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ORIGINAL INVESTIGATION

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Paraoxonase gene Gln192Arg (Q192R) polymorphism is associated with coronary artery spasm

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Abstract We recently reported that oxidative stress is involved in the pathogenesis of coronary spasm. We hypothesized that oxidative-stress-related genetic factors and certain polymorphisms in the paraoxonase gene (PON1) and platelet-activating factor acetylhydrolase (PAF-AH) might influence the pathogenesis of coronary spasm. We therefore examined the possible association between the PON1 Q192R or PAF-AH V279F polymorphisms and coronary spasm in 214 patients with coronary spasm and 212 control subjects. Genotypes were determined by polymerase chain reaction/restriction fragment length polymorphism analysis. The incidence of the PON1-192R allele was significantly higher in the coronary spasm group than in the control group (65% vs 53%; P=0.0005). The PAF-AH-279F allele was not associated with coronary spasm (15% vs 16%; P=0.8781). Multiple logistic regression analysis with forward stepwise selection involving the PON1-192R allele and the environmental risk factors revealed that the most predictive independent risk factor for coronary spasm was the PON1-192R allele (significance=0.0016, OR=2.52), followed by cigarette smoking (significance=0.0007, OR=2.01). We also measured plasma levels of TBARS (thiobarbituric acid-reactive substances) as a marker of oxidative stress. TBARS levels were higher in R/R types than in Q/Q types (2.115±0.086 nmol/ml [n=25] vs 1.676±0.102 nmol/ml [n=11], P<0.01). Thus, there is a significant association between the PON1-192R allele and coronary spasm; the PON1-192R allele may play an important role in the genesis of coronary spasm, probably by attenuating the suppression of oxidative stress.

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Introduction

Coronary spasm plays an important role in the pathogenesis not only of variant angina, but also of ischemic heart disease in general, including other forms of angina pectoris, acute myocardial infarction, and sudden death (Hillis and Braunwald 1978; Yasue et al. 1983; Maseri et al. 1990). However, the precise mechanism(s) responsible for coronary spasm remains unclear.

We have shown that impairment of endothelium-dependent vasodilation may play a key role in the genesis of coronary spasm (Kugiyama et al. 1996a; Okumura et al. 1996). In this regard, both basal and acetylcholine (ACh)-induced endothelial function are improved in coronary spasm patients by intracoronary injection of vitamin C, an antioxidant (Kugiyama et al. 1998). Similarly, coronary spasm is suppressed by administration of vitamin E, another antioxidant, plasma levels of which are low in coronary spasm patients (Miwa et al. 1996; Motoyama et al. 1998). Cigarette smoking impairs endothelium-dependent coronary arterial dilation in humans (Zeiher et al. 1995; Kugiyama et al. 1996b; Motoyama et al. 1997). Indeed, smoking is the single most important environmental risk factor for coronary spasm (Caralis et al. 1992; Sugiishi and Takatsu 1993; Kugiyama et al. 1996b; Takaoka et al. 2000). These findings suggest that oxidative stress may contribute to the genesis of coronary spasm.

In addition, the prevalence of coronary spasm is higher among the Japanese population than among Caucasians (Bertrand et al. 1982; Yasue and Kugiyama 1990) suggesting that genetic factors are also involved in its pathogenesis. We have previously reported that a missense Glu298Asp variant in exon 7 and a $T^{-786}\rightarrow C$ mutation in the 5'-flanking region of the endothelial nitric oxide synthase (eNOS) gene are associated with coronary spasm (Yoshimura et al. 1998, 2000; Nakayama et al. 1999).

