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CD14 and *TLR4* Gene Polymorphisms in Adult Periodontitis

ABSTRACT

Bacterial deposits, smoking, and host genetic factors play a major role in an individual's predisposition to periodontitis. Bacterial components are recognized by CD14 and toll-like receptor 4 (TLR4), resulting in a NF-κB-based inflammatory response. We hypothesized that functional *CD14* and *TLR4* polymorphisms contribute to periodontitis susceptibility. We aimed to investigate the occurrence of *CD14*-260C>T, *TLR4* 299Asp>Gly, and 399Thr>Ile gene polymorphisms in adult periodontititis. DNA was collected from 100 patients with severe periodontitis and from 99 periodontally healthy controls. The gene polymorphisms were determined by the PCR technique. The presence of the periodontal pathogens *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans,* and whether the subjects smoked, was included in the analyses. The *CD14*-260T/T genotype was found in 34.0% of periodontitis patients and in 20.2% of controls. Logistic regression analysis adjusted for gender, age, smoking, and prevalence of *P. gingivalis* and *A. actinomycetemcomitans* showed an association between the *CD14*-260T/T genotype and periodontitis (P = 0.004, OR 3.0, 95% CI 1.4-6.9). We conclude that the *CD14*-260T/T genotype contributes to the susceptibility to severe periodontitis in Dutch Caucasians.

KEY WORDS: *CD14, TLR4,* gene polymorphisms, periodontitis, innate immunity.

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INTRODUCTION

Adult periodontitis is a chronic inflammatory disease of the tooth-supporting tissues and the alveolar bone. Specific micro-organisms of the subgingival plaque, including *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*, and many other mainly Gram-negative strict anaerobic rods initiate the disease (Haffajee and Socransky, 1994). In addition to the microbial component, behavioral factors such as smoking and stress are risk factors for periodontitis (Bergström, 1989; Genco *et al.*, 1999). Immune responses against microbiological agents are partly genetically determined (Girardin *et al.*, 2003) and appear to be important in an individual's susceptibility to periodontitis (Michalowicz, 1994; Laine *et al.*, 2001).

CD14 is a co-receptor involved in the recognition of bacterial lipopolysaccharide (LPS). CD14 forms a complex with LPS and the LPS-binding protein. A membrane-anchored CD14 can be cleaved to a soluble serum protein (sCD14). CD14 is involved in the phagocytosis of bacteria (Grunwald *et al.*, 1996) and LPS-mediated bone resorption (Amano *et al.*, 1997). Increased serum levels of sCD14 have been associated with periodontitis (Hayashi *et al.*, 1999).

Toll-like receptors (TLRs) mediate intracellular signaling and antimicrobial responses upon recognition of pathogen-associated molecular patterns (PAMPs) of micro-organisms, and play a central role in innate immunity (Medzhitov *et al.*, 1997).

Candidate gene approaches investigating the effects of functional polymorphisms in genes involved in an immune response against microbiological agents have shown the importance of the *IL-1* gene cluster in periodontitis (Kornman *et al.*, 1997; Laine *et al.*, 2001). TLR2 and TLR4, both with CD14 as a co-receptor, are involved in Gram-positive and Gram-negative PAMP recognition, making them interesting targets for the candidate gene approach. Activation of these receptors results in the activation of NF-κB, followed by the transcription of various pro-inflammatory cytokine genes, such as *TNF-A, IL-1A,* and *IL-1B,* which code for proteins that have been associated with periodontitis (Wang *et al.*, 2003). Expression of CD14, TLR4, and TLR2 in periodontal tissues supports the importance of these receptors in periodontitis (Wang *et al.*, 2003).

Although TLR2 has been reported to recognize LPS of *P. gingivalis* (Werts *et al.*, 2001), and polymorphisms in the TLR2 gene have been described (Lorenz *et al.*, 2000; Kang and Chae, 2001), they either do not exist in the Caucasian population or occur at very low frequencies. However, both *CD14* and *TLR4* functional gene polymorphisms have frequently been described in Caucasian populations (Agnese *et al.*, 2002; Holla *et al.*, 2002).

