TNFRSF1B in Genetic Predisposition to Clinical Neuropathy and Effect on HDL Cholesterol and Glycosylated Hemoglobin in Type 2 Diabetes

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OBJECTIVE — Genetic variation in the tumor necrosis factor (TNF) receptor 2 gene (TNFRSF1B) has shown association with insulin resistance in type 2 diabetes, hypercholesterolemia, coronary artery disease, and essential hypertension. Here we tested the TNFRSF1B marker used in the latter studies in type 2 diabetes patients.

RESEARCH DESIGN AND METHODS — A case-control study of a microsatellite marker with five alleles (CA13–CA17) in intron 4 of TNFRSF1B was performed in 337 well-characterized white patients and 183 healthy control subjects.

RESULTS — The CA16 allele was associated with clinical neuropathy (frequency = 27% in 69 patients with the condition versus 16% in 230 subjects without the condition; χ² = 9.0, P = 0.011; odds ratio = 2.1 [95% CI 1.2–3.8]). No association was seen with other complications or diabetes itself. The CA16 allele tracked with elevation plasma HDL cholesterol (1.3 ± 0.2, 1.7 ± 0.2, 2.1 ± 0.2 for CA16/CA16, CA16/–, and –/–, respectively; n = 9, 110, and 218, respectively; P = 0.009) and reduction in plasma glycosylated hemoglobin (6.6 ± 0.3, 8.3 ± 0.2, and 8.1 ± 0.1 for CA16/CA16, CA16/–, and –/–, respectively; n = 9, 102, 205, respectively; P = 0.007). Significance remained after Bonferroni correction for multiple testing.

CONCLUSIONS — Genetic variation in or near TNFRSF1B may predispose clinical neuropathy, reduced glycosylated hemoglobin, and increased HDL cholesterol in type 2 diabetes patients. The latter could be part of a protective response.

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Damage to neural and other tissues in type 2 diabetes may be contributed by genetic factors other than those responsible for the disease itself. Such damage arises from hyperglycemia-induced accumulation of end products of glycosylation, and inflammatory mediators are of interest in the underlying pathology. One of these is tumor necrosis factor (TNF-α), the action of which involves local paracrine effects mediated by two receptors (TNF receptor 1 [TNF-R1] and TNF receptor 2 [TNF-R2]) that are expressed by most cells in the body. TNF-R2 responds to TNF-α by markedly upregulating its mRNA, whereas TNF-R1 mRNA is unchanged (1,2). The N-terminal extracellular domain of the 75-kDa, 415-residue TNF-R2 is then shed by hydrolysis at amino acid 211 (3) to give 40-kDa plasma soluble TNF-R2 (sTNF-R2) (4). sTNF-R2 neutralizes TNF-α at high concentrations but, when low, preserves TNF activity and helps sequester TNF to its membrane receptors to increase long-term effects (5). TNF-R2, but not TNF-R1, is increased in peripheral vascular disease and other conditions involving cell damage (6). Recombinant sTNF-R2 is, moreover, used therapeutically to inhibit TNF-α (7). TNF-R2 has higher ligand affinity and faster dissociation and may synergize with TNF-R1 to enhance its effects (8). This promotes NF-κB activation and apoptosis (8). TNF-R2 also has independent (9) slow, long-term effects (1), which include cell proliferation (9), and mediates the strong stimulation by the transmembrane (pro) form of TNF (10).

