R/R Genotype of Human Paraoxonase (PON1) is More Protective against Lipoprotein Oxidation and Coronary Artery Disease in Japanese Subjects

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Human paraoxonase (PON1) is an high-density lipoprotein (HDL) -associated enzyme that is proposed to protect against the oxidation of lipoproteins. Recently, the association of coronary artery disease (CAD) and PON1 activity was reported. Furthermore, the R/R genotype of PON1 has been related to the risk for CAD. In this study we investigated the PON1 genotype and susceptibility to lipoprotein oxidation to elucidate the contribution of PON1 to atherosclerosis in Japanese subjects. We studied 179 patients who underwent coronary angiography and their PON1 genotypes were determined. Lipoproteins were obtained from a patient's blood after at least 12 hours fasting and were separated with sequential ultracentrifugation. We analyzed the thiobarbituric acid reactive substances (TBARS) and continuously monitored the copper-induced oxidation three genotype groups. Genotype frequencies of Q/Q, Q/R, and R/R were 21.2%, 36.9%, and 41.9%, respectively. PON1 polymorphism clearly determined the lipid oxidation. The R/R genotype of PON1 had significantly lower levels of plasma and HDL TBARS and significantly retarded the initiation of oxidation in HDL and low-density lipoprotein (LDL). The R/R genotype was related to the lower prevalence of CAD. The PON1 genotype clearly determined the oxidative modification of lipoproteins and may play a role in the pathogenesis of atherosclerosis via its protective effect against lipoprotein oxidation in Japanese subjects. *J Atheroscler Thromb*, 2003; 10: 85–92.

Key words: PON1, Lipoprotein oxidation, Coronary artery disease

Introduction

Oxidative stress has an important role in numerous disease processes, in particular, the oxidation of lipoproteins is considered to be the main initiating factor of atherosclerosis and the aggravating factor for advanced atherosclerotic lesions. *In vitro* studies, oxidized lipoproteins such as oxidized low-density lipoprotein (LDL) induce the endothelial cell (EC) injury, monocyte and macrophage activation, smooth muscle cell (SMC) proliferation and foam cell formation (1, 2). Recently, it was suggested that high-density

lipoprotein (HDL) has an antioxidant effect on LDL oxidation. In the interaction between LDL and HDL particles, peroxides in the LDL particles are transferred to HDL particles where antioxidant property neutralizes this oxidant. Some HDL-associated enzymes are involved in these processes, such as lecithin-cholesterol acyltransferase (LCAT), platelet-activating factor acetylhydrolase (PAF-AH), and paraoxonase. Each of these enzymes modulates the process of oxidation in different mechanisms *in vitro*.

Human paraoxonase (PON1) is an HDL-associated enzyme, it hydrolyzes organophosphates such as paraoxon, carbamates, and nerve gases, therefore it was initially investigated in the field of toxicology (3, 4). In particular, PON1 prevents the lipoprotein oxidation of HDL, preventing LDL oxidation by the antioxidant effect of HDL *in vitro* (5–7). Lower paraoxonase activity in subjects with myocardial infarction was reported in a clinical study (8). The

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paraoxonase gene family is located on chromosome 7q21.3–22.1 and contains at least 3 members, including PON1, PON2, and PON3 (9–13). This polymorphic distribution of PON1 is determined by a single amino acid substitution at position 192 (Gln to Arg; Q/R192, Q corresponds to A genotype and R to B genotype, respectively) which is believed to exist near the enzymatically active site. PON1 genetic polymorphism may determine the enzymatic activity of PON1 representing the low activity isoform as Q (Gln192) and high activity isoform as R (Arg192) (12). Recently, the association of coronary artery disease (CAD) and the PON1 genotype was reported and, surprisingly, the R/R genotype with high enzymatic activity was related to high prevalence of CAD in Caucasians (14–16). Furthermore, the R/R genotype of PON1 has been reported to have a less protective effect on lipoprotein oxidation (17–19). However, the association of the PON1 genotype with antioxidant activity and the prevalence of CAD are still very controversial.

In this study we focused on the differences in antioxidant activity among the isoforms of the PON1 genotype, and its association with CAD prevalence in Japanese subjects to clarify the role of PON1 in the pathogenesis of atherosclerosis.

