Lower Rate of Tumor Necrosis Factor-α −863A Allele and Higher Concentration of Tumor Necrosis Factor-α Receptor 2 in First-Degree Relatives of Subjects With Type 2 Diabetes

A. Costa, J.M. Fernández-Real, J. Vendrell, M. Broch, R. Casamitjana, W. Ricart, and I. Conget

Allelic variants of the tumor necrosis factor-α (TNF-α) gene seem to contribute to insulin resistance increasing the transcription rate of TNF-α. The TNF-α −863A allele is associated with a lower expression of TNF-α gene and less secretion of the cytokine. To investigate whether an abnormal TNF-α system regulation may contribute to early impairment of insulin action in first-degree relatives of patients with type 2 diabetes mellitus (DM), we studied the TNF-α -863C/A polymorphism and the soluble fraction of TNF-α receptor-2 (sTNFR2) concentration in these subjects in comparison to a control group. A total of 52% of subjects in the relatives' group showed an abnormal oral glucose tolerance (either as impaired glucose tolerance [IGT] or diabetes) and had more features of the insulin resistance syndrome, despite showing similar body composition as controls. The plasma concentration of the sTNFR2 was higher and insulin sensitivity (%S) was lower in the relatives' group than in the controls. Likewise, the TNF-α −863A allele was more commonly detected in the control group (10 of 41) than in the relative's group (2 of 36, $P = .029$). In a multivariate linear regression analysis, neither TNF-α −863A allele nor sTNFR2 independently determined %S. Only body mass index (BMI) and the presence of a positive family history of DM were independent determinants of insulin resistance. In summary, our study showed a lower rate of TNF-α −863A allele and higher concentrations of sTNFR2 in first-degree relatives of DM subjects. These findings could be included among the genetic, metabolic, and clinical heterogeneity that characterizes the pathophysiology of DM. The presence of abnormalities in the TNF-α pathway could predispose to the development of DM in subjects at risk for the disease.

THERE IS increasing evidence supporting the concept that in the presence of an insulin resistance phenotype allelic variants of the tumor necrosis factor-α gene (TNF-α) could contribute to impairment of insulin action and to type 2 diabetes mellitus (DM) through increased transcription rates of TNF-α. In that sense, TNF-α has been shown to play a key role in mediating insulin resistance as a result of obesity.[1,2] The plasma concentration of its soluble receptor-2 (sTNFR2), a surrogate of TNF-α action, circulates in proportion to the impairment of insulin action, independently of body composition.[3,4]

TNF-α −863C/A polymorphism has been recently identified as an important modulator of TNF-α secretion from adipose tissue among non-obese subjects,[5] suggesting that this mutation may have a functional role in the adipokinin network. In fact, the presence of the −863A allele induces a low expression of TNF-α gene and thereby less secretion of TNF-α cytokine.[6] Interestingly, sTNFR2 concentration is lower and insulin sensitivity (%S) significantly higher in healthy subjects carrying the −863A allele.[7]

In offspring of DM patients, some studies suggest that TNF-α does not play a major role in insulin resistance.[8] However, TNF-α values do not usually give precise information about its action in some conditions, such as obesity. In contrast, plasma sTNFR2 concentration is a much more stable protein and serves as a precise predictor of TNF-α system activation.[3] A family history of DM is associated with an increased risk of having diabetes.[9,10] In such a group of subjects, it is possible to identify early metabolic disturbances even before abnormal glucose tolerance becomes clinically significant.[11-13] In addition, it is well demonstrated that features of insulin resistance occur more frequently in first-degree relatives of DM patients than in subjects without a family history.[14] In fact, it is well known that DM is an heterogeneous disorder with strong genetic and environmental components. Its inheritance is polygenic, and it is likely that the simultaneous presence of several susceptible genes is required for the development of the disease.[10,15,16] Thus, it seems that an abnormal TNF-α system regulation might contribute to the pathophysiology of diabetes in first-degree relatives of DM patients. To test this hypothesis, we studied the TNF-α −863C/A polymorphism and sTNFR2 concentration in first-degree relatives of DM patients in comparison to a normal control group.