The *CD14*-260C>T promoter polymorphism enhances the transcriptional activity of the *CD14* gene (Hubacek *et al.*, 1999). Individuals carrying the T/T genotype have significantly higher serum levels of sCD14

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and increased density of CD14 in monocytes than those carrying the C/C or C/T genotype (Hubacek *et al.*, 1999).

The *TLR4* 299Asp>Gly gene polymorphism (+896A>G) has been correlated with hyporesponsiveness to inhaled lipopolysaccharide (Arbour *et al.*, 2000), sepsis, and infections caused by Gram-negative bacteria (Agnese *et al.*, 2002). The *TLR4* 299Asp>Gly polymorphism is in close linkage with the *TLR4* 399Thr>Ile gene polymorphism (+1196C>T), a point mutation that results in threonine-to-isoleucine substitution.

We have recently shown that simultaneous carriage of the rare alleles of the *IL-1* cluster genes is associated with periodontitis (Laine *et al.*, 2001) in non-smoking patients without detectable *P. gingivalis* and *A. actinomycetemcomitans*. This makes the present study of the PAMP-sensing system, by analyses of the functional *CD14* and *TLR4* gene polymorphisms, relevant in relation to periodontitis susceptibility. We hypothesized that patients with *CD14*-260T, *TLR4* 299Gly, and 399Ile polymorphisms have an enhanced inflammatory response to LPS or other components of Gram-negative bacteria, contributing to the development of periodontitis. The obtained genetic information may give more insight into the pathogenesis of adult periodontitis and may identify genetic markers for susceptibility and progression of destructive periodontal disease.

MATERIALS & METHODS

Subjects

The study was performed in unrelated Dutch Caucasian adult patients with severe periodontitis (n = 100) and in periodontally healthy controls (n = 99) (Table 1). Patients who were current smokers, or had stopped smoking ≤ 1 yr previously, were defined as 'smokers', and patients who had never smoked, or had stopped smoking > 1 yr previously, were defined as 'non-smokers'. The study was approved by the Medical Ethical Committee of the VUMC, Amsterdam, and all participants gave informed consent to participate in the study.

Clinical and Microbiological Measurements

The periodontal status was assessed on the basis of radiographic and clinical examination, as described elsewhere (Laine *et al.*, 2001). Briefly, patients with severe periodontitis were required to have ≥ 7 interproximal sites with ≥ 50% bone loss. None of the controls had pocket-probing dept > 4 mm, and there were no sites with radiographic evidence of alveolar bone loss.

For the microbiological examination, subgingival paper-point samples were taken from the deepest subgingival site in each quadrant of the dentition, and from the mesio-buccal sulcus of each first molar of the controls. The samples were analyzed for the presence of *A. actinomycetemcomitans*, with the use of trypticase soy-serum-bacitracin-vancomycin agar plates (Slots, 1982), and for *P. gingivalis* with the use of non-selective blood agar plates (van Winkelhoff *et al.*, 1986).

DNA Isolation

Mouthwash samples were obtained, and DNA was isolated by the mouthwash method, as described previously (Laine *et al.*, 2000).

Analysis of Polymorphisms in Genes

PCR of 35 cycles was performed on a thermal cycler GeneAmp9700 (Perkin-Elmer Cetus, Norwalk, CT, USA). Primers were synthesized (Invitrogen Life Technologies, Breda, The Netherlands), and enzymes were obtained (New England Biolabs, Herts, UK). Overnight-restricted fragments were analyzed on a 4% agarose gel (2% Invitrogen + 2% Nusieve low melting agarose, Tebu-bio, Heerhoevaar, The Netherlands).

CD14-260C>T Gene Polymorphism

Genotyping (NCBI SNP CLUSTER ID: rs2569190) was performed with forward primer 5′-TCACCTCCCCACCTCTCTT-3′ and reverse primer 5′-CCTGCAGAATCCTTCCTGTT-3′. The cycle program consisted of 30 sec at 95°C, 30 sec at 59°C, and 1 min at 72°C. *Hae*III digestion resulted in two fragments of 83 bp and 24 bp (C allele) or 107 bp (T allele).

