A Variant of p22\textsuperscript{phox}, Involved in Generation of Reactive Oxygen Species in the Vessel Wall, Is Associated With Progression of Coronary Atherosclerosis

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Abstract—A series of pro-oxidant and antioxidant enzymes, such as the NADPH oxidase system, maintain the redox state in the vessel wall. A major component of NADPH oxidase is p22\textsuperscript{phox}, which is implicated in atherosclerosis. We prospectively studied the association of the histidine (H)\textsuperscript{72}→tyrosine (Y) mutation in p22\textsuperscript{phox} with the severity and progression/regression of coronary artery disease (CAD), plasma lipid levels, clinical events, and response to treatment with fluvastatin in a well-characterized population. Genotypes were determined by polymerase chain reaction and restriction digestion with Rsal enzyme in 368 subjects in the Lipoprotein and Coronary Atherosclerosis Study (LCAS). Fasting plasma lipids and quantitative coronary angiograms were obtained at baseline and 2.5 years after randomization to fluvastatin or placebo. Subjects with CC genotype (n=157) were identified by the presence of 396-bp and 113-bp products on gel electrophoresis. Those with TT (n=39) were identified by the presence of 316-bp, 113-bp, and 80-bp products, and those with CT (n=172) by the presence of 396-bp, 316-bp, 113-bp, and 80-bp products. Baseline and final plasma levels of lipids and the baseline severity of CAD were not significantly different among the genotypes. In the placebo group, subjects with the mutation had a 3- to 5-fold greater loss in mean minimum lumen diameter (MLD) (TT: −0.15±0.15; CT: −0.17±0.26; and CC: −0.03±0.22 mm; \(P=0.006\)) and lesion-specific MLD (TT: −0.15±0.06; CT: −0.18±0.03; and CC: −0.06±0.03 mm; \(P=0.038\)) than those without. Progression was also more (TT: 8/17 [47%]; CT: 35/73 [48%]; and CC: 17/62 [27%]) and regression less (TT: 0/17 [0%]; CT: 1/73 [1%]; and CC: 11/72 [18%]) common in those with the mutation (\(P=0.029\)). The C\textsuperscript{242}T mutation in p22\textsuperscript{phox}, involved in maintaining the redox state in the vessel wall, is associated with progression of coronary atherosclerosis in the LCAS population. (Circ Res. 2000;86:391-395.)

Key Words: atherosclerosis ■ genetics ■ coronary disease ■ reactive oxygen species ■ polymorphism

Reactive oxygen species (ROS) are implicated in the pathogenesis of a variety of human diseases including atherosclerosis.\textsuperscript{1,2} ROS have a variety of biological functions that comprise induction of gene expression,\textsuperscript{3} promotion of cell proliferation and hypertrophy,\textsuperscript{4,5} apoptosis,\textsuperscript{6,7} and anoikis.\textsuperscript{8} ROS are also involved in oxidation of LDL,\textsuperscript{9} which is considered a fundamental step in initiation and progression of coronary atherosclerosis.\textsuperscript{10,11} Collectively, these data signify the role of ROS in atherosclerosis.

The delicate balance between oxidation and reduction (redox) state is maintained by a series of pro-oxidant and antioxidant enzymes and molecules. The most commonly studied pathway is the plasma membrane–associated enzyme NADPH oxidase,\textsuperscript{12} which is the most important source of superoxide anion, the precursor to a variety of potent oxidants, in intact vessel walls.\textsuperscript{13–16} A major component of NADPH oxidase is p22\textsuperscript{phox} protein, which in conjunction with gp91 forms a membrane-bound heterodimeric protein referred to as flavocytochrome b\textsubscript{558}.\textsuperscript{12,17} The latter is considered the redox center of the NADPH oxidase.\textsuperscript{17} The p22\textsuperscript{phox} protein is essential for the assembly and activation of the NADPH oxidase\textsuperscript{18} and plays a major role in NADPH-dependent O\textsubscript{2} production in the vessel wall.\textsuperscript{19}

