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# 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

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*Background and Objective:* Epidemiological studies have established that patients with diabetes have increased prevalence and severity of periodontal disease. However, the periodontal expression of inflammatory cytokines and matrix metalloproteinases (MMPs) in diabetic patients has not been well characterized. The objective of this study was to determine the difference in the periodontal expression of MMP-1, MMP-8, interleukin-6, tumor necrosis factor-α and interleukin-1β between diabetic and nondiabetic patients.

*Material and Methods:* Periodontal tissue specimens were collected from nine nondiabetic patients without periodontal disease (group 1), from 11 nondiabetic patients with periodontal disease (group 2) and from seven diabetic patients with periodontal disease (group 3). The expression of MMP-1, MMP-8, interleukin-6, tumor necrosis factor-α and interleukin-1β was quantified using real-time polymerase chain reaction.

*Results:* The nonparametric Kruskal–Wallis test showed that the difference in interleukin-6 expression among the groups was statistically significant ($p = 0.04$). Furthermore, the generalized Kruskal–Wallis nonparametric linear-by-linear association test showed a statistically significant trend of increase in the expression of interleukin-6 from group 1 to group 2 to group 3 ($p = 0.02$) and a suggestion of such a trend for MMP-1 ($p = 0.05$). No increase in MMP-8 expression was observed in patients in group 3 compared to patients in groups 1 and 2. Although the average expression levels of MMP-1, interleukin-1β and tumor necrosis factor-α were increased from group 1 to group 3, the differences were not statistically significant.

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*Conclusion:* A trend of increased interleukin-6 expression in periodontal tissues was observed across patients with neither diabetes nor periodontal disease, patients with periodontal disease alone, and patients with both diseases.

Epidemiological studies have established that patients with either type 1 or type 2 diabetes have an increased prevalence and severity of periodontal disease (1). In addition to cardiovascular disease, nephropathy, retinopathy, neuropathy and peripheral vascular diseases, periodontal disease has been considered as another diabetic complication (2). To explain why diabetic patients have an increased risk of periodontal disease, previous studies have proposed that altered immune responses, impaired host defense and increased susceptibility to infection in diabetic patients may be responsible(1). Also, the role of protein glycosylation in diabetic complications has been extensively studied since the mid-1990s and it is generally accepted that advanced glycosylation end-products contribute to diabetic complications, including periodontal disease (3,4). In recent years, an increasing number of studies have reported that inflammation plays a crucial role in the periodontal disease of diabetic patients (5,6). It has been shown that diabetic patients with periodontal disease have significantly higher levels of inflammatory mediators such as interleukin-1β, tumor necrosis factor-α and prostaglandin $E_2$ in the gingival crevicular fluid compared with nondiabetic patients, and mononuclear phagocytes isolated from diabetic patients have exaggerated inflammatory responses to lipopolysaccharide (5). It has also been shown that the expression of matrix metalloproteinases (MMPs), a group of proteases responsible for periodontal tissue destruction, is increased in gingival tissues isolated from diabetic rats compared to tissue from nondiabetic rats (7). These studies indicate that diabetes may promote periodontal disease by increasing the production of pro-inflammatory cytokines and MMPs. Furthermore, increasing evidence has indicated that inflammatory cytokines derived from diseased periodontal tissue may be responsible not only for destructive periodontitis, but also for insulin resistance, diabetes and diabetic complications (8,9).

Although it has been well documented that inflammation plays a key role in periodontal disease in diabetic patients, the periodontal expression of pro-inflammatory cytokines and MMPs in diabetic patients has not been well characterized. In this study, we collected periodontal tissue specimens from 27 diabetic or nondiabetic patients at the time of necessary surgical intervention and determined the expression of tumor necrosis factor-α, interleukin-1β, interleukin-6, MMP-1 and MMP-8 by quantitative real-time polymerase chain reaction (PCR) in these specimens. The results showed that, among these genes, periodontal interleukin-6 expression showed a trend of increase across the patients with neither diabetes nor periodontal disease, patients with periodontal disease alone, and patients with both diseases.

