ELSEVIER

An Association in Non-Insulin-Dependent Diabetes Mellitus Subjects Between Susceptibility to Retinopathy and Tumor Necrosis Factor Polymorphism

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ABSTRACT: In IDDM an association between diabetic retinopathy and polymorphic markers of MHC has been described. However, these associations are complicated by a primary association between the MHC and IDDM. Because the pathogenesis of retinopathy is likely to be the same in IDDM and NIDDM, NIDDM subjects with retinopathy would be the ideal population to study for an association with MHC markers. The following South Indian subjects were therefore studied: unselected NIDDM (n = 76), unselected IDDM (n = 99), non-diabetic controls (n = 96), NIDDM subjects with maculopathy (MAC), n = 55, NIDDM subjects with proliferative retinopathy (PR), n = 53, and without retinopathy (LTD), n = 46. DNA was amplified and studied using a microsatellite polymorphism located 3.5 kb upstream of TNF-β within the MHC class III region on the short arm of chromosome 6. No differences in allelic distribution were observed between the random NIDDM subjects and controls (p = 0.17). Differences in allelic distribution were found between unselected IDDM and controls (p = 0.016) and between the NIDDM subjects with maculopathy and/or proliferative retinopathy and no retinopathy (p = 0.006). This association could be accounted for by those patients with proliferative retinopathy (MAC vs LTD, p = 0.23; MAC vs PR, p = 0.07; and PR vs LTD, p = 0.002).

ABBREVIATIONS

CI	99% confidence interval
IDDM	insulin-dependent diabetes
LI	Likelihood ratio test
LTD	long-term NIDDM subjects without retinopathy
MAC	exudative maculopathy
MHC	major histocompatibility complex
NIDDM	non-insulin-dependent diabetes
PR	proliferative retinopathy
TNF	tumour necrosis factor

INTRODUCTION

Diabetic retinopathy is a significant complication of both insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). The duration of diabetes and glycemic control are the most important factors in the development of retinopathy [1, 2]. However, the duration of disease and glycemic control does not explain the overall distribution of retinopathy, which may be absent from patients with poor glycemic control over a long period, while others may develop retinopathy in a short period despite good glycemic control. This variation may reflect differences in genetic predisposition. A genetic component to retinopathy is further suggested by twin and population studies [3–5]. In IDDM subjects, associations have been described between gene markers of the major histocompatibility complex (MHC) and diabetic retinopathy [6–18]. However, the associations found between the MHC and retinopathy may not be directly related to retinopathy per se but represent a primary association between IDDM and the MHC. Because the etiology of retinopathy is likely to be similar in IDDM and NIDDM, NIDDM subjects with retinopathy would be the best population to study the described association. The MHC

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contains the genes for human tumor necrosis factor (TNF-α and TNF-β), which have been mapped 350 kb centromeric to HLA-B and 340 kb telomeric to the C2/Bf complex [19]. A role for TNF in the pathogenesis of retinopathy can be postulated; furthermore, there is linkage of TNF alleles with certain DR haplotypes [20–23]. In this study we used a TNF microsatellite polymorphism [24] to investigate a group of South Indian NIDDM subjects for an association between TNF and the various forms of retinopathy. Proliferative retinopathy only affects 10%–20% of NIDDM subjects [25], whereas some form of background retinopathy is present in the majority of NIDDM patients with a long duration of disease. We tested the hypothesis that susceptibility to proliferative retinopathy has a genetic component.

SUBJECTS AND METHODS

Population Study

All diabetic patients were unrelated Dravidian (South Indian) subjects recruited via the MV Hospital for Diabetes and MV Diabetes Specialities Centre in Madras. Clinical details are presented in Table 1.

Control Subjects

Ninety-six unrelated Dravidian control subjects without personal or first-degree family history of diabetes were recruited from among blood donors (n = 34) or from staff and spouses of patients at the MV Hospital for Diabetes (n = 62). All the latter subjects had random blood glucose estimations below 6.7 mmol/l.

