Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 688–694

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01286.x

Expression of periodontal interleukin-6 protein is increased across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases

J. H. Ross[1,*], D. C. Hardy[1,*], C. A. Schuyler[2,*], E. H. Slate[3], T. W. Mize[1], Y. Huang[2,4]

[1]Division of Periodontics, Department of Stomatology, College of Dental Medicine, Medical University of South Carolina, Charleston, SC, USA, [2]Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC, USA, [3]Department of Biostatistics, Bioinformatics & Epidemiology, Medical University of South Carolina, Charleston, SC, USA and [4]Division of Endocrinology, Diabetes and Medical Genetics, Department of Medicine, Medical University of South Carolina, Charleston, SC, USA

Ross JH, Hardy DC, Schuyler CA, Slate EH, Mize TW, Huang Y. Expression of periodontal interleukin-6 protein is increased across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases. J Periodont Res 2010; 45: 688–694. © 2010 John Wiley & Sons A/S

Background and Objective: Epidemiological studies have established that patients with diabetes have an increased prevalence and severity of periodontal disease. Interleukin (IL)-6, a multifunctional cytokine, plays a role in the tissue inflammation that characterizes periodontal disease. Our recent study has shown a trend of increase in periodontal IL-6 expression at the mRNA level across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases. However, the periodontal IL-6 expression at the protein level in these patients has not been investigated.

Material and Methods: Periodontal tissue specimens were collected from eight patients without periodontal disease and diabetes (group 1), from 17 patients with periodontal disease alone (group 2) and from 10 patients with both periodontal disease and diabetes (group 3). The frozen sections were prepared from these tissue specimens and IL-6 protein expression was detected and quantified.

Results: The nonparametric Kruskal–Wallis test showed that the difference in IL-6 protein levels among the three groups was statistically significant ($p = 0.035$). Nonparametric analysis using the Jonckheere–Terpstra test showed a tendency of increase in periodontal IL-6 protein levels across group 1 to group 2 to group 3 ($p = 0.006$). Parametric analysis of variance (ANOVA) on IL-6 protein levels showed that neither age nor gender significantly affected the difference of IL-6 levels among the groups.

Conclusion: Periodontal IL-6 expression at the protein level is increased across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases.

Yan Huang, MD, PhD, Ralph H. Johnson Veterans Affairs Medical Center, and Division of Endocrinology, Diabetes and Medical Genetics, Department of Medicine, Medical University of South Carolina, 114 Doughty St., Charleston, SC29403, USA
Tel: (843) 789 6824
Fax: (843) 876 5133
e-mail: huangyan@musc.edu

*Authors who contributed equally to the work presented in this article.

Key words: periodontal diseases; diabetes mellitus; interleukin-6 (IL-6); gene expression

Accepted for publication February 3, 2010

It has been well documented that interleukin (IL)-6 is involved in periodontal disease (1). Our recent study on periodontal expression of genes known to be important in periodontal disease showed a trend for an increase in IL-6 mRNA expression in periodontal tissue across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases ($p = 0.02$) (2). By contrast, no trend of increase across the three groups was found for IL-1β, tumor necrosis factor-α (TNF-α) and MMP-8, while a marginal trend of increase was observed for MMP-1 ($p = 0.05$). Given that periodontal disease in diabetic patients is more severe than that in nondiabetic patients, these findings further underscored the importance of IL-6 in the progression of periodontal disease.

Besides this report, previous studies have also provided evidence supporting that IL-6 is an important cytokine involved in the pathogenesis of periodontal disease. Interleukin-6 is abundant in the inflammatory lesions in periodontal tissues and activates fibroblasts in the presence of soluble IL-6 receptor (3). Interleukin-6 is a potent stimulator for MMP production (1). In addition to periodontal disease, it has been well established that IL-6, along with other proinflammatory cytokines (such as TNF-α and IL-1β), is involved in the production of the chronic and low-grade inflammation that contributes to other diabetic complications such as cardiovascular diseases (4–6). It has been proposed that IL-6 released from diseased periodontal tissue enters the blood circulation and stimulates both immune and nonimmune-related cells by activating the Janus kinase (JAK)/signal transducers and activator of transcription (STAT) and the Src homology 2-containing tyrosine phosphatase (SHP-2)/ERK/MAPK pathways and upregulating gene expression involved in inflammation (5).

