV, and anti-HIV antibodies, and denied ever having used hepatotoxic drugs or herbals, or having abused alcohol or injected drugs. All subjects consented at the time of interview to participation in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Local Ethical Committee of the University Hospital of Larissa (Larissa, Greece).

Total RNA Isolation. PBMCs were isolated from whole blood according to standard procedures and total cellular RNA was extracted using Trizol (Life Technologies, Paisley, UK; Ref. 29). Tissue samples from patients with chronic hepatitis were obtained by needle biopsy. Samples were immediately frozen and stored at –80°C until use. RNA was further purified using the RNase-free DNase kit (Qiagen, Manheim, Germany). The presence of 28S and 18S rRNA species was used to assess RNA integrity. Only samples with prominent 28S and 18S rRNA components were included in the study.

Real-time, Quantitative RT-PCR of *OB-Rl* and *OB-Rs* mRNA. Transcription to cDNA was performed using the AMV Kit (Roche, Indianapolis, IN). Retinoic acid receptor-α (*RAR*-α) cDNA sequences were amplified in separate reactions as positive cDNA controls. Quantification was performed by real-time RT-PCR (LightCycler Instrument; Roche Molecular Systems, Alameda, CA) with 0.2 μl cDNA per sample using the LightCycler FastStart DNA Master HybProbe kit (Roche, Penzberg, Germany) according to the manufacturers' instructions. The oligonucleotide primers used for OB-Rl were 5′-GCTATTTTGGGAAGATGT-3′ (forward, bases 288–306 in exon 19) and 5′-TGCCTGGGCCTCTATCTC-3′ (reverse, bases 553–570 in exon 20), for OB-Rs were 5′-TGTTGTGAATGTCTTGTGCC-3′ (forward, bases 402–421 in exon 6) and 5′-TGCTCCAGTCACTCCAGATTCC-3′ (reverse, bases 649–668 in exon 8), and for leptin were 5′-TTCTTGTGGCTTTGGCCCTA-3′ (forward, bases 81–100 in exon 2) and 5′-GGAGACTGACTGCGTGTGTGTGAA-3′ (reverse, bases 191–212 in exon 2). The thermal cycling conditions for *OB-Rl* were 58°C annealing for 47 cycles, and for *OB-Rs* and leptin were 57°C and 55°C annealing, respectively, for 45 cycles. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide staining. Specific PCR products were identified by direct sequencing (Lark Technologies, Ltd., Essex, UK). A 100–base pair DNA ladder (Gibco BRL, Paisley, UK) was used as a molecular weight standard. The expected PCR products were 132 base pairs (bp) for leptin, 501 bp for *OB-Rl*, and 394 bp for *OB-Rs*. Five different dilutions of cDNA from adipose tissue were used to obtain a standard curve. All samples were analyzed in duplicate and the average value was used for quantification. Variation between the two measurements for each sample was ranged from 0.1% to 10%. If the variation exceeded 10%, measurements were carried out in triplicate for that sample. Data were expressed as a ratio between the target gene mRNA level and the mRNA level of the housekeeping gene porphobilinogen deaminase (PBGD) (*OB-Rl* or *OB-Rs* mRNA copies/*PBGD* copies), which was used as an internal control.

Serum Leptin Determination. Venous blood samples were taken in the morning after a 12-hr fasting period and serum samples were stored at –80°C until assayed; assays were performed in duplicate. As we described previously (33), serum leptin concentration was measured using a commercial sandwich enzyme-linked immunosorbent assay (ELISA; DRG Leptin EIA-2395; DRG Interna-

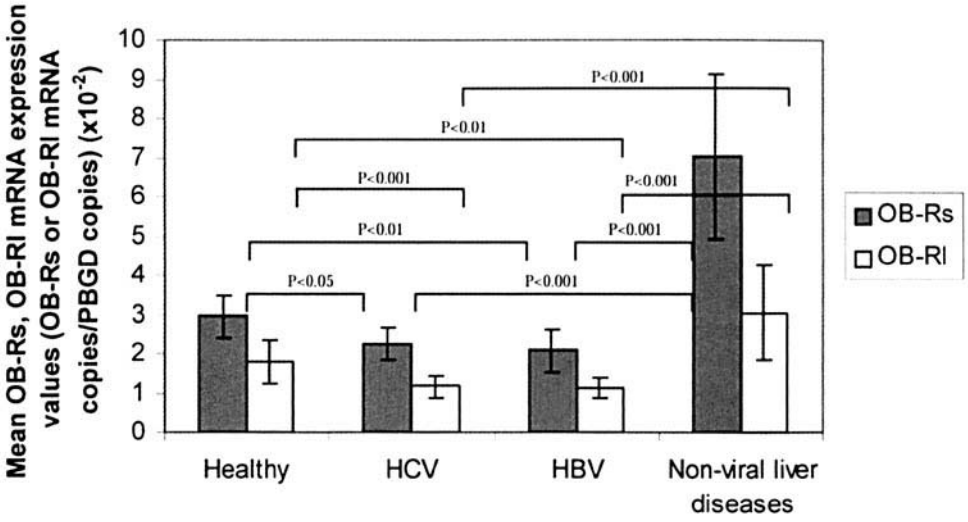

Figure 1. Comparison of PBMCs from healthy individuals, and HCV, HBV and nonviral liver disease patients with respect to mean OB-Rs and OB-Rl expression levels (*OB-Rs* or *OB-Rl* mRNA copies/*PBGD* copies) after real-time RT PCR analysis. Bars, means ± standard deviation.

tional Inc., Marburg, Germany) with a limit of detection of 1.0 ng/ml. The intra- and inter-assay coefficients of variation were 6.91% and 8.66%, respectively.

OB-R Detection on PBMCs by Flow Cytometry. PBMCs isolated from whole blood were incubated with 10 μl monoclonal anti-human leptin receptor (OB-R) antibody (R&D Systems, Minneapolis, MN) or mouse IgG2a Isotype Control (R&D Systems) and 10 μl goat anti-mouse IgG2a conjugated to fluorescein isothiocyanate (IgG2a-FITC; R&D Systems). Flow cytometry was performed using the EPICS XL-MCL Counter (Beckman Coulter, Miami, FL) and data were acquired and analyzed with CellQuest software. Data were acquired from 10,000 cells (events) and the proportion of PBMCs with cell-surface OB-R was determined. Results are displayed as percentage of events positive for OB-R staining.

