ACE Gene Polymorphism and Proliferative Retinopathy in Type 1 Diabetes

Results of a case-control study

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OBJECTIVE — To evaluate the relationship between the ACE insertion/deletion polymorphism and proliferative diabetic retinopathy in patients with type 1 diabetes of long duration. Based on epidemiological and pathophysiological findings, risk factors apart from glycemic control and duration of disease are likely to be involved in the development of proliferative retinopathy.

RESEARCH DESIGN AND METHODS — In this case-control study, we compared 81 patients with longstanding (≥20 years) type 1 diabetes who had nonproliferative (mild or moderate background) retinopathy with 95 patients with diabetes of similar duration and HbA_{1c} who had proliferative retinopathy. To avoid the confounding effect of nephropathy, patients with overt nephropathy were excluded, and microalbuminuria was introduced into the multiple logistical regression model. The polymorphic region in intron 16 of the ACE gene (17q23) was analyzed using the polymerase chain reaction.

RESULTS — The ACE genotype distribution in patients with proliferative retinopathy (DD 39.4%, ID 48.9%, II 11.7%) was significantly different ($P < 0.001$) from that of patients with nonproliferative retinopathy (DD 17.3%, ID 54.3%, II 28.4%). In a multiple logistical regression analysis, the adjusted relative risk for proliferative retinopathy in a patient with a DD genotype compared with a patient with an II genotype was 6.6 (95% CI 2.2–19.5), $P = 0.0026$. In addition to genotype, systolic blood pressure (odds ratio 1.027 [95% CI 1.0–1.1], $P = 0.0093$) but not microalbuminuria (≤20 vs. ≥20 μg/min) reached statistical significance in the multiple regression model. Because subjects were matched regarding diabetes duration and HbA_{1c}, we did not interpret the respective parameter estimates.

CONCLUSIONS — These data provide evidence that deletion in the ACE gene is associated with the prevalence of proliferative retinopathy in type 1 diabetes and suggest that the DD genotype confers susceptibility to proliferative retinopathy independent of diabetic nephropathy.

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Abbreviations: I/D, insertion/deletion; OR, odds ratio; PCR, polymerase chain reaction.

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

Diabetic retinopathy is the most frequent complication of type 1 diabetes and is closely related to the time and extent of glycemic exposure. Diabetic retinopathy is a heterogeneous disease that is divided into two clinical manifestations: nonproliferative (background) retinopathy and proliferative retinopathy. Although changes indicative of retinopathy can be seen in 97.5% of diabetic patients with a duration of disease >15 years, proliferative retinopathy is seen in only 67% of patients with type 1 diabetes for ≥35 years (1). Epidemiological and pathophysiological differences suggest the involvement of factors other than poor glycemic control and long duration of disease in the development of proliferative retinopathy. Genetic factors, hemodynamic alterations accompanying severe hyperglycemia (2,3), various growth factors (4,5) and the renin-angiotensin system (6–15) have been implicated. In fact, there is evidence that the systemic and the intraocular renin-angiotensin system (15–17) may contribute to the development of proliferative retinopathy. Elevated serum levels of prorenin, renin (13,14), and ACE (6–12) have been found in type 1 diabetic subjects with retinopathy, especially in those with proliferative retinopathy (6,7,11,14).

Recently, EUCLID (EURODIAB Controlled Trial of Lisinopril in Insulin-Dependent Diabetes Mellitus) has shown that the ACE inhibitor lisinopril significantly reduces the progression of retinopathy in nonhypertensive patients with type 1 diabetes (18). The level of plasma ACE is associated with a variation found in the gene encoding the ACE (19). An insertion/deletion (I/D) polymorphism situated in intron 16 of the ACE locus accounts for 50% of interindividual ACE level differences (20), and the DD genotype is associated with a significant elevation in ACE plasma levels (19).

An association between the ACE polymorphism and nephropathy and coronary heart disease in diabetic patients has been reported in several studies (21–27). This suggests that the ACE polymorphism confers susceptibility to microangiopathic as

well as macroangiopathic late complications in diabetes, but other investigators have failed to confirm this relationship (28–30).

This case-control study was designed to investigate the distribution of the I/D polymorphism in relation to the severity of diabetic retinopathy and to determine whether the DD genotype is associated with the prevalence of proliferative retinopathy. This study was specifically designed to examine this relationship independent of the influence of other risk factors and to avoid the confounding effect of diabetic nephropathy.

