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MMP-8 Expression in Periodontal Tissues Surgically Removed from Diabetic and Nondiabetic Patients with Periodontal Disease

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Abstract

Background—Although it is known that periodontal MMP-8 expression is associated with periodontal disease, the information concerning the periodontal MMP-8 expression in diabetic patients with periodontal disease is insufficient.

Materials and Methods—Periodontal tissue specimens were collected from 7 patients without periodontal disease and diabetes (Group 1), 15 patients with periodontal disease alone (Group 2) and 10 patients with both periodontal disease and diabetes (Group 3). The frozen sections were prepared and MMP-8 protein expression was detected using immunohistochemistry and quantified. For *in vitro* study, human U937 mononuclear cells were pre-exposed to normal or high glucose and then treated with LPS.

Results—The nonparametric Kruskal-Wallis test showed that the difference in MMP-8 protein levels among the three groups were statistically significant ($p = 0.003$). Nonparametric analysis using Jonckheere-Terpstra test showed a tendency of increase in periodontal MMP-8 levels across Group 1 to Group 2 to Group 3 ($p = 0.0002$). *In vitro* studies showed that high glucose and LPS had a synergistic effect on MMP-8 expression.

Conclusion—Our current study showed an increasing trend in MMP-8 protein expression levels across patients without both periodontal disease and diabetes, patients with periodontal disease alone and patients with both diseases.

Keywords

Periodontal diseases; Diabetes mellitus; MMP-8; Gene expression

Patients with either type 1 or type 2 diabetes have increased prevalence and severity of periodontal disease when compared with nondiabetic subjects (Mealey, 1999). Thus,

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Conflict of Interest
There is no conflict of interest to declare.

periodontal disease has been considered as another diabetic complication besides cardiovascular disease, nephropathy, retinopathy, neuropathy and peripheral vascular diseases (Grossi and Genco, 1998). Although earlier studies proposed that altered immune response, impaired host defense and increased susceptibility to infection in diabetic patients were the underlying mechanisms for greater risk for periodontal disease in diabetic patients (Mealey, 1999), increasing evidence in recent years has indicated a crucial role of pathogen-triggered inflammatory response from host in the pathogenesis of diabetes-associated periodontal disease (Salvi et al., 2005, Salvi et al., 1998, Nishimura et al., 2007, Genco et al., 2005). It has been shown that diabetic patients with periodontal disease have significantly higher levels of inflammatory mediators such as interleukin-1 (IL-1)β, tumor necrosis factor (TNF)α, and prostaglandin E_2 (PGE_2) in the gingival crevicular fluid (GCF) as compared to nondiabetic patients, and mononuclear phagocytes isolated from diabetic patients have exaggerated inflammatory responses to lipopolysaccharide (LPS) (Salvi et al., 1998). The studies from our group also showed that periodontal IL-6 mRNA and protein levels increased across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone, and patients with both diseases (Cole et al., 2008, Ross et al., 2010). In supporting these findings from patient studies, our *in vitro* studies have shown that high glucose in culture medium increased proinflammatory cytokine production by mononuclear cells in response to LPS (Maldonado et al., 2004).

Both LPS and proinflammatory cytokines such as IL-6, IL-1β and TNFα are potent stimulators for matrix metalloproteinase (MMP) expression by mononuclear cells (Li et al., 2010). MMPs belong to a family of zinc-dependent endopeptidases and are capable of degrading collagen and other matrix proteins in connective tissue (Davidson et al., 2002). Numerous studies have shown that elevated levels of MMPs, especially MMP-8 (neutrophil collagenase), in GCF, saliva and mouth rinse samples are associated with the progression of periodontal disease (Mancini et al., 1999, Uitto et al., 2003, Sorsa et al., 2004, Leppilahti et al., Lee et al., 1995, Mantyla et al., 2006, Mantyla et al., 2003). Although the pathological role of MMP-8 in periodontal disease is well known, the information regarding the relationship between periodontal MMP-8 expression and diabetes remains controversial.

By analysis of MMP-9 expression in gingival tissue using Western blot and gelatin zymography, Kumar et al. reported that MMP-8 level was increased by 2 folds in chronic periodontitis patients with diabetes compared to chronic periodontitis patients without diabetes (Kumar et al., 2006). Collin et al. also reported that advanced periodontitis in type 2 diabetes was related to elevated salivary MMP-8 levels (Collin et al., 2000). However, a recent study by Buduneli and coworkers showed that diabetes did not significantly affect GCF levels of MMP-8 (Kardesler et al., 2010). Costa et al. also reported that salivary MMP-9 concentrations were elevated regardless of periodontal inflammation in patients with diabetes (Costa et al., 2010). Thus, more investigations are necessary to determine if the state of diabetes in patients with periodontal disease is associated with increased periodontal MMP-8 expression.