Materials and Methods

Subjects

We analyzed 179 consecutive patients, who underwent coronary angiography, to evaluate coronary artery disease. All the patients were Japanese and patients taking lipid-lowering drugs and antioxidant vitamins were excluded. We defined the existence of coronary artery disease in this study as greater than 50% stenosis in one of the major segments of the coronary arteries. Informed consent was obtained from all subjects for this study.

PON1 genotype analysis

We obtained genomic DNA from a patient's blood using a DNA Extractor WB Kit (Wako-Junyaku Co., Osaka, Japan). The isolated DNA was stored at 4°C until analysis. For the genotyping of PON1 we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. We used the following primers described by Humbert *et al.* (12) for the 5' primer; 5'-TATTGTTGCTGTGGGACCTGAG-3', and for the 3' primer; 5'-CACGC TAAACCCAAATACATCTC-3'. PCR was performed with 30 cycles of amplification with annealing at 64°C. PCR products were digested with AlwI restriction enzyme, separated by 2.5% agarose gel (NuSieve) electrophoresis, and visualized using of ethidium bromide. Genotype Q (Gln192) corresponded to a 99 bp and genotype R (Arg192) to 65 and 34 bp fragments.

Clinical data

Serum samples were obtained by venipuncture with

vacutainer tubes after at least 12 hours' fasting to measure the serum lipid profile of all patients. Serum total cholesterol (TC), HDL cholesterol, and triglycerides (TG) were measured by automated enzymatic methods. Apolipoprotein (apo) A-I and apoB were measured by enzyme-linked immunosorbent assay (ELISA) method.

Lipoprotein separation

Lipoproteins were obtained from a patient's arterial at the time of coronary angiography blood via a catheter sheath into test tubes containing ethylenediaminetetraacetic acid (EDTA) disodium salt (final concentration 1.5 mg/ml). Plasma was immediately separated by centrifugation at 3,000 rpm for 10 minutes. To obtain LDL after removing very low-density lipoprotein (VLDL) using ultracentrifugation, samples taken from the bottom of the tubes were adjusted in density to 1.063 mg/dl with adequate potassium bromide (KBr) solution and then ultracentrifuged at 100,000 rpm for 4.5 hours at 15°C (Optima™ TL Ultracentrifuge, Beckman TL100.4 rotor) as previously described by Havel *et al.* (20). After ultracentrifugation, LDL was removed and then HDL was obtained in same manner after adjusting the density to 1.21 mg/dl, and it was ultracentrifuged at 100,000 rpm for 6 hours at 15°C. To remove KBr and EDTA, the stock samples were dialyzed over 18 hours against three changes of 2,000 ml volume of phosphate-buffered saline (PBS) with pH 7.4 at 4°C in a dark place. After dialysis, the protein levels in each lipoprotein were measured by the Lowry method (21), and stock samples were stored at 4°C until used for all oxidation studies.

TBARS

Plasma and lipoproteins containing lipid peroxides levels were determined by thiobarbituric acid reactive substances (TBARS) in the plasma samples and in each lipoprotein fraction by using a lipoperoxide test kit (Wako-Junyaku Co., Osaka, Japan).

Copper induced lipid oxidation

Copper-induced lipid oxidation was measured during incubation at 37°C using 100 µg of each lipoprotein fraction with 5 µM CuSO₄ in a final volume of 1 ml PBS. The diene production during copper-induced lipid oxidation was monitored by the change in 234 nm absorbance at 5 minute intervals for 240 minutes with a spectrophotometer (Beckman DU650). We calculated the lag time (min) from these results.

Statistical analysis

To compare CAD patients and patients without CAD by age, body mass index (BMI) and lipid levels, we used Student's *t* test. Allele frequencies were determined by the gene counting method, and Hardy-Weinberg equilibrium was tested by the chi-square test. We used ANOVA for the proportional difference of PON1 genotype and age,

BMI, lipids, TBARS, and lag time. Post-hoc analysis was performed in ANOVA analysis. For the nominal parameters we used chi-square analysis. A value of $p < 0.05$ was considered statistically significant. All data were expressed as the mean $\pm$ SD. The analysis was performed by StatView 4.5J and SPSS 5.1 for Macintosh computers.

