Serum Paraoxonase Activity and Its Relationship to Diabetic Complications in Patients With Non–Insulin-Dependent Diabetes Mellitus

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Paraoxonase (PON) is an esterase associated with high-density lipoprotein (HDL). Serum PON activity is affected by PON gene polymorphism (*L/M*, Leu-Met54, and *Q/R*, Gln-Arg191). We investigated PON activity and polymorphism in 108 patients (53 men and 55 women) with non–insulin-dependent diabetes mellitus (NIDDM) and 161 control subjects (82 men and 79 women) matched to the patients by age and gender. Serum PON activity was determined using paraoxon as a substrate. PON gene polymorphisms were detected by the restriction fragment length polymorphism method after a polymerase chain reaction. The mean PON activity in the patients was significantly lower than in the controls (116 ± 55 and 162 ± 57 U/L, respectively, $P < .001$). The distribution of each genotype showed no difference between the patient and control groups, and PON activity increased in the order of the *QQ* < *QR* < *RR* genotype and *MM* < *LM* < *LL* genotype in both groups. However, among each genotype subgroup, the activity was lower in patients than in controls. Forty-one patients with retinopathy had lower PON activity than those without the complication (94 ± 36 and 129 ± 61 U/L, respectively, $P < .002$). There was also a significant difference in PON activity between patients with and without overt proteinuria (93 ± 38 and 122 ± 58 U/L, respectively, $P < .05$). Logistic analysis showed that serum PON activity was one of the significant factors for retinopathy. These results suggest that decreased PON activity in patients with NIDDM is involved in diabetic vascular complications.

HUMAN PARAOXONASE (PON) is an esterase that hydrolyzes aromatic carboxylic acid esters, organophosphates, and carbamates.[1] PON is associated with high-density lipoprotein (HDL) containing apolipoprotein (apo) A-I and apo J,[2] and has been shown to decrease the susceptibility of low-density lipoprotein (LDL) to lipid peroxidation in vitro.[3,4] The serum activity of PON, which may be involved in atherosclerosis,[5] varies among individuals.[6] This protein has polymorphic sites: Leu-Met at position 54 of the amino acid sequence (*L/M*) and Gln-Arg at position 191 (*Q/R*).[7] *Q/R* polymorphism is associated with the PON activity, and PON activity in subjects with the *QQ* genotype is lower than in those with the *RR* genotype.[8] This polymorphism has been associated with coronary heart disease (CHD) among French Caucasians with non–insulin-dependent diabetes mellitus (NIDDM),[9] and is thought to be a risk factor for CHD in the American general population.[10] However, we reported that among the Japanese, the frequency of the *R* allele is higher than in Western study populations, and did not prove the relationship between CHD and the polymorphism.[11] Another polymorphism of *L/M* at position 54 was also recently shown to be associated with serum PON activity and to be closely related with the serum concentration of PON.[12]

Several reports have shown that PON activity in patients with diabetes mellitus is decreased,[13] and Abbott et al[14] showed that this decrease in PON activity was involved in diabetic neuropathy independently of the *Q/R* polymorphism. In the present study, we investigated PON activity and the two gene polymorphisms *L/M* and *Q/R* in NIDDM patients and controls, and examined whether PON activity or PON gene polymorphism are associated with diabetic complications including microangiopathy and macroangiopathy.

SUBJECTS AND METHODS

Subjects

Patients with NIDDM (n = 108, 53 men and 55 women) were recruited from our outpatient clinic between June and October 1996. They had no ketoacidosis, renal failure (serum creatinine > 2.5 mg/dL), liver disorder, or recent history of cardiovascular disease. The age range was 30 to 70 years. Clinical characteristics of the patients are shown in Table 1.

The NIDDM diagnosis was made according to World Health Organization criteria.[15] Diabetic nephropathy was defined as a urine protein concentration of greater than 0.30 g/L at two or more consecutive measurements separated by an interval of 4 weeks or longer. The concentration was measured by a dipstick test (Uriflet II; Kyoto Daiichi Kagaku, Kyoto, Japan). Diabetic retinopathy was assessed by a consultant ophthalmologist by direct ophthalmoscopy with the pupils dilated. A patient with simple, preproliferative, or proliferative retinopathy was defined as having retinopathy. Diabetic neuropathy was defined if the patient had at least one of the symptoms, pain, tingling, burning, or loss of sensation, and at least one of the objective signs on examination, decreased or absent patellar tendon reflex, decreased vibratory sensation, or impaired position sense. Macroangiography included CHD, cerebrovascular disease (CVD), and peripheral atherosclerosis obliterans (ASO). CHD was defined according to the criteria of the Diabetes Control and Complications Trial.[16] CVD was defined as neurological abnormalities with definite evidence on computed tomography or magnetic resonance imaging. ASO was defined as evidence of claudication with objective findings including arteriography.

