recent discovery of other CYP2C9 allelic variants (10, 11), adaptations of this method could allow detection of these variants.

This project was supported in part by NIH Research Grants R01 HL68834 and MH63458, funded by the National Institute of Mental Health and the Office of Dietary Supplements, and NIH M01 RR00082.

References


DOI: 10.1373/clinchem.2004.040071

CYP2C9*2 and CYP2C9*3 Alleles Confer a Lower Risk for Myocardial Infarction, Marion Funk,1 Georg Endler,1 Renate Freitag,1 Johann Wojta,2 Kurt Huber,3 Christine Mannhalter,1, and Raute Sundel-Plassmann1* (1 Institute of Medical and Chemical Laboratory Diagnostics and 2 Department of Internal Medicine II, Division of Cardiology, Medical University of Vienna, Vienna, Austria; 3 3rd Medical Department for Cardiology and Emergency Medicine, Wilhelminenspital, Vienna, Austria; * address correspondence to this author at: Institute of Medical and Chemical Laboratory Diagnostics, Waehringer Guertel 18-20, A-1090 Vienna, Austria; fax 43-1-40400-5761, e-mail Raute.Sunder-Plassmann@meduniewien.ac.at)

Cytochrome P-450 (CYP) genes encode for membrane-bound, heme-containing enzymes that catalyze the oxidation of various drugs and endogenous substrates such as vitamin D, steroids, or fatty acids, including arachidonic acid (AA). CYP enzymes of the P-450 2C9 subfamily are produced in the liver, are responsible for 50% of the epoxygenase activity in human liver, and metabolize a wide variety of clinically important drugs, including losartan, torsemide, and S-warfarin (1). Furthermore, CYP2C9 may also play a role in the regulation of vascular tone. In addition to nitric oxide (NO) and prostacyclin, endothelial cells synthesize and release endothelium-derived hyperpolarizing factor (EDHF), which causes hyperpolarization of underlying vascular smooth muscle cells via activation of C2+-activated K+ channels (2). EDHF has been described as an important regulator of vascular tone under certain pathologic conditions and in certain vascular beds, such as the coronary microcirculation (3). Interestingly, EDHF production seems to be inhibited by NO and/or prostacyclin. There is now compelling evidence that the hyperpolarizing factor produced by coronary arteries is a CYP epoxygenase-derived metabolite of AA. Recently, it has been shown that decreased concentrations of CYP2C2 attenuate EDHF-mediated vascular response in porcine coronary artery endothelial cells (4). This effect appears to be directly attributable to the regulation of an enzyme homologous to CYP2C9 and the generation of the CYP metabolite 11,12-epoxyicosatrienoic acid (5).

Changes in the amino acid sequence of CYP2C9 can affect both the activity and substrate specificity of CYP2C9. Previously, three alleles were identified in the Caucasian population: CYP2C9*1, CYP2C9*2, and CYP2C9*3. The CYP2C9*1 allele encodes the wild-type protein, and the CYP2C9*2 allele contains a C-to-T transition, leading to substitution of cysteine by arginine at amino acid position 144. The CYP2C9*3 allele is defined by an A-to-C nucleotide substitution that leads to an exchange of leucine by isoleucine at amino acid position 359. Both variant alleles are associated with significantly reduced enzyme activity (6, 7). We hypothesized that reduced CYP2C9 activity attributable to genetic alterations may modulate vascular function and influence the risk of vascular disease.
To study the role of the CYP2C9*2 and CYP2C9*3 variants in the pathogenesis of cardiovascular disease, we performed a case-control study on 834 consecutive, extensively clinically characterized Caucasian individuals referred to the Department of Cardiology, Medical University of Vienna, for evaluation of coronary artery disease. Patients were divided into two groups according to clinical findings, patient histories, and coronary angiography. The first group [myocardial infarction (MI) group] included 403 patients [97 females, 306 males; median age, 61 years; interquartile range (IQR), 52–72 years] who had a history of MI according to WHO criteria or who presented with this clinical diagnosis. The second group (control group) included 431 individuals without MI (220 females, 211 males; median age, 58 years; IQR, 46–68 years). In these individuals, the presence of coronary artery disease was excluded by angiography, where indicated, or other objective tests (exercise stress test and/or thallium-persantin scintigraphy). Members of this group were referred to the center for different reasons, including valvular heart disease, nonischemic cardiomyopathy, infectious diseases, and nonischemic arrhythmias. Detailed characteristics and the distribution of cardiovascular risk factors of the MI patients and non-MI patients are given in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue12. The study was approved by the local ethics committee, and all individuals participating in the study gave written informed consent.

