Insulin-Like Growth Factor-I, Inflammatory Proteins, and Fibrosis in Subjects With Nonalcoholic Fatty Liver Disease

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Context: Inflammation may have a pathogenic role in the progression of nonalcoholic fatty liver disease (NAFLD); by contrast, the role of anti-inflammatory molecules has not been addressed. Low circulating levels of the anti-inflammatory molecule IGF-I have been described in subjects with NAFLD.

Objective: The aim of the study was to elucidate the clinical significance of IGF-I in NAFLD and its relationship with inflammatory biomarkers and fibrosis.

Design and Setting: We conducted a cross-sectional study and in vitro experiments on hepatic HepG2 cells at the Internal Medicine and Gastrointestinal and Liver Units of the Universities of Catanzaro and Palermo.

Subjects: A total of 221 individuals with NAFLD diagnosed on ultrasonography (cohort 1) and 50 subjects with biopsy-proven NAFLD (cohort 2) participated in the study.

Intervention: Liver ultrasonography was performed on cohort 1, and hepatic biopsies were obtained from cohort 2.

Main Outcome Measures: NAFLD fibrosis and Kleiner scores were calculated. IGF-I and inflammatory biomarker plasma concentrations were assessed with specific assays. In the in vitro study, real-time RT-PCR was used to assess the mRNA expression levels of acute-phase reactants.

Results: In the first cohort, circulating IGF-I levels showed an inverse correlation with NAFLD fibrosis score and inflammatory biomarkers; similarly in the second cohort, liver IGF-I mRNA levels and the fibrosis score showed a negative relationship. Finally, we showed that IGF-I was able to directly modulate the expression of acute-phase reactants, decreasing C-reactive protein and fibrinogen levels and up-regulating albumin expression in HepG2 cells.

Conclusions: The present data suggest that evaluation of circulating IGF-I and proinflammatory markers might be useful to assess comprehensively the severity of the disease in individuals with NAFLD. (J Clin Endocrinol Metab 98: E304–E308, 2013)

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide (1, 2). NAFLD encompasses a wide spectrum of conditions ranging from simple steatosis to the necroinflammatory form of nonalcoholic steatohepatitis (NASH), which is characterized by hepatocyte damage, inflammation, and fibrosis and is a potentially serious condition associated with increased risk of liver-related morbidity and mortal-

Abbreviations: CRP, C-reactive protein; GLM, general linear model; IGFBP, IGF binding protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
ity (3–5). Increasing evidence suggests that inflammation may have a role in the progression from NAFLD to NASH (6–8), and it is possible to hypothesize that an unpaired balance between proinflammatory and anti-inflammatory proteins may contribute to this phenomenon. Interestingly, in a small cohort of morbidly obese subjects, it has been reported that the proinflammatory molecule, IL-6, and the anti-inflammatory factor, IGF-I, are independent prognostic markers of liver steatosis and NASH (9). More recently, IGF-I has been shown to prevent the development of NASH in a genetically modified animal model prone to the disease (10). Here we investigated the clinical significance of IGF-I in the liver and its relationship with inflammatory biomarkers and severity of fibrosis in 2 independent cohorts of individuals with NAFLD and assessed whether IGF-I is able to directly modulate the expression of acute-phase reactants.

**Subjects and Methods**

**Human subjects**

The first cohort consisted of Caucasian subjects participating in the CATAnzaro MEtabolic Risk factors (CATAMERI) Study, a cross-sectional study assessing cardiometabolic risk factors in subjects carrying at least 1 risk factor (11–13). A total of 221 subjects for whom data on both IGF-I and inflammatory factor plasma levels were available have been included in the present study; no differences were observed between this subgroup and the whole cohort examined in previous studies. Liver ultrasound scanning was performed in all participants by the same trained operator, blind to patients’ clinical characteristics, using a Toshiba Apio 50 ultrasound apparatus equipped with a 3.5-MHz linear transducer, as previously described (11). Fifty subjects with biopsy-proven NAFLD, recruited at the Gastrointestinal and Liver Unit at the University Hospital in Palermo, were included to assess liver expression of IGF-I mRNA (cohort 2). Liver samples were processed as described in the Supplemental Data (published on The Endocrine Society’s Journals Online web site at http://jecm.endojournals.org). The protocol was approved by local ethical committees (Comitato Etico Azienda Ospedaliera “Mater Domini” and Comitato Etico Policlinico Paolo Giaccone), and informed written consent was obtained from all participants. Clinical definitions used in this study are detailed in the Supplemental Data.

**Analytical determinations**

Total serum IGF-I concentrations were determined by chemiluminescent immunoassay (Nichols Institute Diagnostic, San Juan Capistrano, California). Serum samples were acidified to separate IGF-I from IGF binding proteins (IGFBPs), and excess IGF-II was added in the assay to block the IGFBP binding sites from recombining with the released IGF-I. The detection limit of this assay was 5 nmol/L, and the interassay coefficient of variation was 6% (at 7 nmol/L) to 4% (at 55 nmol/L). Additional analytical determinations are detailed in the Supplemental Data.