We have recently published in this issue shows conventional and Systeme International (SI) units and conversion factors for many substances.
Table 1—Characteristics of type 2 diabetes patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All patients</th>
<th>With neuropathy</th>
<th>Without neuropathy</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>357</td>
<td>69</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>8.0 ± 7.6</td>
<td>9.9 ± 7.7</td>
<td>7.5 ± 7.6</td>
<td>0.036</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62 ± 12</td>
<td>68 ± 11</td>
<td>61 ± 12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age of onset (years)</td>
<td>55 ± 13</td>
<td>59 ± 13</td>
<td>54 ± 13</td>
<td>0.017</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>30.5 ± 6.6</td>
<td>30.6 ± 5.5</td>
<td>30.4 ± 7.1</td>
<td>0.79</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>82 ± 10</td>
<td>83 ± 9</td>
<td>81 ± 10</td>
<td>0.41</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>141 ± 17</td>
<td>145 ± 18</td>
<td>140 ± 17</td>
<td>0.019</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.6 ± 0.06</td>
<td>5.6 ± 0.16</td>
<td>5.6 ± 0.08</td>
<td>0.68</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.2 ± 0.02</td>
<td>1.1 ± 0.04</td>
<td>1.2 ± 0.02</td>
<td>0.29</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.1 ± 0.07</td>
<td>2.2 ± 0.16</td>
<td>2.0 ± 0.08</td>
<td>0.25</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.5 ± 0.05</td>
<td>3.4 ± 0.13</td>
<td>3.5 ± 0.06</td>
<td>0.34</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>0.10 ± 0.002</td>
<td>0.11 ± 0.004</td>
<td>0.09 ± 0.003</td>
<td>0.007</td>
</tr>
<tr>
<td>Glycosylated hemoglobin (%)</td>
<td>8.1 ± 0.1</td>
<td>8.2 ± 0.2</td>
<td>8.0 ± 0.1</td>
<td>0.39</td>
</tr>
<tr>
<td>Microalbuminuria (mg/mmol creatinine)</td>
<td>7.8 ± 1.7</td>
<td>16.7 ± 7.6</td>
<td>6.1 ± 1.7</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Data are means ± SD for the first six parameters and means ± SE for the remaining seven parameters. *P values are for comparison of those with clinical neuropathy with those without clinical neuropathy.

Australia) regularly during May 1996 through May 1997 for ongoing management and checkup. Diagnosis was by National Diabetes Data Group criteria. Complications, based on both clinical symptoms and biochemical parameters, were determined by endocrinological examination. Clinical neuropathy was assessed by a Biothesiometer (Biomedical Instruments, Newbury, OH) reading at each big toe, with clinical neuropathy defined as a reading >25 V (15). Retinopathy was determined by indirect ophthalmoscopy followed by fundus photography if an abnormality was suspected. Microalbuminuria was diagnosed from the first urine sample collected in the morning as urinary albumin-to-creatinine ratio >2.5 (for men) and >3.5 (for women) but <30 mg/mmol creatinine; two or more abnormal readings were required. If the albumin-to-creatinine ratio was >30 mg/mmol, microalbuminuria was diagnosed and 24-h urinary protein was determined. Patients were said to have had stroke or myocardial infarction if according to their medical history they had been hospitalized for either of these previously. Patient characteristics are shown in Table 1. Written consent was obtained from all patients, and the study was approved by the Ethics Committee of the University of New South Wales. The population control group used in testing the TNFRSF1B marker for association with diabetes itself involved healthy nondiabetic Anglo-Celtic white subjects recruited from the Sydney Red Cross Blood Bank. These subjects were aged 48 ± 10 years, with a male-to-female ratio of 29:21, BMI 26 ± 4 kg/m², systolic/diastolic blood pressure 120 ± 11/73 ± 8 mmHg, and plasma total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides (means ± SE) 5.2 ± 0.1, 1.3 ± 0.04, 3.2 ± 0.08, and 1.5 ± 0.08 pmol/ml, respectively.

Biochemical measurements
Glycemic control was assessed by glycosylated hemoglobin (HbA₁c) measured by ion exchange high-performance liquid chromatography. Serum creatinine, urinary albumin, and other parameters were determined by clinical chemistry.

Genotype determination
TNF receptor 2 gene in diabetes

Table 2—Comparison of allele frequencies of TNFRSF1B intron 4 microsatellite polymorphism in type 2 diabetes patients and control subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Total alleles on all chromosomes</th>
<th>CA13</th>
<th>CA14</th>
<th>CA15</th>
<th>CA16</th>
<th>CA17</th>
<th>X²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes patients</td>
<td>357</td>
<td>91 (13)</td>
<td>20 (3)</td>
<td>457 (64)</td>
<td>135 (19)</td>
<td>13 (2)</td>
<td>12.6</td>
<td>0.014</td>
</tr>
<tr>
<td>Control subjects</td>
<td>183</td>
<td>71 (19)</td>
<td>5 (1)</td>
<td>205 (36)</td>
<td>77 (21)</td>
<td>8 (2)</td>
<td>7.1*</td>
<td>0.07*</td>
</tr>
</tbody>
</table>

Data are n (%). *Value obtained after combining data for rare alleles CA13 and CA14.
Table 3—Association of CA16 allele of TNFRSF1B polymorphism with clinical neuropathy in type 2 diabetes patients

<table>
<thead>
<tr>
<th>Group</th>
<th>n*</th>
<th>CA16/CA16</th>
<th>CA16/–</th>
<th>–/–</th>
<th>χ²</th>
<th>df</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical neuropathy</td>
<td>69</td>
<td>5 (7%)</td>
<td>27 (39%)</td>
<td>37 (54%)</td>
<td>9.0</td>
<td></td>
<td>0.011</td>
</tr>
<tr>
<td>No clinical neuropathy</td>
<td>230</td>
<td>5 (2%)</td>
<td>67 (29%)</td>
<td>16 (69%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are n and n (% total). *n = number of subjects.

