Proinflammatory Cytokines, Insulin Resistance, and Insulin Secretion in Chronic Hepatitis C patients

A case-control study

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OBJECTIVE — The purpose of this study was to explore the initial pathogenic mechanisms of diabetes associated with hepatitis C virus (HCV) infection.

RESEARCH DESIGN AND METHODS — Insulin resistance, proinflammatory cytokines, and β-cell function were evaluated in a case-control study. A total of 28 consecutive nondiabetic patients with chronic hepatitis C were included in the study (anti-HCV$^+$). Fourteen patients with chronic hepatitis other than HCV infection served as the control group (anti-HCV$^-$). Both groups were closely matched by the main clinical variables associated with insulin resistance and the degree of liver fibrosis. In addition, there were no differences between groups regarding hepatic insulin extraction measured by calculating the ratio between C-peptide and insulin. Serum levels of proinflammatory cytokines (tumor necrosis factor [TNF]-α, soluble TNF receptor [sTNFR] 1, soluble TNFR2, and interleukin-6) were measured by enzyme-linked immunosorbent assay. Insulin resistance (homeostasis model assessment [HOMA] of insulin resistance [HOMA-IR]) and insulin secretion at baseline (HOMA-β) and after various stimulus (oral glucose tolerance test, standard food intake, and intravenous glucagon) were determined by previously validated mathematic indexes.

RESULTS — HOMA-IR was higher in anti-HCV$^+$ than in anti-HCV$^-$ patients (4.35 ± 2.27 vs. 2.58 ± 1.74; $P = 0.01$). All the proinflammatory cytokines analyzed were significantly higher in anti-HCV$^+$ patients than in anti-HCV$^-$ patients. In addition, sTNFR1 and sTNFR2 were directly correlated to HOMA-IR. HOMA-β as well as insulin and C-peptide responses after the intravenous glucagon test were significantly higher in anti-HCV$^+$ patients than in anti-HC$^-$ patients.

CONCLUSIONS — Insulin resistance mediated by proinflammatory cytokines, but not a deficit in insulin secretion, could be the primary pathogenic mechanism involved in the development of diabetes associated with HCV infection.

Diabetes Care **29:1096–1101, 2006**

Large community-based studies have shown that hepatitis C virus (HCV) infection is strongly associated with diabetes (1,2). In addition, a high prevalence of both diabetes and impaired fasting glucose has been reported in HCV-infected patients in comparison with other chronic liver diseases (3–5).

Although the specific mechanisms involved in the pathogenesis of diabetes associated with HCV remain to be elucidated, it seems that insulin resistance plays an essential role (6,7). High levels of proinflammatory cytokines have been found in HCV-infected patients and, thereby, they could be involved in the pathogenesis of insulin resistance associated with HCV (8,9). On the other hand, β-cell dysfunction might also be related to glucose abnormalities in HCV chronic hepatitis (10,11). However, there are no studies in which insulin secretion, insulin resistance, and proinflammatory cytokines have been measured in HCV-infected patients with chronic hepatitis in comparison with HCV$^-$ patients with other chronic liver diseases. Because type 2 diabetes is associated with both insulin resistance and a deficit of insulin secretion, as well as with high levels of proinflammatory cytokines, this condition should be excluded when the specific effect of HCV is evaluated.

On this basis, the aim of this study is to explore for the first time the initial mechanisms involved in diabetes development in HCV infection. For this purpose, insulin resistance, proinflammatory cytokines (tumor necrosis factor [TNF]-α, soluble TNF-α receptor [sTNFR] 1 and sTNFR2, and interleukin [IL]-6), and β-cell function were compared between nondiabetic noncirrhotic patients with chronic HCV infection and closely matched HCV$^-$ patients with other chronic liver diseases according to the main clinical variables influencing insulin resistance and the degree of liver fibrosis. This approach has enabled us to further understand the pathogenic mechanisms that could lead to diabetes development in HCV-infected patients.