SUBJECTS AND METHODS

Subject Assessment

After approval by the local medical ethics committee, 40 first-degree relatives of DM patients and 52 control subjects were included in the study. Subjects were recruited from the outpatient clinics of the Diabetes Units, who attended a diabetes-screening program. None had known their oral glucose tolerance state before inclusion in this study. The control group included subjects with normal glucose tolerance (NGT), a body mass index (BMI) between 25 and 35 kg/m^2, and without family history of DM.

Subjects' physical examination included height, weight, BMI, and systolic/diastolic blood pressure (SBP/DBP, the values measured were recorded in triplicate for all included subjects). Percent body fat was determined by bioelectric impedance analysis (Holtain BC Analyzer, Crosswell, UK). Surface electrodes were applied to the skin surface (wrist and ankle). An alternating electrical current was passed through

From the Endocrinology and Diabetes and Hormonology Units, Hospital Clínic i Universitari de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona; Diabetes, Endocrinology and Nutrition Unit, Hospital Universitari de Girona, Girona; and the Endocrinology Unit, Research Unit, Hospital Universitari Joan XXIII de Tarragona, Universitat Rovira i Virgili, Tarragona, Spain.

Submitted October 24, 2002; accepted February 7, 2003.

Address reprint requests to I. Conget, MD, Servei d'Endocrinologia i Diabetes, Hospital Clínic i Universitari, Villarroel 170, 08036, Barcelona, Spain.

0026-0495/03/5208-0046$30.00/0
doi:10.1016/S0026-0495(03)00108-2

Table 1. Clinical Characteristics

	DM Relatives Group	Control Group	*P*	*P**
Gender (M/F)	21/19	21/31	.294	–
Age (yr)	51.4 ± 12.7	43.3 ± 12.3	.002	–
BMI (men, kg/m^2)	27.0 ± 3.8	27.2 ± 4.1	NS	NS
BMI (women, kg/m^2)	29.5 ± 4.7	29.0 ± 4.6	NS	NS
FM, men (kg)	18.7 ± 10.6	16.5 ± 9.0	NS	NS
FM, women (kg)	30.6 ± 9.4	20.8 ± 10.2	.024	NS
FFM, men (kg)	60.9 ± 8.9	65.8 ± 10.8	NS	NS
FFM, women (kg)	48.9 ± 6.1	47.2 ± 5.6	NS	NS
SBP (mm Hg)	120.9 ± 15.1	121.4 ± 10.0	NS	NS
DBP (mm Hg)	77.6 ± 8.9	70.4 ± 9.9	.003	.042

Abbreviations: BMI, body mass index; FM, fat mass; FFM, fat-free mass; SBP, systolic blood pressure; DBP, diastolic blood pressure; NS, not significant.

**P* value after adjusting for age.

the distal electrode, and the proximal electrode detected a lowering in current. The reduction in current, or total body impedance, was recorded and fat mass and fat-free mass were calculated.

Oral Glucose Tolerance Test

After a 12-hour overnight fast, 3 baseline blood samples (−10, −5, and 0 minutes) were collected to measure insulin and glucose levels. Afterwards, a 75-g oral glucose tolerance test (OGTT) was performed. The test was preceded by at least 3 days of unrestricted diet and usual physical activity. Baseline samples were used to calculate %S and β-cell function (%β) using the computerized homeostasis model assessment (HOMA) method. The model assumes that the principal differences between individuals can be expressed in terms of differences in relative β-cell responsiveness to glucose and in peripheral and hepatic sensitivity to insulin and glucose. β-cell function and %S are expressed as percentages of those in a reference young lean population, set at 100%.[17] Two hours glycemia in the OGTT was used to classify subjects as NGT, impaired glucose tolerance (IGT), and diabetes, according to World Health Organization (WHO) criteria.[18] Total cholesterol, triglycerides, leptin, and TNFR2 levels were also measured in baseline samples.