IDDM Subjects

IDDM subjects (n = 99) were defined by an acute onset of symptoms before 35 years of age, susceptibility to ketoacidosis, and continuing need for exogenous insulin. Fibrocalculous pancreatic diabetes was excluded by an abdominal x-ray and pancreatic ultrasound where clinically appropriate. Clinical details on diabetic complications were available from 52 casenotes of the IDDM patients. At the last annual review six patients (12%) had some form of retinopathy by indirect ophthalmoscopy.

NIDDM

Non-insulin dependent diabetes mellitus (n = 76) was defined by an insidious onset of disease and, if on insulin, this treatment had not been initiated within 5 years of the diagnosis; no subjects were insulin dependent or had an episode of diabetic ketoacidosis. Clinical details on diabetic complications were available in 75 of the 76 NIDDM patients. At the last annual review two patients (3%) had some form of retinopathy by indirect ophthalmoscopy.

Retinopathy Groups

All patients were unrelated Dravidian NIDDM subjects recruited at the MV Hospital for Diabetes or the MV Diabetes Specialities Centre. They are not the same subjects as in the NIDDM group. A complete ophthalmologic examination was performed for all the patients in the retinopathy groups. A detailed examination of the fundus was done by direct and indirect ophthalmoscopy. Stereoscopic color photographs of the seven standard fields were carried out in all subjects. Fundus fluorescein angiography was performed in all patients with maculopathy and proliferative retinopathy. The grading of retinal findings was carried out by a retinal specialist (MR) using an adaptation of the modified Airlie House classification of diabetic retinopathy [26]. Type 2 diabetes was diagnosed by insidious onset of disease and no insulin treatment within 5 years of diagnosis. Patients were then subdivided into one of three groups. In all the retinopathy groups there were similar ages, age of onset of disease, BMI, HbA1, and lipids (Table 1). Those patients with proliferative retinopathy were more frequently treated with insulin than the other groups.

TABLE 1 Clinical details of clinical groups studied

					Treatment (%)								Complications (%)			
	n	Male (%)	Age (yr ± ISD)	Onset (yr ± ISD)	D	T	I	T + I	BMI (kg/m^2)	HbA1	Chol (mmol/l)	Trigs (mmol/l)	IHD	PVD	NEPH	NEU
LTD	46	77	59.5 ± 7.8	38.2 ± 7.1	0	46.3	14.6	39.0	24 ± 5	10.0 ± 1.3	5.9 ± 0.9	1.5 ± 0.8	24	10	15	24
MAC	55	59	54.3 ± 6.8	40.6 ± 7.4	0	19.2	23.1	57.7	24 ± 5	10.7 ± 1.5	6.0 ± 1.3	1.4 ± 0.7	13	10	43	55
PR	53	67	57.5 ± 8.1	39.8 ± 10.7	0	27.1	31.3	41.7	26 ± 4	10.3 ± 1.5	5.7 ± 0.9	1.3 ± 0.5	35	22	49	43
CON[a]	96	60	38.5 ± 13.8	—	—	—	—	—	24 ± 3							
NIDDM	76	62	49.4 ± 12.0	42.8 ± 11.0	4	79.5	6	9.6	25 ± 4							
IDDM	99	65	21.4 ± 10.9	16.7 ± 9.3	—	—	100	—	18 ± 4							

n, number of subjects; age, age at time of study; onset, age at time of onset of diabetes; D, diet only; T, hypoglycemic agent; I, insulin only; T + I, oral hypoglycemics and insulin; BMI, body mass index; HbA1C, glycosylated haemoglobin; chol, cholesterol on first attendance at clinic; trigs, triglycerides at first attendance at clinic; IHD, presence of ischemic heart disease; PVD, presence of peripheral vascular disease; NEPH, presence of nephropathy; NEU, presence of neuropathy; LTD, long-term diabetic; MAC, exudative maculopathy; PR, proliferative retinopathy; CON, controls.

[a] Clinical details only available in 62 subjects.