Statistical Analysis. All calculations were performed using SPSS Software (Version 10.0; Chicago, IL). Data were analyzed, were applicable, by unpaired Student's *t* test, Mann-Whitney *U* test (MWU), Kruskal–Wallis, ANOVA, or the Fisher protected least significant difference (PLSD) as the post-hoc test corrected for multiple comparisons. Correlation coefficients were calculated by simple regression analysis (r) and nonparametric Spearman rank correlation (r_s) when appropriate. Two-sided P values of <0.05 were considered statistically significant.

Results

***OB-Rl* and *OB-Rs* mRNA in PBMCs of HBV, HCV, and Nonviral Liver Disease Patients.** In order to test the immunomodulatory role of leptin receptor isoforms in chronic HBV and HCV infections, we quantified using real-time RT-PCR the mRNA levels of leptin receptors in PBMCs from HBV, HCV, and nonviral liver disease patients as well as from healthy individuals. The mean *OB-Rl* and *OB-Rs* levels differed significantly among the four study groups (HBV, HCV, nonviral liver disease, and healthy). In more detail, after post-hoc analysis with the Fisher PLSD test, we observed significantly lower mean *OB-Rl* and *OB-Rs* mRNA levels in patients with chronic HBV ($1.133 \pm 0.361 \times 10^{-2}$ and $2.086 \pm 0.55 \times 10^{-2}$, respectively) and HCV ($1.167 \pm 0.469 \times 10^{-2}$ and $2.267 \pm 0.648 \times 10^{-2}$, respectively) infections compared with healthy individuals ($1.804 \pm 0.629 \times 10^{-2}$ and $2.953 \pm 0.562 \times 10^{-2}$, respectively) and in the nonviral liver disease group ($3.05 \pm 1.292 \times 10^{-2}$ and $7.02 \pm 2.092 \times 10^{-2}$, respectively) (Fig. 1). A representative gel that shows leptin and leptin receptor (*OB-Rl* and *OB-Rs*) expression in all four study groups is presented in Figure 2. *OB-Rl* and *OB-Rs* values did not differ between HBV and HCV patients ($3.11 \pm 1.12 \times 10^{-2}$ and $7.12 \pm 1.954 \times 10^{-2}$, respectively) or between alcoholic and autoimmune liver disease patients ($2.96 \pm 1.576 \times 10^{-2}$ and $6.87 \pm 2.386 \times 10^{-2}$, respectively). Moreover, the average level of *OB-Rs* expression was approximately 2-fold higher than that of *OB-Rl* in all samples (HBV, HCV, healthy, and nonviral liver disease, $P < 0.001$). Mean *OB-Rl* mRNA levels in PBMCs were not associated with the clinical status of the patients (inactive state, chronic HBV and HCV, and HBV and HCV–related cirrhosis) (ANOVA, $P > 0.05$), even if they were divided according to the type of viral infection (Student's *t* test, $P > 0.05$). Interestingly, we found a significant difference in the expression of *OB-Rs* between inactive HBV patients ($n = 14$) and patients with chronic HBV or HBV-related cirrhosis ($n = 17$) ($2.228 \pm 0.764 \times 10^{-2}$ and $1.944 \pm 0.516 \times 10^{-2}$ respectively, $P < 0.05$). The mean *OB-Rl* and *OB-Rs* mRNA levels did not differ between male and female HBV patients ($1.159 \pm 0.86 \times 10^{-2}$ vs. $2.06 \pm 0.618 \times 10^{-2}$ and $1.107 \pm 0.491 \times 10^{-2}$ vs. $2.114 \pm 0.731 \times 10^{-2}$, respectively), HCV patients ($1.194 \pm 0.393 \times 10^{-2}$ vs. $2.311 \pm 0.521 \times 10^{-2}$ and $1.149 \pm 0.713 \times 10^{-2}$ vs. $2.226 \pm 1.027 \times 10^{-2}$, respectively),

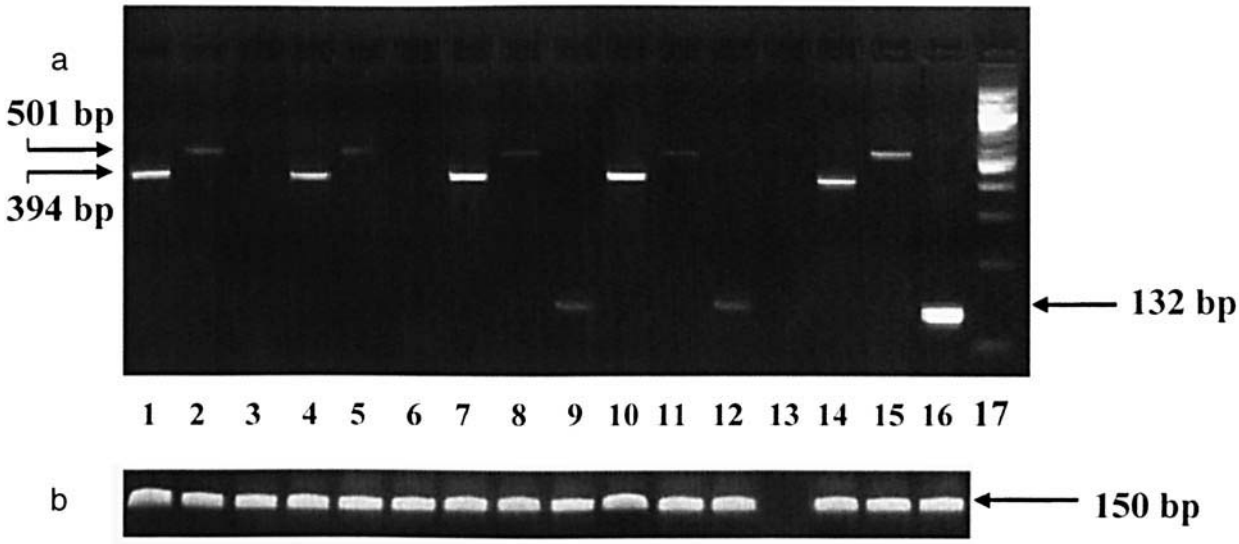

Figure 2. Representative gel showing *OB-RI, OB-Rs,* and leptin mRNA levels in PBMCs from HBV, HCV, and nonviral liver disease groups and healthy groups. (a) (lane 1) OB-Rs, healthy; (lane 2) OB-RI, healthy; (lane 3) leptin, healthy; (lane 4) OB-Rs, HBV; (lane 5) OB-RI, HBV; (lane 6) leptin, HBV; (lane 7) OB-Rs, HCV; (lane 8) OB-RI, HCV; (lane 9) leptin, HCV; (lane 10) OB-Rs, nonviral liver disease; (lane 11) OB-RI nonviral liver disease; (lane 12) leptin, nonviral liver disease; (lane 13) negative control; (lane 14) OB-Rs, positive control; (lane 15) OB-RI, positive control; (lane 16) leptin, positive control; (lane 17) marker, 100 bp ladder. (b) mRNA levels of PBGD.

healthy individuals ($1.787 \pm 0.711 \times 10^{-2}$ vs. $3.026 \pm 0.619 \times 10^{-2}$ and $1.823 \pm 0.557 \times 10^{-2}$ vs. $2.881 \pm 0.441 \times 10^{-2}$, respectively), and nonviral liver disease patients ($3.17 \pm 1.11 \times 10^{-2}$ vs. $2.90 \pm 1.536 \times 10^{-2}$ and $7.16 \pm 2.013 \times 10^{-2}$ vs. $6.85 \pm 2.275 \times 10^{-2}$, respectively).