Although we have demonstrated a trend of increase in periodontal IL-6 expression at the mRNA level in patients with neither periodontal disease nor diabetes, in patients with periodontal disease alone and in patients with both diseases (2), the periodontal expression of IL-6 at the protein level in these patients has not been investigated. While an increase in the mRNA expression level of a gene is frequently associated with an increase in its protein level, the extent of protein expression can be different from that of mRNA expression as a result of the potential translational regulation by the same stimulus. For example, we showed previously that the CD14 expression in U937 histiocytes was stimulated at the mRNA and protein level by 15- and 45-fold, respectively, by lipopolysaccharide (LPS) and high glucose compared to that in U937 histiocytes exposed to LPS and normal glucose (7). Thus, it is important to determine the IL-6 protein level in periodontal tissue to fully elucidate the impact of periodontal disease and diabetes on IL-6 expression.

In this study, we recruited 35 patients, with or without periodontal disease and diabetes, and collected periodontal tissue specimens from these patients during necessary surgical intervention; periodontal IL-6 protein levels were analyzed in these tissue specimens using immunohistochemistry. Quantitative data showed that periodontal IL-6 expression at the protein level is significantly increased across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases.

Material and methods

Patients

Thirty-five patients, including eight patients without periodontal disease and type 2 diabetes (group 1), 17 patients with periodontal disease alone (group 2) and 10 patients with both diseases (group 3), were recruited for this study. Eight patients who had undergone surgery (such as crown lengthening, extractions and periodontal plastic surgery) for dental disorders served as controls in group 1, following periodontal evaluation to exclude periodontal disease. All the tissues collected from these patients were periodontal tissues. Twenty-seven patients in groups 2 and 3 had clinical attachment loss (CAL) of > 5 mm in two or more teeth, which met the diagnostic criteria for chronic periodontitis according to the classification of 1999 (8). The oral examination was conducted as described previously (9). The exclusion criteria were: serum creatinine ≥ 1.6 mg/dL, abnormal hepatic function, hemoglobinopathy, unwillingness to sign the informed consent form or enter the study, aggressive periodontitis, and platelet and coagulation disorders. The patients in groups 2 and 3 received periodontal surgery and the diseased periodontal tissues were removed from sites with the greatest periodontal probing depth or CAL, or both. The hemoglobin A1c (HbA1c) test was performed in patients in group 3 before surgery to document their glycemic control status. HbA1c testing was not performed on patients professing to be nondiabetics. All patients provided informed consent for specimen collection. The study protocol and consent form were approved by the University Institutional Review Board.

Immunohistochemical analysis of IL-6 expression

Periodontal tissue samples were frozen in Tris-buffered saline freezing medium immediately after surgery and stored at −80°C. Using a cryostat, sections of 5 μm were cut and mounted on slides before being placed in 95% ethanol for 10 min and then washed with phosphate-buffered saline (PBS). Sections were blocked with filtered PBS containing 2% normal goat serum and 0.5% nonfat dry milk for 20 min. The sections were incubated with monoclonal antibody against IL-6 (1:150 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min. After washing with PBS, twice, for 5 min each wash, sections were incubated with biotinylated goat anti-mouse IgG (1:250 dilution) (Vector Laboratories, Burlingame, CA, USA) for 30 min. After washing, the sections were incubated with avidin DH and biotinylated horseradish peroxidase H complex (ABC kit; Vector Laboratories) for 30 min and with

diaminobenzidine solution (Sigma, St Louis, MO, USA) for 10 min. The slides were then rinsed in saline containing 0.75% $CuSO_4$. Counterstaining was performed with hematoxylin (Fisher Scientific, Pittsburgh, PA, USA), and the slides were then dehydrated using increasing concentrations of ethanol and xylene, and mounted. Sections stained with normal mouse IgG as the primary antibody were used as negative controls.