TLR4 896A>G (299Asp>Gly) Gene Polymorphism

Genotyping (NCBI SNP CLUSTER ID: rs4986790) was performed with forward primer 5′-TTTACCCTTTCAATAGT CACACTCA-3′ and reverse primer 5′-AGCATACTTAGACTAC TACCTCCATG-3′. The cycle program consisted of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. *Nco*I digestion resulted in two fragments of 80 bp and 22 bp (G allele) or 102 bp (A allele).

TLR4 1196C>T (399The>Ile) Gene Polymorphism

Genotyping (NCBI SNP CLUSTER ID: rs4986791) was performed with forward primer 5′- GCTGTTTTCAAAGTGA TTTTGGGAGAA-3′ and reverse primer 5′-CACTCATTTGTTT CAAATTGGAATG-3′. The cycle program consisted of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec. *Hinf*I digestion resulted in two fragments of 121 bp and 25 bp (T allele) or 146 bp (C allele).

Statistical Methods

The *CD14*-260C>T, *TLR4* 299Asp>Gly, and 399The>Ile allele, genotype, and carrier frequencies were compared between the control and periodontitis groups by Fisher's two-tailed exact test. Subsequently, we performed logistic regression analysis to assess the association of different genotypes with periodontitis, adjusting for gender, age, smoking, and prevalence of *P. gingivalis* and *A. actinomycetemcomitans*. Since prevalence of *P. gingivalis* and *A. actinomycetemcomitans* and smoking have been strongly associated with periodontitis, we investigated whether there was an interaction between these variables and different genotypes. The significant determinants were determined, and adjusted OR and CI were calculated. Differences in genotype frequencies between patient and control subgroups were explored. A p-value < 0.05 was considered statistically significant. Statistical analysis was

Table 1. Demographic and Microbial Characteristics of Adult Patients with Severe Periodontitis and Periodontally Healthy Controls

	Patients (N = 100)	Controls (N = 99)	P value	OR (95%CI)
Mean age (yrs)	46.4	40.5	< 0.0001	
Range	28-66	25-75		
Sex (F/M)	51/49	57/42	NS	
Smokers/Non-smokers	46/54	25/74	0.003	2.5 (1.3- 4.6)
Pg+[a]	41	6	< 0.0001	10.8 (4.3-26.9)
Aa+[b]	31	6	< 0.0001	7.0 (2.7-17.6)
Pg+ and/or Aa+	60	11	< 0.0001	12.0 (5.7-25.2)

[a] Pg+ = *P. gingivalis* detectable.
[b] Aa+ = *A. actinomycetemcomitans* detectable.

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Table 2. Genotype and Allele Frequencies of the *CD14*-260C>T and *TLR4* 299Asp>Gly and 399Thr>Ile Polymorphisms in Adult Patients with Severe Periodontitis and Periodontally Healthy Controls

Genotype	Patients (%), N = 100	Controls (%), N = 99
CD14-260		
C/C	26	28
C/T	40	51
T/T	34	20
Allele T frequency	54.0%	46.0%
TLR4 299Asp>Gly (399Thr>Ile)		
A/A (C/C)	90	90
A/G (C/T)	10	8
G/G (T/T)	0	1
Allele G (T) frequency	5.0%	5.0%

Table 3. Prevalence of the *CD14*-260 T/T genotype *vs.* C/C and C/T Genotypes in Different Subgroups of Patients with Severe Periodontitis and Controls

	CD14-260 T/T Genotype	
Variable	Patients (%)	Controls (%)
Total	34/100 (34.0)	20/99 (20.2)
Non-smokers	21/ 54 (38.9)	16/74 (21.6)
Smokers	13/ 46 (28.3)	4/25 (16.0)
Pg-[a]	25/ 59 (42.4)	19/93 (20.4)
Aa-[b]	23/ 69 (33.3)	19/93 (20.4)
Pg-[a] & Aa-[b]	18/ 40 (45.0)	19/88 (21.6)
Non-smokers and Pg-[a] & Aa-[b]	11/ 22 (50.0)	16/70 (22.9)

a Pg- = *P. gingivalis* not detectable.
b Aa- = *A. actinomycetemcomitans* not detectable.

performed with the use of SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Study Population Characteristics

The mean age of the patients was 46.4 yrs (n = 100; range, 28-66 yrs), and that of the control population was 40.5 yrs (n = 99; range, 25-75 yrs) (Table 1).