Leptin Expression in PBMCs of HBV, HCV, and Nonviral Liver Disease Patients. Leptin was not expressed in PBMCs from any healthy individuals ($n = 36$) or HBV patients ($n = 31$), but was expressed in 3 of 34 samples from the HCV group (8.8%) and in 19 of 25 samples from the nonviral liver disease group (76%). No significant correlation was observed between either type of viral infection and leptin expression ($\chi^2 = 2.868$, $P > 0.05$), while a significant association was observed between leptin expression and the presence of nonviral liver disease ($\chi^2 = 39.7$, $P < 0.001$).

***OB-RI, OB-Rs,* and Leptin Expression in Liver Tissue from HBV and HCV Patients.** There were differences among the mean levels of *OB-RI* and *OB-Rs* mRNA in livers from patients with HBV (0.289 ± 0.12 and $4.04 \pm 1.844 \times 10^{-2}$, respectively), HCV ($0.542 \pm 0.207$ and $4.97 \pm 2.507 \times 10^{-2}$, respectively), and the healthy control group ($1.62 \pm 0.398 \times 10^{-2}$ and $2.93 \pm 0.234 \times 10^{-2}$, respectively) (ANOVA, $P < 0.01$ for all OB-RI comparisons; Fig. 3). Leptin was not expressed in any healthy liver tissues ($n = 6$), but was expressed in five out

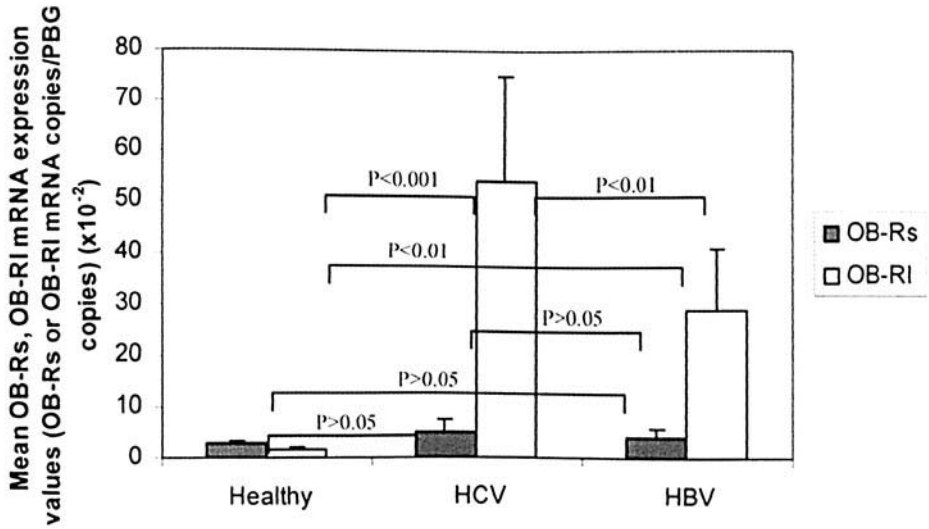

Figure 3. Comparison of liver tissues of healthy individuals, and HCV and HBV patients with respect to mean OB-Rs and OB-RI expression levels (*OB-Rs* or *OB-RI* mRNA copies/*PBGD* copies) obtained after real-time RT PCR analysis. Bars, means ± standard deviation.

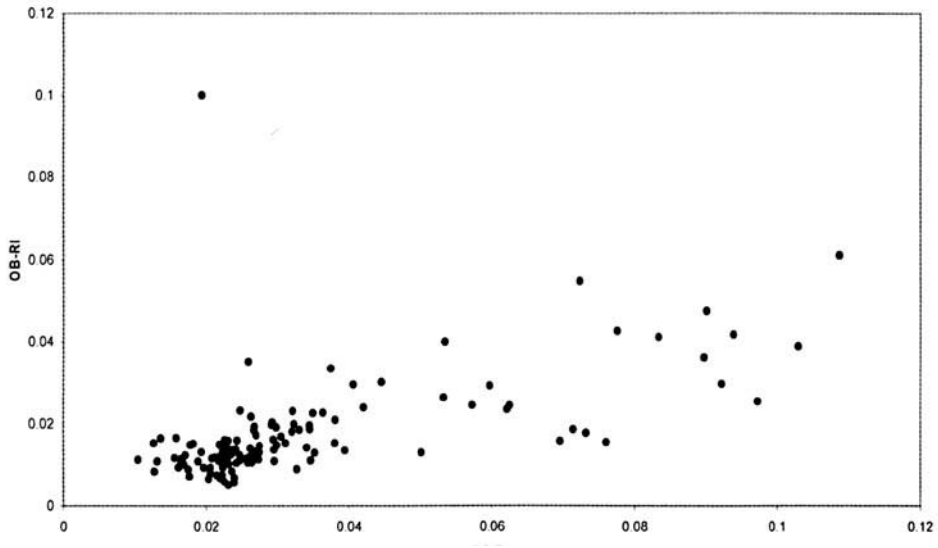

Figure 4. Correlation between *OB-Rs* and *OB-Rl* expression levels (*OB-Rs* or *OB-Rl* mRNA copies/*PBGD* copies) obtained after real-time RT-PCR analysis in all individuals studied ($N = 126$).

of eight tissues of the HBV group (62.5%) and four out of seven samples of the HCV group (57.14%). *OB-Rl* and *OB-Rs* mRNA levels were not associated with age, BMI, sex, disease duration, or level of aminotransferases. Liver leptin expression was significantly associated with HBV and HCV disease ($\chi^2 = 6.3$, $P < 0.05$).