RESEARCH DESIGN AND METHODS

Study population

From among ~800 type 1 diabetic patients who have regularly attended the diabetes outpatient clinic at the Vienna Lainz Hospital for at least 10 years, two groups of patients were recruited, one with evident proliferative retinopathy and one with only background retinopathy. All subjects gave their informed consent after they were given appropriate information concerning study objectives, and the study was approved by the local ethics committee. Screening for retinopathy was performed annually; hence, all patients with retinopathy were preexistant cases when they were enrolled in the study.

Subjects were considered to have proliferative retinopathy based on established features such as preretinal new vessels (disc or elsewhere), fibrous proliferations, and preretinal or vitreous hemorrhage. All of these patients required treatment with photocoagulation. To avoid the confounding effect of impaired kidney function, patients with overt nephropathy were excluded. Of 127 potential subjects, 94 were eligible to be enrolled in the proliferative retinopathy group.

The criterion for acceptance into the control group was the presence of mild or moderate nonproliferative retinopathy as assessed in the multicenter EURODIAB IDDM Complications Study (31). Features included microaneurysms with or without hard exudates, retinal hemorrhages, or early lesions indicative of ischemia (e.g., cotton wool spots, intraretinal microvascular abnormalities, and/or venous beading). To avoid diabetic individuals being misclassified as having no proliferative retinopathy because of a short duration of disease, we excluded patients with diabetes duration <20 years from the main analysis. This recruitment procedure resulted in two groups well matched regarding the clearly established risk factors of HbA_{1c} and duration of diabetes. An additional analysis was performed that included patients with diabetes duration <20 years and/or no signs of retinopathy or overt nephropathy.

Materials and methods

Screening for diabetic retinopathy was performed by direct ophthalmoscopy with a 30°-angle fundus camera (Zeiss FK 50; Oberkochen, Germany) after dilation of the pupils with a mydriaticum (tropicamid and phenylephrin 2.5%). HbA_{1c} levels were determined by high-performance liquid chromatography (Diamat; Bio-Rad, Munich, Germany). The upper limit of the normal range in our laboratory is defined as ≤6%. Arterial blood pressure was measured with a Hawksley random zero sphygmomanometer (Hawksley and Sons, Sussex, U.K.) twice after an interval of at least 10 min in supine position, and the two results were averaged. Blood lipid status was determined with an enzymatic colorimetric test (BM/Hitachi 717; Boehringer Mannheim, Meylan, France). The albumin excretion rate was measured from an overnight urine collection by turbidimetry (Turbitimer; Behring, Marburg, Germany). Microalbuminuria was defined as an albumin excretion rate >20 μg/min (or >30 mg/24 h) on at least two out of three consecutive occasions during the last 6 months. Overt nephropathy was diagnosed in cases of macroalbuminuria (>300 mg/24 h) or any creatinine elevation above the normal range (−1.5 mg/dl).

Detection of the I/D polymorphism

Genomic DNA from leukocytes was prepared with standard techniques. The polymorphic region in intron 16 of the ACE gene (17q23) was amplified with the polymerase chain reaction (PCR) by using primers and PCR-cycling conditions as described previously (32). The two PCR products, a 190-bp fragment in the case of deletion (D), and a 490-bp fragment in the case of insertion (I) were separated by agarose gel electrophoresis. Because the D allele in heterozygous samples is preferentially amplified, each sample found to have the DD genotype was subjected to a second independent PCR amplification with a primer pair that recognizes an insertion-specific sequence (33).

Statistical analysis

Data are means ± SD. Comparisons between groups were performed with an unpaired *t* test for continuous data or with the use of contingency tables (χ^2 test) for analysis of frequencies. A χ^2 test was also performed as a first explorative analysis of differences in genotype distribution between subgroups.

Logistical regression models (34) were used to analyze the influence of known or suspected risk factors (e.g., diabetes duration, HbA_{1c}, systolic blood pressure, microalbuminuria, and ACE genotype) on developing proliferative retinopathy. All analyses were performed in a marginal unadjusted and partial adjusted sense. The strength of the prognostic factors is described by estimates of the odds ratios (ORs) that show the relative risk of developing proliferative retinopathy. In addition, 95% CIs of these estimates and two-sided *P* values were calculated. Although the subjects were matched by diabetes duration and HbA_{1c} in the main analysis, there were still some slight differences between the groups regarding the shape of the distribution of these variables. Thus, we included the prognostic factors, diabetes duration, and HbA_{1c} in the regression model to adjust for this unbalance without interpreting the respective parameter estimates. The role of the qualitative factor ACE polymorphism is described by two ORs that estimate the relative risks of the DD and ID genotypes compared with the II genotype for developing retinopathy.