## Material and methods

### Patients

Twenty-seven patients, including nine patients without periodontal disease and type 2 diabetes (group 1), 11 patients with periodontal disease alone (group 2) and seven patients with both diseases (group 3), were included in this study. Nine patients in group 1 who had surgery for dental disorders such as crown lengthening, extractions and periodontal plastic surgery served as controls. All the tissues collected were periodontal tissues. The eighteen patients in groups 2 and 3 met the following diagnostic criteria for periodontal disease: a periodontal pocket depth of ≥ 6 mm in two or more teeth, or clinical attachment loss of ≥ 5 mm in two or more teeth. The oral examination was conducted as described previously (10). The exclusion criteria were as follows: serum creatinine ≥ 1.6 mg/dL, abnormal hepatic function, hemoglobinopathy, unwillingness to sign the informed consent form or enter the study, juvenile periodontitis, and/or platelet and coagulation disorders. The patients in groups 2 and 3 received periodontal surgery and the periodontal tissues were removed from sites based on the greatest probing depth or clinical attachment loss, or both. The hemoglobin A1c test was carried out on diabetic patients to document their glycemic control status. The hemoglobin A1c test was not carried out on patients professing to be nondiabetics. Hemoglobin A1c levels of > 8% and < 7% were designated as poor and good glycemic control, respectively. All patients provided informed consent for specimen collection. The study protocol and consent form were approved by the University Institutional Review Board. Table 1 shows the patients' clinical data.

### Isolation of RNA and RNA reverse transcription

Total RNA was isolated from periodontal tissue specimens using the RNeasy minikit (Qiagen, Santa Clarita, CA, USA). The first-strand cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) by following the instructions provided by the manufacturer. The reaction was cycled for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C using a PTC-200 DNA Engine (MJ Research, Waltham, MA, USA).

### Quantitative real-time PCR

The reverse transcription reaction mixture from the above experiments was then subjected to PCR amplification. The Beacon Designer Software (PREMIER; Biosoft International, Palo Alto, CA, USA) was used for primer design (Table 2). Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). PCR

*Table 1.* Characteristics of periodontal disease in patients with or without type 2 diabetes

| | 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 | 9 | 11 | 7 |
| Age (years) | 47 ± 18 | 57 ± 13 | 53 ± 20 |
| Gender (male/female) | 4/5 | 7/4 | 5/2 |
| Race | | | |
| White | 8 | 10 | 2 |
| Black | 1 | 1 | 5 |
| Probing depth | NE | 6.9 ± 1.5 | 5.6 ± 1.4 |
| Attachment loss | NE | 7.4 ± 2.3 | 8.1 ± 3.1 |
| Bleeding index | NE | 13.4 ± 9.3 | 12.7 ± 8.6 |

NE, not examined. The data presented represent the mean ± SD.

was carried out in duplicate using a 25-μL reaction mixture that contained 1.5 μL of reverse transcription reaction mixture, 0.2 μM of both primers and 12.5 μL of iQ™ SYBR Green Supermix (Bio-Rad). The PCR reaction was performed using the iCycler™ Real-Time Detection System (Bio-Rad). Forty cycles consisting of denaturation (95°C for 10 s) and annealing/extension (56°C for 45 s) were run. A melt-curve experiment was subsequently performed (incubation at 55°C for 1 min followed by an increase in the temperature by 0.5°C every 10 s) to detect the primer dimers. Data were analyzed using the SMARTCYCLER II software. The average threshold cycle (*Ct*) of fluorescent units was used for analysis. Quantification was calculated using the *Ct* of the target signal relative to that of the glyceraldehyde-3-phosphate dehydrogenase signal in the same RNA sample.

### Statistical analysis

The gender and race of the study participants were presented as counts, and continuous variables were presented as medians (minimum and maximum). Nonparametric analyses testing for any differences in continuous variables among groups were performed using the Kruskal–Wallis procedure. Nonparametric tests to analyze the linear trend across the groups were performed using the generalized Kruskal–Wallis linear-by-linear association test, which is based on a linear combination of the group rank sums (11). The *p*-values reported for these nonparametric tests were determined exactly using the Monte Carlo method with $1 \times 10^6$ samples. Differences in the distributions of the categorical variables gender and race were assessed using Fisher's exact test. A *p*-value of less than 0.05 was considered statistically significant. All analyses were performed in R (version 2.5.1.) (12), with use of the package 'COIN' by Hothorn *et al.* (13; the function Kruskal_test with a factor group variable was used for the nonparametric trend test).