Control subjects (n = 161) who visited a medical center in the same area as our hospital were recruited. The controls were matched to the patients by age and gender (Table 1). They were confirmed to be of normal status by physical and laboratory examinations including a normal resting electrocardiogram. They did not have diabetes mellitus, as confirmed by oral glucose tolerance test, or a history of cardiovascular disease. Informed consent was obtained from each subject prior to the study.

Methods

Serum PON activity was measured using paraoxon as the substrate according to the method described by Eckerson et al.[17] Plasma concentrations of total cholesterol and triglycerides were measured by

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Table 1. Characteristics of the NIDDM Patients and Control Subjects (mean ± SD)

Characteristic	Patients	Controls
Gender, n (male/female)	53/55	82/79
Age, yr (male/female)	58 ± 7/56 ± 10	57 ± 8/58 ± 7
Body mass index (kg/m²)	24.2 ± 4.6	24.4 ± 3.6
Diabetes duration (yr)	9.6 ± 8.7	—
Total cholesterol (mg/dL)	200 ± 40	202 ± 33
Triglycerides (mg/dL)	137 ± 79*	111 ± 68
HDL-C (mg/dL)	51 ± 14*	60 ± 15
Apo A-I (mg/dL)	135 ± 23	138 ± 26
Apo A-II (mg/dL)	33 ± 7	32 ± 5
Apo B (mg/dL)	103 ± 25	103 ± 26
HbA_{1c} (%)	6.7 ± 1.5	ND
Therapy (n)†		
Diet alone	34	—
Sulfonylurea	47	—
Insulin	29	—

Abbreviation: ND, not determined.

*$P < .001$ *v* controls (unpaired *t* test).

†Four patients treated by diet, 13 by sulfonylurea, and 1 by insulin simultaneously took an α-glucosidase inhibitor.

enzymatic methods using an autoanalyzer. The plasma HDL-cholesterol (HDL-C) concentration was determined using a kit based on the dextran sulfate, phosphotungstate, and Mg precipitation method. Apo A-I, apo A-II, and apo B concentrations were measured by turbidometric immunoassays.

Genomic DNA was extracted from whole blood using a commercial kit (SMI test; Sumitomo, Tokyo, Japan). For analysis of the two PON polymorphisms, a polymerase chain reaction and digestion of the amplified fragments with restriction enzymes (*Hsp92II* for *L/M* and *AlwI* for *Q/R*) were performed as described previously.[8,12]

Statistical Analysis

All data are presented as the mean ± SD. A comparison of variables between two groups or among three groups was performed using the unpaired *t* test or one-way ANOVA, respectively. Genotype freqencies were estimated by chi-square test. Logistic analysis was performed using a software program for use with a personal computer (JMP, version 3.1; SAS Institute, Cary, NC). *P* values less than .05 were considered significant.

Table 2. Distribution of the Two PON Gene Polymorphisms in Patients With NIDDM and Controls

	Patients				Controls			
	LL	*LM*	*MM*	Total	*LL*	*LM*	*MM*	Total
QQ	8	2	2	12	13	5	0	18
QR	56	8	1	65	79	14	0	93
RR	31	0	0	31	50	0	0	50
Total	95	10	3	108	142	19	0	161

RESULTS

PON Activity

Serum PON activity in NIDDM patients was significantly lower than in the controls. The ratio of serum PON activity (units per liter) divided by HDL-C (milligrams per deciliter) or by apo A-I (milligrams per decliter) was also significantly lower in the patients than in the controls (Fig 1).

PON Activity and Genotype

The distribution of the two PON gene polymorphisms is shown in Table 2. The distribution of the *Q/R* and *L/M* polymorphisms did not differ between the patient and control groups. The frequency of the *Q* and *R* alleles of the *Q/R* polymorphism was similar between the patient and control groups (0.41/0.59 and 0.40/0.60, respectively). The frequency of the *L* and *M* alleles of the *L/M* polymorphism was also the same among the patient and control groups (0.93/0.07 and 0.94/0.06, respectively), and the frequency of the *M* allele was very small in both groups.