TNF-α Gene Polymorphism Analysis

A 10-mL venous blood sample was collected in an EDTA container in which DNA was isolated from peripheral leukocytes. The transition polymorphism C to A in the −863 position of the gene was studied. A total of 100 ng DNA was used as a template. The primers used were as follows: 5′ GGCTCTGAGGAATGGGTT3′ y 5′ CTACATGGCCCT-GTCTTCGTTACG3′ (GIBCO-BRL, Crewe, UK). The reaction was performed in a final volume of 25 μL containing 0.5 U Taq DNA polymerase (GeneCraft, Munster, Germany), 1 mmol/L $MgCl_2$, 0.2 mmol/L dNTP (Amersham Pharmacia, Uppsala, Sweden), and 0.2 μmol/L of each primer. DNA was amplified during 35 cycles with 30 seconds denaturalization at 94°C, 1 minute annealing at 63.2°C, and 2 minutes extension at 72°C. Finally, a 72°C, 5-minute extension was performed. The amplified fragment (126 bp) was digested with Bsa A I restriction enzyme (New England Biolabs, Beverly, MA) at 37°C for 2 to 4 hours and electrophoreses on a 2.5% agarose gel. This enzyme produced 3 bands of different sizes: a 126-bp fragment corresponding to the −863C allele (restriction side absent) and a set of 103 and 23 bp corresponding to the −863A allele (restriction side present).

Analytical Procedures

Glucose levels were measured using the hexokinase method. Enzymatic methods were used to measure cholesterol and triglyceride levels (cholesterol esterase and cholesterol oxidase for the former and glycerol kinase and glycerol phosphate oxidase for the latter). The levels of these 3 constituents were measured in a DAX 72 analyzer (Bayer Diagnostics, Tarrytown, NY) using the reagents supplied by the man-

Table 2. Metabolic Characteristics

	DM Relatives Group	Control Group	*P*	*P**
Fasting glycemia (mmol/L)	5.8 ± 1.0	5.0 ± 0.6	.000	.001
HbA_{1c} (%)	5.3 ± 0.6	4.7 ± 0.3	.000	.017
Cholesterol (mmol/L)	5.8 ± 1.0	5.2 ± 0.9	.002	.048
HDLc (mmol/L)	1.5 ± 0.4	1.4 ± 0.4	NS	NS
LDLc (mmol/L)	3.7 ± 1.0	3.3 ± 0.8	NS	NS
Triglycerides (mmol/L)	1.6 ± 1.5	1.2 ± 0.6	NS	NS
Insulinemia (pmol/L)	86.4 ± 58.7	66.5 ± 38.0	.053	NS
β%	108.7 ± 55.9	120.0 ± 42.7	NS	NS
S%	67.8 ± 31.0	88.1 ± 41.5	.011	.074
Leptin, men (ng/mL)	9.5 ± 7.5	6.6 ± 5.9	.020	NS
Leptin, women (ng/mL)	31.3 ± 16.7	23.5 ± 14.9	NS	NS
sTNFR2, men (ng/mL)	8.7 [2.3-13.2]	3.0 [1.0-5.1]	.000	.001
sTNFR2, women (ng/mL)	10.0 [3.0-19.1]	3.5 [1.0-5.9]	.000	.001

NOTE. Triglycerides, leptin, and sTNFR2 were log-transformed for statistics. However, their results are presented untransformed to improve comprehension.

Abbreviations: β%, beta-cell function; S%, insulin sensitivity.

**P* value after adjusting for age.

ufacturer. Insulin levels were determined by immunoradiometric assay (IRMA) (Medgenix Diagnostics, Fleurus, Belgium) with a coefficient of variation within and between assays of 5.2% and 6.9%, respectively. No cross-reaction with proinsulin was detected. Serum leptin concentrations were measured by radioimmunoassay (Linco Research, St Charles, MO). The lower limit of detection was 0.5 ng/mL. Coefficients of variation intra- and interassay were $< 7\%$ and $< 8\%$, respectively. The radioimmunoassay for leptin does not present cross-reaction with human proinsulin, insulin, or glucagon. Plasma levels of sTNFR2 were determined by a solid-phase enzyme-amplified sensitivity immunoassay (ELISA) performed on a microtiter as described elsewhere.[19] The intra- and interassay coefficients of variation were $< 7\%$ and $< 9\%$, respectively.