## Results

### Study population

*Age* — The ages of subjects in groups 1, 2 and 3 ranged from 23 to 74, 40 to 82 and 26 to 80 years, respectively, with a mean ± standard deviation of 47 ± 18, 57 ± 13 and 53 ± 20, respectively.

*Gender* — The ratios of men/women in groups 1, 2 and 3 were 4/5, 7/4 and 5/2, respectively.

*Race* — All patients recruited were either non-Hispanic blacks or whites. The ratios of blacks vs. whites in groups 1, 2 and 3 were 1/8, 1/10 and 5/2, respectively.

*Periodontal disease* — The probing depths in groups 2 and 3 were 6.9 ± 1.5 and 5.6 ± 1.4, respectively. The attachment loss values in groups 2 and 3 were 7.4 ± 2.3 and 8.1 ± 3.1, respectively. The bleeding index values in groups 2 and 3 were 13.4 ± 9.3 and 12.7 ± 8.6, respectively.

*Diabetes* — The results from the hemoglobin A1c tests indicated that among the participants with diabetes, five patients had good glycemic control (hemoglobin A1c < 7%) whereas two patients had poor glycemic control (hemoglobin A1c > 8%).

### Expression of pro-inflammatory cytokines and MMP in periodontal tissue

The expression of MMP-1, MMP-8, interleukin-6, interleukin-1β and tumor necrosis factor-α in periodontal tissue specimens was successfully quantified using real-time PCR. As shown in Table 3, although the median expression levels of MMP-1, interleukin-6, interleukin-1β and tumor necrosis factor-α were increased from group 1 to group 3, the differences were not statistically significant. No increase in MMP-8 expression was observed

*Table 2.* The primer sequences for real-time polymerase chain reaction

| Genes | 5′ primer sequence | 3′ primer sequence |
|---|---|---|
| MMP-1 | CTGGGAAGCCATCACTTACCTTGC | GTTTCTAGAGTCGCTGGGAAGCTG |
| MMP-8 | AACGCACTAACTTGACCTACAG | CTCCAGAGTTCAAAGGCATCC |
| TNF-α | CCCCAGGGACCTCTCTCTAA | TTTGCTACAACATGGGCTACAG |
| IL-1β | CTGTACGATCACTGAACTGC | CACCACTTGTTGCTCCATATC |
| IL-6 | AACAACCTGAACCTTCCAAAGATG | TCAAACTCCAAAAGACCAGTGATG |
| GAPDH | GAATTTGGCTACAGCAACAGGGTG | TCTCTTCCTCTTGTGCTCTTGCTG |

IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP, matrix metalloproteinase; TNF-α, tumor necrosis factor-α.

*Table 3.* Periodontal expression of matrix metalloproteinase (MMP)-1, MMP-8, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in patients with or without type 2 diabetes

| | Group 1 (control) | Group 2 | Group 3 | Nonparametric analyses testing for any differences in groups (Kruskal–Wallis test) | | Nonparametric test for linear trend across the three groups | |
|---|---|---|---|---|---|---|---|
| | Without periodontal disease and diabetes | With periodontal disease, without diabetes | With both periodontal disease and diabetes | | | | |
| Number of patients | 9 | 11 | 7 | Test statistic[b] | *p*-value[a] | Test statistic[c] | *p*-value[a] |
| MMP-1 | 0.06 (0.01, 0.63) | 0.15 (0.03, 1.52) | 0.17 (0.12, 2.71) | 3.9 | 0.14 | 3.9 | 0.05 |
| MMP-8 | 1.05 (0.12, 17.54) | 0.69 (0.14, 3.14) | 2.18 (0.64, 5.92) | 2.7 | 0.27 | 1.3 | 0.26 |
| IL-6 | 0.32 (0.03, 1.92) | 0.45 (0.02, 20.52) | 2.19 (0.34, 39.41) | 6.2 | 0.04 | 5.7 | 0.02 |
| IL-1β | 0.57 (0.17, 3.28) | 0.91 (0.52, 3.53) | 0.70 (0.43, 7.21) | 3.2 | 0.20 | 1.3 | 0.26 |
| TNF-α | 3.06 (0.59, 6.47) | 4.03 (1.48, 13.12) | 5.19 (0.39, 25.17) | 1.7 | 0.44 | 1.5 | 0.23 |

