Porphyromonas gingivalis HmuY-Induced Production of Interleukin-6 and IL-6 Polymorphism in Chronic Periodontitis

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Background: In chronic periodontitis (CP), the gene polymorphism of interleukin-6 (IL-6) to 174C/G has been associated with the altered production of this cytokine. The aim of this pilot study is to compare the allelic and genotypic frequencies in patients with CP with control individuals without periodontitis (NP) and to measure the production of IL-6 by whole blood cells stimulated with *Porphyromonas gingivalis* HmuY protein.

Methods: DNA was isolated from peripheral blood cells of 49 patients with CP and 60 control individuals classified as NP, and genotyping was performed by polymerase chain reaction using sequence-specific primers. Whole blood cells from 29 patients with CP and 30 control individuals were stimulated for 48 hours with HmuY, and IL-6 levels were measured using enzyme-linked immunosorbent assay.

Results: The proportion of individuals carrying the G allele at position –174 of the IL-6 gene was higher in the group with CP (85.7%) than in the normal control group (73.3%; $P < 0.03$). *P. gingivalis* HmuY-induced production of IL-6 was higher in the group with CP ($P < 0.05$).

Conclusions: Our findings suggest that *P. gingivalis* HmuY may be associated with increased IL-6 production during CP. Furthermore, patients with periodontitis and individuals with higher HmuY-induced production of IL-6 show a high frequency of the G allele at position –174. *J Periodontol 2013;84:650-655.*

KEY WORDS

Interleukin-6; periodontitis; *Porphyromonas gingivalis*.

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Periodontitis is a group of infectious diseases initiated by periodontopathogens, which elicit a pronounced host response, resulting in collagen breakdown and alveolar bone loss. *Porphyromonas gingivalis* is a black-pigmented Gram-negative anaerobic bacterium that has been implicated as a major etiologic agent in the development and progression of chronic periodontitis (CP).[1] *P. gingivalis* produces several virulence factors that lead to direct connective tissue destruction and alveolar bone loss, as well as increased host immune response. The bacterium can enter gingival epithelial cells and remain viable and capable of spreading among host cells,[2,3] thus contributing to its persistence in the oral cavity. Also, the ability of *P. gingivalis* to participate in biofilm formation on oral surfaces increases the potential for periodontal tissue destruction.[4]

During chronic periodontal infection, bacteria or their components induce monocytes/macrophages to secrete proinflammatory mediators and chemokines, which recruit neutrophils and lymphocytes to infected sites. These cells subsequently produce a number of immunoregulatory and proinflammatory agents, including interleukin-6 (IL-6).[5,6]

doi: 10.1902/jop.2012.120230

Among the various bacterial immunostimulating components are lipoproteins (LPSs). *P. gingivalis* HmuY is an outer-membrane LPS produced at higher levels when planktonic bacteria grow under low-iron/heme conditions or form a biofilm.[7] The protein is associated with the outer membrane, but it may also be released from the bacterial cell in a soluble form or in the form of outer-membrane vesicles.[8] HmuY production under biofilm conditions and its release to the surrounding environment could be of significance in periodontal pockets, where the biofilm provides persistent bacterial colonization on tooth surfaces. HmuY is also resistant to proteolysis caused by *P. gingivalis* gingipains,[8] which may result in its accumulation in the periodontal pocket. In addition, outer-membrane vesicles and free-soluble material may readily gain access into the abundant blood or lymph vascular network immediately under the epithelium, which then opens the route for the systemic spread of the protein.

The nature of the host immune response to the components of periodontopathogens may vary among individuals.[9-12] Many studies have suggested the gene polymorphism of cytokines as putative risk indicators for CP.[13] Genetic research on periodontitis has been mainly focused on gene polymorphisms that play a role in immunoregulation or metabolism, such as cytokines, chemokines, cell-surface receptors, and enzymes that are related to antigen recognition.[13] Interindividual differences in cytokine profiles appear to be due, at least in part, to allelic polymorphism within regulatory regions of a cytokine gene.[14-18]

It has been reported that in CP, the –572 (G/C) polymorphism of the IL-6 gene may be one of the protective factors associated with lower susceptibility to periodontitis.[19] In contrast, the polymorphism at –174 (G/G) of the IL-6 gene may be associated with higher susceptibility to the disease.[14,15,20] In addition, multiple logistic regression analysis revealed that the –6,106 and –174 (G/C) IL-6 gene polymorphisms were associated with detection of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*;[12] these findings confirm the hypothesis that complex interactions between the microbiota and host genome are part of the basis of susceptibility to periodontitis. There is also increasing evidence that periodontitis plays a role in systemic diseases,[21,22] and an association of IL-6 gene polymorphism with rheumatoid arthritis and bone mineral density has been demonstrated.[11]