RESULTS — Allele frequencies of the TNFRSF1B marker in type 2 diabetes patients and control subjects are shown in Table 2. These were similar to those reported for this marker in 78 unrelated probands from Center d’Etude du Polymorphisme Humaine pedigrees (0.19, 0.02, 0.54, 0.24, and 0.01, respectively) (17), indicating that the study groups did not contain any ascertainment bias, at least as far as the TNFRSF1B polymorphism was concerned. Moreover, Hardy-Weinberg equilibrium was observed. Although a significant P value was obtained when comparing TNFRSF1B allele frequencies between patients and control subjects, this was contributed by the rare CA13 and CA14 allele, and after removing this effect by combining data for CA13 and CA14 alleles, no association with diabetes was seen. Comparison of patients with and without clinical neuropathy revealed a significant association, with elevation in CA16 frequency in the clinical neuropathy subgroup (Table 3). In a stepwise logistic regression model in which other factors including age, sex, and duration of diabetes since diagnosis were controlled for, the CA16 genotype still significantly predicted clinical neuropathy. The odds ratio (OR) to have clinical neuropathy for CA16 allele carriers (one or two CA16 alleles) compared with patients lacking a CA16 allele was 2.1 (95% CI 1.2–3.8). The OR for CA16/CA16 versus CA16/– was 2.5 (95% CI 0.6–11.2) and for CA16/CA16 versus –/– was 4.8 (95% CI 1.1–2.0). There was no significant association, however, with nephropathy, retinopathy, or other vascular complications.

The CA16 allele tracked with elevation in HDL cholesterol (Fig. 1) and a decrease in glycosylated hemoglobin (Fig. 2). This remained significant after Bonferroni correction for multiple comparisons (P = 0.013 for HDL cholesterol and P = 0.008 for glycosylated hemoglobin). The significant association between CA16 genotypes and HDL cholesterol were further confirmed (F = 4.7, P = 0.009) in a factorial design of univariate ANOVA in which age, sex, and duration of diabetes were controlled for as independent variables. Similarly, in a factorial design of univariate ANOVA in which age, sex, and duration of diabetes were controlled for, the CA16 genotype was still predictive of glycosylated hemoglobin (F = 4.7, P = 0.009).

CONCLUSIONS — The present study finds that TNFRSF1B genotype is associated with clinical neuropathy in type 2 diabetes patients. The genotype that predisposed to clinical neuropathy was also associated with better glycemic control and more favorable lipid profile, to the extent that glycosylated hemoglobin was reduced and HDL cholesterol was elevated, respectively. The lack of association with other complications of diabetes, such as retinopathy and nephropathy, is consistent either with heterogeneity in the etiology of each of these complications of diabetes, earlier onset of neutral as opposed to other complications of diabetes in response to TNFRSF1B genotype, or tissue-specific differences in contribution of TNF-R2 to organ damage. Not unexpectedly, our diabetes patients with clinical neuropathy were older than the patients without this complication; genetic factors may combine with other factors, such as age, to increase the rate of progression to complications, not necessarily the ultimate eventuality of such pathological consequences of diabetes. Patients with clinical neuropathy also had higher creatinine levels and microalbuminuria, which is consistent with greater renal damage.

Inflammatory vasculopathy is important in proximal diabetic clinical neuropathy (18), and inhibitors of TNF-α production block the development of peripheral neuropathy in streptozotocin-induced diabetic rats (19). TNF-α has also been implicated in pathogenesis and progression of central neuropathies (20). Our findings thus add a genetic perspective to the role of the TNF system in diabetic clinical neuropathy.