Image analysis

Images were taken using a computer-operated Zeiss Axiovert 200M inverted microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) with a Photometrix Cascade 512 CCD digital camera (Roper Scientific, Inc., Trenton, NJ, USA). Images were opened and analyzed using the Photoshop software (version 10; Adobe Systems, San Jose, CA, USA). The method of using the 'Similar' feature to select a particular color stain on a digitized immunohistochemical image has previously been described in detail (10). Briefly, a standard was created by selecting an area of 0.5×1 cm from a tissue section that had the desired brown color from IL-6 immunostaining. The cursor of the Magic Wand tool was clicked on the standard to make a selection, and the area of the standard was highlighted. To specify how broad a range of color the Magic Wand tool should include in the selection, the 'Tolerance' value in the Magic Wand Options palette was set to 100. Using the 'Similar' command, all the areas with the brown color that was similar to the standard on an image being determined were highlighted. The quantification was carried out using the 'Histogram' command in the Image menu, which showed the pixels of the highlighted area. The pixels of the highlighted area were normalized to the total tissue area.

Statistical analysis

Nonparametric analysis of variance (ANOVA) testing for any differences in continuous variables among groups was performed using the Kruskal–Wallis procedure. A rank-based ANOVA was performed to explore the effects of age and gender on the above nonparametric analysis. To test for monotonicity across the groups nonparametrically, the Jonckheere–Terpstra trend test was performed. Analyses were performed using R version 2.9.1. (11–13). A p-value of < 0.05 was considered statistically significant.

Results

Clinical data

The clinical data for patient age, gender, race, periodontal probing depth and CAL are presented in Table 1.

Age— The ages of subjects in groups 1, 2 and 3 ranged from 26 to 70, 30 to 71 and 35 to 74 years with mean ± standard deviation of 59 ± 15, 50 ± 13 and 57 ± 12, respectively. No significant difference in mean age was found among the three groups.

Gender— The ratios of female/male gender in groups 1, 2 and 3 were 7/1 (7.0), 6/11 (0.55) and 4/6 (0.67), respectively. There was a significant difference in the proportion of female subjects between group 1 and group 2 and between group 1 and group 3. No significant difference of gender was found between group 2 and group 3.

Race— The ratios of white people vs. black people in groups 1, 2 and 3 were 8/0, 10/6 and 6/4, respectively. There was a significant difference in the ratio of white people vs. black people between group 1 and group 2 and between group 1 and group 3. No significant difference was found in the ratio of white people vs. black people between group 2 and group 3 (1.67 vs. 1.50).

Smoking status— The smoker/nonsmoker ratios in groups 1, 2 and 3 were 0.6 (3/5), 0.9 (8/9) and 0.25 (2/8), respectively. Statistical analysis showed that no significant difference was present in smoker/nonsmoker ratios between group 1 and group 2 ($p = 1.0$), group 1 and group 3 ($p = 0.61$) and group 2 and group 3 ($p = 0.23$).

Periodontal disease— The periodontal probing depth in group 2 and group 3 was 6.8 ± 3.1 and 5.9 ± 2.5, respectively. The CAL in group 2 and group 3 was 6.2 ± 3.3 and 7.3 ± 2.4, respectively. No significant differences of periodontal probing depth and CAL were found between group 2 and group 3.

Diabetes— All patients in group 3 had type 2 diabetes. The results of HbA1c tests indicated that among the participants with diabetes, nine patients had

Table 1. Patients and periodontal disease in three groups

	Group 1: patients without diabetes and periodontal disease	Group 2: patients with periodontal disease alone	Group 3: patients with diabetes and periodontal disease
Patient number	8	17	10
Age (mean ± SD)	59 ± 15	50 ± 13	57 ± 12
Gender			
Female	7	6	4
Male	1	11	6
Female : Male	7.0	0.55	0.67
Race and ethnicity			
White (nonhispanic)	8	10	6
Black (nonhispanic)	0	6	4
Hispanic	0	1	0
Smoking status			
Smokers	3	8	2
Nonsmokers	5	9	8
PPD	NE	6.8 ± 3.1	5.9 ± 2.5
CAL	NE	6.2 ± 3.3	7.3 ± 2.4

