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A functional variant of the myeloperoxidase gene is associated with cardiovascular disease in end-stage renal disease patients

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A functional variant of the myeloperoxidase gene is associated with cardiovascular disease in end-stage renal disease patients. Cardiovascular disease (CVD) is the leading cause of mortality in end-stage renal disease (ESRD) patients and there is emerging evidence that genetic factors may contribute to the development of atherosclerosis. Myeloperoxidase (MPO) is an abundant enzyme involved in the production of free radicals. A functional G→A single nucleotide polymorphism (SNP) has been identified at position −463, where the A allele is associated with lower MPO expression. To analyze the association between this SNP and inflammation, oxidative stress, and CVD, we studied a cohort of 155 ESRD patients (52 ± 1 years, 62% males, 22% diabetics) shortly before the initiation of dialysis treatment. CVD was defined by medical history criteria; plasma interleukin-6 (IL-6) was used as a marker of inflammation, and plasma pentosidine as an estimation of oxidative protein damage. DNA from leukocytes was used for genotyping, performed by the pyrosequencing reaction. Only five patients (3%) had the genotype AA at the −463 position, whereas 38 (25%) had the GA and 112 (72%) had the GG genotype. No differences were noted in plasma IL-6 levels between the genotype groups, whereas the pentosidine levels were higher in the GG group (28.4 pmol/mg albumin [range, 8.5 to 123 pmol/mg albumin]) compared to the other two groups (21.4 pmol/mg albumin [range, 7.6 to 384 pmol/mg albumin; $P < 0.05$]). Patients with the GG genotype had a higher prevalence of positive serology for *Chlamydia pneumoniae* (51%) when compared to the carriers of the A allele (24%) ($P < 0.05$). The prevalence of CVD was lower in the AA (0%) and GA genotypes (18%), compared to the GG genotype (35%). The GG genotype was still associated with CVD after correction for age, diabetes, smoking, malnutrition, and inflammation. Our findings suggest that the −463 G→A SNP, which supposedly results in lower MPO activity, is associated with a lower prevalence of CVD in ESRD patients. It could be speculated that this effect is mediated by a decreased oxidative stress due to lower production of free radicals.

End-stage renal disease (ESRD) is considered a pro-inflammatory state, and oxidative stress has been implicated as one important cause of the sustained inflammatory activity [1]. Owing to the putative pro-atherogenic effects of intravascular inflammation, we hypothesize that factors regulating the inflammatory and oxidative stress response may contribute to the high prevalence of cardiovascular disease in ESRD.

Myeloperoxidase (MPO) is a hemoprotein found mainly in neutrophils, but to a lower extent also in monocytes. The main biological function of MPO is the defense of the organism, catalyzing the production of hypochlorous acid, and enhancing antimicrobial activity. Apart from its diagnostic relevance in acute myeloid leukemia, MPO has recently attracted significant attention, since it seems to be also involved in a broad range of noninfectious diseases, such as lung cancer, Alzheimer's disease, multiple sclerosis, atherosclerosis, and vasculitis [2]. It could be hypothesized that MPO may be linked to these pathologic states through its strong oxidative activity and/or its genetic variations characterized by differential expression of the protein. Recently, an important functional single nucleotide polymorphism (SNP) has been identified in the promoter region of the MPO gene, consisting of a G to A substitution (−463G→A). Previous studies have demonstrated that the G allele (in contrast to A allele) creates a strong SP1 binding site, which is correlated with a 25-fold transcriptional enhancement of the gene [3].

The description of a highly functional genetic variation in the MPO gene offers an opportunity for an evaluation of the hypothesis that the oxidative stress response plays a role in the development of atherosclerotic cardiovascular disease in ESRD patients. For this purpose, we assessed the associations between the −463G→A MPO SNP and plasma pentosidine, inflammation, chronic infection (*C. pneumoniae*), and cardiovascular disease (CVD) in a group of incident ESRD patients starting dialysis treatment.

Key words: end-stage renal disease, oxidative stress, myeloperoxidase, genetic polymorphism.

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METHODS

Study subjects

This study is part of an ongoing prospective study designed to evaluate risk factors for the development of complications in ESRD patients. One hundred and fifty five ESRD patients were recruited shortly before the initiation of renal replacement therapy. The glomerular filtration rate (GFR) was estimated by the mean of urea and creatinine clearances. Information on CVD (coronary artery disease, cerebrovascular disease, and peripheral artery disease) was obtained from a detailed medical history. Malnutrition was defined as a subjective global assessment (SGA) >2.

