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Association of the tumour necrosis factor α –308G/A polymorphism with the risk of diabetes in an elderly population-based cohort

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Ample evidence supports a role for tumour necrosis factor α (TNFα) in the development of type 2 diabetes and cardiovascular disease. TNFα expression was found to be influenced by a –308G/A polymorphism in the promoter of the gene encoding TNFα (TNF). We investigated the contribution of this polymorphism to diabetes and cardiovascular mortality in a population-based cohort of 664 subjects aged 85 years and over (Leiden 85-plus Study). The –308G/A TNF promoter polymorphism was associated with the prevalence of diabetes in old age (P = 0.006). The risk of diabetes among subjects homozygous for the A-allele was estimated to be 4.6-fold (95% CI, 1.6–13.3) higher than among subjects homozygous for the common G-allele. The promoter polymorphism did not, however, predict mortality from all causes, cardiovascular diseases, cancer or infectious diseases during a 10-year follow-up period. In addition to the promoter polymorphism, TNFa and TNFc microsatellite genotypes were determined but these polymorphisms were not associated with morbidity or mortality. In conclusion, the –308G/A polymorphism in the TNF promoter is strongly associated with the risk of diabetes but not cardiovascular mortality in old age.
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Introduction

Ample evidence supports a role of tumour necrosis factor α (TNFα) in the development of cardiovascular disease. TNFα is expressed in atherosclerotic plaques but not in healthy vessels.[1] In atherosclerotic plaques, TNFα may contribute to foam cell formation, to T-lymphocyte activation and to the expression of matrix metalloproteinases that may destabilise the plaque by degrading the extracellular matrix.[2] Detailed studies also implicated TNFα in the aetiology of insulin resistance, a key feature of type 2 diabetes and a major risk factor for cardiovascular disease in the elderly. TNFα mRNA expression is increased in adipose tissue of severely obese and insulin resistant *fa/fa* rats,[3] while deficiency in the gene encoding TNFα (*TNF*) results in an increased peripheral insulin sensitivity in obese mice.[4] In humans, there is a strong positive association between levels of TNFα mRNA in adipose tissue and the extent of hyperinsulinaemia,[5] and TNFα plasma levels are increased in patients with type 2 diabetes.[6] The molecular mechanism underlying these correlations is that TNFα inhibits the insulin induced tyrosine kinase activity of the insulin receptor.[4,7]

The A allele of a common –308G/A polymorphism in the promoter region of the *TNF* gene is associated with higher reporter gene activity[8,9] and TNFα production in whole blood cell cultures.[10] In addition, several short tandem repeat polymorphisms have been identified at the *TNF* locus, of which TNFa and TNFc were suggested to be associated with differences in TNFα secretion by human monocytes.[11] We have assessed the contribution these polymorphisms to diabetes and all-cause and cause-specific mortality in a population-based cohort of 664 subjects aged 85 years and over.

Results

The genotype distribution of *TNF* –308G/A polymorphism was 65.4% (G/G), 32.2% (G/A) and 2.4% (A/A) in the cohort of 664 subjects aged 85 years and over and was in the Hardy-Weinberg equilibrium. The polymorphism was significantly associated with the risk of diabetes ($P = 0.006$; Table 1). Adjusted for age and gender, the risk of diabetes associated with the G/A and the A/A genotypes were estimated at 0.9-fold (95% confidence interval (CI), 0.5–1.5) and 4.6-fold (95% CI, 1.6–13.3) increased, respectively. The risk estimate for the A/A

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Table 1 *TNF* –308G/A genotype distributions in subjects aged 85 years and over with and without diabetes

TNFα –308G/A Genotype	*Controls (n = 577)*	*Diabetes patients (n = 79)*
G/G	378 (65.5%)	51 (64.6%)
G/A	189 (32.8%)	22 (27.8%)
A/A	10 (1.7%)	6 (7.6%)

Test for different genotype distributions $P = 0.006$.

genotype was 6.5 (95% CI, 1.3–33.1) among men and 3.0 (95% CI, 0.8–12.7) among women. There was no indication for heterogeneity of the association among subjects born in Leiden (55%) and elsewhere in The Netherlands (test for heterogeneity: $P = 0.51$ and $P = 0.31$ for the G/A and A/A genotypes, respectively).

The cohort was followed for mortality over a 10-year period. *TNF* –308G/A genotypes were not associated with all-cause mortality (89%), cardiovascular mortality (37%), or death from cancer (15%) and infectious diseases (9%) (Table 2). Similar risk estimates were obtained when men and women were analysed separately.