In this study, we recruited 32 patients with or without periodontal disease and diabetes and collected periodontal tissue specimens at the time of necessary surgical intervention and analyzed periodontal MMP-8 protein levels using immunohistochemistry. Results showed that periodontal MMP-8 expression is significantly increased across patients with neither periodontal disease nor diabetes, patients with periodontal disease alone, and patients with both diseases.

Materials and Methods

Patients

Thirty-two patients including 7 patients without periodontal disease and diabetes (Group 1), 15 patients with periodontal disease alone (Group 2), and 10 patients with both diseases (Group 3) were recruited for this study. Seven patients who had surgeries for dental disorders such as crown lengthening, extractions, and periodontal plastic surgery served as controls in Group 1. Periodontal evaluation was conducted in these patients to exclude periodontal disease and periodontal tissues were collected. Twenty-five patients in Groups 2 and 3 who presented clinical attachment loss (CAL) > 5 mm in 2 or more teeth at the 6-week non-surgical reevaluation appointment, which met the diagnostic criteria for chronic periodontitis according to the classification of 1999 (Flemmig, 1999), were recruited for the study. The oral examination, including full mouth periodontal examination such as periodontal probing depth (PPD) and CAL measurements using the cement-enamel junction (CEJ) as a reference point, was conducted as previously described (Salvi et al., 1997). 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, platelet and coagulation disorders. The patients in Groups 2 and 3 received periodontal surgery and the diseased periodontal tissue was collected from the site with greatest PPD or CAL or both. Each patient provided one tissue sample that was removed from one periodontal site. Patients in Group 3 had hemoglobin A1c (HbA1c) test before their surgeries to document glycemic control status. HbA1c was not evaluated for patients professing to be nondiabetics. All patients provided informed consent for the specimen collection. The study protocol and consent form were approved by the University Institutional Review Board.

Immunohistochemical Analysis of MMP-8 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 PBS. Sections were blocked with filtered PBS containing 2% normal goat serum and 0.5% non-fat dry milk for 20 minutes. The sections were incubated with monoclonal antibody against MMP-8 (1:150 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA) for 45 minutes. After washing with PBS twice for 5 minutes, sections were then 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 minutes and with diaminobenzidine solution (Sigma, St. Louis, MO) for 10 minutes. Counterstaining was preformed with hematoxylin (Fisher Scientific, Pittsburgh, PA). Sections stained with normal mouse IgG as primary antibody were used as negative control. Five slides from each sample were used for analysis.

Image Analysis

Images were taken using a computer-operated Zeiss Axiovert 200M inverted microscope with a Photometrix Cascade 512 CCD digital camera. Images were analyzed with the Photoshop software (version 10; Adobe Systems, San Jose, CA). The method to use “Similar” feature to select a particular color staining on a digitized immunohistochemical image has previously been described in detail (Lehr et al., 1997). Briefly, a standard was created by selecting an area of 0.5 cm × 1.0 cm from a tissue section that had desired brown color from MMP-8 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.

Cell Culture

U937 mononuclear phagocytes (Sundstrom and Nilsson, 1976) were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in a 5% CO_2 atmosphere in RPMI 1640 medium (GIBCO, Invitrogen Cop. Carlsbad, CA) containing normal glucose (5 mM) or high glucose (25 mM), 10% fetal calf serum, 1% MEM non-essential amino acid solution, and 0.6 g/100 ml of HEPES. Five and 25 mM of glucose have been used commonly as the concentrations of normal and high glucose, respectively (Nareika et al., 2008, Nareika et al., 2007, Sundararaj et al., 2009).

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from cells using the RNeasy minikit (Qiagen). First-strand complementary DNA (cDNA) was synthesized with the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed in duplicate using 25 μl of reaction mixture containing 1.0 μl of RT mixture, 0.2 μM of both primers (5' sequence: AACGCACTAACTTGACCTACAG; 3' sequence: CTCCAGAGTTCAAAGGCATCC) and 12.5 μl of iQ™ SYBR Green Supermix (Bio-Rad Laboratories). Real-time PCR was run in the iCycler™ real-time detection system (Bio-Rad Laboratories). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (5' primer sequence: GAATTTGGCTACAGCAACAGGGTG; 3' primer sequence: TCTCTTCCTCTTGTGCTCTTGCTG). The average starting quantity (SQ) of fluorescence units was used for analysis.