Laboratory methods

The plasma levels of the IL-6 and pentosidine were determined in plasma EDTA samples stored at −70°C. Plasma IL-6 was measured using ultrasensitive ELISA kits (Boerhinger Mannheim, Mannheim, Germany). Pentosidine was measured using reverse phase high-pressure liquid chromatography (HPLC) and adjusted for serum albumin concentration (expressed as pmol/mg albumin). S-albumin was determined using the bromocresol method. *C. pneumoniae*, IgG, and IgA antibodies obtained from Thermo Labsystems (Helsinki, Finland) were measured by the microimmunofluoresence method with elementary bodies of *C. pneumoniae* strain IOL 207 used as a representative *C. pneumoniae* strain type. IgG and IgA titers of 1/64 or greater were considered positive.

Genotyping methods

At recruitment, a 5 mL EDTA sample of peripheral blood was drawn, from which DNA was extracted using QIAamp® DNA kit (Qiagen Inc., Valencia, Lousiana). Samples were stored at −20°C. The design of the sequencing primers was performed using the software Primer Designer 4 for Windows, version 4.1©, (scientific and educational software, Durham, NC) and all oligonucleotides were synthesized by Interactiva® (Ulm, Germany). Sequence amplification was performed using the polymerase chain reaction (PCR) on a PTC-225 Thermocycler (MJ Research, Inc., Cambridge, MA, USA). The PCR reaction volume was 50 μL, containing 20 to 50 ng of DNA, 10 pmol of forward and reverse primers, 0.2 mM of each dNTP, 0.3 U of DyNAzyme™ II (DNA Polymerase; Finnzymes, California, USA), 10 mM of Tris-HCl, 1.5 mM of $MgCl_2$, 50 mM of KCl, and 0.1% Triton X-100. The primers used for the PCR reaction were 5′-CGGT ATAGGCACACAATGGTGAG-3′ (forward primer) and 5′-GCAATGGTTCAAGCGATTCTT-3′ (reverse primer). After electrophoresis size separation, the PCR product was confirmed by ultraviolet (UV) transilluminination of gels stained by EtBr (1.5% agarose). The pyrosequencing reaction was performed on a PSQ96™ Instrument from Pyrosequencing AB (Uppsala, Sweden), as described elsewhere [4]. The sequencing primer used was 5′-CCTGACCTCAAGTGATCCACC-3′.

Statistical analysis

Comparisons between groups of patients were performed using the Student *t* test or Mann Whitney test when appropriate, whereas comparisons between two groups for nominal variables were made with chi-square or Fisher's exact test. Due to the low prevalence of the AA genotype, these patients were grouped with the GA genotype for statistical analysis. The association between the genotypes and CVD was examined using chi-square test, and confirmed with logistic regression following a two-step approach. In the first step, we analyzed, in an univariate fashion, the following risk factors: age, male gender, diabetes, smoking, total cholesterol, pulse pressure, and systolic and diastolic blood pressure (traditional risk factors), as well as the nontraditional risk factors inflammation (plasma IL-6) and malnutrition (SGA >2). All parameters that were significantly associated with CVD in the univariate model (MPO GG genotype, age, diabetes, smoking, IL-6, and malnutrition) were then entered into a second logistic regression model. Values are presented as mean ± SEM or medians with $P < 0.05$ indicating significance. All the results were analyzed using StatView for Windows© (SAS, Inc., Cary, NC, USA).

RESULTS

The study population consisted of 155 patients (62% males) with a mean age of 52 ± 2 years. The primary renal disease was diabetic nephropathy in 35 patients (23%), chronic glomerulonephritis in 40 patients (26%), polycystic kidney disease in 16 patients (10%), and other, or unknown causes in 64 patients (41%). Cardiovascular atherosclerotic disease, as defined by medical history, was present in 46 patients (30%). The median IL-6 concentration was 6.1 pg/mL (range, 0.9 to 44.9 pg/mL).