Statistical Analysis

The results are presented as mean ± SD, median, minimum, maximum, and proportions. Non-Gaussian-distributed variables were log-transformed to achieve normality. This applied to serum leptin, sTNFR2, and triglycerides. The different groups were compared using the Student's t test, Mann-Whitney U test, or the χ^2 test for comparisons of proportions. Subjects with the TNF-α −863A allele (C/A and A/A) were grouped in the same category, because both showed decreased TNF-α gene expression.[5] The relationship between variables was determined by Pearson's correlation coefficient and multiple linear regression analysis. The last one was performed to evaluate the contribution of the insulin resistance syndrome variables, TNF-α −863A allele, sTNFR2, and family history of DM to the degree of %S. All cases located beyond the Cook distance were excluded from analysis. Significance was defined as $P < .05$ for all statistical tests, and these were performed using version 6.1.3 for Windows of the SPSS (SPSS, Chicago, IL) computer program package.

RESULTS

All control subjects were NGT, whereas among the group of relatives, 21 subjects showed abnormal oral glucose tolerance, either as IGT (35%) or as diabetes (17%). Tables 1 and 2 show the clinical and the metabolic characteristics of the different groups.

Total plasma cholesterol concentration and age were significantly higher in the relatives' group. Although not statistically significant, they displayed a trend towards higher basal insulinemia and triglyceridemia ($P = .066$). Despite subjects from the relatives' group having the same body composition as controls, the relatives showed more features of the insulin resistance syndrome; higher rates of DBP, fasting glucose, and lower %S. After controlling for age, the relatives' subjects still maintained significant higher values of DBP, fasting glucose, and a trend towards a lower %S ($P = .074$).

TNF-α −863A allele was overrepresented in the control group (10 of 41) in comparison to the relative's group (2 of 36, $P = .029$). Circulating sTNFR2 concentration was significantly higher in the relatives' group than in the controls, but it was similar in TNF-α −863A and TNF-α −863C allele carriers, as a whole.

Serum sTNFR2 concentration positively correlated with BMI and leptin and inversely with %S (Fig 1). A multiple regression analysis showed that BMI ($r = -.305$, $P = .042$) and family history of DM ($r = -.219$, $P = .050$) inversely contributed to the variance of %S ($R^2 = .35$ $P = .001$). By contrast, neither the TNF-α −863 polymorphism nor the sTNFR2 concentration, age, DBP, leptin and triglycerides, independently determined the %S.

