Serum Paraoxonase Activity and Genotype Distribution in Japanese Patients with Diabetes Mellitus

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We investigated the serum paraoxonase (PON) activity and genotype distribution in 139 patients with non-insulin-dependent diabetes mellitus and in 240 control subjects in a Japanese population. There were no significant differences in PON genotype frequencies between the diabetic and control subjects, but the PON activity was significantly less in diabetic subjects ($p < 0.01$). PON activity was significantly related to levels of total cholesterol and high density lipoprotein (HDL) cholesterol in the control subjects, but not in the diabetic subjects. In diabetic subjects with the genotype AA, the mean maximum intimal-medial thickness (IMT) of the carotid artery was significantly greater than in subjects with genotype AB or BB. PON may play an important role in the development of atherosclerosis associated with diabetes mellitus. (Internal Medicine 37: 581–584, 1998)

Key words: non-insulin-dependent diabetes mellitus (NIDDM), atherosclerosis, intimal-medial thickness (IMT), high density lipoprotein (HDL) cholesterol, polymorphism of paraoxonase gene

Introduction

Paraoxonase [EC 3.1.8.1] is an high density lipoprotein (HDL)-associated esterase that hydrolyzes paraoxon (1). Many studies have indicated that the existence of a genetic polymorphism of paraoxonase is a determinant of its activity (2, 3). This polymorphism results from an amino acid substitution in the active site of the enzyme, giving rise to low- and high-activity isoform. The Gln192(A) isoform is not highly active toward paraoxon; in contrast, the Arg192(B) isoform hydrolyzes paraoxon more rapidly. Previous studies in Caucasians have shown that patients with diabetes mellitus have reduced serum paraoxonase activity (4, 5), and that paraoxonase gene polymorphism is associated with a predisposition to coronary atherosclerosis (6, 7). This suggests that the decrease in the activity of serum paraoxonase associated with diabetes mellitus may play a role in the increased incidence of premature atherosclerosis associated with this disease.

In Caucasian populations, the frequency of the low activity phenotype and genotype seems to be high (8–10). In contrast, the frequency of the low activity phenotype seems to be low in ethnic groups such as Chinese, Filipinos and Dravidian Indians (11), and the relationship of paraoxonase and diabetes mellitus in these groups, as well as in Japanese, is unknown. Here, we

investigate the serum paraoxonase activity and genotype distribution in Japanese patients with diabetes mellitus.

Subjects and Methods

Subjects

The control population consisted of 240 apparently healthy individuals not taking medication, who were present at a routine health check-up. The experiment population was made up of patients at the Third Department of Internal Medicine, Ehime University Hospital, and comprised 139 men and women with non-insulin-dependent diabetes mellitus (NIDDM). Diabetes mellitus was diagnosed according to the criteria of the Japan Diabetes Society. In our assessment of macroangiopathy, we took measurements of the mean maximum intimal-medial thickness (IMT) of the carotid artery by ultrasonography (12, 13). The imaging protocol involved obtaining a single longitudinal lateral view of the distal 10 mm of the right and left common carotid arteries. All subjects gave informed consent in accordance with the Helsinki Declaration.

Laboratory measurements

Venous blood samples were drawn after an overnight fast. Serum total cholesterol, HDL-cholesterol (HDL-C, measured

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after the precipitation of lower density lipoproteins with phosphotungstate/MgCl₂), and triglyceride were enzymatically determined using an autoanalyzer (TBA-80S; Toshiba Inc., Tokyo). Plasma glucose concentration upon fasting was measured by the glucose oxidase method. Glycosylated hemoglobin (HbA1c) was measured by high performance liquid chromatography assay (Kyoto Daiichi Scientific Equipment Inc., Kyoto). The fructosamine (FRA) concentration was measured by nitrobluetetrazolium (NBT) reduction method with fructosamine assay kit II (Roche Japan, Tokyo). Serum C-peptide (CPR) concentration was measured by radioimmunoassay with the C-Peptide Test (Shionogi Research Laboratories, Osaka).

Paraoxonase enzyme activity

Paraoxonase activity was measured by adding serum to 1 ml Tris-HCl buffer (100 mmol/l, pH 8.5) containing 2 mmol/l CaCl₂ and 6.0 mmol/l paraoxon (Sigma Chemical Company, London). The generation of p-nitrophenol was measured at 405 nm and 37°C in a continuously recording spectrophotometer (Pharmacia Ultraspec III, Cambridge, England). A unit was defined as a 1 µmol paraoxon hydrolyzed per minute under the above assay conditions (14).

DNA analysis

Paraoxonase was genotyped by restriction isotyping; the nucleotide substitution corresponding to position 192 (Gln-Arg) creates an AlwI restriction site. White blood cells were isolated from blood and DNA extracted using standard procedures. A 99 base-pair fragment covering the region containing the mutation was amplified by polymerase chain reaction (PCR), with primers described by Humbert et al (3). It involved a primary amplification of 30 cycles with annealing at 61°C. PCR products were digested with AlwI, separated by agarose gel (3%) electrophoresis and visualized by use of ethidium bromide. Allele A (glutamine) corresponded to a 99 base pair fragment and allele B (arginine) to 65 and 34 base pair fragments (Fig. 1).