RESEARCH DESIGN AND METHODS

— A total of 28 consecutive nondiabetic patients with chronic hepatitis due to HCV infection who were attending the outpatient liver unit of our university hospital were included in the study. Fourteen patients with chronic hepatitis other than HCV infection and

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Received for publication 21 December 2005 and accepted in revised form 31 January 2006.

Abbreviations: AUC, area under the curve; HCV, hepatitis C virus; HOMA, homeostasis model assessment; HOMA-IR, HOMA of insulin resistance; IL, interleukin; OGTT, oral glucose tolerance test; TNF, tumor necrosis factor; sTNFR, soluble TNF receptor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

DOI: 10.2337/dc05-2509

Table 1—*Clinical features and biochemical parameters of the nondiabetic patients with chronic hepatitis classified according to anti-HCV status*

	Anti-HCV$^-$	Anti-HCV$^+$	*P* value
n	14	28	
Age (years)	48.4 ± 14.3	48.5 ± 11.6	0.97
Sex (male/female)	5/9	10/18	0.63
BMI (kg/m^2)	25.9 ± 4.3	25.8 ± 3.2	0.99
Waist-to-hip ratio	0.8 ± 0.08	0.8 ± 0.06	0.68
Family history of type 2 diabetes (%)	3 (21.4)	7 (25)	0.13
Fasting glucose (mmol/l)	5.10 ± 0.61	5.27 ± 0.47	0.44
Triglycerides (mmol/l)	1.1 (0.54–2.14)	0.85 (0.50–2.27)	0.17
Aspartate aminotransferase (IU/l)	38.6 ± 15.7	37.4 ± 14.4	0.81
Alanine aminotransferase (IU/l)	58.8 ± 36.7	52.2 ± 27.6	0.52
Degree of liver fibrosis	2.57 ± 0.85	2.53 ± 0.96	0.90
Hepatic insulin extraction			
C-peptide 0 min/insulin 0 min ratio	12.80 ± 7.38	12.60 ± 8.23	0.962
C-peptide 30 min/insulin 30 min ratio	4.48 ± 1.58	5.26 ± 2.13	0.440
ΔC-peptide/Δinsulin ratio	6.48 ± 8.03	4.72 ± 1.17	0.550

Data are means ± SD or median (range).

attending the same outpatient liver unit served as control group. Both groups were carefully matched by age, sex, BMI, waist-to-hip ratio, family history of type 2 diabetes, fasting blood glucose, serum levels of triglycerides and transaminases, and the degree of hepatic fibrosis. Entry criteria included *1*) absence of diabetes and glucose intolerance (fasting blood glucose <126 and <140 mg/dl after an oral glucose tolerance test [OGTT]) and *2*) liver biopsy performed within 12 months before inclusion showing liver fibrosis stage ≤4 based on Ishak's classification (12). The reason for excluding patients with advanced liver fibrosis was that cirrhosis per se could lead to insulin resistance independently of HCV status. Exclusion criteria were *1*) active alcohol consumption (>40 g/day for men and >20 g/day for women) or features of alcoholic disease in the liver biopsy; *2*) previous treatment with interferon [because interferon can produce a transient increase of insulin resistance (13), followed by a subsequent phase of recovery or even favorable effects on insulin sensitivity in long-term treatment (14)], *3*) treatment with corticosteroids or any other medication known to affect glucose tolerance or insulin secretion, and *4*) the presence of other concomitant diseases or conditions such as HIV infection, hemochromatosis, chronic pancreatitis, neoplasia, renal failure, or other serious medical problems.

Informed written consent was obtained from all participants. The study was approved by the hospital's human ethics committee.

Laboratory assessments

After an overnight fast of 12 h, venous blood was collected from the antecubital vein. Serum glucose, alanine aminotransferase, aspartate aminotransferase, and triglyceride levels were determined by standard laboratory techniques used in clinical chemistry laboratories. Anti-HCV was measured using a second-generation commercial enzyme immunoassay (Abbott Laboratories, Chicago IL). All anti-HCV$^+$ patients were HCV-RNA$^+$ as confirmed by HCV-RNA qualitative testing (Amplicor; Roche Molecular Systems, Branchburg, NJ).

