C242T polymorphism of the p22 phox gene is not associated with peripheral arterial occlusive disease

Wilfried Renner b,*, Katharina Schallmoser a, Patricia Gallippi b, Clemens Krauss b, Hermann Toplak a, Thomas C. Wascher a, Ernst Pilger b

a Department of Medicine, Karl Franzens University, Graz, Austria
b Division of Angiology, Karl Franzens University, Graz, Austria

Received 20 April 1999; received in revised form 20 September 1999; accepted 28 October 1999

Abstract

Formation of reactive oxygen metabolites is vital for the microbicidal activity of phagocytes. As an unwanted side effect, these metabolites may contribute to oxidative stress in the vasculature and thus lead to arteriosclerosis. p22 phox, a component of the NADH/NADPH oxidase in phagocytes and vascular smooth muscle cells, is essential for production of reactive oxygen metabolites. Recently, a C/T polymorphism at position 242 of the p22 phox gene has been associated with coronary artery disease (CAD), suggesting a protective effect of the 242 T allele on the vasculature. In the present study, we analysed the relation of this polymorphism to peripheral arterial occlusive disease (PAOD). C242T polymorphism was determined by restriction fragment polymorphism (RFLP) analysis in 324 patients with documented PAOD and 295 control subjects without any known arterial disease. p22 phox 242 T allele frequencies and genotype distributions were not significantly different between patients and controls; the adjusted relative risk associated with the 242 T allele was 1.14 (95% CI 0.84–1.54, P = 0.39), assuming an additive effect of the T allele. C242T polymorphism was not associated with the age of patients at the onset of the disease. Our data indicate that C242T polymorphism of the p22 phox gene is not associated with PAOD. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Genes; Peripheral arterial occlusive disease; p22 phox; Risk factors.

1. Introduction

Arterial diseases are the major cause of morbidity and death in western societies. Depending on the site of the occluded arteries, they present as coronary artery disease (CAD), cerebrovascular disease (CVD) or peripheral arterial occlusive disease (PAOD). In addition to major risk factors, such as smoking, hypertension, diabetes and hyperlipidemia, genetic risk factors are supposed to contribute to the development of arterial diseases.

Formation of reactive oxygen metabolites is vital for the microbicidal activity of phagocytes. p22 phox is an essential component of the NADH/NADPH oxidase, a critical enzyme for ·O2 production in phagocytes and vascular smooth muscle cells [1,2]. The NADH/NADPH oxidase and its components are highly conserved across mammalian species [3,4].

NADPH oxidase activity may be related to vascular disease by several different mechanisms. First, oxidation of low density lipoprotein (LDL), an important process in early atherogenesis, is supposed to be associated with NADPH oxidase activity. Recently, Aviram et al. have shown that NADPH-cytochrome P450 reductase is able to oxidize LDL in vitro [5]. Second, oxidative metabolites such as ·O2 are involved in endothelial dysfunction, which is thought to be a major component in the etiology of atherosclerosis [6,7]. Third, NADPH oxidase activity is responsible for the elimination of infective agents by phagocytes. Infections may be a cause of atherosclerosis [8,9], thus diminished NADPH oxidase activity leading to more frequent infections could increase the risk of vascular...
diseases. These potentially opposing effects of NADPH oxidase activity on cardiovascular risk make it difficult to predict the role of oxidative processes for the etiology of atherosclerosis.

The human p22 phox gene carries a common C→T polymorphism at nucleotide position 242, leading to a histidine→tyrosine exchange at amino acid position 72 [10]. It has been suggested that this exchange alters a potential heme-binding site of the protein, and might thus modulate its enzymatic activity. Recently, Inoue et al. have reported a strong association of this C242T polymorphism with CAD in a study including 402 Japanese subjects, suggesting a protective effect of the 242 T allele on coronary risk [11]. This effect was proposed to be a result of a potentially diminished enzymatic activity of p22 isoform coded by the 242 T allele. In another study, Gardemann et al. [12] did not find any association of this polymorphism with the presence or extent of CAD in 2205 male German subjects who had undergone coronary arteriography. No data have been available about the polymorphism and PAOD.

The present study was designed to investigate the potential association of the p22 phox C242T gene polymorphism with PAOD.

2. Methods:

2.1. Human subjects

324 patients with documented PAOD at Fontaine stage II (claudicatio intermittens), III (rest pain) or IV (gangrene) were included in our study and 295 subjects without any known cardiovascular disease served as a primary control group (controls I). Patients and controls were recruited between December 1997 and July 1999 from those admitted to the Department of Internal Medicine, Universitaetsklinik Graz, Austria; all subjects were Austrian. An additional control group (controls II) of 115 healthy volunteers was recruited from the medical staff of the same department. The study was performed according to the Austrian Gene Technology Act and to the guidelines of the Ethical Committee of the Universitaetsklinik Graz; written informed consent was obtained from all participating subjects.