One hundred patients and 99 periodontally healthy individuals were typed for the *CD14*-260C>T, the *TLR4* 299Asp>Gly, and 399The>Ile gene polymorphisms (Table 2). Each individual allele for each locus of *CD14*-260C>T, *TLR4* 299Asp>Gly, and 399The>Ile gene polymorphisms was in Hardy-Weinberg equilibrium in the control group.

CD14-260C>T Gene Polymorphism

The *CD14*-260T/T genotype was found significantly more often than the C/T and C/C genotypes in the total group of patients as compared with controls (34.0% *vs.* 20.2%, respectively; P = 0.038, OR 2.0, 95% CI 1.1-3.9) (Table 3). Allele frequencies for the *CD14*-260 polymorphism did not differ between the patients and controls (Table 2).

Logistic regression analysis adjusted for gender, age, smoking, and prevalence of *P. gingivalis* and *A. actinomycetemcomitans* showed an association between the *CD14*-260T/T genotype and periodontitis (P = 0.004, OR 3.0, 95% CI 1.4-6.9) (Table 4).

TLR4 299Asp>Gly and 399The>Ile Gene Polymorphisms

TLR4 299Asp>Gly and 399The>Ile genotyping results were in 100% concordance. The overall genotype, allele, and carriage frequencies for the *TLR4* 299Asp>Gly and 399The>Ile gene polymorphisms did not differ between the control population and patients (Table 2).

No significant differences were found in the genotype and allele frequencies for the *TLR4* 299Asp>Gly and 399The>Ile gene polymorphisms in any of the subgroups. Furthermore, logistic regression analysis showed no association between the *TLR4* genotypes and periodontitis.

DISCUSSION

The major finding of the present study is the association between the *CD14*-260T/T genotype and severe periodontitis. The association was found without any corrections but was the strongest when adjusted for age, gender, smoking, and presence of *P. gingivalis* and *A. actinomycetemcomitans*. We found the *CD14*-260T/T genotype to be a risk factor for periodontitis, with the odds ratio 3.0, which was comparable with that of smoking (OR 2.1). The observed *CD14*-260T/T genotype odds ratio is high in comparison with other published genetic risk factors in multifactorial diseases (OR 1.1-1.5) (Ioannidis *et al.*, 2003). No associations between the *TLR4* polymorphisms analyzed and periodontitis were found.

We compared our findings with those from another investigation on the prevalence of the *CD14*-260T/T genotypes in a Dutch Caucasian population (Murillo, 2003). In this control population (N = 169), with unknown periodontal status, 23.7% were homozygous for the *CD14*-260T allele, which is similar to findings in our periodontally healthy group (20.2%), and further validates the reported association with periodontitis.

The frequency of the *CD14*-260T/T genotype in our periodontitis population is comparable with that in Japanese patients (25.0%) (Yamazaki *et al.*, 2003) but higher than in Czech patients (19.2%) (Holla *et al.*, 2002), in whom no association was found between this polymorphism and periodontitis. The frequency of the T/T genotype in our periodontally healthy group (20.2%) was comparable with that found in other control populations (15.6%-34.6%) (Hubacek *et al.*, 1999; Holla *et al.*, 2002; Yamazaki *et al.*, 2003).

The two previous studies did not find any association between the *CD14*-260T/T genotype and periodontitis (Holla *et al.*, 2002; Yamazaki *et al.*, 2003). The different clinical selection criteria, the ethnic background of the study populations, and the age range of subjects may explain the discrepancies. For example, the age limit of the present study was 25 yrs, whereas the previous studies had a limit of 35 yrs for periodontitis. Moreover, the previous studies did not take into account the presence of the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans*. Finally, since a

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specific SNP frequency, as for *CD14*-260, might also be different in various ethnic populations, differences in linkage disequilibrium with other genetic markers may explain the contradictory results between reported studies.