A further analysis of the complete sample that additionally included patients with diabetes duration <20 years, patients with no signs of retinopathy, and patients with overt nephropathy was performed with polychotomous logistical regression analysis. To test for a possible interacting effect of diabetes duration and ACE genotype, an interaction term was included and tested within the regression model.

A *P* value of <0.05 was considered statistically significant, and all *P* values are the results of two-sided tests. All calculations were performed with a commercially available program (BMDP; SAS Institute, Cary, NC).

RESULTS

Characteristics of the patient sample and clinical findings

Some descriptive characteristics of all subjects are shown in Table 1. The two groups were comparable regarding the established

Table 1—*Comparison of clinical characteristics of 175 patients with type 1 diabetes and diabetes duration ≥20 years who had nonproliferative or proliferative retinopathy*

	Type 1 diabetic subjects		
	Nonproliferative retinopathy	Proliferative retinopathy	*P* value
n	81	94	—
Sex (M/F)	47/34	55/39	NS
Age (years)	47.7 ± 11.5	47.2 ± 9.9	NS
BMI (kg/m²)	24.7 ± 2.4	25.4 ± 3.5	NS
Diabetes duration (years)	29.7 ± 8.8	31.5 ± 8.2	NS
HbA_{1c} (%)	8.32 ± 1.3	8.46 ± 1.3	NS
Systolic blood pressure (mmHg)	130.6 ± 17.4	141.1 ± 22.6	<0.05
Diastolic blood pressure (mmHg)	79.9 ± 10.9	81.8 ± 10.7	NS
Blood pressure therapy	29 (37)	51 (54)	<0.05
Intensive insulin treatment	62 (80)	57 (61)	<0.01
Microalbuminuria (≤20 vs. >20 μg/min)	30 (38)	50 (54)	<0.001
Creatinine (mg/dl)	1.02 ± 0.18	1.08 ± 0.23	NS
Microalbumin excretion rate (μg/min)	31.0 ± 56.6	83.7 ± 109.9	<0.001
Triglycerides (mg/dl)	110.1 ± 121.2	135 ± 130.1	NS
Total cholesterol (mg/dl)	216.7 ± 42.5	226.8 ± 59.7	<0.01
HDL cholesterol (mg/dl)	80.1 ± 107.4	65.7 ± 26.6	<0.001
LDL cholesterol (mg/dl)	129.1 ± 45.0	136.3 ± 43.9	NS

Data are *n*, means ± SD, or *n* (%). *n* = 175.

risk factors of diabetes duration and HbA_{1c}. No significant differences could be found regarding sex, age, BMI, diastolic blood pressure, creatinine, and triglycerides.

Patients with proliferative retinopathy had elevated systolic blood pressures and increased microalbumin excretion rates compared with patients without proliferative retinopathy.

Patients with nonproliferative retinopathy were more frequently treated with intensive insulin therapy (at least four blood glucose measurements a day and insulin applications according to blood glucose levels and meal intake). Despite this difference in the treatment strategies, metabolic control was comparable be-tween groups. Because metabolic control in most subjects was well documented for 10 years (1986–1996), we were able to compare actual HbA_{1c} level in 1997 with the median over 10 years in both groups. Long-term HbA_{1c} values were slightly lower (8.4 ± 1.4 and 8.0 ± 1.0% in subjects with proliferative and nonproliferative retinopathy, respectively) but not statistically different when compared with actual HbA_{1c}. Of the control subjects, 37% were on antihypertensive therapy, versus 51% for the proliferative retinopathy group.

Frequency of genotype, association between genotype and phenotype, and importance compared with other risk factors

The genotype frequencies of our study population did not significantly differ from the Hardy-Weinberg equilibrium. Frequencies of genotypes in all subjects are shown in Table 2. The genotype distribution in subjects with proliferative retinopathy (*n* = 94; DD 39.4%, ID 48.9%, II 11.7%) deviated significantly ($P < 0.001$) from those with nonproliferative retinopathy (*n* = 81; DD 17.3%, ID 54.3%, II 28.4%). In univariate logistical regression analysis, the genotype (in particular DD vs. II), systolic blood pressure, and microalbuminuria were shown to have a significant effect on the development of proliferative retinopathy. Subsequent multivariate logistical regression analysis was performed to identify the independent effects of selected variables found to be predictive for proliferative retinopathy (Table 3). The relative risk for developing proliferative retinopathy conferred by the DD genotype compared with an II genotype assessed by OR (95% CI) was 6.6 (2.2–19.5) ($P = 0.0026$), which indicates a 6.6-fold increase in the risk after adjustment for the effects of the other risk factors (Table 3). Apart from the genotype, systolic blood pressure ($P = 0.0093$), but not microalbuminuria, had a significant effect on proliferative retinopathy in the multiple regression analysis.