Long-term NIDDM patients without retinopathy (LTD). Forty-six patients were recruited with a minimum duration of diabetes of 15 years and without evidence of retinopathy clinically (i.e., no microaneurysms or exudates).

Exudative maculopathy (MAC). Fifty-five patients were recruited with exudative maculopathy. This condition was diagnosed where there was evidence of circinate or scattered exudates, plaques in the macular region with or without thickening of the retina.

Proliferative retinopathy (PR). Fifty-three patients were recruited with proliferative retinopathy. This was diagnosed when there was evidence of new vessel formation on the disc or in any of the quadrants of the retina.

Complications in Retinopathy Groups

Ischemic heart disease (IHD) was considered to be present where there was a clear history of angina pectoris or myocardial infarction and/or the ECG demonstrated evidence of myocardial infarction. Peripheral vascular disease (PVD) was deemed to be present if there was a history of intermittent claudication or if one or more peripheral pulse (dorsalis pedis or posterior tibial) was absent to palpation. Neuropathy (NEU) was defined as the absence of ankle jerks bilaterally and/or a glove and stocking neuropathy. Nephropathy (NEPH) was defined by either (a) estimating the 24-hour urinary protein excretion by the sulphosalicylic acid method; those with values greater than 500 mg, in the absence of urinary tract infection or severe hypertension, were considered to have nephropathy or (b) a serum creatinine of greater than 133 μmol/l.

The study was approved by the Ethical Committee of the Diabetes Research Centre and MV Diabetes Specialities Centre (Madras) and informed consent was obtained from all subjects.

METHODS

DNA was extracted from thawed blood either by standard methods (phenol/chloroform extraction) or by preparation of crude lysate [27]. The TNFa microsatellite was amplified in 10-μl volumes containing 0.1 μg of genomic DNA or 0.5 μl of lysate, 5 pmol of each of the primers (5′ GCC TCT AGA TTT CAT CCA GCC ACA G 3′ and 5′ CCT CTC TCC CCT GCA ACA CAC A3′) [28], 0.5 μmol/l dNTP mix, 1 μl 10× PCR buffer (1× = 10 mmol/l Tris-HCl [pH 9.0], 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.1% Triton X-100), and 1 μCi ($\alpha^{-32}P$). The mixture was overlaid with 15 μl of mineral oil. After initial denaturation of the template DNA at 94°C for 8 minutes, 0.1 units of Taq DNA polymerase (Cetus, Norwalk, CT, USA) was added and 18 cycles of amplification carried out comprising 1 minute at 94°C (denaturation), 1 minute at 63°C (annealing), and 30 seconds at 72°C (extension); the final extension step was prolonged by 7 minutes. The PCR product (1 μl) was mixed with 3 μl of formamide containing a stop solution and electrophoresed in 8% polyacrylamide (19:1) 0.4 sequencing gel containing 48% urea, 89 mmol/l Tris borate, and 2 mmol/l EDTA at 45 W for 4 hours. The gel was dried and autoradiographed for 1–24 hours. TNF alleles were sized using DNA sequence ladders derived from a known sequence and internal controls.

STATISTICS

Because expected cell frequencies were frequently low due to the large number of TNF alleles, exact p values were preferred over asymptotic p values calculated using exact permutational analyses (Statexact Turbo, Cytel Corporation, Cambridge, MA, USA). Cross tabulations were calculated using the likelihood ratio test using a Monte Carlo estimate of the p values with 99% confidence limits (CI); for 2 by 2 tables the two-tailed Fisher exact test with correction of the p value for the number of alleles tested was used.

RESULTS

In South Indian subjects 15 alleles of the AC/GT dinucleotide repeat (TNF-α) were identified. The length of consecutively numbered TNFa alleles differed by two nucleotides, the size ranging from 97 bp (B1) to 125 bp (B15). The alleles identified in South Indian subjects were essentially the same as those reported in French samples [21] except the B8 allele was absent and South Indian subjects possessed additional alleles designated B9, B14, and B15. Overall no differences were found between unselected NIDDM, unselected IDDM, and controls (p = 0.065). A priori a difference in TNF allele distribution would be expected between unselected IDDM and controls given the well-documented association between the MHC and IDDM; this was confirmed (p = 0.016; 99% CI 0.013–0.019; Table 2). Examining each allele separately and correcting the p value by the number of alleles tested, no single allele is responsible for the significant overall p value. No differences in TNF allele distribution were found between unselected NIDDM and controls (p = 0.17).