Results

Clinical characteristics

CAD patients were older and, had a high prevalence of cigarette smoking and hypertension (HT) (Table 1). Clas-

sical risk factors other than BMI, TC and the prevalence of diabetes showed a statistically significant difference.

Genotype frequencies

In the 179 patients, the genotype frequencies were Q/Q; 21.2%, Q/R; 36.9%, and R/R; 41.9%. The allele frequencies were Q (Gln); 0.40 and R (Arg); 0.60. The prevalence of each allele was consistent with the Hardy-Weinberg equilibrium. The proportion of the R allele was 2 to 3 times higher than previously reported in Caucasian populations (14–16, 22–26), but identical to the Chinese population (14, 27) (Table 2).

Genotypes and plasma lipid profile

Among the genotypes there were no differences in the prevalence of classical risk factors and lipid levels (Table 3). Figure 1 shows the PON1 genotype and TBARS levels. Plasma and HDL TBARS were determined by the PON1 genotype. The R/R genotype of PON1 had significantly lower levels of plasma and HDL TBARS than the Q/Q genotype ($p < 0.01$), but TBARS values of VLDL and LDL did not

Table 1. Baseline characteristics of patients with or without CAD.

	CAD (+) (n = 124)	CAD (-) (n = 55)	P-value
Age (y)	62.9 $\pm$ 9.4	56.1 $\pm$ 11.2	< 0.0001
BMI	24.1 $\pm$ 9.5	22.9 $\pm$ 4.2	0.313
TC	191.5 $\pm$ 32.3	183.0 $\pm$ 36.1	0.114
TG	146.8 $\pm$ 82.5	120.1 $\pm$ 60.7	0.032
HDL-C	44.8 $\pm$ 13.6	53.2 $\pm$ 18.2	0.001
apo A-I	114.6 $\pm$ 21.5	125.0 $\pm$ 27.1	0.010
apo B	104.2 $\pm$ 21.2	96.3 $\pm$ 21.7	0.035
Gender (male%)	87.5	65.0	< 0.0001
DM (%)	32.2	18.2	0.055
HT (%)	60.9	35.8	0.008
SM (%)	71.4	63.0	0.031

All data were indicated the mean $\pm$ SD. $p < 0.05$ was considered to be statistically significant. BMI: body mass index (kg/m²), TC: total cholesterol (mg/dl), TG: triglycerides (mg/dl), HDL-C: high-density lipoprotein-cholesterol (mg/dl), apo A-I: apolipoprotein A-I (mg/dl), apo B: apolipoprotein B (mg/dl), DM: diabetes mellitus, HT: hypertension, SM: current smoker.

Table 2. Genotype and allele frequency of patient groups.

	Patients (n = 179)
Genotype frequency	
Q/Q	21.2% (38/179)
Q/R	36.9% (66/179)
R/R	41.9% (75/179)
Allele frequency	
Q (Gln)	0.40
R (Arg)	0.60

Q/Q: homozygous low activity genotype, Q/R: heterozygous genotype, R/R: homozygous high activity genotype.

Table 3. Classical coronary risk factors and lipid profile of patients with different genotypes.

	Q/Q	Q/R	R/R	P-value
Age (y)	59.6 $\pm$ 9.9	62.8 $\pm$ 9.9	59.6 $\pm$ 11.7	0.155
BMI	23.0 $\pm$ 3.4	22.9 $\pm$ 2.8	24.3 $\pm$ 12.5	0.585
TC	196.8 $\pm$ 27.0	185.0 $\pm$ 33.1	192.0 $\pm$ 35.2	0.258
TG	128.1 $\pm$ 51.8	141.8 $\pm$ 62.0	140.3 $\pm$ 89.6	0.708
HDL-C	50.8 $\pm$ 15.6	45.4 $\pm$ 14.4	47.7 $\pm$ 16.6	0.340
apoA-I	121.3 $\pm$ 20.0	113.5 $\pm$ 24.8	119.6 $\pm$ 24.5	0.298
apoB	102.1 $\pm$ 22.9	101.2 $\pm$ 21.3	102.8 $\pm$ 21.9	0.934
Gender (male%)	9.5	82.1	75.0	0.581
DM (%)	28.6	37.5	26.2	0.432
HT (%)	51.6	46.7	60.0	0.308
SM (%)	71.8	74.2	75.7	0.904