Cardiovascular risk factors and previous or current cardiovascular disease were identified using the following sources: detailed self-reported medical and medication history, medical records provided by general practitioners, and medical records from the Universitaetsklinikum Graz. Measurement of ankle/brachial index (ABI) was performed according to [13]. Subjects were diagnosed to have PAOD if they had: (I) clinical symptoms of PAOD (claudicatio intermittens, rest pain, or gangrene); (II) an ABI < 1; and (III) significant stenoses of leg arteries confirmed by Doppler sonography and/or angiography. Subjects were also classified as PAOD patients if they had previous aortofemoral or femoropopliteal bypass surgery. Subjects were thought to be eligible as controls if they had (I) no clinical symptoms of PAOD, CAD, or CVD, and (II) no medical history of PAOD, CAD, or CVD.

Furthermore, subjects were considered to be hypertensive if their blood pressure was > 140 mm Hg systolic, or > 90 mm Hg diastolic, or they had already been treated with antihypertensive drugs. Diabetes was diagnosed according to the criteria of the World Health Organisation [14].

2.2. Determination of p22 phox C242T polymorphism

Venous blood was collected in 5 ml EDTA tubes, genomic DNA was isolated using a Nucleospin Blood kit (Macherey–Nagel) and stored at 4°C. Genotyping of p22 phox C242T polymorphism was performed by RFLP analysis, as described previously [11]. Briefly, a 348 bp DNA fragment, containing the C242T polymorphic site, was amplified by polymerase chain reaction (PCR) and digested with restriction enzyme RsaI (New England Biolabs). The 242 C allele remained uncut, while the 242 T allele was cut into two fragments of 160 and 188 bp. Fragments were separated on 2% agarose gels and visualised by use of ethidium bromide.

2.3. Statistics

Normal distribution of data was analysed by the Komogorov–Smirnov normality test. To enable comparison of two groups, numeric values were analysed by Student’s t-test and data showing unequal variance or no normal distribution were analysed by a Mann–Whitney rank sum test. Categorical values were compared by χ² test. Odds ratios (OR) and 95% confidence intervals were determined by multiple logistic regression analysis. Association of p22 phox genotype with age of patients at onset of PAOD was analysed by one-way ANOVA. The criterion for statistical significance was $P = 0.05$.

3. Results

Baseline data of patients and controls are given in Table 1. Of the PAOD patients, 74 (22.8%) also had CAD, and 93 (28.7%) had CVD. Fontaine stage of the disease was II in 258 (79.6%) patients, III in 22 (6.8%) patients, and IV in 44 (13.6%) patients. In both control groups, mean age and prevalence of male subjects, smokers, diabetics and hypertensives was significantly lower than among patients. Mean total cholesterol and
triglycerides of controls I, but not controls II, were significantly lower than those of patients. Among controls II, total cholesterol and triglyceride values were available for only 53 subjects.

Frequency of alleles and distribution of genotypes are summarised in Table 2. Genotype frequencies did not deviate from the Hardy–Weinberg equilibrium in patients, or either control group ($\chi^2$ test). No significant difference of 242 T allele frequency, or either p22 phox genotype frequency between patients, controls I, controls II or a combination of controls I and II could be detected.

OR of potential risk factors for PAOD were determined by multiple logistic regression analysis, including patients and controls I. For calculation of OR, ordinal variables were assigned to the p22 phox genotype, assuming an additive effect of the 242 T allele (CC = 0, CT = 1, TT = 2). OR for the 242 T allele was 0.94 (95% CI 0.74–1.18, $P = 0.25$); after adjustment for age, sex, diabetes, hypertension, smoking, total cholesterol and triglycerides, the OR remained not significant (OR = 1.1, 95% CI 0.84–1.54, $P = 0.39$).

Mean age at onset of PAOD was 64.9 $\pm$ 10.5 years for carriers of the CC genotype, 65.8 $\pm$ 10.6 years for CT genotype and 66.3 $\pm$ 9.1 years for TT genotype. Using one-way ANOVA, we found no significant association of p22 phox genotype with age at onset of PAOD ($P = 0.69$).

In further analyses, we compared genotype and allele frequency of subgroups of patients and controls using different inclusion criteria. Subgroups were male, female, low risk group (non-smokers and non-diabetics), hyperlipidemics, and subjects < 65 years at onset of PAOD. In none of these groups could a significant difference of genotype distribution or allele frequency between patients and controls I or controls II be detected (data not shown).

Among PAOD patients who additionally had CAD ($n = 74$), 26 (35.1%) carried the CC genotype, 36 (48.6%) the CT genotype, and 12 (16.3%) the TT genotype. Again, genotype frequencies and allele frequency were not significantly different from PAOD patients without CAD ($\chi^2$ test, $P = 0.28$), controls I ($P = 0.67$) or controls II ($P = 0.37$).