We performed an additional evaluation that included patients with a short duration of disease and/or no signs of retinopathy or overt nephropathy who had all been excluded from the main analysis because of the matching criteria. The 327 patients were categorized by retinopathy status into three groups: no signs of retinopathy, background retinopathy, and proliferative retinopathy. The ACE genotype distribution in subjects with proliferative retinopathy (*n* = 127; DD 39%, ID 49%, II 12%) was significantly different ($P < 0.001$) from patients with background retinopathy (*n* = 121; DD 20%, ID 54%, II 26%) or without retinopathy (*n* = 79; DD 21.5%, ID 58%, II 20.5%). In the subsequent multivariate polychotomous logisti-

Table 2—*Comparison of frequency of ACE genotypes*

	Background retinopathy	Proliferative retinopathy
n	81	94
Genotype		
DD	14 (17.3)	37 (39.4)
ID	44 (54.3)	46 (48.9)
II	23 (28.4)	11 (11.7)

Data are *n* (%). $P = 0.001$, χ^2 test.

Table 3—*Multiple logistical regression analysis of the independent effects of selected variables found to be predictive of proliferative diabetic retinopathy*

Risk factor	Relative odds (95% CI)	*P* value
ACE genotype		0.0026
ID vs. II	2.401 (0.943–6.114)	
DD vs. II	6.606 (2.215–19.54)	
Systolic blood pressure (continuous)	1.027 (1.007–1.048)	0.0093
Microalbuminuria (≤20 vs. >20 μg/min)	1.335 (0.644–2.768)	0.4372

Subjects were matched with respect to duration of diabetes and HbA_{1c}.

cal regression analysis, the adjusted relative risk for proliferative retinopathy in a patient with a DD genotype versus an II genotype was 5.8 (1.4–25.0) (P = 0.012).

Testing for interaction

This additional analysis was applied to evaluate the effect of duration of diabetes depending on the expression of the polymorphism and vice versa. No statistically significant (P = 0.958) interaction of diabetes duration with the DD genotype in proliferative retinopathy was found in this study population.

CONCLUSIONS — In this case-control study of type 1 diabetic patients with a long duration of disease, we demonstrated that the DD genotype is strongly associated with proliferative retinopathy. Several studies have provided evidence that multiple factors determine the risk of proliferation. Some factors may be metabolic, others may be genetic systemic conditions, or local factors in the eye itself may be responsible.

Because identical twins with type 1 diabetes have similar levels of retinopathy (35), and sibling pairs with diabetes show concordance for proliferative retinopathy (36), genetic factors have been involved in further research. Familial clustering of retinopathy was found in multiplex type 1 diabetic families in the Diabetes Control and Complications Trial cohort, and there is some evidence that certain HLA genotypes contribute to the development of proliferative retinopathy (37–39). ACE is a strong candidate gene with a reasonable probability of involvement in the development of proliferative retinopathy and microvascular complications. An association of the ACE polymorphism with nephropathy has been reported in many studies (21,24,26,27) and confirmed in a meta-analysis published recently (40).

There are several lines of evidence indicating that the renin-angiotensin system plays a key role in the pathophysiological process leading to proliferative retinopathy. Increased plasma ACE levels have been associated with diabetes per se, microvascular complications, and retinopathy in particular (6–12). Furthermore, ACE levels are particularly high in patients with proliferative retinopathy (6,7,11), which suggests that elevated serum ACE levels may be a potential cause of retinal vascular damage in diabetes. ACE levels are under genetic control, the polymorphism in the ACE gene contributes greatly to the variability of ACE plasma levels, and homozygosity for the D allele is strongly linked to the highest plasma concentrations (19,20). Renin and its inactive precursor prorenin are further components of the renin-angiotensin system and have been identified as markers for an increased risk of retinopathy (13) and of proliferative retinopathy in particular (14).