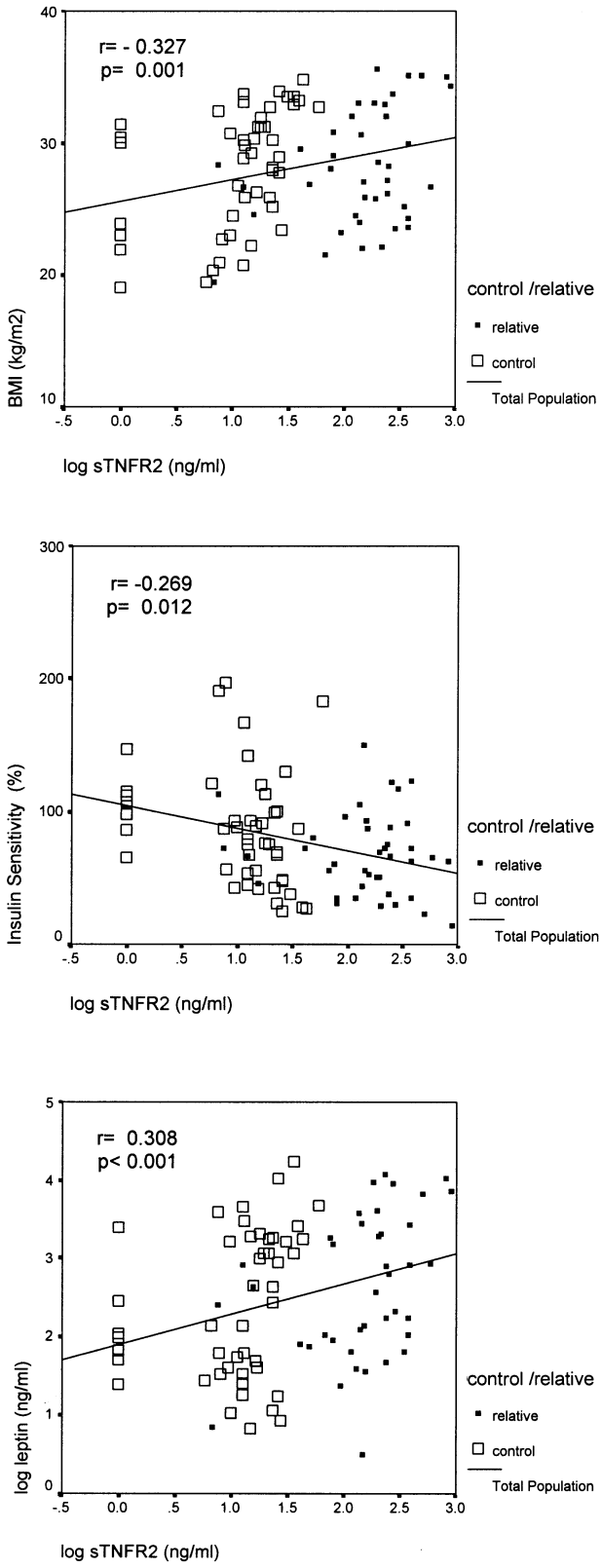

Fig 1. sTNFR2 correlations with BMI, %S, and leptin.

DISCUSSION

In the present study, we found that in a group of first-egree relatives of DM patients, the protective TNF-α −863A allele was less frequent that in NGT subjects without a family history of DM. In contrast to the TNF-α –308 gene polymorphism, the −863A allele was not associated with differences in body composition in control subjects or in relatives.[20] We also showed that sTNFR2 concentration, according to gender, was higher in the group of relatives than in control subjects. In contrast to these results, neither the TNF-α −863C/A polymorphism nor the sTNFR2 concentration independently determined %S. However, an overadjustment of highly associated variables cannot be excluded.

It has been reported that the TNF-α −863A allele is linked to a decreased TNF-α system activation, represented by sTNFR2 concentration.[5,7] This uncommon allele leads to a more insulin-sensitive phenotype, which could be protective against DM risk.[7] Previous data are in agreement with present results showing a very low proportion of the protective −863A allele in DM relatives, who already displayed an important rate of impaired glucose homeostasis. At the same time, the sTNFR2 concentration was higher in the group of relatives despite similar body composition as control subjects. Our results are also consistent with recent observations in lean offspring of DM subjects who showed higher concentrations of sTNFR2 negatively associated with %S.[21] Nevertheless, we could not attribute a preponderant contribution of TNF-α −863C/A polymorphism to the final sTNFR2 concentration.

Interestingly, first-degree relatives also displayed more features of insulin resistance, despite showing a similar body composition (BMI, fat mass, and fat-free mass) as control subjects. In this context, no significantly different leptin concentrations were observed between groups despite higher concentrations of sTNFR2 in relatives. Other TNF-α gene promoter polymorphisms are associated with increased fat mass and concomitantly raised circulating leptin levels.[20] According to the present findings, the TNF-α −863A polymorphism does not significantly contribute to differences in body fat or serum leptin concentration.[20] This piece of information probably confirms the importance of other genetic background contributing to differences in TNF-α secretion and/or action. It should be pointed out that one of the possible shortcomings of this study is the small number of TNF-α −863A allele carriers in the relatives' group.