Statistical Analysis

Nonparametric analysis of variance (ANOVA) using the Kruskal-Wallis procedure was used to text for any differences in the continuous MMP-8 levels among groups. To test for monotonicity across the groups nonparametrically, the Jonckheere-Terpstra trend test was performed. A rank-based ANOVA was performed to explore the effects of age and gender. Differences in gender and race among the groups were evaluated using chisquare tests with *p*-value computing using Monte Carlo sampling as appropriate for the small size of this study. Analyses were performed using R version 2.9.1. (Broberg, 2010, Hothorn et al., 2008, R Development Core Team, 2009). A *p*-value less than 0.05 was considered statistically significant. For cell culture studies, data were presented as mean ± SD. Student *t* tests were performed to determine the statistical significance of gene expression among different experimental groups. A *p*-value less than 0.05 was considered statistically significant.

Results

Clinical Data

Table 1 shows the clinical data for patient age, gender, race, smoking status, PPD and CAL.

Age—The mean ± SD in Groups 1, 2 and 3 was 59 ± 15, 50 ± 13 and 57 ± 13, 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 6/1 (6.0), 5/10 (0.50), and 4/6 (0.67), respectively. The proportion of females did not differ significantly among the groups (p=0.15).

Race—The white vs. black ratios in Groups 1, 2 and 3 were 7/0 (7.0), 9/5 (1.8), and 6/4 (1.5), respectively. The proportion of blacks to whites did not differ significantly among the three groups (p=0.16).

Periodontal disease—The means of PPD from the sampled sites in Group 2 and Group 3 were 6.8 ± 3.1 mm and 5.9 ± 2.5 mm, respectively. The means of CAL from the sampled sites in Group 2 and Group 3 were 6.2 ± 3.3 mm and 7.3 ± 2.4 mm, respectively. No significant difference of the means of PPD and CAL from the sampled sites was found between Group 2 and Group 3.

Diabetes—All patients in Group 3 had type 2 diabetes. HbA1c tests indicate that among the participants with diabetes, 9 patients had good glycemic control (HbA1c <7%) while 1 patient had poor glycemic control (HbA1c>8%). The average duration of diabetes for diabetic patients in Group 3 is 10 ± 6 years, ranging from 1 to 20 years.

Analysis of Periodontal Expression of MMP-8 Protein in Three Groups

Nonparametric ANOVA (Kruskal-Wallis) yielded a *p*-value of 0.003, indicating significant differences among the three groups in median MMP-8 protein levels (Fig. 1). Nonparametric analysis using the Jonckheere-Terpstra test showed an increasing trend in MMP-8 protein expression levels across Group 1, Group 2 and Group 3 (p=0.0002). Neither age (p=0.37) nor gender (p=0.24) was significantly associated with MMP-8 levels.

Representative images of periodontal tissue sections with MMP-8 immunostaining are presented in Fig. 2. These images showed that the intensity and area of MMP-8 immunostaining was increased in patients with periodontal disease (Group 2) as compared to control patients (Group 1) and further increased in patients with both periodontal disease and diabetes (Group 3) as compared to patients with periodontal disease alone (Group 2). The most immunostaining of MMP-8 appeared to be associated with cells (Fig. 2).

High Glucose and LPS Have A Synergistic Effect on MMP-8 Expression

To understand the mechanism by which periodontal MMP-8 expression is increased in patients with diabetes, we hypothesized that hyperglycemia may augment MMP-8 expression in response to bacterial LPS. Since mononuclear cells play an important role in periodontal disease, we tested our hypothesis by performing *in vitro* studies using U937 mononuclear cells to determine if increased glucose concentration in culture medium augmented LPS-stimulated MMP-8 expression. Quantitative real-time PCR showed that high glucose and LPS increased MMP-8 expression by 2-fold as compared to normal glucose and LPS (Fig. 3), indicating that high glucose and LPS had a synergistic effect on MMP-8 expression by U937 mononuclear cells.

Discussion

MMP-8, also called neutrophil collagenase, is expressed and released by not only neutrophils (Sorsa et al., 2004), but also a number of other types of cells including monocytes, macrophages, epithelial cells, endothelial cells and plasma cells in response to inflammatory stimuli (Prikk et al., 2001, Schubert-Unkmeir et al., 2010, Wahlgren et al., 2001). In consistence with these previous reports, our present study also showed that MMP-8 was expressed by human U937 mononuclear cells in response to LPS and high

glucose (Figs. 3). Thus, it appears that MMP-8 not only plays an essential role in tissue inflammation in gingivitis that is characterized by dominant infiltration of neutrophils into the periodontal tissue, but also contributes to tissue inflammation in periodontitis that is associated with increased infiltration of monocytes.