In addition to the –308G/A polymorphism, the TNFa (14 alleles) and TNFc (2 alleles) short tandem repeat polymorphisms were measured. The three polymorphisms were in linkage disequilibrium (all pairwise linkage disequilibria $P < 0.00001$). However, the TNFa and TNFc polymorphisms were not associated with diabetes ($P = 0.53$ and $P = 0.54$, respectively) nor were they associated with mortality from any cause or from a specific cause (data not shown). The absence of an association between the short tandem repeats and diabetes despite their strong linkage disequilibrium with the –308G/A polymorphism is explained by the fact that the –308A allele is distributed over several haplotypes (Table 3). For example, all the –308A alleles occur in combination with a TNFc1 allele, but about 70% of the TNFc1 alleles do not occur in combination with this allele; about 65% of the –308A alleles occur in combination with a TNFa2 allele, but about 56% of the TNFa2 alleles do not occur in combination with this allele.

The *TNF* gene is located in the HLA region, which is characterised by strong linkage disequilibrium. The association with diabetes found here might, therefore, have been the result of linkage disequilibrium between the –308G/A polymorphism and variation elsewhere in the HLA region influencing diabetes risk. The occurrence of diabetes was, however, independent of HLA-DR3, DR4 and B8 phenotypes ($P = 0.71$, $P = 0.69$ and $P = 0.80$, respectively), indicating that this was unlikely to be the case.

Table 3 Frequent *TNF* haplotypes in subjects aged 85 years and over

Haplotype			*Frequency*[a]
–308G/A	*TNFc*	*TNFa*	
G	c1	a6	0.131
G	c1	a10	0.088
G	c1	a11	0.192
G	c2	a2	0.149
G	c2	a5	0.054
A	c1	a2	0.114
A	c1	a4	0.063

Haplotype frequencies were estimated on the basis of 1318 chromosomes of 659 subjects who were successfully genotyped for the three polymorphisms. [a]Only those haplotypes with a frequency higher than 0.05 are included in the table (seven of 24 occurring haplotypes).

Discussion

The –308G/A polymorphism in the promoter of the gene encoding *TNF* strongly contributed to the risk of diabetes in a population-based cohort of elderly subjects aged 85 years and over. Homozygosity for A-allele conferred a more than four-fold increased risk of diabetes. This is in agreement with the extensive evidence for a direct role of TNFα in the aetiology of insulin resistance and type 2 diabetes.[4,7]

The *TNF* gene is located in the HLA region that is characterised by especially strong linkage disequilibrium. Although this makes it difficult if not impossible to definitely prove that the –308G/A polymorphism itself is the functional variation underlying the association with diabetes, it is an attractive candidate. First, the A-allele was associated with higher gene expression levels in several studies.[8–10] The absence of this effect in another study[12] may be related to the use of different reporter gene constructs, stimuli, cells or cell culture conditions. Furthermore, the association with diabetes in our study was independent of other genetic variation at the *TNF* locus as measured by two short tandem repeat polymorphisms and the –308A allele does not occur on the same haplotype as rare alleles of other promoter polymorphisms (–238G/A, –851C/T and –857C/A) in Caucasians.[13] Finally, in view of the suggested shared genetic susceptibility of type 1 and type 2 diabetes[14,15] it is notable that

Table 2 Ten-year all-cause and cause-specific mortality risks according to *TNF –308G/A genotype in subjects aged 85 years and over*

TNFα –308G/A Genotype	*No.*	*Cause of death*							
		All causes (n = 591)		*Cardiovascular diseases (n = 248)*		*Cancer (n = 99)*		*Infectious disease (n = 62)*	
		RR	*(95% CI)*	*RR*	*(95% CI)*	*RR*	*(95% CI)*	*RR*	*(95% CI)*
G/G	433	1	–	1	–	1	–	1	–
G/A	213	0.9	(0.8–1.1)	0.8	(0.6–1.0)	0.9	(0.6–1.4)	1.4	(0.9–2.4)
A/A	16	0.9	(0.5–1.5)	0.9	(0.4–2.1)	0.8	(0.2–3.5)	1.4	(0.3–6.0)

linkage disequilibrium of the promoter polymorphism and HLA-DR3, DR4 and B8 was excluded as the underlying cause of the association.

