A New Promoter Polymorphism in the Gene of Lipopolysaccharide Receptor CD14 Is Associated With Expired Myocardial Infarction in Patients With Low Atherosclerotic Risk Profile

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Abstract—Recent findings suggest that inflammation plays a role in atherosclerosis and its acute complications. Cellular response in infections with Gram-negative bacteria is mediated by bacterial lipopolysaccharide (LPS), which activates monocytes to expression of cytokines, growth factors, and procoagulatory factors via LPS receptor CD14. Endothelial cells and smooth muscle cells are stimulated by a complex of LPS and soluble CD14. In this study, LPS receptor CD14 was analyzed to find genetic variants and check them for an association with coronary artery disease or myocardial infarction (MI). When screening the CD14 gene by single-strand conformation polymorphism analysis, a promoter polymorphism was detected and confirmed as a T-to-C exchange at position 2159. We determined the genotypes of 2228 men who had undergone coronary angiography for diagnostic purposes. Within the total study group there was no significant association of either genotype with MI or coronary artery disease. However, in a subgroup with low coronary risk (normotensive nonsmokers), a relative risk for MI in probands homozygous for the T allele could be evaluated (OR, 1.6; 95% CI, 1.0 to 2.4; \( p < 0.05 \)). The association was even stronger in low-risk patients older than 62 years (OR, 3.8; 95% CI, 1.6 to 9.0; \( p < 0.01 \)). In conclusion, we describe a new CD14 promoter polymorphism that is associated with MI, especially in older patients with a low atherosclerotic risk profile. (Arterioscler Thromb Vasc Biol. 1999;19:932-938.)

Key Words: CD14 ■ genetics ■ coronary disease ■ myocardial infarction ■ risk factors

CD14 is a leucine-rich 55-kDa glycoprotein that is expressed in considerable amounts by mature monocytes, macrophages, and activated neutrophil granulocytes. In these cells CD14 is known as a surface marker, being glycosylphosphatidylinositol anchored in the cell membrane (mCD14). In addition, soluble CD14 (sCD14) can be found in serum where 2 major isoforms coexist, differing in molecular weight. CD14 serves as receptor for bacterial lipopolysaccharide (LPS, endotoxin) and mediates cell activation by LPS. The receptor–ligand interaction depends on a serum protein, LPS-binding protein, which complexes LPS and facilitates binding to mCD14 or sCD14. Endothelial cells and smooth muscle cells are activated via sCD14, lacking their own membranous protein and CD14 mRNA. In addition, they are activated indirectly by cytokines from LPS-stimulated monocytes.

Endotoxin-activated monocytes produce proinflammatory cytokines such as tumor necrosis factor-α, interleukin-1 and interleukin-6, and growth factors. In stimulated endothelial cells, expression of endothelial leukocyte adhesion molecule-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 is induced, accompanying cell adhesion to endothelium. LPS increases the levels of LDL and VLDL and supports the oxidation of LDL; HDL levels are decreased. It stimulates smooth muscle cell proliferation and migration by release of platelet-derived growth factor by monocytes. Moreover, LPS promotes procoagulant activity by induction of tissue factor expression in monocytes and endothelial cells. Cell activation, leukocyte adhesion to endothelium and extravasure, smooth muscle cell proliferation and migration, and altered lipoprotein metabolism and coagulability are events with a marked impact in the development of atherosclerosis (for review, see Liao). Assuming that LPS plays a role in atherogenesis, its dependence on CD14 as a receptor molecule led us to investigate CD14.

We wondered whether interindividual variations in susceptibility to LPS stimulation because of genetic polymorphisms of CD14 could contribute to a disposition to coronary artery disease.
disease (CAD) or myocardial infarction (MI). This is of interest, because in some patients there seems to be a genetic disposition in the absence of classic atherosclerotic risk factors.