Serum PON activity in each subgroup classified by the combination of two genotypes among the patients and controls is shown in Fig 2. PON activity increased in the order of the *QQ* < *QR* < and *RR* genotype in both the patient and control groups. The activity increased in the order of the *MM* < *LM* < *LL* genotype. Among all subgroups except *QQ/MM*, *QR/MM*, *RR/MM*, and *RR/LM*, which were not found among the controls, activity was lower in the patients than in the controls. The ratios of PON activity/HDL-C and PON activity/apo A-I in each subgroup among the patients and controls showed almost the same results (data not shown).

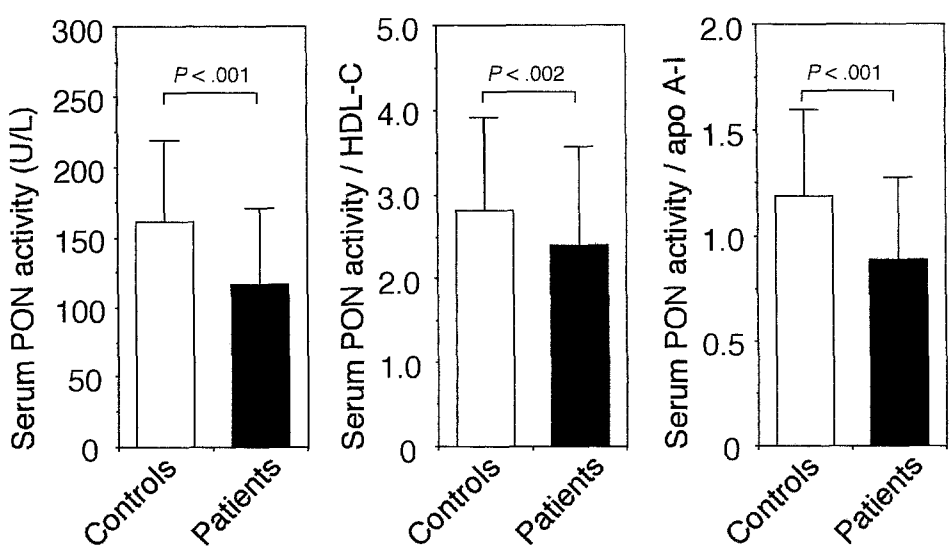

Fig 1. Serum PON activity (U/L), serum PON activity (U/L)/HDL-C (mg/dL) ratio, and serum PON activity (U/L)/apo A-I (mg/dL) ratio in control subjects and patients with NIDDM.

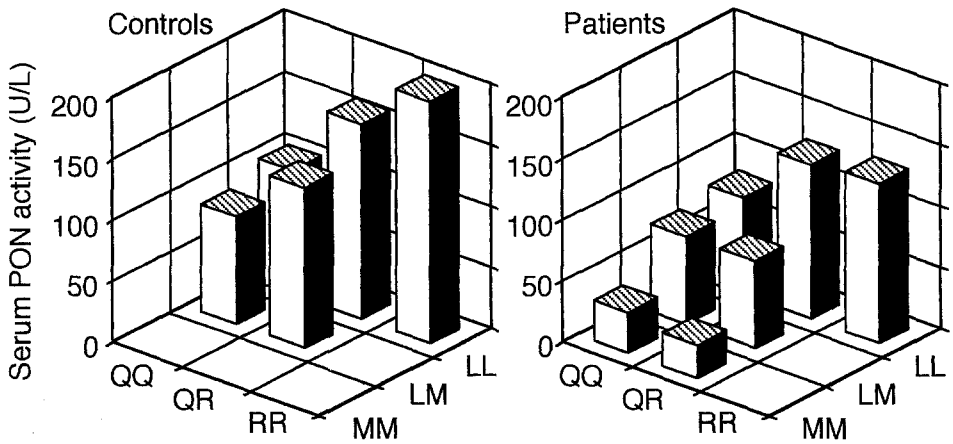

Fig 2. Serum PON activity (U/L) in each subgroup classified by *Q/R* polymorphism and *L/M* polymorphism of PON among the control subjects and patients with NIDDM.