Statistical analysis

Values were expressed as mean ± standard deviation (SD). All data except that for triglycerides were analyzed using the unpaired *t*-test, linear regression, and Scheffe test followed by one-way ANOVA. Triglycerides were analyzed by the Mann-Whitney U test and Kruskal-Wallis test. Categorical variables were compared between groups by use of the Chi-squared test and crude odds ratio. Allele frequencies were estimated by the gene-counting method, and Hardy-Weinberg's equilibrium was tested by the Chi-squared test. Significance was defined at *p*<0.05.

Results

The diabetic subjects had a lower percentage of men and were on average older than the control subjects (Table 1). However, neither of these parameters was related to the serum paraoxonase activity or to genotype in either group of subjects

Figure 1. PCR-RFLP analysis of paraoxonase genotype. Lane 2, 8, 9, and 11 show the band patterns for the BB genotype. Lane 4, 5, 6, and 7 show the band patterns for the AB genotype. Lane 3 and 10 show the band patterns for the AA genotype. Lane 1 is ϕ 174 Hind III digest.

Table 1. Demographic Details of the Populations Studied

	Control (n=240)	NIDDM (n=139)
Sex (male/female)	179/61	65/74**
Age (years)	48.0 ± 8.70	61.7 ± 13.6**
FPG (mmol/l)	5.82 ± 0.77	8.24 ± 3.43**
HbA1c (%)	ND	7.5 ± 1.9
Fructosamine (µmol/l)	ND	353 ± 78.7
C-peptide (nmol/l)	ND	0.73 ± 0.44
Total cholesterol (mmol/l)	5.02 ± 0.90	5.05 ± 0.98
Triglyceride (mmol/l)	1.47 ± 0.91	1.51 ± 1.01
HDL cholesterol (mmol/l)	1.40 ± 0.38	1.32 ± 0.46

HbA1c: glycosylated hemoglobin, FPG: fasting plasma glucose, ND: not determined, NIDDM: non-insulin-dependent diabetes mellitus. Values are mean ± SD. ***p*<0.01 significantly different from control subjects.

(data not shown).

The gene frequencies in control subjects were 0.31 for allele A with low activity and 0.69 for allele B with high activity. In diabetic subjects, the allele A frequency was 0.33 and the allele B frequency was 0.67. There were no significant differences in the gene frequencies between diabetic and control subjects (Table 2). In both groups, the paraoxonase activity was highest in genotype AA, lower in genotype AB, and lower still in BB. Furthermore, the paraoxonase activity was significantly lower in the diabetic subjects of every genotype except genotype AA (Table 3). Paraoxonase activity was significantly related to the levels of total cholesterol and HDL-C in control subjects, but this was not true in diabetic subjects. Triglycerides were not related to paraoxonase activity in either group (Table 4).

In diabetic subjects, paraoxonase activity was not significantly associated with fasting plasma glucose (FPG), HbA1c, FRA, or CPR (Table 5). In an investigation of clinical parameters with different genotypes, the IMT of carotid arteries in

subjects with the genotype AA were significantly greater than in those with genotypes AB and BB (Table 6).

Discussion

To the authors' knowledge, this is the first report of paraoxonase gene frequencies in a Japanese population, and our findings indicate a higher frequency of allele B with high activity than has been found in Caucasian populations (8–10).

Table 2. Paraoxonase Genotype Distributions in Control and Diabetic Subjects

	Control (n=240)	NIDDM (n=139)
Genotype distribution (%) [#]		
AA	24 (10.0)	14 (10.1)
AB	102 (42.5)	63 (45.3)
BB	114 (47.5)	62 (44.6)
Gene frequency ^{##}		
A	0.31	0.33
B	0.69	0.67

AA: a homozygous low activity genotype, AB: a heterozygous genotype, BB: a homozygous high activity genotype, NIDDM: non-insulin-dependent diabetes mellitus. [#] $\chi^2=0.321$, $p=0.852$, ^{##} $p=0.711$.

Table 3. Paraoxonase Activity in Control and Diabetic Subjects

	Control (n=240)	NIDDM (n=139)
Paraoxonase activity (U/l)	568.8 ± 208.3	437.5 ± 170.3**
genotype AA	249.0 ± 41.1	215.9 ± 73.7
AB	478.3 ± 124.2	382.3 ± 110.8
BB	717.0 ± 162.4	543.6 ± 161.6

AA: a homozygous low activity genotype, AB: a heterozygous genotype, BB: a homozygous high activity genotype, NIDDM: non-insulin-dependent diabetes mellitus. Values are mean ± SD. ** $p<0.01$ significantly different from control subjects. # $p<0.01$ significantly different among genotype AA, AB and BB.

Table 4. Linear Regression of Paraoxonase Activity and Serum Lipid

	Control		NIDDM	
	R	P	R	P
Total cholesterol	0.196	0.002	0.042	0.626
Triglyceride	0.008	0.899	0.056	0.516
HDL cholesterol	0.177	0.006	0.005	0.955

NIDDM: non-insulin-dependent diabetes mellitus.