Hemochromatosis gene mutations C282Y and H63D were screened for using enzymatic digestion of PCR products encompassing the mutation sites as previously described (15). The restrictive enzymes used were *Rsa*I for the C282Y mutation and *Bcl*I for the H63D mutation.

Serum insulin was determined by radioimmunoassay (INSI-CTK IRMA; DiaSorin, Reutlinger, Germany), and serum C-peptide was estimated by a competitive immunoassay (C-PEP-RIA-CT; BioSource Europe, Nivelles, Belgium). TNF-α signals through at least two known cell surface receptors (sTNFR1 and sTNFR2), and membranous shedding of these receptors reflects activation of the TNF system. Circulating levels of TNF-α are highly variable but sTNFRs are more stable proteins, thus remaining elevated in the systemic circulation for longer periods of time and, therefore, being better markers for the activation of the TNF-α system than TNF-α itself (16). TNF-α, sTNFR1, sTNFR2, and IL-6 concentrations were measured by enzyme-linked immunosorbent assay (Quantikine; R&D Systems Europe, Abingdon Lane, U.K.).

Insulin resistance was determined by the homeostasis model assessment (HOMA-IR) according to the formula: HOMA-IR = [fasting glucose (millimoles per liter) × fasting insulin (milliunits per liter)]/22.5. Insulin secretion was calculated as the HOMA-β cell index according to the equation: HOMA-β = [fasting insulin (milliunits per liter) × 20]/[fasting glucose (millimoles per liter) − 3.5]. Both models have been previously validated against clamp measurements (17). Apart from the HOMA-β cell index, insulin secretion was also evaluated as the area under the curve (AUC) of insulin and C-peptide obtained from three dynamic tests performed over a period of 14 days. These were *1*) an OGTT with 75 g of glucose, *2*) standard food (200 ml, 150 kcal: 6 g of proteins, 5.8 g of lipids, and 18.4 g of carbohydrates; Fortisip; Nutricia, Madrid, Spain) administered in a 10-min period, and *3*) the intravenous administration of 1 mg of glucagon (Novo Nordisk Pharma, Bagsvaerd, Denmark). Blood samples were collected at 0, 30, 60, 90, and 120 min for OGTT; at 0, 30, 60, 90, 120, and 180 min for the standard food test; and at 0, 5, and 15 min for the glucagon test. The AUC was calculated using the GW-Basic 3.22 package (© 1987 Microsoft) for Windows. In addition, the first (1st PH) and second phase (2nd PH) of insulin release predicted by the OGTT were calculated as follows:

Table 2—*Insulin resistance and β-cell function of the nondiabetic patients with chronic hepatitis classified according to anti-HCV status*

	Anti-HCV−	Anti-HCV$^+$	*P* value
HOMA-IR	2.58 ± 1.74	4.35 ± 2.27	0.01
Insulin (pmol/l)	66.2 ± 41.7	114.2 ± 57.6	0.01
C-peptide (pmol/l)	744 ± 408	1257 ± 275	0.03
HOMA-β	151.5 ± 111.6	229.6 ± 147.1	0.02
1st phase (pmol)	1,380.5 ± 580.8	1,608.6 ± 709.9	0.45
2nd phase (pmol)	365.4 ± 133.1	418.8 ± 167.1	0.45
OGTT	3,482.1 ± 729.3	4,381.9 ± 2969	0.42
AUC C-peptide (pmol · l^{-1} · min^{-1})	521.7 ± 270.8	626.5 ± 439.3	0.54
AUC insulin (pmol · l^{-1} · min^{-1})	63.1 ± 21.5	70.4 ± 25.1	0.31
AUC insulin/glucose (pmol/mmol)			
Standard food intake:	2,173.2 ± 968.2	2,493.2 ± 449.4	0.35
AUC C-peptide (pmol · l^{-1} · min^{-1})	250.2 ± 138	328.6 ± 162.4	0.43
AUC insulin (pmol · l^{-1} · min^{-1})	39.2 ± 20.8	50.4 ± 16.9	0.50
AUC insulin/glucose (pmol/mmol)			
Glucagon test			
AUC C-peptide (pmol · l^{-1} · min^{-1})	1,640.6 ± 882.9	2,295.7 ± 936.6	0.08
AUC insulin (pmol · l^{-1} · min^{-1})	270.0 ± 151.7	405.3 ± 156.5	0.04
AUC insulin/glucose (pmol/mmol)	36.8 ± 24.9	59.6 ± 27.9	0.04

