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EDITORIAL COMMENT

Significance of Vascular Nitric Oxide Synthase Pathways in Coronary Artery Disease

A Multiple-Level Biomarker Strategy*

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Coronary artery disease (CAD) is a polygenic disorder representing the major cause of cardiovascular (CV) mortality. The susceptibility to CAD is affected by the complex interaction of genomic and proteomic interactions. Prognostic studies have shown that endothelial dysfunction with a decreased bioactivity of nitric oxide (NO) is associated with an increased rate of major adverse cardiovascular events in patients with CAD, diabetes, peripheral arterial occlusive disease, and arterial hypertension. Thus elucidating the role of genes, whose products are involved in formation and degradation of NO, a ubiquitous signalling molecule largely responsible for the maintenance of normal endothelial function, seems of major significance. It is clear that endothelial nitric oxide synthase (eNOS) has evolved to be tightly controlled by co-translational and post-translational lipid modifications, phosphorylation, multiple residues, and regulated protein-protein interactions (1). Thus far no prospective studies exist that simultaneously quantify alterations of a single nucleotide in the deoxyribonucleic acid sequence, so-called single nucleotide polymorphism (SNP), that encode for eNOS and metabolites of NO in CAD patients.

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Three SNPs for eNOS have been described thus far: intron-4, Glu298Asp, and $T^{-786}C$ SNPs. The GAG to GAT substitution in exon 7 ($G^{894}T$) determines the conservative replacement of glutamate with aspartate (Glu298Asp), which might cause a tight turn of the α-helix and therefore an increased susceptibility to degradation. The $T^{-786}C$ SNP is located in the promoter region of the eNOS encoding gene. The significance of these SNPs is still controversial (2). The conflicting results may be attributable to different analytical approaches (3), to varying stabilization of gene products in various vascular diseases (4), and most importantly, to the fact that most of the published reports used a cross-sectional rather than a prospective study design and the patient populations were rather small, raising the issue of several statistical bias.

In this issue of the *Journal*, Rossi et al. (5) reported on the impact of eNOS SNPs in 1,086 patients with CAD at high CV risk recruited from the prospective branch of the GENICA (Genetic and Environmental Factors in Coronary Artery Disease) study (5). The extent of CAD in large coronary vessels was graded by the modified Duke Prognostic Index. The investigators tested the hypothesis that the $G^{894}T$ and $T^{-786}C$ eNOS SNPs predict CV deaths in this cohort. Genotyping was performed with a LightCycler (Roche, Milan, Italy) using melting curve analysis. The sum of oxidative NO metabolites in plasma (NOx, that is nitrite and nitrate), nitrotyrosine, and the formation of free radicals as a surrogate for mylo peroxidase activity were estimated using commercial enzyme-linked immunosorbent assay and photometric kits. The Glu298Asp according to SNP in exon 7 ($G^{894}T$), a conservative replacement, showed no effect. In a previous small cross-sectional study, Rossi et al. (5) had already shown that this SNP only affects the flow response to acetylcholine in the forearm circulation of hypertensive individuals through the interaction with $T^{-786}C$ SNP, both being in a linkage disequilibrium. In the present study, the SNP $T^{-786}C$ located in the eNOS gene promoter had a significant effect on CV death-free survival, but not on plasma nitrite/nitrate levels.

The strength and unique character of the present study (5) is supported by several features: it is the first prospective study showing an effect of the $T^{-786}C$ SNP on survival in high-risk patients, even after control for other typical CV risk factors. A large sample size with a high follow-up rate of 98% was studied, providing adequate statistical power to test the hypothesis. Coronary artery risk burden was quantified in every single patient at study entry. Not only were SNPs analyzed, but also surrogate markers of the respective signalling pathways of the vascular wall. Previous medication with NO donors was considered. However, biochemical analysis was done only in a small (but randomly selected) subset of patients, and biochemical assays of questionable specificity were used. This may explain some aspects of the paradoxical finding that the T^{-786} allele was associated with increased CV risk, although surrogates of NO bioactivity such as NOx and nitrotyrosine were directed in the opposite direction.

The steady-state concentration and thus the biological effects of NO are critically determined not only by its rate of formation, but also by its rate of decomposition (6). A series of studies within recent years has unraveled mechanisms by which NO bioactivity in blood might be sustained. Circulating NO pool comprises of various nitrosylated species and the oxidative products nitrite and nitrate (7). The plasma levels of nitrotyrosin compounds are influenced not only by eNOS activity but also by multiple enzymes involved in the metabolism of reactive oxygen species. Therefore, this parameter may not merely reflect changes caused by eNOS

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expression and activity. Although nitrate levels are affected by a variety of eNOS-independent factors (6), plasma nitrite reflects constitutive NOS activity in mammals (8). In the present study by Rossi et al. (5), only the sum of nitrite and nitrate was determined. Thus the high background level of nitrate may have masked directed changes of nitrite as a valuable index of eNOS activity, and thus might explain some of the unexpected results. Apart from plasma, erythrocytes have been shown to represent a major storage site of nitrite in human blood (9) and are capable of producing bioactive amounts of NO through reduction of nitrite, further contributing to the circulating NO pool (10). Very recently these findings have been extended, showing that erythrocytes express a functional eNOS (11). These recent advances should enable us now to perform a comprehensive analyses of the circulating NO pool in humans, together with analysis of SNPs for eNOS.

The scientific field of cardiac biomarkers is rapidly growing (12). A novel approach has evolved by using a multimarker strategy combining biochemical measures of heart failure, ischemia, and inflammation (12). This strategy has been broadened by measuring biochemical markers (12) together with structural (13) and functional (14) markers for endothelial dysfunction and cardiovascular disease. The present study by Rossi et al. (5) offers the avenue for developing new strategies in the scientific field of diagnostic markers of malign across the cardiovascular continuum.

Cardiovascular disease is the consequence of a complex series of pathophysiological events in genetically susceptible individuals resulting in systemic and/or localized tissue damage, ultimately leading to target organ failure, clinical events, or death. Endothelial dysfunction is a key event in these processes. Based on the present findings, a multiple-level marker strategy can be invented targeting specifically pathophysiological alterations along the eNOS pathway in patients by measuring genetic, biochemical, functional, and structural markers in patients simultaneously. Such an approach could comprise the analysis of eNOS SNPs, a comprehensive analysis of the circulating NO pool, measurement of flow-mediated dilation, and intima-media thickness. Because these analytical tools will become available for clinical routine in the very near future, a multiple-level marker strategy focusing on a single key signalling pathway to diagnose different stages of the complex and polygenic cardiovascular diseases seems realistic very soon.

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