Correlation of PBMC *OB-Rl* and *OB-Rs* mRNA with Clinical Characteristics. *OB-Rl* and *OB-Rs* mRNA levels were not associated with age, BMI (Pearson rank correlation, P=NS), sex (Student's t test, $P > 0.05$), disease duration, or level of aminotransferases (Spearman rank correlation, P=NS) when all patients were considered together. However, a significant negative correlation was observed between *OB-Rs* level and age (Pearson rank correlation, $r = -0.269$, $P < 0.05$). Additionally, when patients were considered separately in groups (HBV, HCV, nonviral liver disease), there was a correlation of *OB-Rl* and *OB-Rs* levels with age, BMI, sex, disease duration, and aminotransferase level. A notable negative correlation was observed between *OB-Rs* levels and AST levels in the HBV group ($r = -0.341$, $P < 0.05$) and a positive correlation was observed between *OB-Rl* and AST levels in the autoimmune liver disease group ($r = 0.752$, $P < 0.05$).

Concerning the association between *OB-Rl* and *OB-Rs* mRNA levels and age, BMI, sex, disease duration, and levels of aminotransferases in the chronic viral disease group, we found no significant associations for the inactive state group, the chronic hepatitis group, and the cirrhotic group. Also, no correlation was observed between *OB-Rl* and *OB-Rs* mRNA levels and either HBV or HCV viral loads, measured by HBV DNA and HCV RNA levels, respectively, or HCV virus genotype.

The levels of *OB-Rl* and *OB-Rs* mRNA were associated when all samples were considered together as a single group ($N = 126$; Pearson rank correlation, $r = 0.78$, $P < 0.001$; Fig. 4).

Serum Leptin Measurements. Serum leptin levels were decreased, although not significantly, in HBV (6.522 ± 5.659 ng/ml) and HCV (13.175 ± 17.856 ng/ml) patients compared with healthy individuals (16.822 ± 15.45 ng/ml) and the nonviral liver disease group (17.632 ± 19.629 ng/ml) (Table 1 and Fig. 5). Moreover, females had higher serum leptin levels than males in the HBV patient group (8.412 ± 6.89 ng/ml vs. 5.077 ± 4.154 ng/ml; Student's t test, $P > 0.05$), HCV patient group (19.582 ± 21.06 ng/ml vs. 4.024 ± 2.769 ng/ml; Student's t test, $P < 0.02$), among

Table 1. Oligonucleotide Primers for Real-Time RT-PCR and Their Respective Product Sizes

Transcript		Primer sequence (5′–3′)	Location (bases)	Product size (bp)
OB-Rl	Forward	GCTATTTTGGGAAGATGT	288–306 in exon 19	501
	Reverse	TGCCTGGGCCTCTATCTC	553–570 in exon 20	
OB-Rs	Forward	TGTTGTGAATGTCTTGTGCC	402–421 in exon 6	394
	Reverse	TGCTCCAGTCACTCCAGATTCC	649–668 in exon 8	
Leptin	Forward	TTCTTGTGGCTTTGGCCCTA	81–100 in exon 2	132
	Reverse	GGAGACTGACTGCGTGTGTGTGAA	191–212 in exon 2	

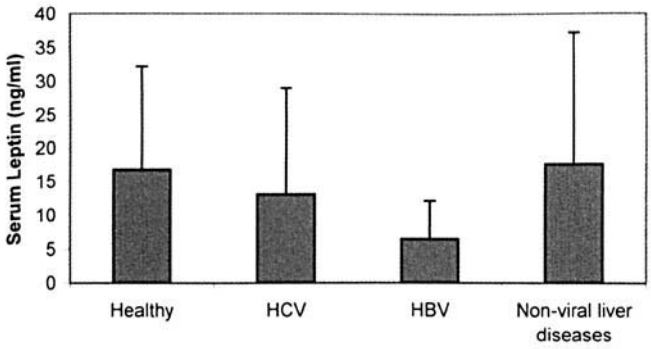

Figure 5. Serum leptin levels in healthy individuals, and HCV, HBV, and nonviral liver disease patients. Bars, means ± standard deviation.

healthy individuals (17.122 ± 14.67 ng/ml vs. 16.403 ± 17.019 ng/ml; Student's t test, $P > 0.05$), and in the nonviral liver disease group (30.12 ± 21.031 ng/ml vs. 7.82 ± 11.641 ng/ml; Student's t test, $P < 0.01$).

Serum leptin levels and BMI showed a significant correlation (Spearman rank correlation, $r = 0.443$, $P < 0.001$) when all samples were considered together ($N = 126$) (Fig. 6). However, no correlation was observed between serum leptin levels and age, disease duration, or the levels of aminotransferases. Also, no correlation was observed between serum leptin levels and *OB-Rl* or *OB-Rs* mRNA levels in groups with HBV ($r = -0.052$, $P > 0.05$ and $r = 0.002$, $P > 0.05$, respectively), HCV ($r = -0.172$, $P > 0.05$ and $r = -0.399$, $P < 0.05$, respectively), healthy individuals ($r = -0.168$, $P > 0.05$ and $r = -0.228$, $P > 0.05$, respectively), and nonviral liver diseases ($r = -0.195$, $P > 0.05$ and $r = 0.071$, $P > 0.05$, respectively). Similarly, when all subgroups where taken together, no correlation was observed between levels of serum leptin and *OB-Rl* expression ($r = 0.026$, $P > 0.05$) or *OB-Rs* expression ($r = 0.135$, $P > 0.05$) (Fig. 7).

Leptin Receptor Expression on PBMCs. Flow cytometric analysis revealed the presence of OB-R on 2.32 ± 0.8% of PBMCs from 10 of 65 (15.3%) patients of the combined HBV and HCV group, 3.1 ± 0.9% of PBMCs from the control group, and 4.57 ± 0.9% of PBMCs from the nonviral liver disease group (Fig. 8). Flow cytometry analysis of the HBV and HCV groups demonstrated an increased monocyte population, consistent with the phenomenon of monocytosis present in chronic viral diseases (Fig. 8e–h).