IL-6 plays a crucial role in bacterial infections, and significant correlations between levels of IL-6 and the severity of inflammation, tissue destruction, and attachment loss have been reported in periodontitis.[23-26] Our previous data suggest that release of *P. gingivalis* components could constitute a mechanism for systemic stimulation and be of particular importance in CP as well as in systemic diseases.[27] In addition, the polymorphism at –174 (G/G) of the IL-6 gene may be associated with higher susceptibility to periodontal disease. Therefore, our hypothesis is that *P. gingivalis* HmuY may elicit IL-6 production, mainly in genetically susceptible individuals. Thus, in this pilot study we determine the ability of *P. gingivalis* HmuY protein to induce IL-6 production by whole blood cells in patients with CP and control individuals. We also investigate the association of –174 (C/G) polymorphism of the IL-6 gene with CP.

MATERIALS AND METHODS

Patients and Control Participants

To evaluate the genetic polymorphism of the IL-6 gene at position –174, 109 systemically healthy Brazilian individuals (Table 1) were recruited from the School of Dentistry, Feira de Santana State University, from 2008 to 2009. This descriptive study includes 49 patients with CP (10 males and 39 females; mean age 36.14 ± 10.33 years) and 60 control individuals without periodontitis ([NP]; 24 males and 36 females; mean age 35.7 ± 11.35 years). To measure IL-6 production by whole blood cells, 29 patients with CP and 30 individuals in the NP group were randomly selected by raffle from the entire group. The following exclusion criteria were established: 1) presence of systemic disease; 2) smoking; 3) pregnancy; 4) previous periodontal treatment 1 year before; 5) treatment with antibiotics ≤6 months; and/or 6) treatment with anti-inflammatory agents ≤2 months before entering the study. Informed written consent was obtained according to guidelines established by the Institutional Ethics Committee of Feira de Santana State University, Bahia, Brazil (007/2004).

Table 1.

Clinical Findings of Patients With CP and Control Individuals Without Periodontitis (NP)

Parameter	NP (n = 60)	CP (n = 49)	*P* Value
Male/female	24/36	10/39	0.040
Age (years)	35.7 ± 11.35	36.14 ± 10.33	0.700
Number of teeth	26.89 ± 2.11	20.32 ± 6.43	0.124
% BOP	15.32 ± 4.13	35.8 ± 5.62	0.032
% PD ≥4 mm	0.0 ± 0.0	43.2 ± 11.8	0.001
% CAL ≥3 mm	0.0 ± 0.0	68.39 ± 16.3	0.001

Values are mean ± SD.

Periodontal examination was performed by only one previously calibrated periodontist (SCT) (κ 0.832 for probing depth [PD]), and 0.824 for clinical attachment level [CAL]) using a periodontal probe[¶] and included bleeding on probing (BOP), CAL, and PD at six sites for each tooth. Individuals who had ≥4 teeth showing ≥1 site with PD ≥4 mm, with clinical attachment loss ≥3 mm and BOP at the same site, were diagnosed as having periodontitis.[28] The chronic character of the disease was based on the American Academy of Periodontology criteria.[29]

IL-6 Genetic Polymorphism

Blood was collected from each participant into a tube containing 0.5 M EDTA, and DNA was isolated using a genomic DNA mini kit.[#] Genotyping for cytokine polymorphism was performed by standard polymerase chain reaction using sequence-specific primers with a cytokine genotyping kit[**] and electrophoretic separation of DNA fragments in 1% agarose gel. Genetic analysis was performed masked to clinical diagnosis, in duplicate, and hidden duplicates were added to each plate to estimate error rates. The results were scored according to the manufacturer's protocol using the visual presence or absence of a specific DNA fragment in a gel. Allele frequencies were estimated from direct genotyping.