The data presented are mean ± standard deviation (SD).
CAL, clinical attachment loss; NE, not examined; PPD, periodontal probing depth.

good glycemic control (HbA1c < 7%) while one patient had poor glycemic control (HbA1c > 8%). The average duration of diabetes for diabetic patients in group 3 was 10 ± 6 years, ranging from 1 to 20 years. For treatment of diabetes, eight patients took metformin alone or in combination with glipizide, nateglinide, rosiglitazone or sitagliptin. One patient took glyburide and one was on a diabetic diet.

Analysis of periodontal expression of IL-6 protein in three groups

A nonparametric ANOVA (Kruskal–Wallis) yielded a *p*-value of 0.037, indicating significant differences among the three groups in median IL-6 levels (Fig. 1). Representative images of periodontal tissue sections with IL-6 immunostaining are presented in Fig. 2 (lower magnification) and Fig. 3 (higher magnification). These images showed that the intensity and area of IL-6 immunostaining was increased in patients with periodontal disease (group 2) compared with control patients (group 1), and further increased in patients with both periodontal disease and diabetes (group 3) compared to patients with periodontal disease alone (group 2). Nonparametric analysis using the Jonckheere–Terpstra test showed a tendency for periodontal IL-6 protein expression levels to increase across group 1 to group 2 to group 3 ($p = 0.006$) (Table 2). Parametric ANOVA of IL-6 expression levels with log- or rank-transformation showed that neither age ($p > 0.85$) nor gender ($p > 0.70$) was significantly associated with IL-6 levels among the three groups.

Discussion

In our previous study (2), we reported that the periodontal IL-6 mRNA expression levels differed significantly ($p = 0.04$) and showed a trend to increase across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and patients with both diseases ($p = 0.02$). Although the mRNA expression of MMP-1, MMP-8, IL-1β

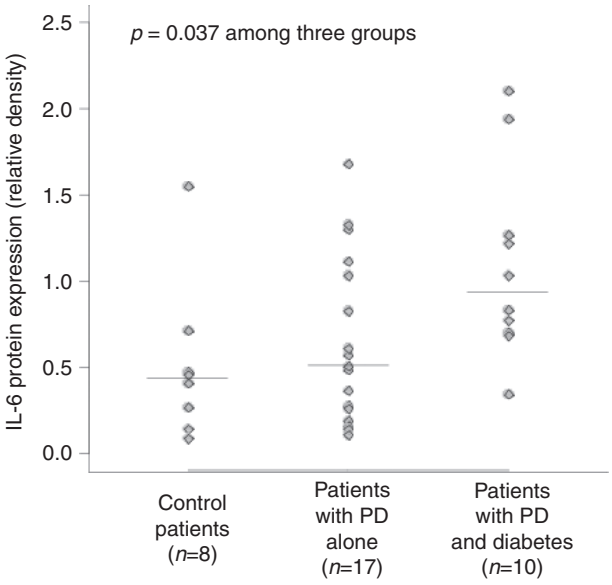

Fig. 1. Expression of periodontal interleukin-6 (IL-6) protein in patients with neither periodontal disease nor diabetes, patients with disease alone, or patients with both diseases. Interleukin-6 protein was detected in periodontal tissue and quantified as described in the Material andmethods. The Kruskal–Wallis test was performed to analyze the difference in IL-6 expression levels among the three groups. Horizontal lines indicate the group median levels. PD, periodontal disease.