Earlier studies (Simon et al. 1990; Anderson et al. 1995) have indicated that low-density lipoprotein (LDL) cholesterol and, in particular, its oxidative derivatives are injurious to the endothelium, whereas high-density lipoprotein (HDL) has been shown to prevent the oxidative modification of LDL. The antioxidant activity associated with HDL is believed to reside in its enzymes, particularly paraoxonase (PON1; Durrington and Mackness 2000; Mackness et al. 2000). PON1 is known to protect LDL against oxidative modification by preventing accumulation of lipid peroxides (Mackness et al. 1993, 1998; Aviram et al. 1998a), and a study by Shih et al. (1998) indicates that mice lacking serum PON1 are susceptible to oxidative stress and atherosclerosis. Furthermore, the use of specific inhibitors has shown that PON1 has a pivotal role in preventing the oxidation of HDL (Aviram et al. 1998b). PON1 activity is known to be genetically determined, at least in part (Humbert et al. 1993; Aviram et al. 1998a, 2000; Mackness et al. 1999). The PON1 Gln192Arg (Q192R) polymorphism yields three genotypes with differing enzymatic activities (Mackness et al. 1998), and the PON1-192R allele confers a lower ability on HDL for preventing the accumulation of lipid peroxides on LDL than does the PON1-192Q allele.

It is of interest to observe changes in the incidence of PON1 Q192R genotypes with area or race; the PON1-192R allele has a higher frequency in the Japanese population than in the populations of the USA, France, and Finland (Yamasaki et al. 1997). This evidence may be associated with the prevalence of coronary spasm being higher among Japanese than among Caucasians (Bertrand et al. 1982; Yasue and Kugiyama 1990). Furthermore, cigarette smoking reduces serum PON1 activity in patients with coronary artery disease (James et al. 2000a). Since cigarette smoking is highly prevalent in the Japanese (Bernhardt et al. 1987; Stehel et al. 1988), the relationship between cigarette smoking and PON1 activity may also be a cause of the prevalence of coronary spasm among Japanese (Bertrand et al. 1982; Yasue and Kugiyama 1990).

Another HDL-associated enzyme, platelet-activating factor acetylhydrolase (PAF-AH) can also retard the oxidation of LDL by preventing the generation of phospholipid hydroperoxides (Van Lenten et al. 1995; Watson et al. 1995). Polymorphisms are also present in the PAF-AH gene, which exhibits a G→T mutation at nucleotide position 994 in exon 9; this nucleotide change results in a Val→Phe (V→F) substitution at amino acid 279 of the mature protein, with the loss of catalytic activity in the catalytic domains in the PAF-AH protein (Stafforini et al. 1996). However, the role of the PAF-AH mutation in the susceptibility to coronary spasm has not been determined.

We therefore hypothesized that the PON1 Q192R polymorphism and the PAF-AH V279F polymorphism might influence the genesis of coronary spasm. To test this hypothesis, we analyzed the genotypes of patients with coronary spasm and compared them with those of control subjects.

Materials and methods

Study subjects

The study protocol was approved by the ethics committee at our institution. The study population included 214 patients with coronary spasm (105 men and 109 women; mean age: 61.3 years; range: 36–79 years) who were admitted consecutively at our institution. All 214 patients with coronary spasm had experienced episodes of spontaneous angina attacks. We defined coronary spasm as an abnormal contraction of epicardial coronary artery resulting in myocardial ischemia (Yasue 1980; Yasue et al. 1983).

In the present study, all medication except sublingual nitroglycerin was withdrawn at least 3 days before the study. Nitroglycerin also was not used at least 12 h before the study. Thus, no patients had received medication that may have affected the redox state during cardiac catheterization.

In all patients, coronary spasms associated with symptoms and ST-segment changes were angiographically documented during spontaneous attacks in 11 patients or after intracoronary injection of ACh in 203 patients. After intracoronary injection of isosorbide dinitrate, the patients' coronary arteries appeared normal and exhibited no significant organic stenosis (<50% luminal diameter). Those who had significant organic stenosis in coronary arteries were excluded from the study.