Variation in the *CD14* gene at the interface between the environment and host may skew the immune response by modulating the impact of pathogen exposure. This impact, however, might be modulated by both the time of first exposure and the bacterial load associated with the exposure, variables which might differ greatly in different ethnic, geographic, and urban *vs*. rural populations (Holla *et al.*, 2002; Yamazaki *et al.*, 2003).

The association found for the *CD14*-260T/T genotype and periodontitis emphasizes the importance of the choice of functional gene polymorphisms. However, the possible causality of the reported association needs to be established in further studies. We hypothesized that, in periodontitis, the CD14-mediated signaling is involved in a pro-inflammatory pathway through the release of cytokines.

A role for TLR4 signaling has been suggested in periodontal disease (Wang *et al.*, 2003). However, our study demonstrated that the *TLR4* polymorphisms studied are not associated with periodontitis. The prevalence of the 299Gly and the 399Ile polymorphisms was 5.0% among Dutch periodontitis patients and controls. In a recent study on these polymorphisms in a Caucasian population, a comparable prevalence for these polymorphisms was reported for periodontitis patients (4.1 to 4.5%) and periodontally healthy controls (3.3% to 3.7%) (Folwaczny *et al.*, 2004). The lack of association could be due to the fact that the heterozygous genotype presents no deficit in the recognition of LPS (Erridge *et al.*, 2003), although the homozygous genotype is functional (Arbour *et al.*, 2000). In our study, we found no patients homozygous for the *TLR4* 299/399 mutant allele.

We have previously genetically identified a patient group with increased prevalence of certain *IL-1* cluster and *CARD15* genotypes (Laine *et al.*, 2001, 2004), characterized by the absence of *P. gingivalis* and *A. actinomycetemcomitans* and by non-smoking habits. The present study provides a new genetic risk marker for severe periodontitis in adults. In agreement with results from our previous studies, the highest prevalence of the *CD14*-260T/T genotype was found in a group of patients without two major risk factors for adult periodontitis, *i.e.*, smoking and the presence of *P. gingivalis* and *A. actinomycetemcomitans* (50.0% *vs*. 22.9% in controls). We speculate that the patients with the *CD14*-260T/T genotype are more susceptible to developing periodontal infections caused by opportunistic pathogens, which otherwise display little pathogenicity.

We hypothesize that periodontitis susceptibility is partly given by the *CD14*-260T/T genotype. In polygenic diseases such as periodontitis, one genetic variation may be insufficient to cause disease. However, a combination of certain environmental factors and gene polymorphisms may determine the susceptibility and resistance to, and the severity of, an inflammatory process. The additive effect of different gene polymorphisms and environmental factors has previously been reported for severe periodontitis (Laine *et al.*, 2001, 2004).

In conclusion, this is the first report to show that a gene polymorphism of the CD14 receptor, which is involved in LPS recognition, is associated with severe periodontitis in adults. *CD14*-260 gene polymorphism and our previously identified genetic markers in the *IL-1* gene cluster (Laine *et al.*, 2001) may be useful to identify a group of at-risk periodontitis patients.

Table 4. Logistic Regression Analysis of *CD14*-260 Genotype in Patients with Severe Periodontitis and Controls

	P value	OR (95%CI)
CD14 genotype[a]	0.004	3.0 (1.4- 6.9)
Age	0.065	1.0 (1.0- 1.1)
Sex	0.61	1.2 (0.6- 2.4)
Smoking	0.038	2.1 (1.1- 4.4)
Pg+[b]	< 0.0001	9.7 (3.5-27.0)
Aa+[c]	0.001	6.0 (2.0-16.7)

[a] T/T *vs*. C/C and C/T genotype.
[b] Pg+ = *P. gingivalis* detectable.
[c] Aa+ = *A. actinomycetemcomitans* detectable.

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