All data were indicated the mean $\pm$ SD. $p < 0.05$ was considered to be statistically significant. BMI: body mass index (kg/m²), TC: total cholesterol (mg/dl), TG: triglycerides (mg/dl), HDL-C: high-density lipoprotein-cholesterol (mg/dl), apoA-I: apolipoprotein A-I (mg/dl), Apo B: apolipoprotein B (mg/dl), DM: diabetes mellitus, HT: hypertension, SM: current smoker.

Fig. 1. PON1 genotype and TBARS values of plasma (A), LDL (B), VLDL (C), and HDL (D). Plasma and lipoproteins containing lipid peroxides levels were determined using a lipoperoxide test kit. We used chi-square analysis for the nominal parameters. $p < 0.05$ was considered to be statistically significant.

Fig. 2. PON1 genotype and lag time of VLDL (A), LDL (B), and HDL (C). The calculated lag time resulting from copper-induced lipid oxidation. Copper-induced lipid oxidation was measured during incubation at 37°C and was then analyzed by a spectrophotometer with detection at 234 nm. We used chi-square analysis for the nominal parameters. $p < 0.05$ was considered to be statistically significant.

differ. Figure 2 shows the calculated lag time results from copper induced lipoprotein oxidation. The HDL lag time was significantly different among the genotypes. The R/R genotype showed significantly retarded initiation of oxidation compared with the Q/Q genotype ($p < 0.01$). The lag time of VLDL and LDL had a similar tendency to HDL. The R/R genotype also showed significantly retarded initiation of oxidation compared with the Q/Q genotype in VLDL and LDL ($p < 0.05$, respectively).

Polymorphisms and incidence of coronary artery disease

Table 4 shows the genotype distribution of the PON1 polymorphism in CAD patients. The genotype distribution was significantly different between CAD patients and patients without CAD ($p = 0.02$). In patients without CAD, the frequency of the R/R genotype was significantly higher than the other genotypes (R/R; 56%, Q/Q; 20%, Q/R; 24%, respectively; $p < 0.05$).

Discussion

In this study, we evaluated the PON1 genotype and susceptibility to oxidation of lipoproteins to elucidate the contribution of PON1 to atherosclerosis in Japanese subjects. In particular we focused on the differences of antioxidant activity using TBARS and lag time measurements among the isoforms of the PON1 genotype and we clearly demonstrated that the R/R genotype had higher activity against HDL and LDL oxidation, subsequently having the lowest prevalence of CAD, compared with other genotypes. These results indicated that PON1 could be considered a determinant factor of lipoprotein oxidation and CAD development.

PON1 is an esterase showing polymorphic enzymatic activity affected by the PON1 genotype. In general, the R/R genotype has high paraoxonase activity and the Q/Q genotype has low activity. However, the active site of PON1 for antioxidation is reported to be related cysteine at position 283 of the amino acid sequence (17), which is different from the enzymatic active site. Therefore, the antioxidant capacity of PON1 is not always identical to PON1 enzymatic activity and the PON1 genotype, which may cause the controversial association of PON1 with CAD. It is clear that HDL-associated PON1 has antioxidant activity against HDL itself, leading to the protective effect of HDL against LDL oxidation indirectly. Moreover,

several studies have reported that PON1 was able to inhibit copper-induced lipid oxidation in LDL (5) and to hydrolyze long-chain oxidized phospholipids isolated from oxidized LDL (6). In addition, recent studies have also reported that specific non-competitive inhibitors of PON1 were able to suppress its protection against the oxidation both of HDL and LDL (28). Regarding the association of the PON1 genotype with lipoprotein oxidation, some studies have reported that the R/R genotype was less efficient at retarding the oxidation of LDL than the Q/Q genotype (17–19). However, in our study, the R/R genotype significantly retarded the initiation of oxidation in HDL and LDL and suppressed the oxidative production of plasma and HDL. One explanation for the antioxidant activity of PON1 associated with HDL against LDL oxidation, is that PON1 causes metabolic changes which may modify the composition or structure of LDL, resulting in changed levels of vitamin E and fatty acids and leading to LDL resistance against oxidation without the presence of HDL. Although a few studies have examined the direct effect of PON1 against LDL oxidation and its association with the PON1 genotype, the association of antioxidant activity of PON1 with the PON1 genotype is still controversial. In order to clarify this inconsistent association, an experiment should be performed to assess the antioxidant activity of HDL from PON1 knockout mice in which recombinant R/R or Q/Q PON1 genotype mutants are incorporated.