Age- and gender-matched patients (n=212) who underwent diagnostic cardiac catheterization, including coronary angiography, for evaluation of chest pain and who had angiographically normal or nearly normal coronary arteries and did not exhibit coronary spasm after intracoronary injection of ACh served as controls. All subjects enrolled in the study gave their informed consent. Furthermore, we compared the association between the PON192-R allele and coronary spasm in both smokers (157 men and 34 women; mean age: 58.9 years; 114 patients with coronary spasm and 77 control subjects) and nonsmokers (50 men and 185 women; mean age: 63.1 years; 100 patients with coronary spasm and 135 control subjects). A smoker was defined as a current smoker or as a smoker who had stopped smoking within 2 years, and a nonsmoker as a subject who had never smoked or had stopped smoking for more than 2 years.

Paraoxonase genotype determination

PON1 genotypes were determined by using polymerase chain reaction (PCR) according to previously published protocols with modifications (Humbert et al. 1993). Genomic DNA was extracted from white blood cells.

The DNA region containing the polymorphic site (a 99-bp DNA fragment) was amplified by PCR, with the two primers described by Humbert et al. (1993): sense primer 5'-TATTGTTGCTGTGGGACCTGAG-3', and antisense primer 5'-CACGCTAAACCCAAATACATCTC-3', followed by restriction endonuclease (*Alw*I) digestion. The samples were then separated by electrophoresis on 3.0% Nusieve gel and visualized by using ethidium bromide.

Screening the plasma PAF-AH Val279Phe polymorphism

PAF-AH genotypes were determined by using PCR according to previously published protocols with modifications (Stafforini et al. 1996). Genomic DNA was extracted from white blood cells.

The DNA region containing the polymorphic site (a 177-bp DNA fragment) was amplified by PCR, with the two primers described by Stafforini et al. (1996): sense primer 5'-CTATAAATTT ATATCATGCT-3' and antisense primer 5'-TTTACTATTCTCTTGCTTTAC-3', followed by restriction endonuclease (*Mae*II) digestion. The samples were then separated by electrophoresis on 3.0% Nusieve gel and visualized by using ethidium bromide.

Biochemical assay of thiobarbituric acid-reactive substances

The content of lipid peroxide in plasma was determined by using thiobarbituric acid-reactive substances (TBARS) as a marker (Buege and Aust 1987). Briefly, 2.0 ml trichloroacetic acid-thiobarbituric acid (TBA)-HCl reagent reagent was added to 1.0 ml sample and vortexed. To minimize peroxidation during the assay procedure, butylated hydroxytoluene was added to the TBA reagent mixture. The results were expressed as malondialdehyde (MDA) equivalent content (nanomoles of MDA per milliliter plasma).

Statistical analysis

Hypertension was operationally defined as blood pressures higher than 140/95 mmHg, diabetes mellitus as fasting blood glucose levels greater than 7.8 mmol/l or greater than 11.1 mmol/l at 2 h in an oral glucose tolerance test. Continuous variables were compared by two-tailed unpaired *t* tests. Categorical variables were compared by χ^2 analysis with Fisher's exact probability. For comparison of plasma TBARS levels in each genotype of the PON1 gene, a two-way analysis of variance (ANOVA) for repeated measures, followed by the Bonferroni multiple comparison test, was used. OR (odds ratios; approximating relative risk) were calculated as an index of the association of the PON1 Q192R polymorphism (Q/Q type, Q/R type, R/R type) or the PAF-AH V279F polymorphism (V/V type, V/F type, F/F type) with the phenotype of coronary spasm. For each OR, we calculated two-tailed probability value and 95% confidence intervals (CIs). The effects of the mutant allele were assumed to be either additive, dominant, or recessive; values for the additive effect were predicted by the Hardy-Weinberg equilibrium.