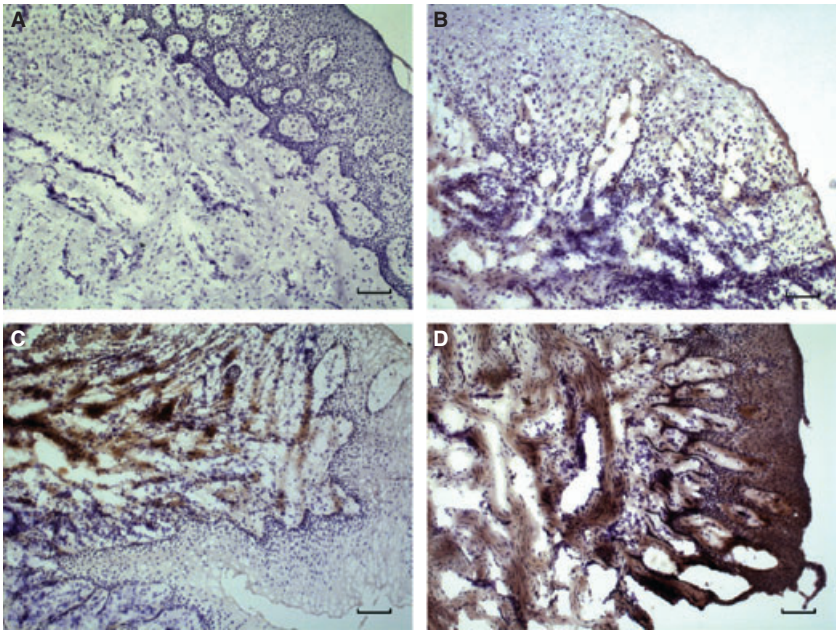

Fig. 2. Representative images of periodontal interleukin-6 (IL-6) protein immunostaining with lower magnification. (A) Negative control for IL-6 immunostaining. Periodontal tissue was collected from a patient with periodontal disease and immunostaining was performed using normal mouse IgG as control primary antibody. (B) Immunostaining of IL-6 in periodontal tissue collected from a patient without periodontal disease and diabetes. (C) Immunostaining of IL-6 in periodontal tissue collected from a patient with periodontal disease alone. (D) Immunostaining of IL-6 in periodontal tissue collected from a patient with both periodontal disease and diabetes. Bar, 100 μm.

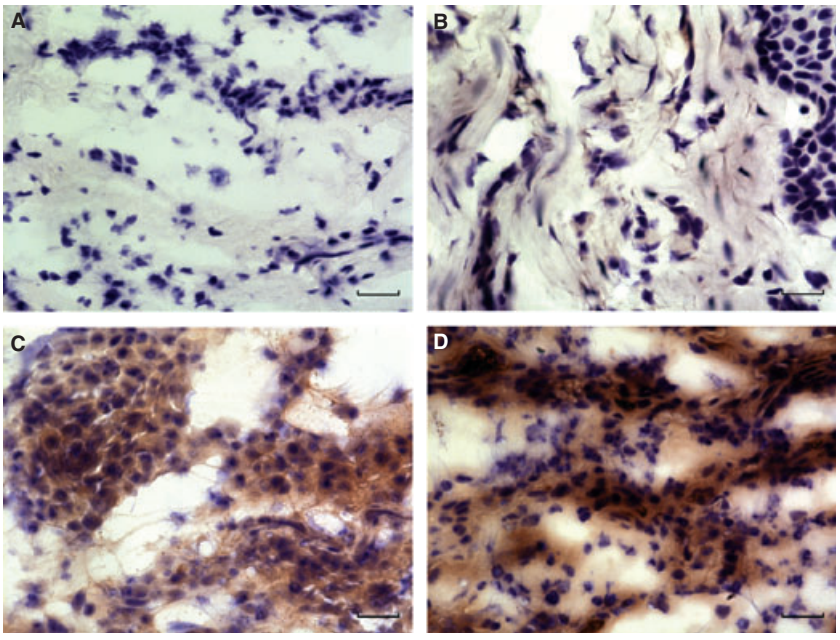

Fig. 3. Representative images of periodontal interleukin-6 (IL-6) protein immunostaining with higher magnification. (A) Negative control for IL-6 immunostaining using the control antibody as described in Fig. 2A. (B) Immunostaining of IL-6 in periodontal tissue collected from a patient without periodontal disease and diabetes. (C) Immunostaining of IL-6 in periodontal tissue collected from a patient with periodontal disease alone. (D) Immunostaining of IL-6 in periodontal tissue collected from a patient with both periodontal disease and diabetes. Bar, 20 μm.

and TNF-α also increased across the three groups, the difference was not statistically significant. Because the periodontal tissue specimens obtained in the above investigation were completely used up for the real-time PCR analysis to determine the mRNA expression, new patients were recruited for the current study to quantify IL-6 protein expression using immunohistochemistry. Thus, the current study was an independent study with a different patient pool.