Discussion

The leptin system is likely to have an important role in inflammatory states such as multiple sclerosis, rheumatoid arthritis, nonalcoholic steatohepatitis, and other diseases, probably by the induction of proinflammatory factors such as TNF-α, IFN-γ, IL-2, Cox-2, and nitric oxide (1, 3, 34, 35). In the present study, we investigated, using quantitative real-time RT-PCR, the mRNA levels of *OB-Rl* and *OB-Rs* in PBMCs from patients with HBV, HCV, and nonviral liver disease in order to investigate the possible immunomodulatory role of leptin in chronic viral liver disease. We observed significantly lower expression levels of *OB-Rl* and *OB-Rs* in HBV and HCV patients compared with healthy individuals and nonviral liver disease patients. Flow cytometric analysis revealed that OB-R protein levels exhibited a pattern of expression similar to that of their mRNAs, with the nonviral liver disease group showing increased levels compared with healthy control, HBV, and HCV groups. The reduction in observed OB-R protein expression in a low number of HBV and HCV samples could have resulted from virus-induced molecular dysregulation and increased sensitivity to apoptosis of PBMCs,

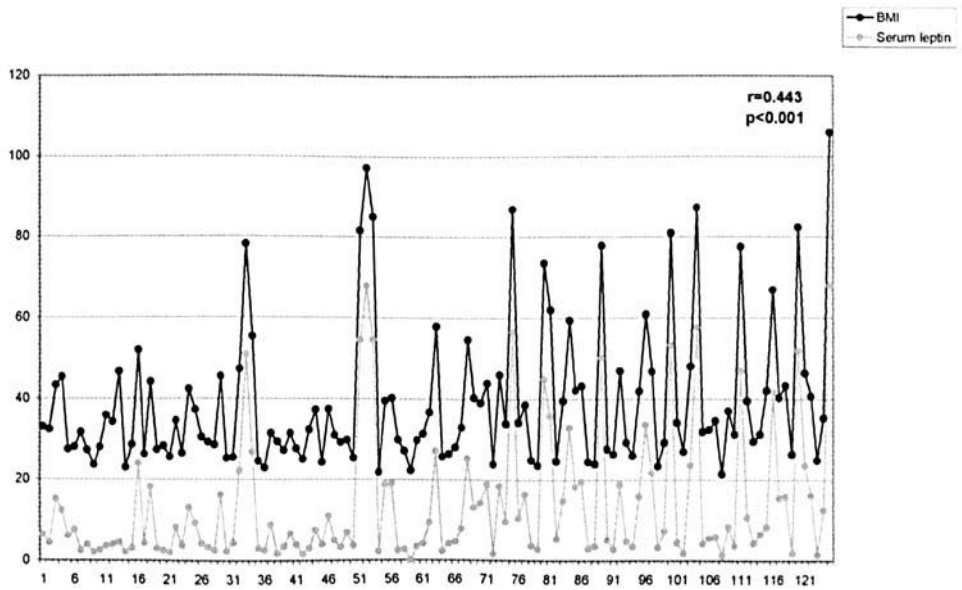

Figure 6. Correlation between serum leptin levels (ng/ml) and BMI in all individuals studied.

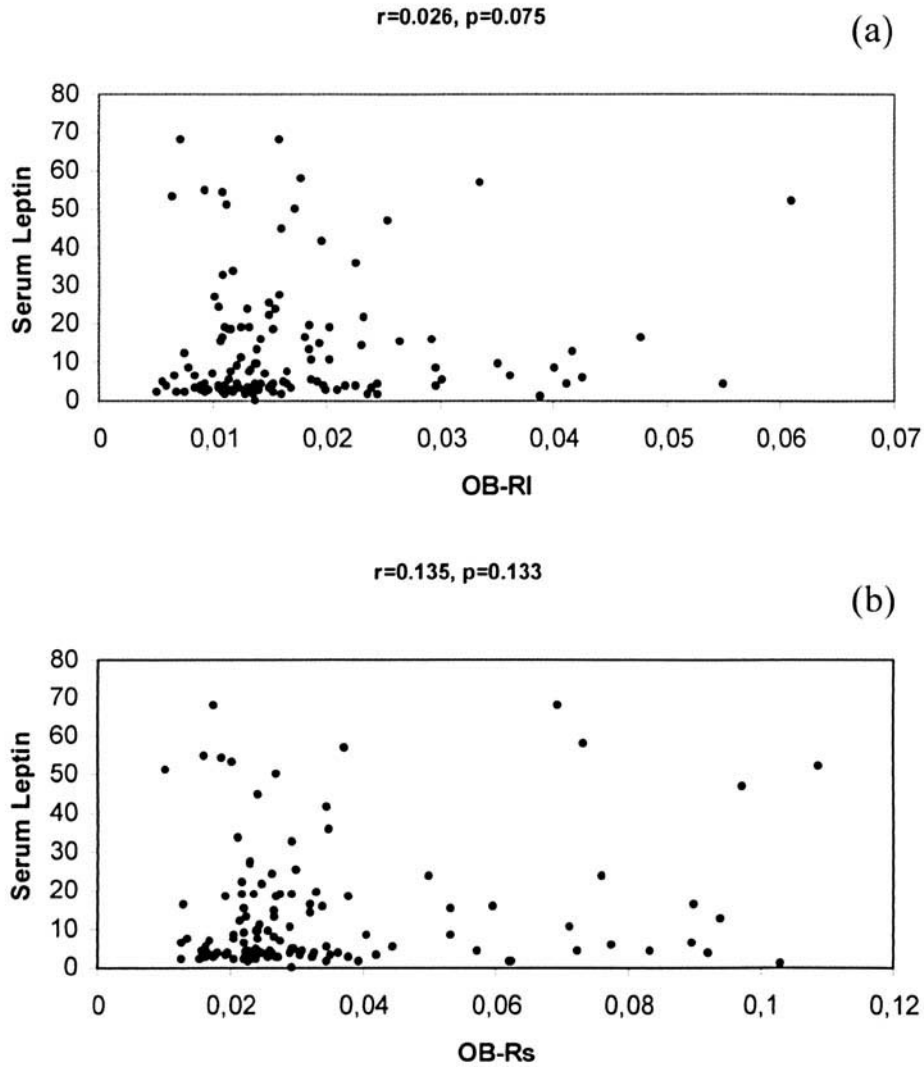

Figure 7. Correlation between serum leptin (ng/ml) and (a) *OB-RI* mRNA levels and (b) *OB-Rs* mRNA levels (*OB-RI* or *OB-Rs* mRNA copies/*PBGD* copies) in all individuals.

which would result reduction or absence of translation (36–38).