Data presented are medians (minimum, maximum).
[a]The reported *p*-values were computed exactly by the Monte Carlo method using $1 \times 10^6$ samples.
[b]The asymptotic reference distribution for the Kruskal–Wallis test statistic is the chi-square on 2 degrees of freedom.
[c]The asymptotic reference distribution for the nonparametric linear trend test statistic is the chi-square on 1 degree of freedom.

across the three groups. A statistically significant expression of interleukin-6 was observed among the groups ($p = 0.04$). Furthermore, the nonparametric trend test showed a statistically significant linear trend of increase in the expression of interleukin-6 from group 1 to group 2 to group 3 ($p = 0.02$) and the suggestion of such a trend in MMP-1 ($p = 0.05$) (Table 3).

## Discussion

Gingival crevicular fluid has been commonly utilized for quantification of the levels of cytokines and MMPs (10,14,15) in the investigation of gene expression in periodontal tissue. Although this is an effective approach, it has a major limitation: the amount of gingival crevicular fluid collected from each patient is very small and often insufficient for quantification of a number of pro-inflammatory cytokines and MMPs. Taking a different approach, our present study utilized periodontal tissue specimens removed from diabetic and nondiabetic patients to quantify the gene expression in periodontal tissue using the real-time PCR technique. This approach has several advantages. First, it allowed us to study gene expression at the mRNA level, which indicates the activation of gene transcription or mRNA stabilization in periodontal tissue. Second, because PCR is a very sensitive method for using to detect gene expression and only requires a small amount of RNA, the RNA isolated from each tissue specimen is sufficient for quantification of a large number of genes. Third, because the periodontal specimens surgically removed from patients with periodontal disease are diseased tissues, the changes in the expression of cytokines and MMPs in the specimens are likely to be associated with periodontal disease.

In the present study, the expression of MMP-1, MMP-8, interleukin-6, interleukin-1β and tumor necrosis factor-α in periodontal tissue specimens obtained from 27 patients in three groups (patients without both diabetes and periodontal disease; patients with periodontal disease alone; and patients with both diabetes and periodontal disease) was quantified using real-time PCR. All of these genes have been implicated previously in periodontal disease (16–18). The objective of this study was to determine if there is a trend of increase in the expression of these genes when the disease status changes from 'no disease' to 'one disease (periodontal disease)' to 'two diseases (periodontal disease and diabetes)'. To the best of our knowledge, this is the first study in which periodontal tissue specimens and the quantitative real-time PCR technique have been utilized to determine the periodontal expression of cytokines and MMPs in diabetic and nondiabetic patients. Although the sample size in this study was relatively small owing to the limited number of diabetic patients who could provide periodontal tissues, our statistical analyses showed that among the five genes examined, interleukin-6 expression differed statistically significantly among the groups ($p = 0.04$) and showed a trend for increased levels across the three groups ($p = 0.02$) (Table 3). In contrast, although the expression of tumor necrosis factor-α, interleukin-1β and MMP-1 increased across the three groups, the trend in these levels was not statistically significant. A larger sample size may enable detection of a difference in the periodontal expression of these genes among these groups of patients.

In this study, the Kruskal–Wallis test was employed for nonparametric assessment of any differences in groups, and the associated linear-by-linear association test was used to test for a linear trend. In the latter procedure, we tested the null hypothesis that the cytokine and MMP expression levels in the three groups have the same location (median) vs. the ordered alternative that the medians increase linearly across groups 1, 2 and 3. Although designed to be sensitive to a linear trend, this test may also detect nonlinear trends, and thus no specific functional form should be attributed to

our finding of an increase in interleukin-6 expression across the three study groups. Differences in distributions besides location shifts can affect these nonparametric procedures (19) and hence it is important also to examine the shape of the data distributions. Our graphical exploration of the data suggests a greater right skewness in the expression values in group 3 (diabetes and periodontal disease) than in the other groups, with the exception of MMP-8, which exhibits greater skewness in group 1. Interestingly, for each of the cytokines, these apparent differences in skewness across the three groups are driven by one expression value. For example, for MMP-1, there was one study participant in group 3 with a value of 2.71, which far exceeds the next largest value of 1.52 obtained from a participant in group 2. It is noteworthy that the cytokine expression values driving the right skewness were not all from the same study participant. Hence, there is no 'outlying' participant, so there is no basis for performing analyses that exclude these largest values, especially in light of the small study size. An additional caution for interpretation arises from the modest amount of multiple testing present in Table 3, for which no adjustment has been made.