All patients were questioned for established cardiovascular risk factors, including arterial hypertension (history of arterial hypertension, systolic blood pressure >140 mmHg or diastolic blood pressure >80 mmHg at repeated measurements, or treatment with antihypertensive drugs), diabetes (fasting glucose ≥126 mg/L according to American Diabetes Association criteria), hypertriglyceridemia (triglyceride concentrations >1800 mg/L after overnight fasting or taking lipid-lowering medication), hypercholesterolemia (baseline cholesterol concentrations >2000 mg/L or serum LDL >1300 mg/L), smoking (>20 cigarettes for more than 5 years), and family history of cardiovascular disease.

For genotyping, 4 mL of blood was drawn from each individual. The blood was kept frozen at −20 °C until isolation of DNA by standard procedures. For the detection of CYP2C9 genetic polymorphisms, we adapted the principle of the mutagenic separated PCR (MS PCR) assay. Before the introduction of MS PCR, many of the patient samples had been tested by a published multiplex PCR combined with restriction enzyme digestion with AlwII and NsiI described by Taube et al. (8). These samples were then evaluated with the easy-to-use one-tube method based on allele-specific primers that differ in length by 9 bp (9). PCR products were generated in 25-μL volumes containing 0.74 U of AmpliTaq Gold (Perkin-Elmer Cetus), 2 mM MgCl2, 200 mM each deoxynucleotide triphosphate (Amersham Pharmacia Biotech), 0.5 pmol of CYP2C9*2 wild-type reverse primer (5'-CGGCTTCTCTTTGACGCAG-3'), 1.5 pmol of CYP2C9*2 mutant reverse primer (5'-ACAAAGCTTTGCTTC-CTCTGATTACA-3'), 3 pmol of CYP2C9*2 common forward primer (5'-CAATGGAAGAAATGGAAGGATT-3'), 1 pmol of CYP2C9*3 wild-type forward primer (5'-TGCAGAAGTCCAGAGTACA-3'), 2 pmol of CYP2C9*3 mutant forward primer (5'-ATGCTTGGAAAAACGAGGTCCAGAGAAACC-3'), and 3 pmol of CYP2C9*3 common reverse primer (5'-AATGATATGTCAATTTGGAAC-CAATGGAAAGAAATGGAAGGAGGT-7), 3 pmol of CYP2C9*3 allele lead to decreased enzyme activity (6, 7), both genotype results were pooled for regression analysis. The following groups were formed: homozygous carriers of wild-type alleles (CYP2C9*1/*1 individuals) and individuals carrying one or two variant alleles (mutant allele carriers). We tested for interactions between the CYP2C9 genotype and traditional vascular risk factors, using multiplicative interaction terms and log likelihood ratio χ² tests. Results of the logistic regression models are given as the odds ratio (OR) and the 95% confidence interval (95% CI). A two-sided P value <0.05 was considered statistically significant.

We observed no significant differences in overall CYP2C9 genotype frequencies between the two patient groups (MI and non-MI). The genotype distribution of CYP2C9 in MI and non-MI individuals is shown in Table 1A. Genotype frequencies were in Hardy–Weinberg equilibrium in both groups and were in very good agreement with those reported in the Viennese population (data not shown) and in other European populations (7). We observed an interaction between the CYP2C9 genotype, gender, and the risk of MI. The mutant CYP2C9 alleles...
were more frequent in male non-MI individuals (42.7% vs 34.3%; P = 0.065). This trend was confirmed in a logistic regression analysis after adjusting for age, diabetes, smoking, hypercholesterolemia, hypertriglyceridemia, hypertonus, and family history of cardiovascular disease after stratification for gender. Males carrying a variant CYP2C9 genotype had a significantly reduced risk for MI. The adjusted OR was 0.56 (95% CI, 0.33–0.95; P = 0.03). In contrast, in females the CYP2C9 genotype was not associated with the risk for MI (OR = 1.12; 95% CI, 0.58–2.12; P = 0.75). A comparison of CYP2C9 allele frequencies in males and females is given in Table 1B. The frequencies of other cardiovascular risk factors such as age, smoking, diabetes mellitus, hypertension, and hypercholesterolemia were not significantly imbalanced between carriers and noncarriers of the mutant alleles.