Multiple logistic regression analysis with forward stepwise selection (Wald) was performed with SPSS Advanced Statistics 6.1 for a Macintosh (SPSS Japan). Independent variables were coded as the following dummy variables: genotype, 0 for Q/Q type and 1 for R/R type or Q/R type; sex, 0 for female and 1 for male; age, 0 for less than 60 years and 1 for equal to or more than 60 years; body mass index, 0 for less than 26 kg/m^2 and 1 for equal to or more than 26 kg/m^2; hypercholesterolemia, 0 for less than 6.24 mmol/l and 1 for more than 6.24 mmol/l; low HDL, 0 for more than 0.91 mmol/l and 1 for less than 0.91 mmol/l; high LDL, 0 for less than 4.16 mmol/l and 1 for more than 4.16 mmol/l; high TG, 0 for less than 1.70 mmol/l and 1 for more than 1.70 mmol/l; cigarette smoking, 0 for nonsmokers and 1 for smokers; hypertension, 0 for normotension and 1 for hypertension; diabetes mellitus, 0 for absence and 1 for presence. Statistical significance was defined as $P<0.05$.

Results

Clinical characteristics of the study patients

The incidences of coronary risk factors, including age, sex, total cholesterol, hypertension, diabetes mellitus, body mass index, and cigarette smoking were compared between the control and coronary spasm groups (Table 1). The incidence of cigarette smoking was significantly higher in the coronary spasm group than in the control group ($P<0.0001$), but there were no significant differences among the other risk factors between the two groups.

Table 1 Clinical characteristics of the study patients. Values are means ± SD (*HDL* high density lipoprotein, *LDL* low density lipoprotein)

Variable	Control (*n*=212)	Coronary Spasm (*n*=214)	*P*-value
Age (years)	61.1±9.8	61.4±10	0.803
Men:women	103:109	105:109	0.921
Cigarette smoking	76/211 (36%)	110/210 (52%)	<0.0001
Diabetes mellitus	27/207 (13%)	34/209 (16%)	0.353
Hypertension	67/201 (33%)	66/207 (32%)	0.724
Total cholesterol (mmol/l)	5.04±0.91	4.97±0.94	0.469
HDL-choloesterol (mmol/l)	1.33±0.42	1.25±0.36	0.093
Triglyceride (mmol/l)	1.40±0.81	1.54±0.85	0.098
LDL-cholesterol (mmol/l)	3.15±1.12	2.99±0.86	0.128
Body mass index (kg/m^2)	23.7±3.2	23.5±3.0	0.538

Association of paraoxonase polymorphism with coronary spasm

The R/R genotype, R/Q genotype, and Q/Q genotype were present in 84 (39%), 109 (51%), and 21 (10%) of the 214 patients with coronary spasm, respectively. On the other hand, the R/R genotype, R/Q genotype, and Q/Q genotype were found in 58 (28%), 109 (51%), and 45 (21%) of the 212 control subjects, respectively. The frequencies of the genotypes were in agreement with those predicted by Hardy-Weinberg equilibrium ($P>0.05$). When the additive, recessive, and dominant effect of the PON1 Q192R polymorphism was analyzed, the incidence of the PON1-192R allele was significantly higher in the coronary spasm group than in the control group ($P=0.001$, 0.009, 0.001, respectively; Table 2).

Association of PAF-AH279F allele with coronary spasm

The association of the V279F polymorphism in the PAF-AH gene with coronary spasm has been investigated in 214 patients with coronary spasm and 212 control subjects. Homozygotes for the PAF-AH-279F allele, heterozygotes, and PAF-AH-279 V homozygotes were present in 6 (3%), 53 (25%), and 155 (72%) of the spasm group. On the other hand, PAF-AH-279F homozygotes, heterozygotes, and PAF-AH-279 V homozygotes comprised 7 (3%), 52 (25%), and 153 (72%) of the control group. When the additive, recessive, and dominant effect of the PAF-AH V279F polymorphism was analyzed, the incidence of the PAF-AH-279F allele was not associated with coronary spasm ($P=0.878$, 0.765, 0.952, respectively).