It is interesting to note that the TNFa (14 alleles) and the TNFc (2 alleles) short tandem repeats were not associated with the risk of diabetes despite their strong linkage disequilibrium with the –308G/A promoter polymorphism. This illustrates that the complexity of haplotype structures may severely hamper the usefulness of linkage disequilibrium mapping as a tool in unravelling the genetic component of complex diseases.

The –308G/A polymorphism was not associated with type 2 diabetes in previous studies among patients with a mean age of 57 years[16] and 39 years.[17] Apart from differences in study design and environmental and genetic background of the subjects studied, this may suggest that other genetic factors contribute to the disease at younger ages. Possibly, the adverse effects of mild alterations in TNFα expression become apparent only in old age. Age differences have also been suggested to underlie the inconsistent results from studies assessing the association of the promoter polymorphism and insulin sensitivity.[18,19] More extensive studies are warranted to more precisely characterise the role of the *TNF* promoter polymorphism in type 2 diabetes.

The –308G/A polymorphism in the promoter of the *TNF* gene was not related to all-cause and cardiovascular mortality during a 10-year follow-up period. In previous studies, the polymorphism was not associated with the risk of myocardial infarction[13] or coronary artery disease.[20,21] This may reflect that the aetiology of cardiovascular diseases is much more heterogeneous than type 2 diabetes.

In conclusion, our study indicates that the TNFα –308G/A polymorphism may be a potent risk factor for diabetes in old age. We did not find evidence that the polymorphism contributes to the risk of cardiovascular mortality.

Methods

Subjects

The Leiden 85-plus Study is a population-based study in which all inhabitants of Leiden, The Netherlands, aged 85 years and over were invited to take part.[22] Out of a total of 1258 eligible subjects, 221 died before enrolment. Of the 1037 remaining subjects, 977 (94%) participated and were medically interviewed at home. Diabetes was diagnosed on the basis of a history, use of anti-diabetic medication and/or a glucose level over 11.0 mmol/l in a non-fasting blood sample. After the exclusion of subjects with a non-Dutch ($n = 29$) or unknown ($n = 69$) place of birth, sufficient cell material was available from 666 (188 men/478 women) subjects for the present genetic study. The study was approved by the Medical Ethics Committee of the Leiden University and informed consent was obtained from all participants.

Genotyping and HLA typing

The *TNF* –308G/A genotypes were determined by PCR-amplification followed by *Nco*I digestion.[23] Digestion products were separated on 7.5% polyacrylamide MADGE-gels (microtitre array diagonal gel electrophoresis).[24] Genomic regions containing the TNFa (located in the promoter of the gene *LTA* that encodes TNFβ and flanks the *TNF* gene) and TNFc (located in intron 1 of *LTA*) short tandem repeats were amplified in a multiplex PCR[11] and alleles were separated with an ALF-express automated sequencer (Amersham Pharmacia Biotech, Picataway, NJ, USA). Because of technical reasons two, four, and five subjects could not be typed for the –308G/A, TNFa, and TNFc polymorphism, respectively. All genotypes were independently assessed by two observers. As a standard laboratory procedure a randomly chosen 10% of the samples was reamplified.

Typing for HLA-DR antigens was performed with a two colour fluorescence test using a set of highly selected alloantisera to class II antigens.

Prospective study

All participants in the Leiden 85-plus Study were followed up for mortality until 1 October, 1996. Among the 666 subjects of the cohort studied, two were lost to follow-up. Primary causes of death were obtained from the Dutch Central Bureau of Statistics and categorised for cardiovascular disease (*ICD-9* codes[25] 390–459), ischaemic heart disease (410–414), cerebrovascular disease (430–438) and cancer (140–239). Death from infection was coded as previously described.[22]

Statistical analyses

Distributions of genotypes, alleles and HLA phenotypes were compared by the χ^2-test. Age and gender adjusted risks for diabetes and 95% CIs were estimated by odds ratios calculated using logistic regression analysis. Heterogeneity of associations was tested using Mantel-Haenszel's test for stratified analyses. Pairwise linkage disequilibria between the three *TNF* polymorphisms and maximum likelihood haplotype frequencies were estimated using Arlequin software version 2.000.[26]

In the prospective study, survival times for subjects were computed from the date of the home visit to the date of one of the following events: death from a specific cause, death from any cause, or 1 October 1996. Age and gender adjusted mortality risks and 95% CIs were estimated with Cox proportional hazards models. Causes of death were assumed to be independent. *P*-values of less than 0.05 were considered to indicate statistical significance and all *P*-values were based on two-sided tests.

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