In the present study, we observed a protective effect of the CYP2C9 mutant genotype for the development of MI in males. This was surprising because one would have expected carriers of CYP2C9 mutant alleles to exhibit reduced CYP2C9 metabolic capacity, leading to decreased endothelial EDHF synthesis and an increased risk for MI. However, CYP enzymes also contribute to oxidative stress through the formation of oxygen radicals in the vasculature (10). Particularly, CYP2C9 has been shown to be a major source of reactive oxygen species (ROS) within coronary artery endothelial cells (11). Lower formation of oxygen radicals in carriers of mutant alleles might explain our findings. Interestingly, in recent studies, inhibition of CYP2C9 enhanced the bioavailability of NO through attenuation of ROS production (12) and reduced ROS production and myocardial infarct size in an isolated perfused rat heart model (13). Therefore, therapeutic inhibition of CYP2C9 could represent a promising option in the prevention of cardiovascular disease. Recently, Yasar et al. (14) described a modest increase in risk for MI in female carriers of mutant CYP2C9 alleles, and deficiency of NO has been shown to activate gender-specific signal transduction (15). Moreover, a new constitutive androstene receptor binding site was identified in the CYP2C9 promoter (16) that regulates the transcription of CYP2C9 and represents the basis for gender-specific findings.

We are aware that the impact of single-nucleotide polymorphisms on a complex disease such as MI is affected by patient selection, ethnic background, and sample size. It is well known that the overall incidence of cardiovascular disease is lower in females than in males. Thus, female patients diagnosed with MI were less frequent in our cross-sectional study population. We cannot exclude that a small effect of the CYP2C9 mutation in females could have been missed. However, because we did not observe a difference in genotype distribution between female patients and controls (Table 1B), a protective effect of the CYP2C9 mutation in females seems unlikely. In addition, our population comprised only survivors of MI consecutively referred to the Department of Cardiology. Patients with MI who died of sudden cardiac death before reaching the hospital were not included. Our findings can therefore be applied only to survivors of MI.

In conclusion, we observed a gender-specific impact of the CYP2C9 genotype on development of MI. Male carriers of the CYP2C9 mutant genotype seem to have a lower risk for suffering MI. If confirmed in independent studies, our findings might help explain at least some of the gender-specific differences in cardiovascular risk profiles. Our newly established MS PCR assay, which allows fast
and economic determination of the CYP2C9 genotype without the need of restriction digestion, might facilitate further studies and the implementation of this test in routine laboratories.

This work was supported by a research grant from the Hochschuljubiläumsstiftung der Stadt Wien (Project H-1458/02).

References


DOI: 10.1373/clinchem.2004.038034

Potential Interferences from Blood Collection Tubes in Mass Spectrometric Analyses of Serum Polypeptides, Steven K. Drake,1 Raffick A.R. Bowen,2 Alan T. Remaley,2 and Glen L. Hortin2* (Departments of 1 Critical Care Medicine and 2 Laboratory Medicine, Warren Magnuson Clinical Center, NIH, Bethesda, MD; * address correspondence to this author at: Department of Laboratory Medicine, NIH, Bldg. 10, Room 2C-407, Bethesda, MD 20892-1508; fax 301-402-1885, e-mail ghortin@mail.cc.nih.gov)

Currently, there are high degrees of both enthusiasm and controversy regarding the potential diagnostic application of matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry (1–7). This technique permits the simultaneous analysis of a large number of polypeptide components in biological fluids. Greatest sensitivity and resolution are achieved by MALDI TOF mass spectrometry in the mass range from ~500 to 20 000 Da; this has led to the recognition that there is a highly complex mixture of peptide components in serum that circulate bound to larger proteins (8, 9). The complex patterns of peptide components are very informative and may contain multiple biomarkers of diagnostic value (1–9).

The present study examined whether different types of blood collection tubes add molecules to specimens that may appear as interfering or confounding peaks during MALDI TOF mass spectrometry. Commercially available blood collection tubes contain multiple components that may shed polymers in the molecular size range of interest. Silicons are commonly used as lubricants for stoppers or coatings for the internal surface of tubes. Polymeric surfactants such as polyvinylpyrrolidones or polyethylene glycols may be added to influence surface wetting. Tubes may contain either clot inhibitors or activators. Serum separator tubes contain polymeric gels with several constituents to adjust viscosity, density, and other physical properties. Rubber stoppers and the plastics comprising tube walls may shed polymeric components or plasticizers. Previous studies have reported effects of blood collection tubes on a variety of laboratory tests (10–14). These effects can arise from adsorption of serum or plasma components to the tube or separator gel, from the release of materials that interfere with specific test procedures, or from variable clotting of specimens. Although the potential effects of blood collection tubes need to be considered for any laboratory test, this may be particularly important for MALDI TOF mass spectrometry-based laboratory tests, which measure a broad spectrum of different components in a single analysis.

We examined the shedding of components from tubes into aqueous solutions by adding 1 mL of phosphate-buffered saline (pH 7.2; KD Medical) to collection tubes and incubating the tubes at room temperature for ~4 h with gentle rocking to allow contact with all surfaces of the tubes. This was considered to simulate typical contact times of blood specimens from collection to processing or analysis. Tubes tested included multiple types from two manufacturers, Becton Dickinson and Greiner Bio-One.