The purpose of this study was to determine if IL-6 expression at the protein level showed an increase similar to that at the mRNA level across the three groups of patients. Our findings from this study provided further evidence indicating that periodontal IL-6 expression increases when the disease status changes from 'no disease' to 'one disease (periodontal disease)' to 'two diseases (periodontal disease and diabetes)'.

Table 2. Summary statistics for interleukin-6 (IL-6) expression for the three groups

Groups	Minimum	First quartile	Median $p = 0.037$[a] $p = 0.006$[b]	Mean	Third quartile	Maximum
1. Control group	0.086	0.236	0.432	0.512	0.532	1.551
2. With periodontal disease alone	0.112	0.264	0.505	0.646	1.033	1.684
3. With both periodontal disease and diabetes	0.344	0.720	0.933	1.091	1.257	2.105

[a]Nonparametric Kruskal–Wallis test indicated differences in the median IL-6 protein level, with a *p*-value of 0.037.
[b]Nonparametric Jonckheere–Terpstra test for increasing trend in IL-6 protein levels was statistically significant, with a *p*-value of 0.006.

Several lines of evidence have indicated an essential role of IL-6 in periodontal disease. First, a number of genetic studies have shown that polymorphisms of IL-6 in its promoter region are associated with periodontitis (14–16). Second, studies from our laboratory and from other laboratories have shown that IL-6 is highly expressed in the periodontal tissue of patients with periodontal disease (2,3) and is associated with severity of periodontal disease (1). Third, *in vitro* studies have shown that IL-6 is a potent stimulator for the expression of MMPs by mononuclear cells that play an important role in periodontal tissue destruction (17,18). Our recent study has also demonstrated that IL-6 derived from fibroblasts is essential for the upregulation of MMP-1 expression by fibroblast/mononuclear cell interaction (18). Fourth, it was found that periodontal disease is associated with increased concentrations of circulating IL-6, which decreased 3 mo after nonsurgical periodontal therapy (19), indicating that periodontal disease contributes to increased levels of circulating IL-6. Thus, it has been proposed that IL-6 derived from periodontal tissue may contribute to inflammation-associated diseases in other systems, such as cardiovascular diseases (5), which is a potential mechanism involved in the links between periodontal disease and cardiovascular diseases (20,21).

It has been well established that periodontal disease is accelerated and more severe in patients with diabetes (22–24). To understand the mechanisms underlying periodontal disease, increasing evidence has suggested that the host immune responses to oral pathogens and gram-negative bacteria-derived pathogenic molecules such as LPS may be amplified by diabetes-associated factors such as hyperglycemia, leading to increased inflammation (25,26). In support of this notion, our recent studies have shown that high glucose augmented LPS-stimulated TNF-α, IL-1β and IL-6 secretion from human U937 macrophages by 6-, 4- and 27-fold, respectively (27,28). Obviously, the augmentation of LPS-stimulated IL-6 secretion by high glucose is much

higher than that of TNF-α and IL-1β secretion, suggesting an important role of IL-6 in diabetes-associated periodontal disease. Furthermore, our current study has suggested a relationship between periodontal IL-6 expression and diabetes.