Methods

Study Population

Study patients were recruited from the Department of Cardiology at the University of Giessen and from the Kerckhoff-Klinik (clinic for heart diseases) at Bad Nauheim. Blood samples and data were collected from 2228 male patients who underwent heart catheterization because of suspected CAD or for preparation of valvular replacement. Mean age of the patients was 62 years. The patients gave their informed consent to participate in this study.

Individual histories were taken concerning diabetes mellitus, arterial hypertension, chest pain, and smoking habits. Whether MI had occurred in the patient’s former history was assessed according to criteria of the World Health Organization. Body mass index (BMI) was calculated as weight in kilograms by height in meters squared. Levels of fibrinogen, cholesterol, apoA1, apoB, and apoE, Lp(a), triglycerides, and C-reactive protein were determined with standard laboratory procedures (after overnight fast).

Coronary angiography was performed according to the Judkins method. Relevant CAD was defined as ≥50% coronary artery stenosis and qualified as 1-, 2-, or 3-vessel disease. In addition, the assessment of CAD was based on the Gensini score as described elsewhere.17 Ventriculography gave information about the ejection fraction and any impairment of left ventricular function.

Within the study group, significant CAD (stenosis, ≥50%) could be excluded in 501 patients (mean age, 58 years); 423 patients had no or mild coronary artery sclerosis, defined by a Gensini score <10; and 1175 patients had never had MI (mean age, 61 years). These probands served as control groups for statistical analyses.

Statistical Analysis

Statistics were calculated by using the SPSS software for Windows 95 (Version 7). The deviation of continuous variables from normal distribution was analyzed by the Kolmogorov–Smirnov goodness-of-fit test. Their relation to the CD14 genotype was checked by Kruskal–Wallis 1-way ANOVA. Binary variables were checked by loglinear analysis of contingency tables. Probability values (significance, 0.05) and 95% CI values were calculated by Pearson’s χ² test and multiple regression or logistic regression with regard to the above-mentioned risk factors.

Molecular Analysis

Genomic DNA was isolated from EDTA-anticoagulated whole blood as described elsewhere.19 Primers (of 18- to 22-bp length) and PCR conditions were designed by using OLIGO primer analysis software (see Table 1). PCR was run in a 20-µL volume in 96-well microtiter plates, using the Biometra UNO-Thermoblock. Samples contained 100 ng of genomic DNA, 10 pmol of each primer, dNTPs at 250 µmol/L each, and 1 U of Taq DNA polymerase in the provided reaction buffer (Boehringer Mannheim). PCR conditions comprised 3 minutes of denaturation at 94°C followed by 30 cycles of 30 seconds at 94°C, 1 minute of annealing at 59°C to 62°C, and 30 seconds of extension at 72°C, and a final extension time of 5 minutes at 72°C. If necessary, the concentration of magnesium chloride was adjusted to 2.5 mmol/L (PCR products 10 and 12) or DMSO was added to 0.05× (PCR product 12).

Single-Strand Conformation Polymorphism (SSCP) Analysis

For screening of PCR products, PCR was performed by adding 37 000 Bq (1 µCi) of [α-32P]dCTP (Amersham) per reaction and reducing nonradioactive dCTP to 20 µmol/L. PCR products were denatured by mixing with a formamide buffer and incubating at 94°C for 1 minute. SSCP analysis was performed on 0.5× modified polyacrylamide gels (Servogel, Serva) in vertical electrophoresis in 0.6× Tris borate/EDTA buffer. Gels were run at 4°C and at room temperature, and the probes were visualized by autoradiography. For typing of 1285 individuals, nonradioactive PCR products of primer pair 2 were run in horizontal electrophoresis on 17% to 5% polyacrylamide gradient gels (49:1, acrylamide:bisacrylamide) with 5% glycercin in 0.5× Tris borate/EDTA buffer. The 17% polyacrylamide solution contained 4% saccharose. Gels were run at 25°C, and DNA bands were visualized by silver staining.