Diabetic Complications and PON Activity

Table 3 shows PON activity, and the ratios of PON activity/HDL-C and PON activity/apo A-I in each group of patients with or without diabetic complications. PON activity was significantly lower in the group with diabetic nephropathy versus the group without, although there was no difference in thePON activity/HDL-C or PON activity/apo A-I ratios. In the group with retinopathy, not only PON activity but also the PON activity/HDL-C and PON activity/apo A-I ratios were significantly lower than in the group without retinopathy. There was no difference in any of these activities between groups with and without neuropathy. The 13 patients with macroangiopathy (six with CHD, five with CVD, and two with ASO) had lower PON activity, but not significantly so.

The result of one model of logistic analysis for the risk of retinopathy, using the independent factors of age, gender, plasma total cholesterol, HDL-C, hemoglobin A_{1c} (HbA_{1c}), duration of diabetes, body mass index, and PON activity, is shown in Table 4. The diabetes duration, serum PON activity, HbA_{1c}, and total cholesterol were found to be significant factors for the risk of retinopathy. The effect of PON activity was not weak in several other models analyzed. However, PON activity was not a significant factor for the risk of nephropathy in the same model of logistic analysis.

There was no difference in the genotype distribution between patients with and without each complication (data not shown).

Table 3. Comparison of PON Activity, PON Activity/HDL-C Ratio, and PON Activity/Apo A-I Ratio Between Patients With and Without Diabetic Complications

Complication	Absent	Present	Unpaired *t* Test (*P*)
Nephropathy	n = 85	n = 23	
PON activity	122 ± 58	93 ± 38	<.05
PON/HDL ratio	2.45 ± 1.27	2.15 ± 0.80	NS
PON/Apo-AI ratio	0.90 ± 0.42	0.77 ± 0.29	NS
Retinopathy	n = 67	n = 41	
PON activity	129 ± 61	94 ± 36	<.002
PON/HDL ratio	2.60 ± 1.33	2.01 ± 0.78	<.01
PON/Apo-AI ratio	0.96 ± 0.45	0.74 ± 0.27	<.01
Neuropathy	n = 74	n = 34	
PON activity	120 ± 55	108 ± 57	NS
PON/HDL ratio	2.39 ± 1.23	2.36 ± 1.10	NS
PON/Apo-AI ratio	0.88 ± 0.41	0.87 ± 0.40	NS
Macroangiopathy	n = 95	n = 13*	
PON activity	119 ± 57	90 ± 33	<.1
PON/HDL ratio	2.43 ± 1.23	2.03 ± 0.76	NS
PON/Apo-AI ratio	0.90 ± 0.41	0.73 ± 0.25	NS

*Six patients had CHD, 5 had CVD, and 2 had ASO.

DISCUSSION

There is a large variation in serum PON activity among individuals,[6] and one cause of this variation has been identified as the *Q/R* polymorphism of the PON gene.[8] PON activity in patients with diabetes mellitus has been shown to be lower.[13,14] Abbott et al[14] reported that the basal activity and specific activity of PON in patients with diabetes is low; however, the *Q/R* genotype of PON polymorphism did not differ in patients with diabetes versus controls. Therefore, patients with diabetes must have another factor influencing PON activity besides the polymorphism. Abbott et al[14] also revealed that the decreased PON activity was involved in diabetic neuropathy. PON has been shown to be associated with the development of atherosclerosis.[5] The oxidative modification of LDL is important in the initiation of atherosclerosis.[18,19] Both purified PON and HDL-associated PON inhibit LDL oxidation in vitro.[3-5] Therefore, HDL-associated PON may protect LDL against oxidation in vivo. These findings prompted us to investigate whether PON activity in patients with diabetes was related to diabetic vascular complications including not only macroangiopathy but also microangiopathy.

We found that serum PON activity in NIDDM patients was significantly lower than in the gender- and age-matched controls. Although the HDL-C concentration was lower in the

Table 4. Logistic Analysis of Various Factors for Diabetic Retinopathy in Patients With NIDDM

Factor	Chi-Square Test	*P*
Duration	20.0	<.001
PON activity	8.43	<.005
HbA_{1c}	6.02	<.02
Total cholesterol	5.21	<.05

$R^2 = .544$, $P < .001$.

NOTE. Independent factors are as follows: age (yr), gender (male = 1 and female = 2), total cholesterol (mg/dL), HDL-C (mg/dL), HbA_{1c} (%), duration of diabetes (yr), body mass index (kg/m^2), and PON activity (U/L).

patients than in the controls, the PON activity/HDL-C and PON activity/apo A-I ratios were also lower in the patients. Therefore, PON activity in the patients did not always result from a decrease of HDLs, which contain PON in the circulation.