The gene coding for p22\textsuperscript{phox} (CYBA) is located on chromosome 16q24 and has several allelic variants,\textsuperscript{20–24} including a C\textsuperscript{242}T transition, that results in replacement of histidine by tyrosine at amino acid position 72 (H\textsuperscript{72}Y), a potential heme-binding site.\textsuperscript{25} The C\textsuperscript{242}T polymorphism has been implicated in susceptibility to coronary artery disease (CAD) in case-control studies, but the results have been conflicting.\textsuperscript{26–28} We performed a prospective study and determined the association of the C\textsuperscript{242}T variants with the severity, progression, and regression of CAD, as determined by serial quantitative coronary angiography, plasma levels of lipids, and clinical events, as well as the response of these variables to treatment with fluvastatin in a well-characterized population.
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Lipids and Apolipoproteins

Total cholesterol, HDL cholesterol, triglyceride, lipoprotein(a), and apolipoprotein levels were measured in all subjects, and LDL cholesterol was calculated at baseline and 2.5 years after randomization.

Angiography

Quantitative coronary angiography was performed at baseline and 2.5 years after randomization. The primary end point was within-subject per-lesion change in the minimum lumen diameter (MLD) of qualifying lesions, defined by MLD ≥25% of the reference lumen diameter at baseline and MLD ≥0.8 mm less than the reference lumen diameter at either baseline or follow-up. Subjects were also categorized as having definite progression (≥1 qualifying lesion with MLD decrease ≥0.4 mm, including new total occlusions, and no qualifying lesion with MLD increase ≥0.4 mm), definite regression (≥1 qualifying lesion with MLD increase ≥0.4 mm, no qualifying lesion with MLD decrease ≥0.4 mm, and no new total occlusion), or mixed change.

Clinical Events

Clinical events monitored were definite or probable myocardial infarction (MI), unstable angina requiring hospitalization, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, and death of any cause.

Genotyping

Laboratory personnel who had no knowledge of the angiographic and clinical data performed the genotyping. Genotyping was per-

formed by polymerase chain reaction (PCR) and restriction digestion

with RsaI restriction endonuclease per published protocol with minor modifications. To avoid possible mistyping of the genotypes, because of incomplete digestion, a second RsaI restriction site was included in the PCR amplification product as an internal control. The sequences of forward and reverse oligonucleotide primers were 5′-CTCTGTTGGTCTTCAGTAG-3′ and 5′-ACTCAC-AGGAGATGCAGGACG-3′, respectively, and the annealing temperature was 65°C. Each genotype was read by two individuals independently; if in conflict, genotyping was repeated.

Materials and Methods

Study Population

All subjects in the Lipoprotein and Coronary Atherosclerosis Study (LCAS) provided informed consent, and the study was approved by the institutional review board. Design and results of LCAS have been published. In brief, 429 subjects who were 35 to 75 years of age and had at least one coronary lesion causing 30% to 75% stenosis and LDL cholesterol of 115 to 190 mg/dL despite diet were randomized to fluvastatin (40 mg daily) or placebo.

Lipids and Apolipoproteins

Total cholesterol, HDL cholesterol, triglyceride, lipoprotein(a), and apolipoprotein levels were measured in all subjects, and LDL cholesterol was calculated at baseline and 2.5 years after randomization.

Statistical Analysis

Continuous variables were expressed as mean±SD, except for the lesion-specific MLD, which was expressed as mean±SE. Differences among the genotypes were compared by ANOVA and between two groups by Student’s t test. Variables that were unsuited for ANOVA, because of inequalities of variance, were analyzed by Kruskal-Wallis test. Distribution of the categorical variables among genotypes was compared using Pearson χ² or Fisher’s exact test. To determine the association of C242T genotypes with response to fluvastatin treatment, mean changes in plasma lipid levels and MLD among the genotypes were compared using ANOVA. Statistical analysis was performed using STATA, version 5.0 (Stata Corporation, College Station, Tex).

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Genotypes

In 54 subjects, no DNA sample was available and in another 7, the PCR reaction did not work. The size of the amplified product by PCR was 509 bp. Subjects with the CC genotype were identified by the presence of two products of 396 bp and 113 bp and those with TT by the presence of three products of 316 bp, 113 bp, and 80 bp. Heterozygous individuals (CT) were identified by the presence of four products of 396 bp, 316 bp, 113 bp, and 80 bp. Genotyping was completed in 368 subjects, and 157 (43%), 172 (47%) and 39 (11%) subjects had CC, CT, and TT genotypes, respectively. The frequencies of the C and T alleles were 0.66 and 0.34, respectively. The distribution of genotypes was according to the Hardy-Weinberg equilibrium.