Restriction Fragment Length Polymorphism Analysis

A new forward primer for PCR product 2 (see Table 1) was designed to obtain larger restriction fragments. PCR conditions did not change.Typing of a further 943 probes was done by overnight digestion of PCR products with 2 U of restriction endonuclease HaeIII at 37°C. Digests were analyzed on 3% NuSieve agarose gels and stained with ethidium bromide.

Sequence Analysis

Distinct PCR products were extracted from 1.2% agarose gels (gel extraction kit, Qiagen) and directly sequenced by using the Thermo Sequenase cycle sequencing kit (Amersham). We performed sequencing of both strands with 5′-[γ-32P]dATP-labeled (Amersham) primers. PCR primers were radiolabeled with T4 polynucleotide kinase (USB). After 2 minutes of denaturation at 94°C, 30 cycles of 20 seconds at 94°C, 30 seconds at 60°C, and 20 seconds at 72°C were run. The sequencing reaction was analyzed on 6% polyacrylamide gels (19:1, acrylamide:bisacrylamide) with 7.5 mol/L urea in 1× Tris borate/EDTA buffer. Gels were autoradiographed.

Results

Screening for Polymorphisms in the Gene of CD14

Fourteen distinct PCR products were analyzed in a series of SSCP gels. From screening, each time, 20 samples of different blood donors, we observed band shifts in the case of primer pairs 2, 9, 13, and 14. Each time, 2 products with different migration patterns were sequenced in sense and antisense directions. In 3 cases, base exchanges could be discerned (PCR products 2, 13, and 14). Two distinct samples of PCR product 9 did not differ in their sequence. Only a deviation of both from the published genomic sequence20 was discovered, which was already described by Simmons et al. Instead of an A, there is a G at position 661 of cDNA, which leads to a change of the predicted amino acid from tyrosine to cysteine (codon 168 of mature protein; TAC to TGC). The polymorphic pattern in SSCP analysis of PCR product 9
probably was caused by an unspecific additional product of the PCR reaction.

PCR product 2 comprises a T-to-C exchange at position 2159, ie, a promoter polymorphism (Figure 1). Genotyping of this polymorphic site in 2228 study patients was done by nonradioactive SSCP analysis (Figure 2A) and by restriction fragment length polymorphism analysis (Figure 2B); 100 probes were genotyped with both techniques, which gave discordant results in 3 cases. In the SSCP gels no further band shift was detected, indicating that a third allele is not likely to exist.

PCR product 13 bears a G-to-C exchange (1202 of cDNA), which changes codon 348 from CTG to CTC. This does not change the predicted amino acid leucine. The T-to-G polymorphic site in PCR product 14 has position 1497 in genomic DNA, which is 2 bp adjacent to the 3' end of cDNA. Polymorphisms of PCR products 13 and 14 were not investigated further.

Distribution of the T/C Polymorphism of CD14 Promoter

Genotype distribution in the study group of 2228 male probands (mean age, 62 years) was 631 CC (28%), 1104 CT (50%), and 493 TT (22%). Within the study group, we compared subgroups with varying extents of CAD, defined as no, or 1-, 2-, or 3-vessel disease. We also checked the subgroup with no or mild CAD (Gensini score, <10) against that with severe CAD (Gensini score, >90). Finally, groups with or without MI in their history were compared. In the above-mentioned subgroups of the study population, the distribution of genotypes did not differ significantly. Allele distributions and their 95% CIs are depicted in Table 2. We also checked the genotypes in subgroups with or without impairment of left ventricular function, which did not differ significantly. The distribution of established risk factors [age, BMI, apoA1, apoB, apoE, total cholesterol, Lp(a), triglycer-
CD14 Promoter Polymorphism and Risk of CAD or MI

ORs were calculated for probands carrying the T allele as well as for T-homozygous ones (considering a dominant or recessive effect of the T allele). Again regarding the total study group, there was no significant relative risk for either genotype. Calculations referred to severeness of CAD (defined as multivessel disease or by Gensini score) and MI. The C allele in no case showed a tendency of a positive association with CAD or MI. In further calculations, subgroups were formed as low-risk groups [excluding probands with hypertension and/or smoking, and/or with continuous variables such as BMI, fibrinogen, Lp(a), cholesterol, triglycerides, apoB, and apoA1 beyond their average values in the total study group]. In all cases, no relation of the CD14 promoter genotype to CAD or severeness of CAD could be discerned.