Determination of IL-6 Levels

Ten milliliters of blood were collected in a tube containing heparin. Whole blood cells were cultured in 24-well flat-bottom plates, with each well containing 1 mL blood and pokeweed mitogen (PWM; 2.5 μg/mL), purified HmuY protein (HmuY; 2.5 μg/mL), or no stimulus. *P. gingivalis* HmuY polypeptide part lacking the first 25 residues (NCBI accession no. CAM 31898) was expressed using pHmuY11 plasmid and *Escherichia coli* ER2566 cells[††] and purified from a soluble fraction of *E. coli* lysate as previously reported.[30] Contaminating endotoxins were removed from HmuY samples using an endotoxin removal kit.[‡‡] The cultures were grown for 48 hours at 37°C, under 5% CO_2 and humidified air. Supernatants were collected by centrifugation and used in duplicate for IL-6 determination by an enzyme-linked immunosorbent assay according to the manufacturer's instructions.[§§]

Better absorbance of the samples compared to a standard curve and values in picograms per milliliter were tested in a receiver operating curve (ROC). Considering 3,822 pg/mL as the cutoff, with sensitivity of 79.9% and specificity of 76.7%, the samples were separated into two groups: high and low production of IL-6 levels stimulated by HmuY.

Statistics

Clinical variables were described in terms of mean ± SD values. The Fisher exact test was used to compare the allelic and genotypic frequencies between groups. The genotype distributions of the investigated polymorphisms were tested according to the Hardy-Weinberg equilibrium. The phenotype cytokine production was evaluated according to Hoffman et al.,[31] and the Mann-Whitney *U* test was used to test differences between groups concerning IL-6 measurements. In addition, an ROC was used to establish a cutoff to the levels of IL-6 in the culture supernatants under HmuY stimulation. $P < 0.05$ was considered significant. A statistical software program[‖‖] was used for analysis.

RESULTS

Clinical Parameters

As shown in Table 1, the two examined groups were comparable with respect to age, but the *P* value related to sex comparison is 0.04, which is below the 0.05 cutoff selected in this study. As expected, the periodontal status showed statistically significant differences between the groups. The population studied was in Hardy-Weinberg equilibrium. No differences regarding socio-demographic, medical, or lifestyle characteristics were found between the two examined groups (data not shown).

Genotype Frequencies and Frequencies of Allele Carriage

The differences in genotypes for the polymorphism of the IL-6 gene observed between the CP and NP groups were not statistically significant ($P = 0.1.263$). In contrast, the frequency of the G allele carriage at –174 position of the IL-6 gene was higher in the CP group than in the NP group, and this difference was statistically significant ($P = 0.03$; Table 2).

Production of IL-6

In both CP and NP groups, whole blood cells cultured in the presence of HmuY produced higher levels of IL-6 than those cultured with no stimulus (data not shown). Whole blood cells of patients with CP cultured with HmuY produced higher levels of IL-6 compared with those of the control individuals without periodontitis ($P < 0.05$; Fig. 1).

Based on the ROC curve, the cutoff was 3.822 pg/mL, with sensitivity of 79.9% and specificity of 76.7%. Thus, values equal to or less than this cutoff were considered low production, whereas values >3,822 pg/mL were considered high production of IL-6.

¶ Williams periodontal probe, Hu-Friedy, Chicago, IL.
# PureLink Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA.
** Cytokine Genotyping Tray Kit, One Λ Incorporation, Canoga Park, CA.
†† New England Biolabs, Ipswich, MA.
‡‡ Detoxi-Gel Endotoxin Removing Columns, Thermo Scientific, Rockford, IL.
§§ R&D Systems, Abingdon, UK.
‖‖ SPSS v.17.0, IBM, Chicago, IL.

Table 2.

Allelic and Genotypic Frequencies of IL-6 (–174) Polymorphism in Patients With CP and Control Individuals Without Periodontitis (NP)

IL-6–174 G/C Genotypic Polymorphism (Phenotype)	NP (n = 60)	CP (n = 49)	*P* Value
G	73.3%	85.7%	0.03*
C	26.7%	14.3%	

* $P \leq 0.05$.

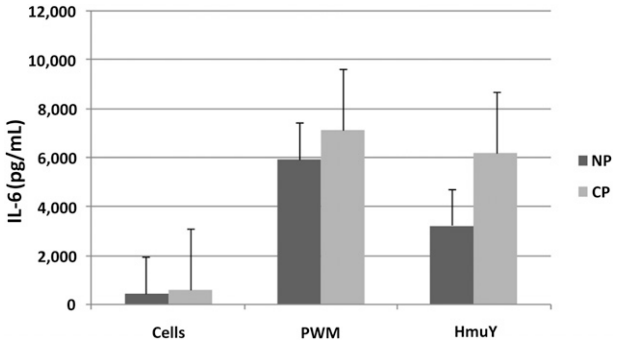

Figure 1.
IL-6 production by whole blood cells derived from control individuals without periodontitis (NP; n = 30) and patients with CP (n = 29) upon 48-hour stimulation with P. gingivalis *HmuY and PWM. Each experiment was carried out ≥3 times in triplicate. Cells = non-stimulated cells.*

In the group showing high production of IL-6 induced by HmuY, the proportion of individuals carrying at least one G allele at region –174 was greater than in the group showing a lower level of this cytokine in the cell cultures ($P<0.001$; Table 3).