Baseline Characteristics

The baseline demographic characteristics of subjects were not significantly different among the three genotypes. The mean number of qualifying lesions and total occlusions, the number of subjects with ≥1 qualifying lesions or total occlusions, and the mean MLD and lesion-specific MLD at baseline also did not differ significantly among the genotypes (see online supplementary information for details, http://www.circresaha.org).

Plasma Levels of Lipids

There were no significant differences in mean plasma total cholesterol, LDL cholesterol, HDL cholesterol, triglyceride, lipoprotein(a), or apolipoprotein levels at baseline or completion of the study among the genotypes (see online supple-
mentary information for details, http://www.circresaha.org). In addition, there was no significant interaction between response of plasma lipids to treatment with fluvastatin and the p22\textsuperscript{phox} genotypes.

**Progression and Regression of CAD**

Angiographic data were available in 313 subjects who were genotyped. In the placebo group, progression of CAD was strongly associated with the presence of the mutant allele. Subjects with the mutation had a 3- to 5-fold greater loss in mean MLD (P=0.006) and lesion-specific MLD (P=0.038) compared with those without the mutation, as shown in the Table. When analyzed for a dominant effect, subjects with the T allele had greater loss in mean MLD (−0.17±0.24 versus −0.03±0.22; P=0.0004) and in mean lesion-specific MLD (−0.17±0.02 versus −0.06±0.03; P=0.0036) in the placebo group. Similarly, the number of subjects with the mutation (CT+TT genotypes) who had definite progression was twice greater and with definite regression was 11-fold less (Table). Only 1 of 90 subjects with the mutation had definite regression in contrast to 11 of 62 (18%) subjects without the mutation (odds ratio=16; 95% confidence interval: 2.0 to 127; P=0.001). In the fluvastatin group, losses in mean MLD (−0.06±0.23 versus −0.01±0.27) and lesion-specific MLD (−0.05±0.03 versus −0.04±0.03) were also greater in those with the mutation. However, the differences were not statistically significant. There were no significant differences in the number of new lesions or new total occlusions among the genotypes either in the placebo or in the fluvastatin groups.

**Clinical Events**

Morbid or fatal clinical events occurred in 53 patients (14%). The distribution of cardiovascular events was similar among the genotypes in the placebo (CC: 12/62; CT: 13/73; and TT: 3/17) and fluvastatin groups (CC: 11/74; CT: 13/70; and TT: 1/12). Similarly, the number of events in the subgroup with available angiography did not differ significantly among the genotypes (data not shown).

**Discussion**

The results of this prospective study show that the 242\textsuperscript{T} allele of the p22\textsuperscript{phox} protein component of the vascular NADPH/NADH oxidase pathway is associated with progression of CAD in the LCAS population. In the presence of the 242\textsuperscript{T} allele, losses in mean MLD and lesion-specific MLD were greater, a greater number of subjects had progression, and a smaller number of subjects had regression of CAD during 2.5 years of follow-up. The association of the C\textsuperscript{242}T genotypes with progression of CAD was stronger when analyzed for a dominant effect of the T allele. In the fluvastatin group, losses in mean MLD and lesion-specific MLD were also greater in those with the T allele. However, the differences were not statistically significant. We note that the number of subjects in each subgroup is relatively small, and a larger sample size is needed to properly address the possible association of the C\textsuperscript{242}T polymorphism with the progression of CAD in the fluvastatin group. It is also possible that fluvastatin by lowering plasma cholesterol levels, which is known to increase NADH-dependent vascular O_2\textsuperscript{−} production, modifies the association of the genotypes with progression of coronary atherosclerosis. There was no association between the C\textsuperscript{242}T genotypes and the baseline characteristics of subjects, including angiographic indices of severity of CAD, plasma levels of lipids, or frequencies of conventional risk factors. The lack of the association of the C\textsuperscript{242}T genotypes with the baseline severity of CAD in LCAS subjects, despite their association with the progression of CAD, raises the possibility of self-selection. We note that the design of LCAS and end points of the study were chosen before the decision to perform genetic analyses. However, the distribution of the genotypes was according to the Hardy-Weinberg equilibrium, which reduces the chance of self-selection. Collectively, these results suggest that the 242\textsuperscript{T} allele is associated with progression of CAD in the LCAS population.