Association between PON192R allele and coronary spasm in smokers and nonsmokers

The present analysis revealed that the PON1-192R allele was significantly associated with coronary spasm both in smokers (OR=1.69, $P=0.013$) and in nonsmokers (OR=1.55, $P=0.022$). We then performed multiple logistic regression analysis with forward stepwise selection by using PON1-192R allele and all the clinical risk factors in this study population. This analysis revealed that the most

Table 2 Frequency of the genotypes of the paraoxonase gene. The odds ratio (*OR*) was calculated as a measure of the association of the paraoxanase genotype (*Q/Q*, *Q/R*, or *R/R*) with the phenotype of the coronary spasm, with the effects of the R allele being assumed as being *Additive* (*R* vs *Q*), *Dominant* (*R/R* and *Q/R* combined vs *Q/Q*), and *Recessive* (*R/R* vs *Q/R* and *Q/Q* combined)

PON Q192R polymorphism	Control (*n*=212)	Coronary spasm (*n*=214)	OR (95% CI)	*P*-value
Q/Q	45/212 (21%)	21/214 (10%)	–	–
Q/R	109/212 (51%)	109/214 (51%)	–	–
R/R	58/212 (28%)	84/214 (39%)	–	–
Additive	–	–	1.62 (1.23–2.14)	0.001
Recessive	–	–	1.72 (1.14–2.58)	0.009
Dominant	–	–	2.48 (1.23–4.33)	0.001

Table 3 Multiple logistic regression analysis: forward stepwise selection (*SE* standard error, *Wald* multiple logistic regression analysis with forward stepwise selection, *df* degrees of freedom, *R* residual, *Exp(β)* exponential distribution)

Variable	β Coefficient	SE	Wald	df	Significant	*R*	Exp (β)
PON192-R allele	0.9252	0.2933	9.9509	1	0.0016	0.1180	2.5233
Smoking	0.6975	0.2046	11.6203	1	0.0007	0.1030	2.0088
Constant	–1.0894	0.2859	14.5166	1	0.0001	14.5166	–

predictive independent risk factor for coronary spasm was the PON1-192R allele, followed by cigarette smoking (Table 3).

Plasma TBARS levels in each genotype of the PON1 gene

Plasma TBARS levels were significantly higher in the R/R type than in the Q/Q type (2.115±0.086 nmol/ml [*n*=25] vs 1.676±0.102 nmol/ml [*n*=11], *P*<0.01), and the TBARS levels in R/R type tended to be higher than those of R/Q type (2.115 nmol/ml±0.086 nmol/ml [*n*=25] vs 1.911±0.060 nmol/ml [*n*=27], *P*=0.05). The TBARS levels in the Q/Q type were not significantly different from those of R/Q type (1.676 nmol/ml±0.102 nmol/ml [*n*=11] vs 1.911±0.060 nmol/ml [*n*=27], *P*=0.08), as shown in Fig. 1.

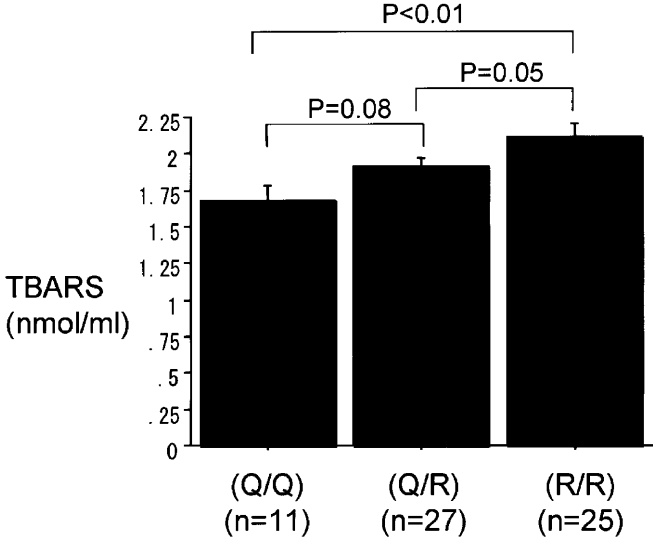

Fig. 1 Comparison of plasma thiobarbituric acid-reactive substance (*TBARS*) levels between genotypes of PON1 gene (Q/Q; *n*=11, Q/R; *n*=27, R/R; *n*=25)