For MI, however, there exists a relative risk of 1.4 (95% CI, 1.0 to 2.0) in T-homozygous normotensive subjects, by applying the $\chi^2$ test ($P<0.05$). Multiple regression analysis did not produce a significant value ($P=0.11$, $n=841$). Subjects without hypertension and also not smoking have a relative risk of MI of 1.6 (95% CI, 1.0 to 2.4; $P<0.05$ in multiple logistic regression; $n=173$, 109 without and 64 with infarction). After subdividing into groups of different ages, it is noteworthy that only in normotensive patients older than 62 years there was a relative risk of MI when they were T homozygous (OR=1.7; 95% CI, 1.0 to 2.9; $P<0.05$ by Pearson’s $\chi^2$ test, $P=0.053$ by multiple logistic regression; $n=379$, 180 without and 199 with infarction). In the small group of patients older than 62 years and without hypertension and not smoking ($n=76$, 46 without and 30 with infarction), the OR for MI and T homozygosity was 3.8 (95% CI, 1.6 to 9.0; $P<0.01$ by multiple logistic regression). Relative risks that were statistically significant are depicted in Table 3. Checking high-risk groups, we did not come to any significant ORs for an association with either genotype.

**Discussion**

We describe a new diallelic promoter polymorphism of the CD14 gene (T$\rightarrow$159C) with an almost equal distribution of both alleles in the white population, with regard to only men with a mean age of 62 years. In the total study group, there is no significant correlation of either genotype with CAD or former MI. But by arranging low-risk groups, the T allele can be detected as an independent risk factor for MI.

A significant correlation of T homozygosity with MI can be found in the low-risk group of normotensive patients without the smoking habit. Within the whole study group of 2228 probands, only 173 patients fulfill that criterion. Divid-

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**Figure 1.** Sequence analysis of 2 distinct homozygous PCR products of primer pairs 2. PCR primer 2 reverse was radiolabeled and used as a sequencing primer (antisense sequence). The arrows indicate an A (in panel A) to G (in panel B) exchange that corresponds to a T-to-C exchange in the strand sequence at position $-159$ of the CD14 promoter (strand sequence not shown).

**Figure 2.** A, SSCP analysis of PCR product 2, which was performed in 1285 patients for genotyping of the CD14 promoter polymorphism. Typing results of 18 distinct probands are depicted. The arrow points correspond to the location of the $-159$C allele on the upper band and the location of the $-159$T allele on the lower band. Both bands are found in heterozygous subjects. PCR products were visualized by silver staining. B, Restriction fragment length polymorphism analysis with restriction endonuclease HaeIII (recognition site, 5’-GGCC-3’), as it was performed in 943 probands for genotyping of polymorphic PCR product 2. With the $-159T$ polymorphic base, the recognition sequence is modified to 5’-GGTC-3’, which is not cut by HaeIII. Lanes 1, 3, and 5 show undigested PCR products of 3 distinct probands, ie, A, B, and C (295 bp). Proband A is homozygous for $-159C$ (lane 2, totally cut, 155 and 140 bp), proband B is heterozygous (lane 4), and proband C is homozygous for $-159T$ (lane 6, uncut, 295 bp). MW, as molecular weight standard served a Sau3A digest of the plasmid pBSIII (base pairs).
ing these into categories of different ages, the association of MI and T homozygosity becomes stronger in the older patients of >62 years. As these subgroups are rather small and because they are arranged arbitrarily, the observed effect can be the result of mere coincidence. The association of the T genotype of the CD14 promoter with MI could be observed in any low-risk group as a tendency, but only significant data were presented.