The association of the C\textsuperscript{242}T genotypes with atherosclerosis and MI has been studied previously in three case-control studies, and the results have been conflicting. Inoue et al\textsuperscript{26} compared the distribution of C\textsuperscript{242}T genotypes in 201 Japanese patients with coronary artery stenosis >75% and 210 subjects without clinical evidence of CAD. They found that the combination of TT+CT variants was less common in the Japanese cases. They concluded that the 242\textsuperscript{T} allele conferred protection against atherosclerosis in a Japanese study population. In contrast, Cai et al\textsuperscript{28} found that the 242\textsuperscript{T} allele was a modest risk factor for the presence of angiographically defined CAD in young (<45 years of age) Australian Caucasian patients but not in the overall population. Li et al\textsuperscript{37} found that the frequency of the C\textsuperscript{242}T mutation was similar in 149 subjects with CAD and 103 subjects without significant CAD but chest pain. They also showed that coronary epicardial or microvascular responses to acetylcholine or sodium nitroprusside were not significantly associated with the genotypes.\textsuperscript{28} The frequency of the 242T allele in the above Caucasian populations was 3 to 4 times higher than that in the Japanese population reported by Inoue et al\textsuperscript{28} and was similar to that in the LCAS population. The conflicting results of the case-control studies may reflect the high rate of spurious association that is intrinsic to case-control polymorphism association studies,\textsuperscript{31} the differences in ethnic backgrounds of the populations, and the criteria used for phenotypic definitions. The present study, unlike previous studies, is a prospectively designed study in a well-characterized population that has undergone extensive phenotypic characterization. The severity, progression, and regression of coronary atherosclerosis were assessed by serial quantitative coronary angiography, and extensive lipid profiles were measured at baseline and at completion of the study. The results of the present study were concordant for two continuous indices of progression of coronary atherosclerosis (MLD and lesion-specific MLD, both showing an association) as well as the trichotomous (progression/regression/mixed) classification of CAD progression. In addition, the strength of the association between the 242T allele and progression of CAD in the LCAS population is strong. Furthermore, the frequencies of the C\textsuperscript{242}T alleles in the LCAS population are similar to those reported in previous studies of Caucasians, and the frequencies of the genotypes are similar to those expected according to the Hardy-Weinberg
equilibrium. Collectively, these data further reduce the likelihood of a false result in the present study. Whether the C242T genotypes are associated with higher clinical event rates remains to be determined. In the LCAS population, the number of clinical events was relatively low (53/368), which precludes a definitive conclusion regarding the possible association of the C242T polymorphism with new clinical events, such as MI or coronary revascularization.

The mechanism underlying the association of the 242T allele with progression of CAD is unknown. The p22phox protein is expressed in endothelial cells and vascular smooth muscle cells. Existing data suggest that p22phox is probably a common component of vascular and phagocytic oxidases. However, despite their similarities, vascular and phagocytic NADH/NADPH oxidases exhibit significant differences in their enzymatic characteristics. In contrast to phagocytic, the vascular oxidase system prefers NADH to NADPH as the primary substrate for its activity and has much lower activity. Vascular p22phox is expressed at low levels in normal coronary vessels and is upregulated in atherosclerosis and in response to trophic factors, such as angiotensin II and cytokines, such as tumor necrosis factor-α. In view of the complexity of the biological function of p22phox in the vessel wall, we can only speculate on the mechanism by which the C242T mutation affects the progression of coronary atherosclerosis. Given the critical role of the p22phox protein in the generation of ROS in the vessel wall, it is likely involved in the differential effects of the C242T variants on production of ROS. Topography of the C242T mutation in a potential heme-binding site favors this notion. However, it is anticipated that mutation in the heme-binding site of the p22phox protein leads to loss of function, rather than gain of function, of the protein and, thus, lesser production of ROS. Therefore, it seems counterintuitive that the loss of function mutation in p22phox is associated with progression of coronary atherosclerosis. There are several potential explanations including the differential effects of the C242T variants on upregulation of antioxidant defenses, gene expression, inflammation, lipid peroxidation, apoptosis, and other biological processes that are mediated by ROS.

In conclusion, the results of this relatively large prospective study show that the 242T allele of the CYBA gene is associated with the progression of CAD, as determined by serial quantitative coronary angiography, in the LCAS population. Treatment with fluvastatin annulled the association of the genotypes with progression of CAD. Thus, the C242T polymorphism in p22phox, involved in the generation of ROS in the vessel wall, is a risk factor for progression of CAD in the LCAS population.

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References


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