### 4. Discussion

The present study was designed to analyse the potential association of the C242T polymorphism of the p22 phox gene with PAOD. Genotype and allele frequencies were not different between patients and two different control groups; in further subgroup analyses we also found no association of the p22 phox C242T polymorphism with PAOD. Where patients with PAOD were concerned, the p22 phox genotype was not associated with age at the onset of the disease, or the presence of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline data of patients and controls$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients ($n = 324$)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>194 (59.9)</td>
</tr>
<tr>
<td>Age, years</td>
<td>68.8 $\pm$ 9.6$^e$</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>133 (41.0)</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>113 (34.9)</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>231 $\pm$ 56$^e$</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>187 $\pm$ 149$^e$</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>200 (61.7)</td>
</tr>
</tbody>
</table>

$^a$ Numeric values are presented as mean $\pm$ S.D. Categorical values were analysed by $\chi^2$ test, numeric values were analysed by rank-sum test.

$^b$ Values were available for 53 subjects.

$^c$ Values not normally distributed.

$^* P < 0.05$, compared to patients.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Prevalence of p22 phox genotypes and 242 T allele frequency$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients ($n = 324$)</td>
</tr>
<tr>
<td>CC genotype</td>
<td>140 (43.2)</td>
</tr>
<tr>
<td>CT genotype</td>
<td>139 (42.9)</td>
</tr>
<tr>
<td>TT genotype</td>
<td>45 (13.9)</td>
</tr>
<tr>
<td>242 T allele frequency</td>
<td>0.353</td>
</tr>
</tbody>
</table>

$^a$ Prevalences of genotypes are presented as n (%). No statistically significant difference of genotype or allele frequency between groups was detected ($\chi^2$ test).
CAD. Our results suggest that this polymorphism is not associated with PAOD.

Some points should be considered when interpreting this result. First, controls I of the present study were recruited from patients admitted to the Department of Internal Medicine, Universitätsklinik Graz, Austria, who did not have any known cardiovascular disease. This has led to an overrepresentation of diabetes, hyperlipidemia and hypertension in controls I and an underestimation of the OR for PAOD caused by these risk factors. Nevertheless, p22 phox genotype distribution among an additional control group (controls II) of 115 healthy volunteers was essentially the same as that among controls I. In recent studies, Gardemann et al. [12] reported a 242 T allele frequency of 0.34 in 499 male subjects without CAD and the same frequency in 1706 patients with documented CAD. Handschug et al. [15] have investigated the p22 phox polymorphism in 134 German patients who had undergone a myocardial infarction, and 242 T allele frequency among them was 0.33. In both studies, allele frequencies of patients or controls were similar to frequencies among patients and controls of the present study. Thus, it seems unlikely that our negative findings have been caused by the use of ‘non-healthy’ subjects as primary control group.

Second, analysis of subgroups in our study was limited by the small number of subjects in some subgroups. We cannot rule out the possibility that in particular populations, with or without distinct risk factors, this polymorphism might be associated with a risk for PAOD. To analyse these potential associations, larger studies restricted to such population groups would be required.

Third, environmental factors, such as life style and diet, as well as the different ‘genetic background’ in other ethnical groups may strongly influence oxidative stress caused by a particular genotype. Our results refer to a Caucasian population in Middle Europe and may vary from results obtained in other geographical or ethnical populations. Interestingly, 242 T allele frequency in controls of the present study was 2.7 times higher than that among 201 Japanese without CAD reported by Inoue et al [11]. To our knowledge, no study besides the present one has been published about the association of the p22 phox gene polymorphism and PAOD.

Flavocytochrome b558 is the membrane bound component of NADH/NADPH oxidase, consisting of p22 phox and gp91 phox [16]. Downregulation of p22 phox gene expression in vascular smooth muscle cells by antisense RNA lead to decreased ·O₂⁻ production, indicating that p22 phox was critical for ·O₂⁻ production in the vasculature [2]. It has been speculated that the histidine → tyrosine exchange caused by the p22 phox C242T polymorphism might change a heme-binding site of the NADPH oxidase, thereby leading to a reduced activity of the enzyme and reduced oxidative stress in the vasculature. Recently, spectral analysis of membrane fractions prepared from transfected COS7 cell lines, expressing either gp91 phox alone or gp91 phox and p22 phox, indicated that gp91 is the sole heme-binding component of flavocytochrome b558 [17]. Thus, functional consequences of the p22 phox polymorphism remain unclear. As has been stated in Section 1, NADPH oxidase activity may be related to oxidative stress and LDL oxidation, as well as infections, leading to potentially opposing effects on cardiovascular risk. Further investigations are necessary to enlighten the role of reactive oxygen metabolites in the etiology of atherosclerosis.

In summary, we have shown that the C242T polymorphism of the p22 phox gene is not associated with the presence of PAOD or age at onset of the disease.

Acknowledgements

The authors would like to thank Dr Antonella de Campo for her help with recruitment of subjects.

References

structure, chromosomal location, and mutations in cytochrome-