Data are means ± SD.

$$1\text{st PH} = 1{,}283 + 1.829 \times \text{Ins}_{30} - 138.7 \times \text{Gluc}_{30} + 3.772 \times \text{Ins}_0$$

$$2\text{nd PH} = 287 + 0.4164 \times \text{Ins}_{30} - 26.07 \times \text{Gluc}_{30} + 0.9226 \times \text{Ins}_0$$

These indexes were used because they were the best correlated with the hyperglycemic clamp ($r = 0.78$ and 0.79, respectively) in an adult nondiabetic white population with an ethnicity and a range of age and BMI similar to patients included in our study (18).

Hepatic insulin extraction was assessed by calculating the molar ratio between C-peptide and insulin in the fasting state and at 30 min after OGTT and by the molar ratio of the incremental areas over 2 h between C-peptide and insulin (19).

Statistical analysis

Comparisons between groups were made using the Student's *t* test for continuous variables and the χ^2 test for categorical variables. Results are expressed as the mean ± SD or as median (range). All *P* values are based on a two-sided test of statistical significance. Significance was accepted at the level of $P < 0.05$. Statistical analyses were performed with the SSPS statistical package.

RESULTS — The clinical and biochemical features of patients included in the study are shown in Table 1. Both groups were well-matched for age, sex, BMI, waist-to-hip ratio, fasting glucose, triglyceride and transaminase levels, degree of liver fibrosis, and hepatic insulin extraction.

The results for insulin resistance and insulin secretion are shown in Table 2. Basal insulin levels were significantly higher in anti-HCV$^+$ patients, thus leading to higher HOMA-IR values in comparison with anti-HCV$^-$ patients (4.35 ± 2.27 vs. 2.58 ± 1.74; $P = 0.01$). HOMA-β was significantly higher in anti-HCV$^+$ than in anti-HCV$^-$ patients (229.6 ± 147.2 vs. 151.5 ± 111.6; $P = 0.02$). The first and second phases of insulin release after OGTT were higher in anti-HCV$^+$ patients but without statistical significance. In addition, we did not detect any significant difference between groups in the insulin secretory indexes after OGTT and the standard food test. However, anti-HCV$^+$ patients showed a higher AUC of insulin and ratio of insulin to glucose after the glucagon test.

Serum levels of TNF-α, sTNFR1, sTNFR2, and IL-6 were higher in anti-HCV$^+$ than in anti-HCV$^-$ patients (Table 3). Furthermore, sTNFR1 and sTNFR2 were directly correlated to HOMA-IR (Fig. 1).

CONCLUSIONS — The mechanisms leading to insulin resistance and diabetes in HCV-infected patients remain to be clarified. In this study we provide the first evidence that nondiabetic HCV-infected patients have higher insulin resistance than patients with other chronic liver diseases in association with the activation of TNF-α system and high IL-6 levels. Because HCV$^+$ and HCV$^-$ patients have been closely matched by the main clinical variables related to insulin resistance, HCV infection is the single feature accounting for the differences in insulin resistance detected between the groups.

Table 3—*Proinflammatory cytokines in anti-HCV$^+$ nondiabetic patients with chronic hepatitis in comparison with anti-HCV$^-$ patients*

	Anti-HCV$^-$	Anti-HCV$^+$	*P* value
n	14	28	
TNF-α (pg/ml)	2.71 ± 3.06	6.47 ± 3.44	0.007
sTNFR1 (pg/ml)	1,494.16 ± 270.63	1,739.58 ± 347.35	0.040
sTNFR2 (pg/ml)	2,417.50 ± 815.59	2,999.16 ± 785.55	0.046
IL-6 (pg/ml)	2.07 ± 1.02	3.78 ± 1.45	0.002

Data are means ± SD.