Table 5. Linear Regression of Paraoxonase Activity and Laboratory Measurement in Diabetic Subjects

	R	P
FPG	0.053	0.537
Fructosamine	0.072	0.401
HbA1c	0.082	0.335
C-peptide	0.038	0.655

FPG: fasting plasma glucose, HbA1c: glycosylated hemoglobin.

Table 6. Clinical Data in Diabetic Subjects with Different Genotypes

		AA (n=14)	AB (n=63)	BB (n=62)
Age	(years)	62.6 ± 13.5	60.4 ± 13.3	62.5 ± 14.1
Sex	(male/female)	5/9	32/31	37/25
BMI	(kg/m ²)	23.1 ± 4.2	24.7 ± 4.8	21.7 ± 6.0
Smokers	(yes/no)	6/8	36/27	40/22
Hypertension	(yes/no)	8/6	40/23	35/27
Diabetes duration	(years)	11.4 ± 12.5	11.7 ± 6.7	13.2 ± 9.1
FPG	(mmol/l)	9.14 ± 3.93	8.50 ± 3.55	7.77 ± 3.17
HbA1c	(%)	7.52 ± 2.07	7.57 ± 1.90	7.49 ± 1.92
C-peptide	(nmol/l)	0.70 ± 0.43	0.70 ± 0.33	0.77 ± 0.53
Total cholesterol	(mmol/l)	4.84 ± 1.15	5.20 ± 0.93	4.95 ± 0.99
Triglyceride	(mmol/l)	1.22 ± 0.43	1.64 ± 1.27	1.45 ± 0.76
HDL-cholesterol	(mmol/l)	1.27 ± 0.71	1.27 ± 0.40	1.38 ± 0.45
IMT	(mm)	0.89 ± 0.38*	0.72 ± 0.17	0.74 ± 0.18

AA: a homozygous low activity genotype, AB: a heterozygous genotype, BB: a homozygous high activity genotype, BMI: body mass index, FPG: fasting plasma glucose, HbA1c: glycosylated hemoglobin, IMT: intimal-medial thickness of carotid artery. * $p<0.05$ compared with genotype AB and BB.

These frequencies are close to those observed in other Asian ethnic populations (11). Our results show that diabetic patients in the Japanese population have reduced serum paraoxonase activity similar to that in the Caucasian population (4, 5). This suggests that the decrease in the activity of serum paraoxonase associated with diabetes is not related to genotype distribution. The cause of the lower paraoxonase activity in diabetic patients is unknown, and further experiments are necessary. Paraoxonase is believed to be anchored to the HDL lipids by its hydrophobic N-terminal end (14). However, the absence of a correlation between paraoxonase activity and HDL cholesterol suggests that the lower paraoxonase activity in diabetes is caused by factors other than the lower HDL cholesterol level. Furthermore, the glycation, or nonenzymatic glycosylation, of several structural and functional proteins has been described previously (15, 16). Glycation often leads to alterations in the conformation and/or function of proteins (17, 18). Paraoxonase activity may be altered as a result of glycation of the protein itself or of HDL. In this cross-study, paraoxonase activity was not significantly associated with glycosylated hemoglobin or fructosamine, but this hypothesis cannot be denied.

The clinical importance of the lower paraoxonase activity in diabetes is unknown. Significant associations were not found between the paraoxonase activity and diabetic microangiopathy (data not shown), but we observed significant differences in IMT among the genotypes. IMT in diabetic patients was significantly greater than in age-matched control subjects (0.77 ± 0.21 vs 0.68 ± 0.18 ; $p < 0.01$). In genotype AA, IMT was significantly greater than in genotypes AB and BB. However, other parameters which are known risk factors for atherosclerosis, such as age, sex, serum total cholesterol, prevalence of hypertension, and smoking were no different among the genotypes. This suggests that the decrease in the activity of serum paraoxonase in diabetes may play a role in the increased incidence of premature atherosclerosis associated with this disease. Two previous studies in patients with coronary heart disease found an increased frequency of the genotype B (6, 7), but there are no detailed studies showing that genotype B seems to be a high activity phenotype that is a risk factor for atherosclerosis. In the present study, we have no explanation for the discrepancy between our findings and those of the two previous studies. A recent study of Finns by Antikainen et al (19) concluded that the paraoxonase gene polymorphism Gln-Arg192 is not associated with a predisposition to coronary atherosclerosis. We therefore think that the explanation for the discrepancy is at the population level, or is related to genetic heterogeneity within or between our study group. Furthermore, paraoxonase *in vitro* seemed to have the capacity to hydrolyze at least some of the products of lipid peroxidation, and under experimental conditions it was able to decrease the extent to which low density lipoprotein (LDL) is oxidized (20). This finding suggests that individuals with a low paraoxonase activity phenotype may have a greater risk than those with high-activity phenotypes of developing a disease such as atherosclerosis, which may involve lipid peroxidation.

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