In summary, our study demonstrates a lower rate of TNF-α −863A allele carriers and higher concentrations of sTNFR2 in first-degree relatives of DM subjects. These findings could be included among the genetic, metabolic, and clinical heterogeneity that characterizes the natural history of DM. The presence of abnormalities in the TNF-α pathway could predispose to the development of DM in subjects at risk for the disease.

REFERENCES

1. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. Science 259:87-91, 1993
2. Saghizadeh M, Ong JM, Garvey WT, et al: The expression of TNF alpha by human muscle. Relationship to insulin resistance. J Clin Invest 97:1111-1116, 1996
3. Aderka D, Engelmann H, Maor Y, et al: Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. J Exp Med 175:323-329, 1992
4. Fernandez-Real JM, Broch M, Ricart W, et al: Plasma levels of the soluble fraction of tumor necrosis factor receptor 2 and insulin resistance. Diabetes 47:1757-1762, 1998
5. Skoog T, Eriksson P, Hoffstedt J, et al: Tumour necrosis factor-alpha (TNF-alpha) polymorphisms-857C/A and - 863C/A are associated with TNF-alpha secretion from human adipose tissue. Diabetologia 44:654-655, 2001
6. Skoog T, Van't Hoof FM, Kallin B, et al: A common functional polymorphism (C → A substitution at position −863) in the promoter region of the tumour necrosis factor-α (TNF-α) gene associated with reduced circulatimg levels of TNF-α. Hum Mol Genet 8:1443-1449, 1999
7. Fernandez-Real JM, Broch M, Vendrell J, et al: Letter. Diabetologia 45:149-150, 2002
8. Kellerer M, Rett K, Renn W, et al: Circulating TNF-alpha and leptin levels in offspring of NIDDM patients do not correlate to individual insulin sensitivity. Horm Metab Res 28:737-743, 1996
9. Martin BC, Warram JH, Krolewski AS, et al: Role of glucose and insulin resistance in development of type 2 diabetes mellitus: Results of a 25-year follow-up study. Lancet 340:925-929, 1992
10. Granner DK, O'Brien RM: Molecular physiology and genetics of NIDDM. Importance of metabolic staging. Diabetes Care 15:369-395, 1992
11. Eriksson J, Franssila-Kallunki A, Ekstrand A, et al: Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus. N Engl J Med 321:337-343, 1989
12. O'Rahilly S, Turner RC, Matthews DR: Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes. N Engl J Med 318:1225-1230, 1988
13. Costa A, Rios M, Casamitjana R, et al: High prevalence of abnormal glucose tolerance and metabolic disturbances in first degree relatives of NIDDM patients. A study in Catalonia, a mediterranean community. Diabetes Res Clin Pract 41:191-196, 1998
14. Stewart MW, Humphriss DB, Berrish TS, et al: Features of syndrome X in first-degree relatives of NIDDM patients. Diabetes Care 18:1020-1022, 1995
15. Turner RC, Hattersley AT, Shaw JT, et al: Type II diabetes: Clinical aspects of molecular biological studies. Diabetes 44:1-10, 1995
16. Aitman TJ, Todd JA: Molecular genetics of diabetes mellitus. Baillieres Clin Endocrinol Metab 9:631-656, 1995
17. Matthews DR, Hosker JP, Rudenski AS, et al: Homeostasis model assessment: Insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28:412-419, 1985
18. Alberti KG, Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15:539-553, 1998
19. Kirchgessner TG, Uysal KT, Wiesbrock SM, et al: Tumor necrosis factor-alpha contributes to obesity-related hyperleptinemia by regulating leptin release from adipocytes. J Clin Invest 100:2777-2782, 1997
20. Fernandez-Real JM, Gutierrez C, Ricart W, et al: The TNF-alpha gene Nco I polymorphism influences the relationship among insulin resistance, percent body fat, and increased serum leptin levels. Diabetes 46:1468-1472, 1997
21. Straczkowski M, Kowalska I, Stepien A, et al: Increased plasma soluble tumor necrosis factor α receptor 2 level in lean nondiabetic offspring of type 2 diabetic subjects. Diabetes Care 25:1824-1828, 2002