Discussion

Endothelial dysfunction and oxidative stress are known to be crucially involved in the pathogenesis of coronary spasm (Yasue et al. 1990; Zeiher et al. 1991; Miwa et al. 1996; Kugiyama et al. 1998; Motoyama et al. 1998). Indeed, we have shown that endothelial dysfunction is improved by intracoronary injection of vitamin C, an antioxidant, and that a coronary spasm attack can be suppressed by vitamin E, another antioxidant (Miwa et al. 1996; Kugiyama et al. 1998; Motoyama et al. 1998).

We have recently showed that a missense Glu298Asp variant in exon 7 and a $T^{-786}\rightarrow C$ mutation in the 5'-flanking region of the eNOS gene are each significantly associated with coronary spasm (Yoshimura et al. 1998, 2000; Nakayama et al. 1999). Considering the possibility that other genetic factors associated with oxidative stress might also be present in patients with coronary spasm, we hypothesized that polymorphisms of PON1 and PAF-AH, HDL-associated antioxidant enzyme genes, would also affect endothelial function by modifying LDL oxidations.

The present study shows that the PON1-192R allele occurs significantly more frequently among coronary spasm patients than among control subjects (65% vs 53%; *P*=0.0005) in the Japanese population. The distribution of the PON1-192R allele is compatible with the Hardy-Weinberg equilibrium, indicating that our screening method was appropriate. The PON1-192R allele confers a lower ability on HDL to prevent the accumulation of lipid peroxides on LDL than does the PON1-192Q allele (Aviram et al. 1998a, 2000; Mackness et al. 1998, 1999). There-

fore, the PON1-192R allele confers a lower antioxidant activity than does the PON1-192Q allele. Indeed, we have measured TBARS as an oxidative stress marker and have shown that the TBARS levels in the R/R genotype of the PON1 gene are significantly higher than those of the Q/Q genotype gene. The results are in agreement with those of Paolisso et al. (2001), but not with those of Bauters et al. (2000). However, the latter study included only a small number of study subjects (n=27). On the other hand, the PAF-AH-279F allele was not associated with coronary spasm in the present study.

Multiple logistic regression analysis with forward stepwise selection involving several risk factors has revealed that the most predictive independent risk factor for coronary spasm is the PON1-192R allele, followed by cigarette smoking. Smoking is the single most important environmental risk factor for coronary spasm (Caralis et al. 1992; Sugiishi and Takatsu 1993; Kugiyama et al. 1996b; Takaoka et al. 2000). Smoking is also associated with reduced serum paraoxonase activity in patients with coronary artery disease (James et al. 2000a). The present analysis has also revealed that the PON1-192R allele is significantly associated with coronary spasm in both groups and that the relation seems to be unaffected by cigarette smoking.

We have not measured PON activity or concentrations in the present study. However, PON activity is not directly related with antioxidant effects (Mackness et al. 1998).

This is the first report showing a significant relationship between coronary spasm and the PON1-192R allele. Recent epidemiological studies indicate that the PON1-192R allele is a risk factor for the development of organic stenosis (Ruiz et al. 1995; Serrato and Marian 1995; Zama et al. 1997). However, other studies have reported no association between organic stenosis and the PON1-192R allele (Herrmann et al. 1996; Suehiro et al. 1996; Ombres et al. 1998). The reason for the differences among these studies has not been clarified, and further investigations are be required with regard to these problems.

The PON1 Q192R polymorphism is in linkage disequilibrium with another PON1 M54L polymorphism (Blatter-Garin et al. 1998) but is not in linkage disequilibrium with PON1 promoter polymorphism (James et al. 2000b). Thus, it will be interesting to determine which of the polymorphisms, PON1 Q192R or M54L, is functional or the more important.

In conclusion, the present study shows that the PON1-192R allele is significantly associated with coronary spasm in the Japanese population.

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