Our recent study showed that IL-6 was released by both cultured gingival fibroblasts and monocytes/macrophages, and LPS stimulated IL-6 expression in both types of cells (18). A previous study by Yamazaki *et al.* (29) showed that epithelial cells in periodontal tissue also express IL-6. In the present study, we found, from the images of IL-6 immunostaining plus hematoxylin counterstaining, that a large number of cells expressed IL-6 in the periodontal tissue of patients with both periodontal disease and diabetes (Fig. 2D and Fig. 3D). Based on the histology, these cells seem to include epithelial cells, fibroblasts and mononuclear cells. As resident cells (such as epithelial cells and fibroblasts) and inflammatory cells (such as mononuclear cells) in periodontal tissue express IL-6, it is expected that the amount of IL-6 released from periodontal tissue, especially in the state of periodontal infection, is considerably high.

The Kruskal–Wallis procedure enables evaluation of whether there is evidence of differences in IL-6 expression levels among the three groups. This procedure produced an asymptotic *p*-value of 0.037, which supported the belief that there were differences in IL-6 (median) values among the three groups. Computation of the *p*-value using Monte Carlo sampling with 100,000 samples yielded a value of 0.034, so the asymptotic value is quite good here. To explore the effects of age and gender on this nonparametric analysis, we performed a rank-based ANOVA. In this case, neither age nor gender emerged as being statistically significant in the model, and the *p*-value associated with the groups was 0.039, again showing significance of the differences in median IL-6 expression levels among the three groups.

In summary, the results from this study showed a significant increase in periodontal IL-6 expression at the protein level across patients with neither diabetes nor periodontal disease, patients with periodontal disease alone and patients with both diseases. This finding is consistent with our previous study reporting a trend of an increase in IL-6 expression at the mRNA level across the three patient groups (2) and strongly indicates an important role of IL-6 in periodontal disease in diabetic patients.

Acknowledgement

This work was supported by NIH grant DE16353 and by a Merit Review Grant from the Department of Veterans Affairs (to Y.H.). This work was also partially supported by COBRE grant P20RR017696 from NIH.

References

1. Irwin CR, Myrillas TT. The role of IL-6 in the pathogenesis of periodontal disease. *Oral Dis* 1998;**4:**43–47.
2. Cole CM, Sundararaj KP, Leite RS *et al.* A trend of increase in periodontal interleukin-6 expression across patients with neither diabetes nor periodontal disease, patients with periodontal disease alone, and patients with both diseases. *J Periodontal Res* 2008;**43:**717–722.
3. Takashiba S, Naruishi K, Murayama Y. Perspective of cytokine regulation for periodontal treatment: fibroblast biology. *J Periodontol* 2003;**74:**103–110.
4. McCarty MF. Interleukin-6 as a central mediator of cardiovascular risk associated with chronic inflammation, smoking, diabetes, and visceral obesity: down-regulation with essential fatty acids, ethanol and pentoxifylline. *Med Hypotheses* 1999;**52:**465–477.
5. Moutsopoulos NM, Madianos PN. Low-grade inflammation in chronic infectious diseases: paradigm of periodontal infections. *Ann N Y Acad Sci* 2006;**1088:** 251–264.
6. Kamimura D, Ishihara K, Hirano T. IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev Physiol Biochem Pharmacol* 2003;**149:** 1–38.
7. Nareika A, Im YB, Game BA *et al.* High glucose enhances lipopolysaccharide-stimulated CD14 expression in U937 mononuclear cells by increasing nuclear factor kappaB and AP-1 activities. *J Endocrinol* 2008;**196:**45–55.
8. Flemmig TF. Periodontitis. *Ann Periodontol* 1999;**4:**32–38.
9. Salvi GE, Yalda B, Collins JG *et al.* Inflammatory mediator response as a potential risk marker for periodontal diseases in insulin-dependent diabetes mellitus patients. *J Periodontol* 1997;**68:** 127–135.
10. Lehr HA, Mankoff DA, Corwin D, Santeusanio G, Gown AM. Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem* 1997;**45:**1559–1565.
11. R Development Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2009. ISBN 3-900051-07-0, URL http://www.r-project.org/, Accessed on February 1, 2010.
12. Broberg P. SAGx: Statistical Analysis of the GeneChip. R package version 1.18.0, 2009 URL http://www.bioconductor.org/packages/2.4/bioc/html/SAGx.html. Accessed on February 1, 2010.
13. Hothorn T, Hornik K, van de Wiel MA, Zeileis A. Implementing a class of permutation tests: the coin package. *J Stat Softw* 2008;**28:**1–23.
14. Trevilatto PC, Scarel-Caminaga RM, de Brito RB Jr, de Souza AP, Line SR. Polymorphism at position -174 of IL-6 gene is associated with susceptibility to chronic periodontitis in a Caucasian Brazilian population. *J Clin Periodontol* 2003;**30:**438–442.
15. Tervonen T, Raunio T, Knuuttila M, Karttunen R. Polymorphisms in the CD14 and IL-6 genes associated with periodontal disease. *J Clin Periodontol* 2007;**34:**377–383.
16. Babel N, Cherepnev G, Babel D *et al.* Analysis of tumor necrosis factor-alpha, transforming growth factor-beta, interleukin-10, IL-6, and interferon-gamma gene polymorphisms in patients with chronic periodontitis. *J Periodontol* 2006;**77:**1978–1983.
17. Irwin CR, Myrillas TT, Traynor P, Leadbetter N, Cawston TE. The role of soluble interleukin (IL)-6 receptor in mediating the effects of IL-6 on matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1 expression by gingival fibroblasts. *J Periodontol* 2002;**73:**741–747.
18. Sundararaj KP, Samuvel DJ, Li Y, Sanders JJ, Lopes-Virella MF, Huang Y. Interleukin-6 released from fibroblasts is essential for up-regulation of matrix metalloproteinase-1 expression by U937 macrophages in coculture: cross-talking between fibroblasts and U937 macrophages exposed to high glucose. *J Biol Chem* 2009;**284:**13714–13724.
19. Marcaccini AM, Meschiari CA, Sorgi CA *et al.* Circulating interleukin-6 and high-sensitivity C-reactive protein decrease after periodontal therapy in otherwise healthy subjects. *J Periodontol* 2009; **80:**594–602.