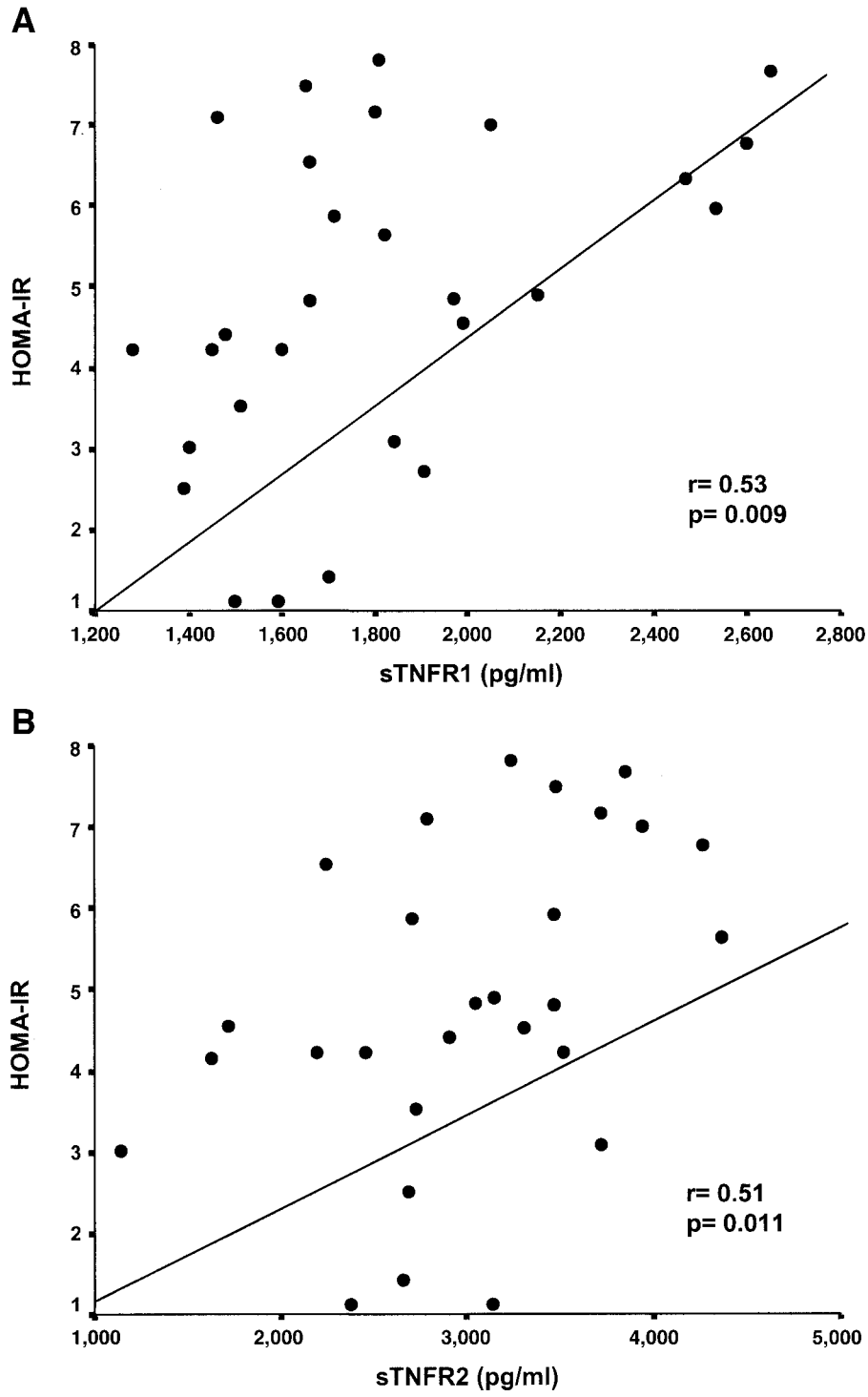

Figure 1—*Correlations of sTNFR1* (A) *and sTNFR2* (B) *with HOMA-IR in anti-HCV$^+$ patients.*

Therefore, it seems that insulin resistance will be crucial in the development of diabetes associated with HCV infection.