Our data suggest that either the polymorphism is itself causal or that it is in linkage disequilibrium with a nearby variant that is responsible for the associations we observed. The location of the variant in an intron of the 26-kb, 10 exon gene and its nature make it more likely that the variant we tested is merely a marker for a causative variant elsewhere. Recent estimates of the extent of linkage disequilibrium range from 3 kb (21) to >100 kb (22), the latter being a more reasonable estimate for Homo sapiens, in that it accords with actual observations and may represent the establishment of new equilibria after cyclical expansions in populations after a bottleneck in the Neo-

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TNF receptor 2 gene in diabetes

lithic (22). Thus, the causative variant could reside in TNFRSF1B itself or in a neighboring gene. Both TNFRSF1B and brain natriuretic factor gene colocalize at chromosome 1p36.2, and the latter is expressed in neural tissue. It is, however, remote, being ~10 cM centromeric to TNFRSF1B (http://www.cedar.genetics.soton.ac.uk/pub/chrom1/map.html).

In the only other study of TNFRSF1B in type 2 diabetes to date, an association was found among the A2 allele (T620C) in the 3′-UTR, elevation in BMI, and leptin in 49 diet-treated diabetes patients and in female nondiabetic subjects but not in the diabetes group overall (14). It was concluded that these effects may predispose individuals to insulin resistance and that TNFRSF1B might be involved in weight-control mechanisms. It is not known whether alleles of the 3′-UTR variant are in linkage disequilibrium with alleles of the TNFRSF1B microsatellite polymorphism we studied, so we cannot speculate on the relationship of our findings to those from this other recent study.

In both obesity and type 2 diabetes, TNF-α provokes insulin resistance (23) and sTNF-R2 is associated with indexes of insulin resistance (24). These include increasing circulating free fatty acids (23) and cholesterol (25) by stimulation of hepatic lipid synthesis and secretion (26) and inhibition of lipoprotein lipase (27). Moreover, TNF-R2, but not TNF-R1, mRNA is increased in adipose tissue and plasma sTNF-R2 levels correlate with hyperinsulinemia and insulin resistance (23,24,28). The latter has been suggested to be a possible side effect of actions of TNF-α that attempt to counter weight gain (29). Thus, effects of the TNF system are a mixture of benefit and harm (30). In this regard, it is not possible to link an effect of CA16 genotype to pathways leading to clinical neuropathy, as one can suggest various scenarios involving TNF-R2 that would result in such effects. This is because cellular stimulation by TNF-α activates both survival- and death-signaling pathways (involving NF-κB-mediated activation of antipoptotic genes) and caspase activation leading to apoptosis (31).

Indeed, consistent with the benefit/harm concept, we found that HDL cholesterol was increased in diabetes patients with the CA16 allele. Such an elevation in HDL cholesterol and apolipoprotein A-I (apoA-I) has also been seen by us in patients with coronary artery disease (12). Moreover, in other studies, we have found the CA16 allele to be associated with lack of sTNF-R2 increase compared with the major (CA15) allele in hypertensive patients (11). Whether this could reflect reduced TNFRSF1B expression, and thus lower cell surface receptor number, or reduced shedding, and thus increased cell surface receptor number, is not clear. The CA16 allele has also shown an association with hypertension (11) and coronary artery disease (12). Cytokines stimulate apoA-I production (32) and thus HDL cholesterol. Moreover, apoA-I inhibits neutrophil activation in inflammation (33). Considering the association of the CA16 allele with clinical neuropathy, it could be that the increased HDL cholesterol is a marker for activation of defensive mechanisms aimed at countering TNF-R2–related pathophysiological changes that may be more prevalent in diabetes patients with the CA16 allele.

Consistent with this hypothesis, TNF-α–induced apoptosis of human vascular endothelial cells is prevented by HDL (34). This involves inhibition by HDL of CPP32-like protease activity and implicates HDL as having a protective role in the “response-to-injury” hypothesis of atherogenesis (34). We speculate that TNF-α might directly upregulate hepatic apoA-I production because apoA-I is, by itself, a factor in the inflammatory response.

We also found that the CA16 allele was associated with reduced glycosylated hemoglobin, consistent with a TNFSF18-mediated mechanism that improves glycemic control in diabetes patients with the CA16 allele. It is clear that more research will be required to uncover the components that mediate the deleterious genotypic effect on neural tissue and the effects on other parameters influenced by TNFRSF1B genotype.

In conclusion, our findings implicate TNFRSF1B as a candidate in clinical neuropathy in type 2 diabetes patients. At the same time, protective mechanisms are activated as reflected in increased HDL cholesterol and reduced glycosylated hemoglobin. Determination of the causative variant, as well as the mechanisms involved, merit further investigation.

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