20. Mustapha IZ, Debrey S, Oladubu M, Ugarte R. Markers of systemic bacterial exposure in periodontal disease and cardiovascular disease risk: a systematic review and meta-analysis. *J Periodontol* 2007;**78:**2289–2302.
21. Paquette DW, Brodala N, Nichols TC. Cardiovascular disease, inflammation, and periodontal infection. *Periodontol 2000* 2007;**44:**113–126.
22. Darwazeh AM. Diabetes mellitus, dental caries and periodontal disease: evidence for a relationship. *Dent Health (London)* 1990;**29:**3–7.
23. Katz PP, Wirthlin MR Jr, Szpunar SM, Selby JV, Sepe SJ, Showstack JA. Epidemiology and prevention of periodontal disease in individuals with diabetes. *Diabetes Care* 1991;**14:**375–385.
24. Yalda B, Offenbacher S, Collins JG. Diabetes as a modifier of periodontal disease expression. *Periodontol 2000* 1994;**6:**37–49.
25. Silva JA, Lorencini M, Reis JR, Carvalho HF, Cagnon VH, Stach-Machado DR. The influence of type I diabetes mellitus in periodontal disease induced changes of the gingival epithelium and connective tissue. *Tissue Cell* 2008;**40:**283–292.
26. Perrino MA. Diabetes and periodontal disease: an example of an oral/systemic relationship. *N Y State Dent J* 2007;**73:** 38–41.
27. Maldonado A, He L, Game BA *et al.* Pre-exposure to high glucose augments lipopolysaccharide-stimulated matrix metalloproteinase-1 expression by human U937 histiocytes. *J Periodontal Res* 2004;**39:**415–423.
28. Nareika A, Maldonado A, He L *et al.* High glucose-boosted inflammatory responses to lipopolysaccharide are suppressed by statin. *J Periodontal Res* 2007;**42:**31–38.
29. Yamazaki K, Nakajima T, Gemmell E, Polak B, Seymour GJ, Hara K. IL-4- and IL-6-producing cells in human periodontal disease tissue. *J Oral Pathol Med* 1994;**23:**347–353.