It could be argued that hyperinsulinemia detected in HCV-infected patients might be due to a diminished hepatic insulin degradation rate by the diseased liver, thus leading to a false increase of the HOMA-IR. However, both groups of patients (anti-HCV$^+$ and anti-HCV$^-$) had a similar degree of liver fibrosis as well as a hepatic insulin extraction index, the latter being in the same range as has been reported in healthy individuals (20).

The relation between inflammation and insulin resistance in the pathogenesis of type 2 diabetes is well known (21). Cytokines such as TNF-α and IL-6 have been related to insulin resistance and type 2 diabetes development (22–25). Several reports have shown an increase in serum levels of TNF-α and its receptors as well as IL-6 in HCV-infected patients (26–31). Moreover, liver biopsy specimens from nondiabetic HCV patients revealed significant impairments in the insulin signaling pathways (32), which were strikingly similar to TNF-α effects (33,34). Shintani et al. (7), using a transgenic mouse model that specifically expressed the HCV core protein in hepatocytes, have shown direct experimental evidence of HCV infection in the development of insulin resistance, which finally leads to the development of type 2 diabetes. In addition, in this study the role of TNF-α in the pathogenesis of the HCV-associated insulin resistance state is strongly suggested. Recently, Romero-Gómez et al. (6) demonstrated in nondiabetic patients that the clearance of HCV after antiviral therapy induces an improvement in insulin resistance, but no data on proinflammatory cytokines was reported. In the present study, proinflammatory cytokines in HCV$^+$ and HCV$^-$ patients with chronic hepatitis without diabetes and without advanced liver fibrosis have been compared for the first time. The increased levels of sTNFR1 and sTNTR2 detected in HCV-infected patients and their relationship with HOMA-IR strongly suggest that TNF-α is a mechanism by which HCV-infected patients are more prone to develop type 2 diabetes than patients with other chronic liver diseases.

A deficit in insulin secretion is another potential mechanism involved in diabetes associated with HCV infection. HCV infection has been linked to immunologic disorders such as cryoglobulinemia, glomerulonephritis, thyroiditis, and Sjögren syndrome (35). In addition, HCV shares regional amino acid homology with pancreatic islet autoantigens (36). Therefore, it might be thought that HCV could trigger an immune reaction against the β-cell, leading to diabetes. However, in a previous study we ruled out autoimmunity phenomena as a mechanism involved in diabetes associated with HCV infection (37). Direct HCV-induced damage in β-cells might be another mechanism involved in insulin secretion impairment. Certain viruses such as mumps and Coxsackie have been found to infect human β-cells (38–40). Although HCV is an hepatotropic virus, it has also been identified in extrahepatic tissues, including the pancreas. Recently,

Masini et al. (10) have detected virus-like particles in pancreatic β-cells from HCV^+ donors associated with morphological changes and a reduced in vitro glucose-stimulated insulin release. However, we have not found a decrease of β-cell function in nondiabetic anti-HCV^+ patients in comparison with anti-HCV^- patients with chronic hepatitis. By contrast, in HCV-infected subjects, we observed an increase of insulin secretion assessed by HOMA-β and the glucagon test. These results suggest that the insulin secretory function is preserved in HCV-infected patients when insulin resistance is already present. Furthermore, insulin secretion is increased in the initial stages of HCV infection to compensate for insulin resistance. Obviously, the transition from normal to impaired fasting glucose and diabetes will require the progressive deterioration of insulin secretion.

In summary, our results support the hypothesis that insulin resistance mediated by proinflammatory cytokines, but not a deficit in insulin secretion, is the primary pathogenic mechanism involved in the development of diabetes associated with HCV infection. However, prospective studies to confirm this issue are needed.

Acknowledgments— This study was supported by grants from the Fundació La Marató TV3 (99/2610), Novo Nordisk Pharma (01/0066), and the Instituto de Salud Carlos III (G03/212, C03/08, and C03/02).

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