It has been suggested that leptin, acting through OB-R, activates the Janus kinase–signal transducers and activators of transcription (JAK-STAT), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) signaling pathways in PBMC, which in turn stimulate cytokine induction by monocytes and macrophages, proliferation of naive T cells, Th1 immune responses, Th2 inhibition, antiapoptotic effects on T cells, and increased cytotoxicity of natural killer (NK) cells (1, 20). Our findings suggest an involvement of OB-RI and OB-Rs in immune dysfunction in patients with chronic HBV and HCV infection, as decreased *OB-R* expression and subsequent reduction of protein on the cell surface diminishes the ability of leptin to stimulate PBMCs, decreases the number of activated immune cells, and possibly increases the number of apoptotic immune cells. Our results provide a model for hepatitis virus–induced downregulation of the immune system that is in accordance with previous studies, which have reported that HBV and HCV antigens directly influence activation-induced cell death in peripheral T cells and generally in PBMCs in both HBV patients and an HCV transgenic murine model (36, 37). A recent study from our group (29) has suggested that immunodeficiency in HBV and HCV patients correlates with the observed reduction in

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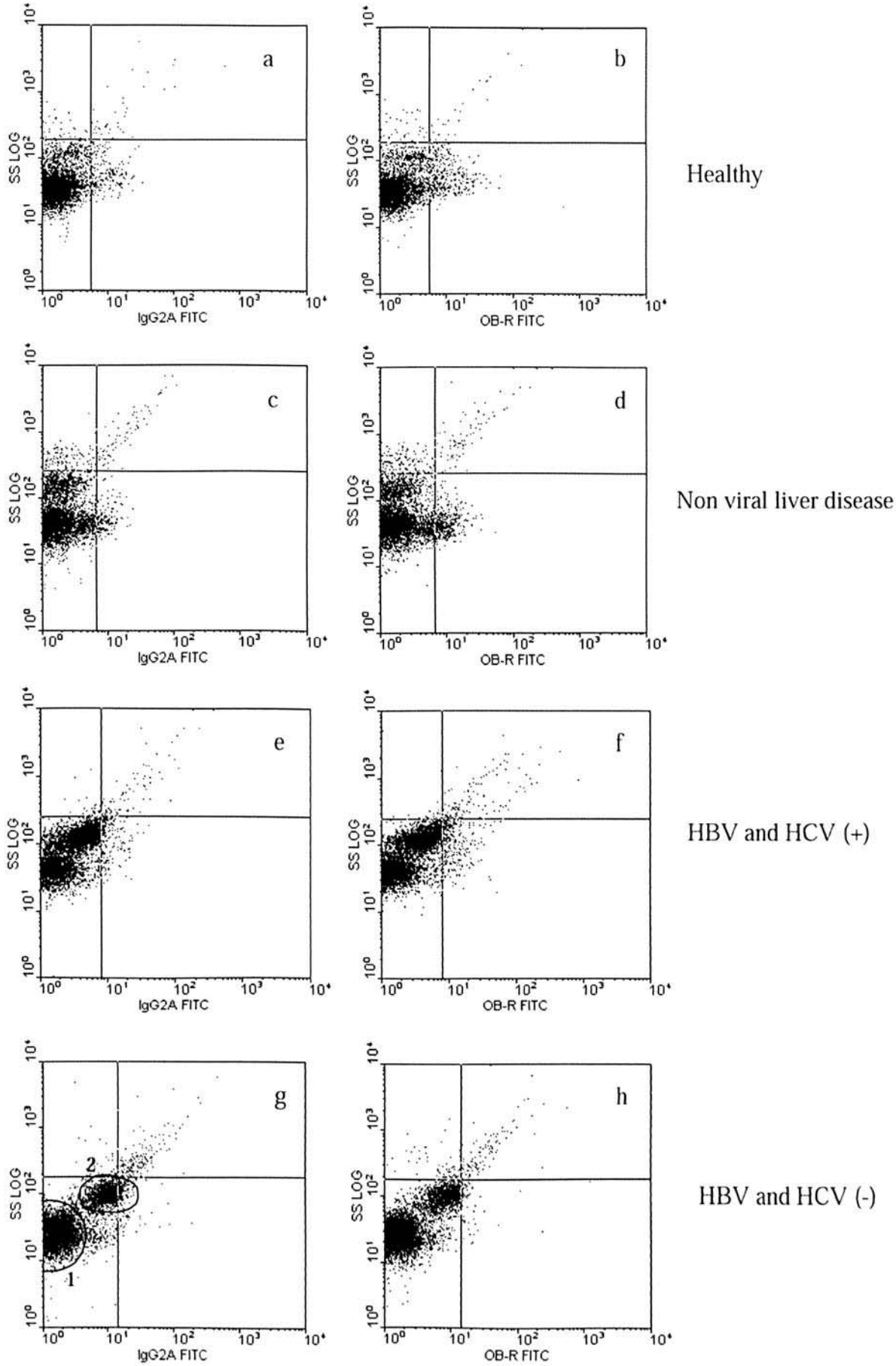
Figure 8. Flow cytometry analysis of OB-R protein on the surface of PBMCs incubated with either IgG2a isotype control (a, c, e, and g) or antibody against OB-R (b, d, f, and h). OB-R–positive and OB-R–negative cells are represented in the lower right and lower left quadrants, respectively. (a and b) Healthy individuals. (c and d) Nonviral liver disease group. (e and f) HBV and HCV OB-R–positive group. (g and h) HBV and HCV OB-R–negative group. Circles in g, the discrete lymphocyte (1) and monocyte (2) populations.


a
b
c
d
e
f
g
h
SS LOG
IgG2A FITC
OB-R FITC
1
2
Healthy
Non viral liver disease
HBV and HCV (+)
HBV and HCV (-)

human telomerase reverse transcriptase (hTERT) mRNA in PBMCs from infected patients.

In the present study, a significant positive correlation was observed between *OB-Rl* and *OB-Rs* expression (expression of *OB-Rs* was approximately twice that of *OB-Rl*) when all patients and healthy controls were taken together. The above correlation indicates a stable and parallel expression pattern of OB-Rl and OB-Rs, suggesting that OB-Rs could serve either as an auxiliary to leptin signaling through the OB-Rl isoform in PBMCs or as a regulator of serum leptin clearance from the circulation through the soluble leptin receptor (10, 11). Based on the latter possibility, the observed reduction of serum leptin in HBV and HCV patients could lead to the downregulation of *OB-Rs* expression in PBMCs. Regarding serum leptin levels in chronic hepatitis patients, there are conflicting data in the literature (33, 39). Our results are in agreement with a report that indicates that serum leptin levels are lower in chronic hepatitis patients compared with healthy individuals (39). It is possible that a reduction of leptin secretion from adipose tissue is part of a mechanism to reverse the negative energy balance, caused by either reduced energy intake or increased energy expenditure, that occurs in the course of chronic viral liver disease.

We detected leptin mRNA in 3 of 34 samples from the HCV group (8.8%) and in 19 of 25 samples from the nonviral liver disease group (76%). The observed elevated leptin expression in PBMCs from patients with nonviral liver disease, accompanied with the increase of *OB-Rl* and *OB-Rs* mRNA and protein levels, suggests that leptin could be part of an autocrine signaling loop in PBMCs. This may reflect the immunoactivity that might be responsible for hepatocyte cytotoxicity in autoimmune liver diseases and possibly in alcoholic liver disease (40). In addition, elevated leptin secretion from PBMCs may contribute to the elevated serum leptin levels observed in the nonviral liver disease group. Similarly, leptin expression was observed in activated inflammatory Th1 lymphocytes during experimental autoimmune encephalomyelitis, in an animal model of multiple sclerosis (41). The relationship between HBV and HCV viral diseases and the autocrine role of leptin will need to be evaluated in future studies.