**In vitro studies with HepG2 cells**

To study the effects of IGF-I on IL-6-induced expression of C-reactive protein (CRP), fibrinogen, and albumin, confluent human hepatoma HepG2 cells were incubated for 16 hours in serum free DMEM containing 1% BSA, followed by incubation for 24 hours in the presence or absence of human recombinant IL-6 (20 ng/mL), with or without human recombinant IGF-I 10 or 100 nM and processed as described in the Supplemental Data.

**Statistical analysis**

Continuous data are expressed as means ± SD. Categorical variables were compared by \( \chi^2 \) test. A general linear model (GLM) with post hoc Bonferroni correction for multiple comparisons was used to compare differences of continuous variables between groups. Partial correlation coefficients adjusted for confounders were computed between variables. To compare IGF-I mRNA expression levels of subjects from cohort 2, SD scores were computed from real-time RT-PCR data and analyzed using a GLM adjusted for age and gender. A \( P \) value <.05 was considered statistically significant. All analyses were performed using SPSS software version 16.0 for Windows (SPSS Inc., Chicago, Illinois).

**Results**

The main clinical and biochemical features of the first cohort are described in Supplemental Table 1. When subjects were stratified according to the NAFLD fibrosis score, individuals classified as at high risk of fibrosis showed an overall impaired metabolic profile and were more likely to have elevated fasting glucose \( (P < .0001) \) and IGF/type 2 diabetes \( (P < .0001) \) and metabolic syndrome \( (P < .0001) \). Notably, the highest values for IGF-I were observed in individuals classified as at low risk of fibrosis, and the lowest values were in those at high risk of fibrosis. The differences observed were little affected by further adjustment for medications for diabetes and dyslipidemia and the presence of metabolic syndrome, in addition to age and gender (Supplemental Table 1). Partial correlations between IGF-I and inflammatory biomarkers with adjustment for confounders are reported in Table 1. After adjusting for age and gender, IGF-I levels were inversely correlated with plasma high-sensitivity CRP, fibrinogen, and erythrocyte sedimentation rate and positively correlated with albumin. These correlations were little affected by further adjustment for medications for diabetes and dyslipidemia and the presence of metabolic syndrome (or diagnosis of type 2 diabetes) in addition to age and gender (Table 1). The main clinical and biochemical features of the second cohort are described in Supplemental Table 2. When subjects were classified using the Kleiner score \( (14) \), 19 subjects showed absence of fibrosis, 1 had stage 1 perisinusoidal or portal fibrosis, 18 had stage 2 perisinusoidal and portal/periportal fibrosis, 9 had stage 3 septal or
bridging fibrosis, and 3 had stage 4 cirrhosis. Stages 1 and 2 and stages 3 and 4 were thus pooled to obtain three groups with a comparable number of subjects for statistical purposes. IGF-I mRNA levels and the fibrosis score showed a negative relationship, with IGF-I expression decreasing with an increasing degree of fibrosis ($P_{<0.01}$, after correction for age and gender) (Figure 1A). Next, we wished to determine whether IGF-I directly exerts an anti-

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Abbreviations: hsCRP, high-sensitivity CRP; ESR, erythrocyte sedimentation rate.

Figure 1. IGF-I mRNA expression levels in 50 subjects with biopsy-proven NAFLD (A) and mRNA expression levels of acute-phase proteins in HepG2 hepatoma cells incubated in the presence of IGF-I (B–D). A. To compare IGF-I mRNA levels of cohort 2 subjects with different stages of fibrosis, SD scores were computed from the real-time RT-PCR data and analyzed using a GLM adjusted for age and gender ($P_{<0.01}$). Group 1, n = 19; group 2, n = 19; group 3, n = 12. B–D. HepG2 cells were incubated (black bars) or not (white bars) with IL-6 in the presence or absence of IGF-I (IGF-I 10 nM, vertical striped bars; or IGF-I 100 nM, horizontally striped bars). CRP (B), fibrinogen (C), and albumin (D) mRNA levels were assessed by RT-quantitative PCR. Data on graphs represent mean ± SD of 5 experiments run in duplicate. ***$P_{<0.001}$ IL-6 vs control; #$P_{=0.0102}$ IGF-I 10 nM + IL-6 vs IL-6; $\ddag P_{=0.0023}$ IGF-I 100 nM + IL-6 vs IL-6; $\ddagger P_{=0.0013}$ IGF-I 10 nM + IL-6 vs IL-6; &$P_{=0.0004}$ IGF-I 100 nM + IL-6 vs IL-6; *$P_{=0.05}$ IL-6 vs control; ^$P_{=0.05}$ IGF-I 10 nM + IL-6 vs IL-6; +$P_{=0.05}$ IGF-I 100 nM + IL-6 vs IL-6.
inflammatory effect in hepatic cells. IL-6 is a master cytokine controlling the expression of several hepatic acute-phase reactants including CRP, fibrinogen, and albumin (15). To gain insight into the possible anti-inflammatory role of IGF-I in hepatic acute-phase reactant expression in response to IL-6, we incubated HepG2 cells, a well-differentiated human hepatoma cell line, with IL-6 in the presence or absence of IGF-I. As compared with control cells, incubation with IL-6 resulted in a significant increase in the copy number of CRP and fibrinogen mRNA transcripts (Figure 1, B and C) and a decrease in the copy number of albumin mRNA transcripts (Figure 1D). Incubation with IGF-I, at physiological or supraphysiological concentrations, reverted the effects of IL-6, causing a down-regulation of CRP and fibrinogen mRNA transcripts, and an up-regulation of albumin mRNA transcripts (Figure 1, B–D).