In the retinopathy group, a difference in TNF allelic frequency distribution was found between LTD, MAC, and PR (p = 0.006), with a suggestion of a primary association with PR; LTD vs MAC (p = 0.23), PR vs LTD (p = 0.002), PR vs MAC (p = 0.07) (Table 3). After correction for the number of alleles studied, only the B9 allele had a significantly different distribution between patients with proliferative retinopathy and those without

TABLE 2 TNFa allelic frequencies in South Indian diabetic subjects compared to controls

Allele	IDDM (n = 198) No. (%)	NIDDM (n = 152) No. (%)	Control (n = 192) No. (%)
B1	0	2 (1.3)	0
B2	38 (19.2)	30 (19.7)	30 (15.5)
B3	1 (0.5)	0	1 (0.5)
B4	4 (2.0)	3 (2.0)	4 (2.0)
B5	24 (12.1)	10 (6.6)	11 (5.7)
B6	32 (16.2)	24 (15.8)	20 (10.4)
B7	12 (6.1)	13 (8.6)	19 (9.9)
B9	8 (4.0)	5 (3.3)	17 (8.9)
B10	42 (21.2)	41 (27.0)	54 (28.1)
B11	19 (9.6)	12 (7.9)	24 (12.5)
B12	2 (1.0)	2 (1.3)	0
B13	16 (8.1)	8 (5.3)	9 (4.7)
B14	0	1 (0.7)	3 (1.6)
B15	0	1 (0.7)	0

Overall likelihood ratio test (LI) = 36.7; p = 0.065 (99% CI 0.059–0.072).
Controls vs IDDM LI = 24.5; p = 0.016 (CI 0.013–0.019).
Controls vs NIDDM LI = 19.2; p = 0.17 (CI 0.18–0.20).

retinopathy (p = 0.04). Because a difference in TNF allele distribution existed between unselected IDDM patients and control subjects, NIDDM patients with PR and unselected IDDM patients were compared; differences in TNF allelic distribution were found (p = 0.001), indicating that the TNF allele association with proliferative retinopathy is different from that with IDDM.

TABLE 3 TNFa allelic frequency in South Indian NIDDM subjects with and without diabetic retinopathy

Allele	MAC (n = 110) No. (%)	PR (n = 106) No. (%)	LTD (n = 92) No. (%)
B1	0	7 (6.5)	0
B2	10 (9.0)	15 (14.0)	10 (11.0)
B3	2 (2.0)	0	0
B4	4 (3.5)	3 (3.0)	3 (3.5)
B5	6 (5.5)	3 (3.0)	3 (3.5)
B6	22 (20.0)	18 (17.0)	24 (26.0)
B7	8 (7.5)	8 (7.5)	9 (10.0)
B9	13 (12.0)	7 (6.5)	19 (20.5)
B10	26 (23.5)	28 (26.5)	14 (15.0)
B11	9 (8.0)	5 (4.5)	5 (5.5)
B12	0	0	2 (2.0)
B13	9 (8.0)	12 (11.5)	3 (3.0)
B14	1 (1.0)	0	
B15	0	0	0

Overall LI (13 × 3) = 47.34; p = 0.0057 (CL = 0.0038–0.0076; MAC vs PR LI = 19.85; p = 0.0728 (CI = 0.0661–0.0795); MAC vs LTD LI = 15.72; p = 0.226 (CI = 0.215–0.237); PR vs LTD LI = 29.71; p = 0.0017 (CI = 0.0006–0.0028). B9 allele; PR vs LTD. Two-sided p value corrected p = 0.044: PR vs MAC and MAC vs LTD; p value not significant.