Of the 155 ESRD patients tested, 112 (72%) were homozygous for the GG genotype, 5 (3%) for the AA genotype, and 38 (25%) were heterozygous. As expected, our results do not deviate from Hardy-Weinberg Equilibrium (chi-square 0.62, $P = NS$). The main characteristics of the patients, according to the genotype, are described in Table 1. There were no differences between the groups regarding age, gender, nor residual renal function, IL-6 levels, and the prevalence of diabetes mellitus. In contrast, the concentration of plasma pentosidine was higher in the GG genotype (28.4 pmol/mg albumin [range, 8.5 to 123 pmol/mg albumin]) when compared to the GA and AA groups combined (21.4 μMol/L [range, 7.6 to 384 pmol/mg albumin; $P < 0.05$]). Additionally, patients with the GG genotype presented a higher prevalence of positive serology for *C. pneumoniae* (51%) when

Table 1. Main characteristics of the study population divided into genotypes

	Genotype groups		
	GG	GA and AA	*P* value
Patients *N*	112	43	
Age *years*	51 ± 1	51 ± 6	NS
Males *%*	64	51	NS
GFR *mL/min*	7 ± 0.2	6.5 ± 0.4	NS
CVD *%*	35	16	<0.05
Malnutrition *%*	33	31	NS
Diabetes *%*	27	21	NS
IL-6 *pg/mL*	5.9 (range, 0.9–26)	5.9 (range, 1.3–45)	NS
Pentosidine *pmol/mg albumin*	28.4 (range, 8.5–123)	21.4 (range, 7.6–384)	<0.05
Chlamydia pneumoniae seropositivity *%*	51	24	<0.05

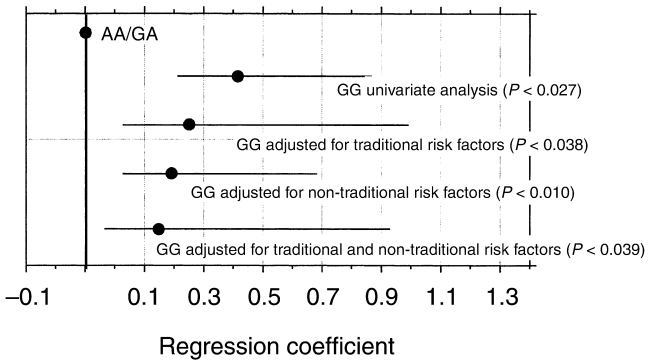

Fig. 1. Associations between the MPO genotypes and cardiovascular disease. Dots represent the regression coefficient and the lines represent the 95% confidence interval. Traditional risk factors included in the multivariate regression model were age, diabetes mellitus, and smoking, and nontraditional risk factors included inflammation (plasma IL-6) and malnutrition (SGA >2).

compared to the carriers of the A allele (24%) ($P < 0.05$). The prevalence of CVD as defined by medical history was also significantly higher in the GG group when compared to GA and AA groups combined. In the univariate analysis performed as a first step of the logistic regression model, the GG genotype was significantly associated with CVD (chi-square 4.8; regression coefficient 0.36, CI 0.14% to 0.89%; $P < 0.05$). The traditional risk factors age, diabetes mellitus and smoking, and also the nontraditional risk factors, IL-6 and malnutrition, were significantly associated with CVD in the univariate model. In contrast, male gender, serum cholesterol, and blood pressure parameters were not significantly associated with CVD, and therefore were excluded from the multivariate step of the regression model. After adjustment for age, diabetes mellitus, smoking, malnutrition, and inflammation, the GG genotype remained significantly associated with the CVD (chi-square 4.1, regression coefficient 0.12, CI 0.01% to 0.92%; $P < 0.05$) (Fig. 1).

DISCUSSION

The prevalence of atherosclerotic CVD is extremely high in patients with ESRD, probably as a result of a high prevalence of both traditional risk factors, such as hypertension and diabetes mellitus, and nontraditional risk factors, such as inflammation and oxidative stress [5]. MPO is released by activated polymorphonuclear neutrophils as part of the defense system of the organism, resulting in production of hypochlorous acid, a potent oxidant [6]. In light of the links between oxidative stress, inflammation, and CVD, we hypothesize that factors affecting the expression of MPO may be involved in the development of atherosclerosis. In this investigation, we provide unique evidence indicating that a highly functional genetic variation in the MPO gene (−463G→A SNP) is associated with the decreased presence of cardiovascular disease in ESRD.

Several studies have previously applied the same approach to investigate associations between the −463G→A SNP and other disease states. Indeed, the frequency of the G allele was also associated with a higher risk of Alzheimer's disease [7], lung cancer [8], and coronary artery disease in non-uremic individuals [9]. In the present study, we report a very similar genotype distribution of the variants in the MPO gene compared to the description in other population groups [7, 9, 10]. These findings suggest that the SNP analyzed does not vary significantly among different populations around the world. We believe that our findings have at least two major implications: a) they facilitate the identification of biologic pathways involved in atherosclerosis; and b) the use of the −463G→A SNP in the risk stratification for CVD in ESRD.

