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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

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Abstract

Background and objectives—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 differences in IL-6 protein levels among the three groups were statistically significant ($p = 0.035$). Nonparametric analysis using 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.

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Keywords

Periodontal diseases; Diabetes mellitus; IL-6; Gene expression

It has been well documented that interleukin (IL)-6 plays an essential role in periodontal disease (1). Our recent study on periodontal expression of genes known to be involved in periodontal disease showed a trend of 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). No trend of increase across the three groups was found for IL-1 β , 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 suggest that IL-6 may contribute to 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 pro-inflammatory cytokines (such as TNF- α and IL-1 β), 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 across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone and 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 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 due to the translational regulation by the same stimulus. For example, we showed previously that the stimulation of CD14 expression at the mRNA and protein levels in U937 histiocytes by lipopolysaccharide (LPS) and high glucose was 15-fold and 45-fold as compared to those by LPS and normal glucose, respectively (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 at the time of necessary surgical intervention and analyzed periodontal IL-6 protein expression levels 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.

MATERIALS & METHODS

Patients

Thirty-five patients, including 8 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 in group 1 who had 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) > 5 mm in 2 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 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 was not performed for 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 TBS freezing media immediately after surgery and stored at -80°C . Using a cryostat, 5 μm sections were cut and mounted on slides before being placed in 95% of ethanol for 10 minutes and then washed with phosphate-buffered saline (PBS). Sections were blocked with filtered PBS containing 2% normal goat serum and 0.5% non-fat dry milk for 20 min. The sections were incubated with monoclonal antibody against IL-6 (1:150 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 min. After washing with PBS, twice, for 5 min each wash, sections were incubated with biotinylated-goat anti-mouse antibody (1:250 dilution) (Vector Laboratories, Burlingame, CA) for 30 minutes. 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) for 10 min. The slides were then rinsed in saline containing 0.75% of CuSO_4 . Counterstaining was performed with hematoxylin (Fisher Scientific, Pittsburgh, PA, USA) and the slides were then dehydrated using increasing concentrations of ethanol and xylenes, and mounted. Sections stained with normal mouse IgG as primary antibody were used as negative control.

Image Analysis

Images were taken using a computer-operated Zeiss Axiovert 200m inverted microscope with a Photometrix Cascade 512 CCD digital camera. Images were opened and analyzed with the Photoshop software (version 10; Adobe Systems, San Jose, CA, USA). The method of using “Similar” feature to select a particular color staining on a digitized immunohistochemical image has previously been described in detail (10). Briefly, a standard was created by selecting an area of 0.5 cm $\times$ 1 cm from a tissue section that had 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 is similar to the standard on an image being determined were highlighted. The quantification was done 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 variable among groups were 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 $\pm$ SD of 59 ± 15 , 50 ± 13 and 57 ± 12 , respectively. No significant difference in mean age was found among the 3 groups.

Gender—The ratios of female/male in groups 1, 2 and 3 were 7/1 (7.0), 6/11 (0.55), and 4/6 (0.67), respectively. There is a significant difference in the proportion of females 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 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 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 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 procedure (Kruskal-Wallis) yielded a p -value of 0.037, indicating significant differences among the 3 groups in median IL-6 levels (Fig. 1). Representative images of periodontal tissue sections with IL-6 immunostaining are presented in Fig. 2. 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 (group 2). Nonparametric analysis using Jonckheere-Terpstra test showed a tendency of increase in periodontal IL-6 protein expression levels across from 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 had a trend of 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-1 β , and TNF- α also increased across the three groups, the difference was not statistically significant. Because the periodontal tissue specimens were totally used for real-time PCR analysis to determine the mRNA expression level of genes in the above investigation, 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 patient groups. 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)”.

Several lines of evidence have indicated an essential role of IL-6 in periodontal disease. First, a number of genetic studies have shown that IL-6 polymorphisms in its promoter region are associated with periodontitis (14–16). Second, studies from our and other laboratories have shown that IL-6 is highly expressed in periodontal tissue of patients with periodontal disease (2,3) and 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 upregulation of MMP-1 expression by fibroblast/mononuclear cell interaction (18). Fourth, it was found that periodontal disease is associated with increased circulating concentrations of IL-6, which decreased 3 mo after non-surgical periodontal therapy (19), indicating that periodontal disease contributes to increased 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 underlying mechanisms, 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-1 β , 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-1 β secretion, suggesting an important role of IL-6 in diabetes-

associated periodontal disease. Furthermore, our current study has suggested a relationship between the 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 of IL-6 immunostaining plus hematoxylin counterstaining, that a large number of cells expressed IL-6 in the periodontal tissue of patients with 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 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 to be 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, results from this study showed a significant increase in periodontal IL-6 expression at the protein levels 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 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.

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Figure 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 and methods. 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.

Figure 2.

Representative images of periodontal IL-6 protein immunostaining. Panel 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. Panel B: Immunostaining of periodontal tissue collected from a patient without periodontal disease and diabetes. Panel C: Immunostaining of periodontal tissue collected from a patient with periodontal disease alone. Panel D: Immunostaining of periodontal tissue collected from a patient with both periodontal disease and diabetes.

Figure 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 μ m.

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
Number of patients	8	17	10
Age	59 ± 15	50 ± 13	57 ± 12
Gender (female/male)	7/1 (7.0)	6/11 (0.55)	4/6 (0.67)
Race and ethnicity			
White (nonhispanic)	8	10	6
Black (nonhispanic)	0	6	4
Hispanic	0	0	0
Smoking Status			
Smokers	3	8	2
Nonsmokers	5	9	8
Periodontal Probing Depth (PPD)	NE	6.8 ± 3.1	5.9 ± 2.5
Clinical Attachment Loss (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.

Table 2

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

Group #	Minimum	1 st Quartile	Median p = 0.037* p = 0.006 ⁺	Mean	3 rd Quartile	Maximum
1, control	0.086	0.236	0.432	0.512	0.532	1.551
2, perio	0.112	0.264	0.505	0.646	1.033	1.684
3, perio + diab	0.344	0.720	0.933	1.091	1.257	2.105

* Nonparametric Kruskal-Wallis test indicated differences in median IL-6 protein levels with p = 0.037.

⁺ Nonparametric Jonckheere-Terpstra test for increasing trend in IL-6 protein levels was statistically significant with p = 0.006.