Furthermore, the association of the PON1 genotype with CAD prevalence is also inconsistent in clinical studies. In recent basic research, Shih *et al.* demonstrated that reduced serum levels of PON1 activity are associated with higher lipoprotein oxidation and more pronounced atherosclerotic lesions in the PON1 knockout mice and PON1 knockout/apoE knockout mice model (29, 30). This suggests that reduced PON1 expression may increase the risk for CAD. In our clinical study, the R/R genotype of PON1 showing high antioxidant activity had a lower prevalence of CAD in Japanese subjects, compared with the other genotypes, which is the very compatible result with the PON1 genotype, its antioxidant activity, and CAD. However, most studies have shown that the R/R genotype of PON1 had a higher prevalence of CAD in Caucasians (14–16) and Japanese (31–33) and some studies have failed to find such a relationship (14, 24–27, 34). Mackness *et al.* (35) reported the meta-analysis of the PON1 genotype and the prevalence of CAD. They concluded that PON1 activities toward paraoxon and PON1 concentration are lower in patients with CAD than the control subjects regardless of the PON1 genotype. This indicates that PON1 genetic polymorphisms may determine neither the antioxidant activity of PON1, nor the enzymatic activity and concentration of PON1. Recently, Suehiro *et al.* (36) reported that PON1 concentration was decreased but paraoxonase and arylesterase activities were increased in hemodialyzed patients with no significant as-

Table 4. Genotype distribution in patients with or without CAD.

	CAD (–)	CAD (+)
Q/Q	11 (20%)	28 (22%)
Q/R	13 (24%)	53 (43%)
R/R	31 (56%)	43 (35%)

sociation of serum the PON1 concentrations with the PON1 genotype.

Finally, the association of the PON1 genotype with antioxidant activity and the prevalence of CAD are still very controversial. However, some clinical studies in Caucasians have demonstrated that the R/R genotype had lower antioxidant activity of PON1 leading to a higher prevalence of CAD (17–19). Our results totally oppose these results, showing that the R/R genotype has a protective effect against oxidation of HDL and LDL and development of CAD. Although previous studies have not examined the association of antioxidant activity and genotype of PON1 with CAD simultaneously, this discrepancy may result from some factors affecting PON1 activity, such as race, Diabetes mellitus, and smoking. In contrast to most studies in Caucasians, all our subjects were Japanese undergoing coronary angiography to evaluate CAD without taking lipid-lowering drugs. In the analysis of the PON1 genotype in diabetic patients, one study demonstrated increased intima-media thickness (IMT) on carotid artery echogram in the Q/Q genotype (37), which is consistent with our results, but the other study showed no association between the IMT and PON1 genotypes (38). Another possibility related to factors affecting the antioxidant activity of PON1 is smoking. James *et al.* (39) reported that cigarette smoking is independently associated with significant decreases in serum PON1 activities and concentrations, diminishing its antioxidant capacity. Among smokers, the Q/Q genotype at low risk for CAD has been demonstrated to have increased the susceptibility to CAD (40). In our study, two thirds of the subjects were current smokers, therefore we analyzed our data in smokers and non-smokers separately. However, we could find no association of its antioxidant activity with the PON1 genotype.

Our study was a relatively a small sample size, therefore our results were insufficient to reach conclusive results. Further studies are needed to clarify these complex issues.

In conclusion, the PON1 genotype clearly determined the oxidative modification of lipoproteins and may play a role in the pathogenesis of atherosclerosis via its protective effect against lipoprotein oxidation in Japanese subjects.

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