**Discussion**

The present results raise the possibility that lowered protection against inflammation, ie, lower IGF-I levels, resulting in an unbalance between proinflammatory and anti-inflammatory proteins, may have a role in the development of NASH. It is, however, also possible to speculate that lowered IGF-I levels are a consequence of the presence of both NASH/advanced fibrosis and features of metabolic syndrome (abdominal obesity and insulin resistance), as indeed we have recently demonstrated that hepatic insulin resistance may affect IGF-I levels by modulating GH-stimulated synthesis of hepatic IGF-I (11). Furthermore, the increased hepatic content of inflammatory cytokines can directly affect IGF-I secretion from hepatocytes, as has been suggested in previous studies (15, 16).

Our data are in agreement with those of previous studies exploring the association of circulating IGF-I levels with hepatic fibrosis (17, 18). Interestingly, Colak et al (17) observed that serum concentrations of IGFBP-5 were significantly higher in patients with advanced fibrosis than in patients with early fibrosis; in addition, they found a significant and positive correlation between lobular inflammation and IGFBP-5. On the other hand, it has been suggested that a low IGF-I/IGFBP-3 ratio may lead to development of fibrosis in NAFLD patients (18). Unfortunately, data on serum levels of IGFBP-5, IGFBP-3, or other IGFBPs are not available for our cohorts, and their pathogenic role in the development of fibrosis in NAFLD cannot be tested in this study.

It should also be noted that, whereas the liver contributes significantly to its circulating levels, IGF-I is widely synthesized and other organs may also contribute to the levels seen in circulation, and while using liver biopsies, we were able to directly demonstrate an association between hepatic IGF-I expression and the severity of fibrosis, it is impossible to exclude that a correlation between IGF-I of a nonhepatic origin and NAFLD also exists.

The present study has several strengths, including the relatively large sample size with detailed clinical characterization, the ability to adjust for multiple confounders, the exclusion of confounding conditions such as heavy drinking, positivity for antibodies to hepatitis C virus or hepatitis B surface antigen and cirrhosis, the use of liver biopsies to confirm the association between hepatic IGF-I expression and severity of fibrosis, and the combination of in vivo, ex vivo, and in vitro approaches to assess the anti-inflammatory role of IGF-I in the liver. Nonetheless, some limitations of this study must be considered. First, in the larger cohort, NAFLD was assessed by ultrasonography, which is the most common method of diagnosing in clinical practice, although its sensitivity is suboptimal when hepatic fat infiltration is $<33\%$ (19) and the severity of fibrosis was determined applying the NAFLD fibrosis score (20), a noninvasive index for assessing the severity of fibrosis in NAFLD based on a combination of 6 clinical features and routine laboratory variables. However, we confirmed these results, assessing the association between hepatic IGF-I expression and severity of fibrosis, in a second cohort where liver biopsy, the “gold standard” method, was employed for the assessment of hepatic fibrosis. The present study is an observational investigation based on outpatients seen at a referral university hospital, representing individuals at increased risk for cardiometabolic disease, and therefore, liver biopsy may not be an appropriate investigation for many of these subjects. Noninvasive surrogate measures such as the NAFLD fibrosis score (20), which can reliably diagnose or exclude advanced fibrosis, would be clinically beneficial to reduce the need for liver biopsy. Next, to avoid an unpredictable modification of the characteristics of the original sample, we chose not to exclude the subjects treated with glucose and lipid-lowering therapies, which may attenuate inflammation-related markers. However, controlling for medication intake did not affect the results. Furthermore, although our findings are clinically and biologically plausible, causality cannot be inferred due to the cross-sectional design of the study, which should be considered hypothesis generating and requiring confirmation by further prospective studies. Finally, all subjects in the present study were Caucasians. Thus, the present results cannot be simply extrapolated to other ethnic groups. Nevertheless, we consider our results important in attempting to understand the pathophysiological interaction between inflam-
mation and IGF-I activity during progression from simple steatosis to the necroinflammatory form of NASH. Furthermore, our data suggest that the evaluation of IGF-I circulating levels may represent a noninvasive prognostic marker that may help to achieve a comprehensive assessment of the severity of the disease in individuals with NAFLD.

Acknowledgments

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