In the present study, a significant increase in *OB-Rl* mRNA and a high percentage (60%) of leptin expression was observed in liver tissue from patients with chronic viral hepatitis compared with healthy livers. The functional OB-Rl isoform exhibited an approximately 10-fold higher expression in HBV and HCV liver samples compared with healthy tissues. Recent reports have also shown that leptin induces leptin receptor expression in the liver (42), acts as a profibrogenic factor (26), increases susceptibility to hepatotoxicity by regulating cytokine production and T cell activation (24), and upregulates proinflammatory and proangiogenic cytokines in human liver (43). In leptin-deficient *ob/ob* mice, protection from concanavalin A (Con A)–induced hepatitis has been associated with reduced production of proinflammatory cytokines, increased levels of the protective cytokine IL-10, diminished T cell activation, and a decrease in the number of hepatic NK T cells (44, 45). There is therefore increasing evidence for an extended inflammatory role of leptin in liver tissue.

When we grouped patients according to their clinical status, there was a significant difference between patients with inactive HBV infection and those with chronic HBV or HBV-related cirrhosis with respect to *OB-Rs* mRNA levels. Moreover, expression of the *OB-Rs* isoform was negatively correlated with AST levels in the HBV group. These results may indicate that OB-Rs functions differently in HBV disease, with a role that extends beyond that of leptin transport and degradation. We also observed a negative correlation between *OB-Rs* mRNA level and age in the patients group, which might reflect an age-dependent influence of chronic HBV and HCV infection on the immune system. A positive correlation between serum leptin and BMI and the increase in leptin levels observed in females support a role for adipose tissue in regulating leptin production.

In conclusion, for the first time to our knowledge, we have used quantitative real-time RT-PCR and flow cytometry to observe decreased *OB-Rl* and *OB-Rs* expression in PBMCs, and increased expression of *OB-Rl* and leptin in the liver, of HBV and HCV infected patients. This suggests a possible involvement of the leptin system in immunopathogenesis of these viral liver diseases. There is increasing evidence that chronic HBV and HCV diseases are characterized by a complicated immunopathology and promote, through a multilateral mechanism, immunodeficiency, which increases chronicity, accelerates liver disease, and induces mortality. Further studies are needed to confirm the involvement of the leptin system in the immune response to HBV and HCV disease pathogenesis.

1. La Cava A, Matarese G. The weight of leptin in immunity. Nat Rev Immunol 4:371–379, 2004.
2. Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. Nature 395:763–770, 1998.
3. Otero M, Lago R, Lago F, Casanueva FF, Dieguez C, Gomez-Reino JJ, Gualillo O. Leptin, from fat to inflammation: old questions and new insights. FEBS Lett 579:295–301, 2005.
4. Fantuzzi G. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol 115:911–919, 2005.
5. Flier JS. Leptin expression and action: new experimental paradigms. Proc Natl Acad Sci U S A 94:4242–4245, 1997.
6. Zhang Y, Wilsey JT, Frase CD, Matheny MM, Bender BS, Zolotukhin S, Scarpace PJ. Peripheral but not central leptin prevents the immunosuppression associated with hypoleptinemia in rats. J Endocrinol 174:455–461, 2002.
7. La Cava A, Alviggi C, Matarese G. Unraveling the multiple roles of leptin in inflammation and autoimmunity. J Mol Med 82:4–11, 2004.
8. Matarese G, Moschos S, Mantrosos CS. Leptin in immunology. J Immunol 174:3137–3142, 2005.
9. Margetic S, Garrela C, Pegg GG, Hill RA. Leptin: a review of its peripheral actions and interactions. Int J Obes 26:1407–1433, 2002.
10. Bjorbaek C, Uotani S, da Silva B, Flier JS. Divergent signaling capacities of the long and short isoforms of the leptin receptor. J Biol Chem 272:32686–32695, 1997.