Atherosclerosis is a chronic inflammatory process in which oxidative damage within the artery wall is implicated in the pathogenesis of the disease. Although most of the data concerning atherosclerosis as an inflammatory disease comes from animal models, evidence from a recent clinical study in non-renal patients shows that widespread inflammation (increased MPO in neutrophils) is present regardless of the location of stenotic sites [11]. There is growing evidence of the important role of MPO in the pathogenesis of atherosclerosis, starting from identification of the enzyme in the atherosclе-

rotic plaques, where it was catalytically active and responsible for the oxidation of low-density lipoprotein (LDL) [12]. In fact, multiple distinct products of MPO, such as 3-chlorotyrosine, are enriched in human atherosclerotic lesions and LDL recovered from human atheroma [13]. Moreover, in a recent study, MPO was linked to the modulation of the vascular signaling and vasodilatory functions of nitric oxide (NO) during acute inflammation, impairing the endothelium-dependent relaxant response through the catalytic consumption of NO by the free radicals generated by MPO [14]. Therefore, MPO activity may be implicated in the development of cardiovascular disease through at least two distinct pathways: 1) increased oxidative stress in the atherosclerotic plaques; and 2) interference in the endothelium relaxant response. Unfortunately, in the present study we did not measure MPO activity or the mRNA expression, and the indirect evidence that the genotype affects the enzyme function is extrapolated from previous in vitro models. This potential limitation does not allow us to rule out that the −463 G→A SNP is in linkage disequilibrium with other SNPs in the MPO gene, and that those SNPs could be affecting the transcriptional or translational status, or the enzyme activity itself, instead of the herein investigated SNP.

Himmelfarb et al [15] recently showed that the formation of MPO-catalyzed 3-chlorotyrosine is increased also in dialysis patients and related to oxidative stress. Although several putative markers of oxidative stress have been used previously, there is no established golden standard. In this study, we used pentosidine as an indirect marker of oxidative protein damage, since pentosidine can be generated as a result of increased MPO activity through the increased production of aldehydes [6]. Recent studies suggest that AGEs might form in the inflamed foci under experimental conditions, and that the generation of Nε-(carboxymethyl)lysine is driven by the myeloperoxidase pathway using α-amino-acids as substrate [16]. Indeed, Miyata et al [17] observed that carboxymethyllysine (CML) and pentosidine production is accelerated under oxidative stress in ESRD patients. The same group proposed that AGEs could be considered markers of oxidative stress damage to proteins [18]. Our results, showing an association between the genetic variation related to lower activity of MPO and lower plasma levels of pentosidine, demonstrate a positive in vivo pathophysiologic relationship between MPO activity and the biological effects of the oxidative stress (namely AGE formation), previously shown only in experimental studies [6]. The findings of higher prevalence of *C. pneumoniae* seropositivity in our study indicate links between chronic infection, MPO activity, and CVD in ESRD patients. Further investigation of these associations is warranted.

It is noteworthy that no relationship was found between inflammation markers and the genotypes for MPO in the present study. This might reflect the fact that inflammatory markers are quite variable and influenced mostly by nongenetic factors, such as age, residual renal function, gender, comorbidities, and intermittent clinical events, including infections.

The chief finding of the present study is the association between the MPO genetic variation and the presence of CVD based on medical history in incident predialysis patients, confirming results previously described by Nikpoor et al [9] in a group of non-uremic patients with coronary artery disease. In line with these findings, a study by Kutter et al [19] demonstrated that patients with inherited MPO deficiency presented a protection against CVD. It should be emphasized that despite the relatively low number of patients included in our study, the predictive value of the MPO genetic variation evaluated appeared to be quite strong, holding its significance even after adjustment for several comorbidities.

In summary, we found that the presence of the A allele (−463G→A SNP) is associated with lower prevalence of CVD in predialysis ESRD patients. Furthermore, the prevalence of *C. pneumoniae* seropositivity was lower in the GA and AA groups, and plasma pentosidine concentration was lower in the carriers of the A allele, which may be a consequence of a reduced production of free radicals resulting from lower MPO activity in this group of patients. Our data provide epidemiologic support for the hypothesis that increased oxidative stress related to MPO activity is associated with atherosclerotic CVD in ESRD patients.

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