DISCUSSION

According to the present results, patients with periodontitis and individuals with higher HmuY-induced production of IL-6 show higher frequency of G allele at position –174 than individuals with no disease. Several studies have been published on whether the IL-6 polymorphisms predispose to periodontitis.[14,19,32-35] It has been demonstrated that individuals carrying the GG genotype at position –174 were most severely affected by periodontal disease,[20] whereas the CC genotype was associated with susceptibility to CP in a German population.[15] Inasmuch as several reports have demonstrated that polymorphism at –174 (G/G) may be associated with higher susceptibility to periodontal disease,[14,15,20] in this study we analyze this feature in patients with CP and control individuals

Table 3.

Allelic and Genotypic Frequencies of IL-6 (–174) Polymorphism in Patients With High (HP/IL-6) and Low (LP/IL-6) Production of IL-6 After *P. Gingivalis* HmuY Stimulation

IL-6–174 G/C Genotypic Polymorphism (Phenotype)	HP/IL-6 (n = 32; NP = 4, CP = 28)	LP/IL-6 (n = 27; NP = 26, CP = 1)	*P* Value
G	93.1%	46.7%	0.00*
C	6.9%	53.3%	

* $P \leq 0.05$.

in a Brazilian population. However, this difference in the G allele carriage is not reflected in genotypic frequencies. The small sample size of this pilot study is an important limitation, especially in a Brazilian mixed-race population. The continuation of the sampling will allow us to better evaluate these aspects as well as to perform an adjusted analysis (e.g., sex, age, smoking, and ethnicity).

The available evidence indicates that IL-6 is involved in the pathogenesis of periodontitis, because *P. gingivalis*–induced production of IL-6 in peripheral mononuclear cells is higher in patients with periodontitis.[24] *P. gingivalis* LPS, but not *E. coli* LPS, has been shown to activate monocytes/macrophages to produce IL-6.[36] However, specific mechanisms and particular bacterial components stimulating its expression have not been clearly identified. Because *P. gingivalis* HmuY is a surface-exposed LPS and may be released into the host environment in the form of outer-membrane vesicles and a soluble protein,[7,8] it seems that it would be available for recognition by the immune response during CP and may cause increased cytokine production in vivo. Indeed, we demonstrate here that HmuY stimulated higher production of IL-6 by peripheral blood cells from diseased individuals compared to cells from individuals without periodontitis.

Ex vivo stimulation of whole blood cells, used in this study, appears to represent a more physiologic environment where the immune cells are present in natural ratios and can interact with each other. As no isolation procedure beyond the drawing of blood is required, the assay is characterized by some preparation artifacts and standardized performance. In addition, the cellular interactions are preserved and the presence of various plasma components are maintained.[37] In addition, despite the local aspect of periodontitis, primed lymphocytes and monocytes recirculate between lymphoid and non-lymphoid tissues and can be found in peripheral blood.

CONCLUSIONS

IL-6 production by peripheral blood cells is induced by the polypeptide part of *P. gingivalis* HmuY, suggesting that the protein plays a role in CP. The G allele at position –174 of the IL-6 gene can be associated with CP. However, although among patients with CP the number of G allele carriers was higher than among control individuals without periodontitis, the role of IL-6 gene polymorphism must be further investigated.

ACKNOWLEDGMENTS

This study was supported by grant 120/2006 from the Research Support Foundation of the State of Bahia, Salvador, Brazil (RM), and by grant N N303 518438 from the Polish Ministry of Science and Higher Education (TO). The authors also thank the Laboratory of Immunology, Health Sciences Institute, Federal University of Bahia, Bahia, Brazil, and Coordination of the Improvement of Higher Education Personal – CAPES for student fellowships. The authors report no conflicts of interest related to this study.

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Submitted April 9, 2012; accepted for publication June 8, 2012.