11. Yamashita T, Murakami T, Otani S, Kuwajima M, Shima K. Leptin receptor signal transduction: OBRa and OBRb of fa type. Biochem Biophys Res Commun 246:752–759, 1998.
12. Murakami T, Yamashita T, Iida M, Kuwajima M. A short form of leptin receptor performs signal transduction. Biochem Biophys Res Commun 231:26–29, 1997.
13. Tsiotra CP, Pappa V, Raptis SA, Tsigos C. Expression of the long and short leptin isoforms in peripheral blood mononuclear cells: implications for leptin's actions. Metabolism 49:1537–1541, 2000.
14. Lord GM, Matarese G, Howard JK, Baker RJ, Bloom SR, Lechler RI. Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 394:897–901, 1998.
15. Ozata M, Ozdemir IC, Licinio J. Human leptin deficiency caused by a missense mutation: multiple endocrine defects, decreased sympathetic tone, and immune system dysfunction indicate new targets for leptin action, greater central than peripheral resistance to the effects of leptin, and spontaneous correction of leptin-mediated defects. J Clin Endocrinol Metab 84:3686–3695, 1999.
16. Gainsford T, Wilson TA, Metcalf D, Handman E, McFarlane C, Ng A, Nicola NA, Alexander WS, Hilton DJ. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. Proc Natl Acad Sci U S A 93:14564–14568, 1996.
17. Santos-Alvarez J, Goberna R, Sánchez-Margalet V. Human leptin stimulates proliferation and activation of human circulating monocytes. Cell Immunol 194:6–11, 1999.
18. Loffreda S, Yang SQ, Lin HZ, Karp CL, Brengman ML, Wang DJ, Klein AS, Bulkley GB, Bao C, Noble PW Lane MD, Diehl AM. Leptin regulates proinflammatory immune responses. FASEB J 12:57–65, 1998.
19. Martin-Romero C, Santos-Alvarez J, Goberna R, Sanchez-Margalet V. Human leptin enhances activation and proliferation of human circulating T lymphocytes. Cell Immunol 199:15–24, 2000.
20. Sanchez-Margalet V, Martin-Romero C, Santos-Alvarez J, Goberna R, Najib S, Gonzalez-Yanes C. Role of leptin as an immunomodulator of blood mononuclear cells. Clin Exp Immunol 133:11–18, 2003.
21. Sanchez-Pozo C, Rodriguez-Bano J, Dominguez-Castellano A, Muniain MA, Goberna R, Sanchez-Margalet V. Leptin stimulates the oxidative burst in control monocytes but attenuates the oxidative burst in monocytes from HIV-infected patients. Clin Exp Immunol 134:464–469, 2003.
22. Koziel MJ. Cytokines in viral hepatitis. Semin Liver Dis 19:157–169, 1999.
23. Schumann J, Tiegs G. Pathophysiological mechanisms of TNF during intoxication with natural or man-made toxins. Toxicology 138:103–126, 1999.
24. Sennello JA, Fayad R, Morris AM, Eckel RH, Asilmaz E, Montez J, Friedman JM, Dinarello CA and Fantuzzi G. Regulation of T cell–mediated hepatic inflammation by adiponectin and leptin. Endocrinology 146: 2157–2164, 2005.
25. Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation and hematopoiesis. J Leukoc Biol 68:437–446, 2000.
26. Ikejima K, Lang T, Zhang Y, Yamashina S, Honda H, Yoshikawa M, Hirose M, Enomoto N, Kitamura T, Takei Y, Sato N. Expression of leptin receptors in hepatic sinusoidal cells. Comp Hepatol 3(Suppl 1): S12, 2004.
27. Dalekos GN, Christodoulou D, Kistis KG, Zervou EK, Hatzis J, Tsianos EV. A prospective evaluation of dermatologic side effects during alpha-interferon therapy for chronic viral hepatitis. Eur J Gastroenterol Hepatol 10:933–939, 1998.
28. Dalekos GN, Achenbach K, Christodoulou D, Liapi GK, Zervou EK, Sideris DA, Tsianos EV. Idiopathic dilated cardiomyopathy: lack of association with hepatitis C virus infection. Heart 80:270–275, 1998.
29. Satra M, Dalekos GN, Kollia P, Vamvakopoulos N, Tsezou A. Telomerase reverse transcriptase mRNA expression in peripheral lymphocytes of patients with chronic HBV and HCV infections. J Viral Hepatol 12:488–93, 2005.
30. EASL jury. EASL International Consensus Conference on Hepatitis B. 13–14 September, 2002: Geneva, Switzerland. Consensus statement (short version). J Hepatol 38:533–540, 2003.
31. Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WGE, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston ALWF, Fainboim L, Heathcote J, Homberg J-C, Hoofnagle J H, Kakumu S, Krawitt EL, Mackay IR, MacSween RNM, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Büschenfelde K-H, Mieli-Vergani G, Nakanuma Y, Nishioka M, Penner E, Porta G, Portmann BC, Reed WD, Rodes J, Schalm SW, Scheuer PJ, Schrumpf E, Seki T, Toda G, Tsuji T, Tygstrup N, Vergani D, Zeniya M. International autoimmune hepatitis group report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol 31:929–938, 1999.
32. Rigopoulou EI, Davies ET, Pares A, Zachou K, Liaskos C, Bogdanos D-P, Rodes J, Dalekos GN, Vergani D. Prevalence and clinical significance of isotype specific antinuclear antibodies in primary biliary cirrhosis. Gut 54:528–532, 2005.
33. Zografos TA, Rigopoulou E, Liaskos C, Togousidis E, Zachou K, Gatselis N, Germenis A, Dalekos GN. Alterations of leptin levels during IFN-alpha treatment in patients with chronic viral hepatitis B and C: evidence of an IFN-alpha–mediated suppression of leptin production. J Hepatol 44:848–855, 2006.
34. Otero M, Lago R, Lago F, Reino JJ, Gualillo O. Signaling pathway involved in nitric oxide synthase type II activation in chondrocytes: synergistic effect of leptin with interleukin-1. Arthritis Res Ther 7: R581–R591, 2005.
35. Mehebik N, Jaubert AM, Sabourault D, Giudicelli Y, Ribiere C. Leptin-induced nitric oxide production in white adipocytes is mediated through PKA and MAP kinase activation. Am J Physiol Cell Physiol 289: C379–C387, 2005.
36. Soguero C, Joo M, Chianese-Bullock KA, Nguyen DT, Tung K, Hahn YS. Hepatitis C virus core protein leads to immune suppression and liver damage in a transgenic murine model. J Virol 76:9345–9354, 2002.
37. Nakamura K, Yuh K, Sugyo S, Shijo H, Kimura N, Okumura M. Apoptosis observed in peripheral T lymphocytes from patients with chronic hepatitis B. Gastroenterology 111:156–164, 1996.
38. Taya N, Torimoto Y, Shindo M, Hirai K, Hasebe C, Kohgo Y. Fas-mediated apoptosis of peripheral blood mononuclear cells in patients with hepatitis C. Br J Haematol 110:89–97, 2000.
39. Testa R, Franceschini R, Giannini E, Cataldi A, Botta F, Fasoli A, Tenerelli P, Rolandi E, Barreca T. Serum leptin levels in patients with viral chronic hepatitis or liver cirrhosis. J Hepatol 33:33–37, 2000.
40. Ichiki Y, Aoki C, Bowlus C, Shimoda S, Ishibahi H, Gershwin ME. T cell immunity in autoimmune hepatitis. Autoimmun Rev 4:315–321, 2005.
41. Sanna V, Giacomo A, La Cava A, Lechler R, Fontana S, Zappacosta S, Matarese G. Leptin surge precedes onset of autoimmune encephalomyelitis and correlates with development of pathogenic T cell responses. J Clin Invest 11:241–250, 2003.
42. Cohen P, Yang G, Yu X, Soukas AA, Wolfish CS, Friedman JM, Li C. Induction of leptin receptor expression in the liver by leptin and food deprivation. J Biol Chem 280:10034–10039, 2005.
43. Aleffi S, Petrai I, Bertolani C, Parola M, Colombatto S, Novo E, Vizzutti F, Anania FA, Milani S, Rombouts K, Laffi G, Pinzani M, Marra F. Upregulation of proinflammatory and proangiogenic cytokines by leptin in human hepatic stellate cells. Hepatology 42:1339–48, 2005.
44. Faggioni R, Jones-Carson J, Reed DA, Dinarello CA, Feingold KR, Grunfeld C, Fantuzzi G. Leptin-deficient (*ob/ob*) mice are protected from T cell-mediated hepatotoxicity: role of tumor necrosis factor-a and IL-18. Proc Natl Acad Sci U S A 97:2367–2372, 2000.
45. Siegmund B, Lear-Kaul KC, Faggioni R, Fantuzzi G. Leptin deficiency, not obesity, protects mice from Con A–induced hepatitis. Eur J Immunol 32:552–560, 2002.