Clinical and experimental trials with ACE inhibitors have had beneficial effects on the progression of diabetic retinopathy. ACE inhibitors have been found not only to delay a progressive breakdown of the blood-retina barrier (41) and consecutively reduce leakage but also to reverse preproliferative retinopathy (42). In fact, improvement by one or more grades of retinopathy was seen in diabetic patients after long-term treatment with ACE inhibitors. This improvement was not consistently related to a decrease in blood pressure, better glycemic control, or a reduction in albumin excretion rate (42). Recently, a multicenter study (EURODIAB) demonstrated a reduction in retinopathy progression in normotensive type 1 diabetic patients treated with the ACE inhibitor lisinopril (18).

The objective of this case-control study was to examine the relationship between the ACE polymorphism and proliferative retinopathy. Our data provide evidence that, in addition to Hba_{1c} and diabetes duration, the DD genotype is an independent risk factor for proliferative retinopathy in type 1 diabetic patients with a long duration of disease. Consequently, the findings are limited to retinopathy occurring mainly in the third and fourth decades of disease. After diabetes duration of >20 years, patients without any signs of retinopathy are rare, so we excluded this small group from further analysis. Moreover, all subjects were selected from a population in one institution; thus, metabolic control in terms of education, knowledge, and motivation was quite homogenous between groups.

Previous studies that attempted to assess the risk for diabetic retinopathy related to the I/D polymorphism of the ACE gene failed to find an association (21,22,26,28,29,43). In most of these studies, patients without retinopathy and with any stage of retinopathy were compared (40), and sample sizes of patients with proliferative retinopathy were small for detecting statistically significant associations (21,28,43). Besides, the effects of differences in metabolic control (43) or diabetes duration as well as the confounding influence of nephropathy were not considered (29). Other studies with data from Japanese patients with type 2 diabetes (22,26) reported a lack of association with myocardial infarction, nephropathy, or retinopathy. However, due to ethnic differences and differences in the type of diabetes, these results are not comparable with our findings. Recently, Tarnow et al. (29) examined the ACE genotype distribution in 155 type 1 diabetic patients with proliferative retinopathy and compared it with 67 patients with no signs of diabetic retinopathy. The investigators found no association between the DD genotype and patients with proliferative retinopathy or without retinopathy. In this study, patients with proliferative retinopathy had significantly higher HbA_{1c} levels (9.6 ± 1.6%) compared with patients without retinopathy (8.4 ± 0.9%; $P <$ 0.001), and diabetes duration was significantly longer (P = 0.01). Due to the huge differences in HbA_{1c} levels and duration of disease between the two patients groups, a possible genetic susceptibility to diabetic retinopathy might be overlapped by the influence of these main risk factors and the fact that multiple regression analysis was not applied to the evaluation of retinopathy. Besides, the confounding effect of nephropathy was not considered by Tarnow et al. (29): nephropathy was present in 88% of their patients with proliferative retinopathy. These important differences in study design may be responsible for the conflicting results.

The case-control design of this study, which was matched in terms of the main clinical parameters (diabetes duration and HbA_{1c}), was chosen to avoid bias caused by the influence of known risk factors. Case-control studies may nonetheless suffer from several biases that may lead to false-positive and false-negative results (44). The association between the ACE deletion polymorphism and proliferative retinopathy is strongly supported by the multiple logistical regression analysis used to examine the independent influence of the predictive parameters. Further evidence of the association was provided by the complete sample analysis. Because microalbumin excretion may contribute to the risk of proliferation in patients with background retinopathy, we introduced microalbuminuria as an additional covariate into the logistical regression analysis. The risk associated with the DD genotype remained highly significant after adjustment for the effects of other predictive factors.

On the other hand, a survival bias cannot be avoided in a disease association study, and prospective studies in diabetic families will be necessary to confirm the role of the ACE locus. It is possible that early mortality of diabetic subjects because of the D allele could lead to an underestimation of this gene in our study.

We conclude that the development of the proliferative form of diabetic retinopathy is determined by four independent risk factors: diabetes duration, metabolic control, ACE genotype, and systolic blood pressure. The clinical effect of this study could be the identification of subjects susceptible to the development of proliferative retinopathy. Hence, the potential value of identifying a patient with a DD genotype could be the advantages of early therapeutic intervention and reducing further progression of diabetic retinopathy. Further studies are necessary to clarify whether early ACE inhibitor therapy for normotensive patients with a DD genotype could have beneficial effects in preventing proliferative retinopathy.

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