Among our recorded variables, race emerged as the most likely potential confounder. To investigate this possibility, we utilized two modeling methods permitting adjustment for the effects of race. The first method was parametric analysis of variance of the log-transformed cytokine expression levels to adjust for race (i.e. analysis of covariance). The log transformation served to reduce the right skewness, making the homogeneity and normality assumptions of this parametric procedure more tenable. The second method we used was analysis of covariance of the rank-transformed cytokine expression (20). The results of these two analyses were consistent. Race did not contribute significantly to the model for any of the cytokines. Interestingly, even when adjusted for race, the interleukin-6 levels differed significantly among the three groups ($p = 0.045$ for log-transform; $p = 0.038$ for rank-transform), although no differences were evident for the remaining cytokines.

It has been well documented that interleukin-6 plays an essential role in periodontal disease (21). Interleukin-6 is abundantly present in the inflammatory lesions in periodontal tissues, activates fibroblasts in the presence of soluble interleukin-6 receptor (22) and is involved in periodontal tissue destruction (21). Besides periodontal disease, studies have established in detail that interleukin-6, along with other pro-inflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, is involved in chronic and low-grade inflammation that contributes to diabetes and diabetic complications (23–25). It was proposed that interleukin-6 released from diseased periodontal tissue enters the circulation and interacts with both immune-related and nonimmune-related cells by activating the Janus kinase/signal transducers and activator of transcription and the Src homology 2-containing tyrosine phosphatase/extracellular signal-regulated kinase/mitogen-activated protein kinase pathways and stimulates gene expression involved in inflammation (24).

Our *in vitro* studies may provide some clues to understand why, among the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β and interleukin-6 tested in the current study, only interleukin-6 expression significantly increased across the three study groups. Recently, we reported that high glucose levels augmented lipopolysaccharide-stimulated tumor necrosis factor-α, interleukin-1β and interleukin-6 secretion from human U937 macrophages by 6-, 4- and 27-fold, respectively (26). Obviously, the augmentation of lipopolysaccharide-stimulated interleukin-6 secretion by high glucose levels is much higher than that of tumor necrosis factor-α and interleukin-1β secretion. As it is known that gram-negative bacteria-derived lipopolysaccharide is a potent stimulator for interleukin-6 expression, and hyperglycemia is a major metabolic abnormality in both type 1 and type 2 diabetes, this finding may provide an explanation, at least partially, of why periodontal interleukin-6 expression was significantly increased across the three study groups.

Besides interleukin-6, the results of this study also suggested that MMP-1 expression had an increasing trend across patients with neither diabetes nor periodontal disease, patients with periodontal disease alone, and patients with both diseases ($p = 0.05$). Irwin *et al*. have demonstrated *in vitro* that interleukin-6, in combination with soluble interleukin-6 receptor, significantly stimulated MMP-1 expression by gingival fibroblasts (27), suggesting that the increased periodontal interleukin-6 expression may contribute to the increase in MMP-1 expression. In contrast to MMP-1, MMP-8 expression was not significantly different between the three groups ($p = 0.27$). This finding was not unexpected because it has been reported that the expression of MMP-8, a proteinase mainly released by neutrophils, was increased not only in periodontitis, but also in other oral diseases, such as dental caries and oral cancer (28). It is possible that some patients in the group 1 had dental caries and hence increased MMP-8 expression.

In summary, the results from this study showed a trend of increased interleukin-6 expression in periodontal tissues across patients with neither diabetes nor periodontal disease, patients with periodontal disease alone, and patients with both diseases. The finding from this study warrants further investigations on the role of periodontal interleukin-6 expression in diabetic patients in larger studies.

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