DISCUSSION

The MHC associations with retinopathy in IDDM have not been consistent. In IDDM subjects with retinopathy increased frequencies of HLA-B8 [11–13] and DR3 and/or DR4 [5, 6] has been described, whereas other groups have reported to HLA-B antigen association [14–16], and one group reported decreased frequencies of HLA-B7 in proliferative retinopathy [17]. Cruickshanks and colleagues [18] reported that type 1 diabetic subjects who possessed HLA-DR4/not HLA-DR3 were more likely to have proliferative retinopathy compared with those who were DR4 negative [18]; this is one of the few groups to study proliferative retinopathy specifically. The only studies of the class III region have been by Mijovic and colleagues [10], who found an association between the fourth component of complement (C4B3) and retinopathy. These conflicting results may be related to small sample size in many studies, different diagnostic criteria, methodology, and heterogeneity of retinopathy associated with diabetes. Our study has important differences compared to many previously published studies. First, we restricted ourselves to the study of NIDDM subjects, and second, we very carefully characterized our retinopathy groups by the use of ophthalmoscopy, 7-field retinal photography, and fluorescein angiography. Last, our hypothesis was that the differences in TNF allele distribution would be found in those patients with proliferative retinopathy rather than with any form of retinopathy. We found an association between TNF alleles and retinopathy in NIDDM subjects and furthermore this is likely to be explained by a primary genetic susceptibility to proliferative retinopathy. Although strong linkage disequilibrium of the TNF alleles exists with alleles of the HLA class I and class II genes the detailed linkage disequilibrium relationships are yet to be worked out for the TNF microsatellite polymorphism studied in this ethnic group; previously, studies have been done in the French [21] and Danish [20] populations. Further studies should be directed at MHC halpotypes (including markers of the class I, class III, and class II regions).

TNF is implicated in the etiology of some autoimmune diseases [20, 28, 29]. The tumor necrosis factors (TNF-α and TNF-β) are cytotoxic proteins which have similar biologic activities and biochemical characteristics, sharing 30% amino acid homology [30]. TNFs have been recognized as essential mediators of the inflammatory process, immune reactions, and hematopoiesis [31, 32]. Animal studies demonstrate that TNF has a major effect in vivo in protecting non-obese diabetic (NOD) mice from developing autoimmune insulitis and diabetes [33]. Furthermore, TNF-α and interferon γ (IFN-γ) can induce the expression of class II MHC molecules on pancreatic β cells in vitro that are normally MHC class II

negative [34]. TNF gene polymorphisms have been shown to determine TNF-α levels in response to phytohemagglutinin, suggesting a functional link between genotype and disease pathogenesis [20, 35].

The early histopathologic changes of diabetic retinopathy are pericyte loss and thickening of the capillary basement membrane. It has been suggested that TNF inhibits endothelial cells and stimulates pericytes of retinal vessels in vitro [36]. Therefore, it is possible that TNF secretion determined by TNF genotype might lead to an alteration in the pericyte/endothelial cells ratio, thus leading to the development of retinal microvascular changes. TNF might also act in the pathogenesis of retinopathy by inducing a hypercoagulatory state through an effect on platelet-activating factors, thromboxane, and protein kinase C [37]. In our study we could not find an association between TNF and the unselected NIDDM patients; however, a strong association was found in NIDDM patients with retinopathy ($p = 0.002$), with the TNFa-B9 allele conferring protection from proliferative retinopathy. The strong association between TNF polymorphism and retinopathy in our South Indian NIDDM subjects suggests that the MHC association with retinopathy is not a spurious finding. It also raises the possibility that TNF may play an important role in the pathogenesis of diabetic retinopathy and, in particular, proliferative retinopathy, but this can only be proven by functional studies.

In conclusion, our results, together with our previous studies of the immunoglobulin heavy chain switch region polymorphisms [38], indicate a genetic component in the etiology of proliferative retinopathy in South Indian NIDDM subjects. In addition to the importance of glycemic control, immunogenetic factors may play an important role in the pathogenesis of this important diabetic complication.

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