Our results also showed that the two different polymorphisms were associated with PON activity in both NIDDM patients and control subjects. PON activity increased in the order of the *QQ* < *QR* < *RR* genotype within the *Q/R* polymorphism and in the order of the *MM* < *LM* < *LL* genotype within the *L/M* polymorphism in both the patient and control groups. *Q/R* genotype distribution in the patients did not differ significantly from that in the controls, a finding previously obtained by Abbott et al[4]; therefore, this genetic polymorphism may not cause the difference in PON activity between the patient and control groups. *L/M* polymorphism was associated not only with PON activity but also with the serum PON concentration, and the PON concentration in subjects with the *L* allele has been shown to be high.[12] However, our results showed no difference in the distribution of *L/M* polymorphism between the patients and controls. Therefore, this genetic polymorphism is not involved in decreased PON activity in patients with NIDDM.

Among NIDDM patients, PON activity was significantly decreased in subjects with diabetic nephropathy or retinopathy compared with those without the complications. The PON activity in patients with macroangiopathy tended to be low, although the difference was not significant. These results suggest that decreased PON activity is involved in diabetic vascular complications. We have three speculations regarding the decreased activity in NIDDM patients with such complications. First, some factors that cause vascular damage in diabetes may decrease PON activity. The diabetic state may affect lipoprotein composition or metabolism.[20] For example, glycation of apo A-I or PON itself may alter the function of PON on HDLs and reduce the enzyme activity. Second, the development of microangiography or macroangiopathy disturbs the chemical and physical function of the vascular wall. Factors derived from the vascular wall, such as certain cytokines, may have a secondary effect on the function of circulating PON. Finally, PON has been shown to play a role in the decrease of LDL oxidation in vitro; PON may thus be an important factor in the protection against oxidation in vivo. Therefore, a decreased PON activity may affect the oxidation of lipoproteins or other circulating factors and thus make a diabetic patient susceptible to vascular complications. Decreased PON function appears to be one of the risk factors for vascular damage in patients with diabetes. The strength of the association of PON activity and nephropathy was weak compared with the association of PON activity and retinopathy. This result suggests that the association with nephropathy was weakened under the influence of stronger factors affecting the progression of nephropathy such as hypertension or dietary protein. Our results showed no relationship between PON activity and diabetic neuropathy, which differs from the report by Abbott et al.[14] This discrepancy may be caused by different criteria used to define diabetic neuropathy. We included patients with mild neuropathy, but Abbott et al recruited only patients with moderate or severe neuropathy. Therefore, we cannot exclude the possibility that PON activity shows some relationship to neuropathy by increasing the number of patients and selecting only patients with severe complications. A larger study is necessary to resolve these problems.

The *RR* genotype of PON polymorphism has been reported to be a risk factor for CHD.[9,10] The *RR* genotype has also been shown to be linked to the *LL* genotype of another polymorphism of PON, and the *LL* genotype may be the more important risk factor for CHD.[12] Our results also showed that the *RR* genotype is linked to the *LL* genotype, and PON activity of the *RR* and *LL* genotypes was higher than that of the other genotypes in each polymorphism. PON genotypes with higher activity may be associated with the development of CHD, which is a slightly contradictory phenomenon. However, Mackness et al[21] showed in vitro that HDL with the *RR* genotype, which has high serum PON activity, had less ability to protect against LDL oxidation with time compared with that of the *QR* or *QQ* genotype. This may explain the discrepancy between PON activity and the PON effect on CHD. PON activity reveals its enzymatic function with the use of paraoxon, which is never detected in the human body, as a substrate. The actual substrate of PON in humans is not yet known; therefore, it may not be accurate to estimate PON function in vivo using paraoxon as a substrate. However, the decrease of PON activity detected using paraoxon as the substrate in the present NIDDM patients group suggests that PON itself or something related to PON is changed in NIDDM. The mechanism of the PON change in NIDDM appears to differ from that in genetic polymorphisms. The fundamental mechanism of the decrease in PON or the changed PON itself in diabetic patients may be harmful to vessels.

In conclusion, our results showed that PON activity was decreased in patients with NIDDM, and PON activity in patients with diabetic vascular complications was lower than in patients without such complications. The PON associated with HDL in patients with NIDDM is probably changed and may be involved in diabetic vascular complications. In the future, we must clarify the mechanism underlying decreased PON in diabetes and the manner in which PON affects diabetic complications.

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