The T allele may also work as a protective factor, because it occurs more frequently in older patients who have survived MI. But being a protective factor, its association with MI would be expected to be stronger in a high-risk group rather

<table>
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<tr>
<th>TABLE 2. Distribution of CD14 Promoter Genotypes and Allele Frequencies in the Total Study Group of 2228 Male Patients Having Been Subjected to Coronary Angiography and in Subgroups With Different Clinical Conditions</th>
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Odds ratios (ORs) and 2-tailed P values were calculated by Pearson’s χ² test and by multiple logistic regression with adjustment for established cardiovascular risk factors. NS indicates not significant.
than in a low-risk group. What we initially had in mind, however, was to check whether that genetic variant of the CD14 gene represents a genetic risk factor, i.e., contributes to the so-called familial risk in patients lacking a classic risk profile. Assuming that case, an association with MI in a large population should be stronger in low-risk patients who in addition are relatively young. The population that underlies our epidemiological study might omit that interesting collective of patients although it is rather extensive. In addition, it is noteworthy that this is a retrospective study design with regard to MI. A prospective study, with a case group of young low-risk patients going through acute MI, is in progress.

We also cannot exclude uncontrolled factors to be associated with the CD14 genotype. In particular, the control groups of probands without CAD and MI include many patients with valvular disease, which might falsify our results. On the other hand, one can conclude from the study design that all control patients are angiographically classified, which guarantees the most reliable results concerning CAD.

Familial or genetic risk may be comprised by a single factor responsible for, for example, a phenotypic metabolic disorder such as hyperhomocysteinemia or familial hypercholesterolemia. On the other hand, genetic risk might be composed of several genetic markers that, isolated, have a functional impact that is scarcely measurable. Many polymorphisms already have been found with an isolated, only weak or controversial influence on coronary syndromes.\textsuperscript{22–27} There might be an additive effect of gene polymorphisms that concerns molecules involved in adhesive, coagulatory, and proliferative processes, or in vasoconstriction, and that is of a complexity beyond our knowledge until now.

Authors of several studies have suggested a role of chronic inflammation in genesis and progression of atherosclerosis. Acute-phase proteins like C-reactive protein and fibrinogen are markers of inflammation in genesis and progression of atherosclerosis.\textsuperscript{31,32} As this is an epidemiological study, we cannot provide evidence of whether the MI promoter polymorphism effects an altered expression of CD14 and susceptibility to LPS stimulation. According to investigations on the CD14 promoter by Zhang et al.,\textsuperscript{21} the polymorphic base at \( -159 \) lies 49 bp adjacent to an experimentally detected binding site for transcription factor Sp1 at \(-110\) and 1 bp adjacent to a putative Ap2 site at \(-158\). Sp1 was found to be critical for the expression of CD14, whereas purified Ap2 did not interact with the CD14 promoter. Nuclear extracts of Mono Mac 6 cells interacted with 4 sites of the CD14 promoter, the nearest beginning at position \(-154\). These experiments made use of a clone exhibiting a T at position \(-159\) of the CD14 gene. One can speculate that with a C adjacent to the Ap2 consensus sequence, nuclear extracts would have interacted. In constructs of luciferase gene with truncated CD14 promoter, a construct up to position \(-227\) had the highest promoter activity (including the polymorphic site), and a construct up to \(-128\) had less activity (including only the Sp1 site). These findings suggest that the newly detected polymorphic site lies within or near a functional region of the CD14 promoter. Ongoing studies will show whether the T\(-159\)C polymorphism affects the expression of LPS receptor.

In conclusion, we found a new T/C polymorphism of the CD14 promoter at position \(-159\). Homozygosity of the T allele is associated with a history of MI in the subgroup of older low-risk patients. Whether homozygosity of the T allele acts as a risk factor for MI or as a protective factor to survive MI must be clarified in further studies.

References


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