Although MMP-8, MMP-1 and MMP-13 as collagenases target similar matrix proteins such as collagen I, II, III, VII, VIII, X, it was found that MMP-8 level was higher than MMP-1 and MMP-13 levels in GCF collected from patients with periodontal disease (Ingman et al., 1996, Lee et al., 1995). Furthermore, patient studies have shown that treatment of periodontal disease decreases MMP-8 level and its activity in GCF (Sorsa et al., 1999). Based on the findings from a large number of studies, MMP-8 was considered as a good marker for periodontal disease and thus a MMP-8 specific chair-side test has been developed to monitor periodontal health and disease (Sorsa et al., 1999). Clinical studies have provided strong evidence that MMP-8 plays an important role in periodontal disease.

Our present study utilized periodontal tissue specimens removed from diabetic and nondiabetic patients to study the relationship between diabetes and periodontal expression of MMP-8. Because the periodontal specimens surgically removed from patients with periodontal disease are diseased tissues, the change in MMP-8 expression in these specimens is likely to be associated with periodontal disease. Our findings are consistent with the previous studies that utilized GCF or saliva collected from nondiabetic and diabetic patients and showed that MMP-8 level in GCF or saliva was higher in diabetic patients than that in nondiabetic patients (Kumar et al., 2006, Collin et al., 2000). We found a significant difference among 3 groups (p=0.003) and a significant trend of MMP-8 increase among 3 groups (p=0.0002). These findings, therefore, suggest that MMP-8 may be involved in diabetes-associated periodontal disease.

The data from our *in vitro* studies (Fig. 3) have provided clue that may partially explain why diabetic patients have increased periodontal MMP-8 expression. Since our previous studies have shown that U937 mononuclear cells and human monocytes have similar response to LPS and high glucose in their expression of genes involved in inflammation (Samuvel et al., 2010, Sundararaj et al., 2009), we used U937 cells in this study. Results from these studies showed that high glucose and LPS exert a synergistic effect on MMP-8 expression by U937 cells, indicating that hyperglycemia may be a key factor in MMP-8 upregulation in periodontal tissue. Besides hyperglycemia, patients with type 2 diabetes may also have other pathological factors such as hypercholesterolemia and hypertriglyceridemia, which are also associated with increased proinflammatory cytokines (Chan et al., 2002, Wisse, 2004). Indeed, it has been shown that the expression of proinflammatory cytokines such as IL-6 have increased expression in periodontal tissue in diabetic patients (Cole et al., 2008, Salvi et al., 1998) and proinflammatory cytokines are potent stimulators for MMP-8 expression (Wahlgren et al., 2001, Li et al., 2010). Thus, diabetic patients with a good glycemic control may also have increased periodontal MMP-8 expression if they have dyslipidemia. This may explain why the diabetic patients in this study had increased MMP-8 expression despite most of them had good glycemic control.

In summary, our study has shown an increasing trend in MMP-8 protein expression levels across patients without both periodontal disease and diabetes, patients with periodontal disease alone and patients with both diseases. To the best of our knowledge, this is the first study in which periodontal tissue removed from surgery were used to determine the periodontal MMP-8 expression in diabetic and nondiabetic patients. Although the sample size in this study was relatively small due to the limited number of diabetic patients who had surgery, our statistical analyses showed a significantly increased MMP-8 expression in

diabetic patients. The findings from this study warrant further investigations in a larger population.

Clinical Relevance

Scientific Rationale for Study

It has been well documented that periodontal MMP-8 expression is increased in patients with periodontal disease as compared to patients without periodontal disease. However, it remains unclear if periodontal MMP-8 expression is further increased in patients with both periodontal disease and diabetes as compared to patients with periodontal disease alone.

Principal Findings

The nonparametric Kruskal-Wallis test showed that the difference in MMP-8 protein levels among the three groups were statistically significant ($p = 0.003$). Nonparametric analysis using Jonckheere-Terpstra test showed a tendency of increase in periodontal MMP-8 levels across Group 1 to Group 2 to Group 3 ($p = 0.0002$). *In vitro* studies showed that high glucose and LPS had a synergistic effect on MMP-8 expression.

Practical Implications

MMP-8 inhibition is a potential therapeutic strategy and further studies need to prove its application and efficacy.

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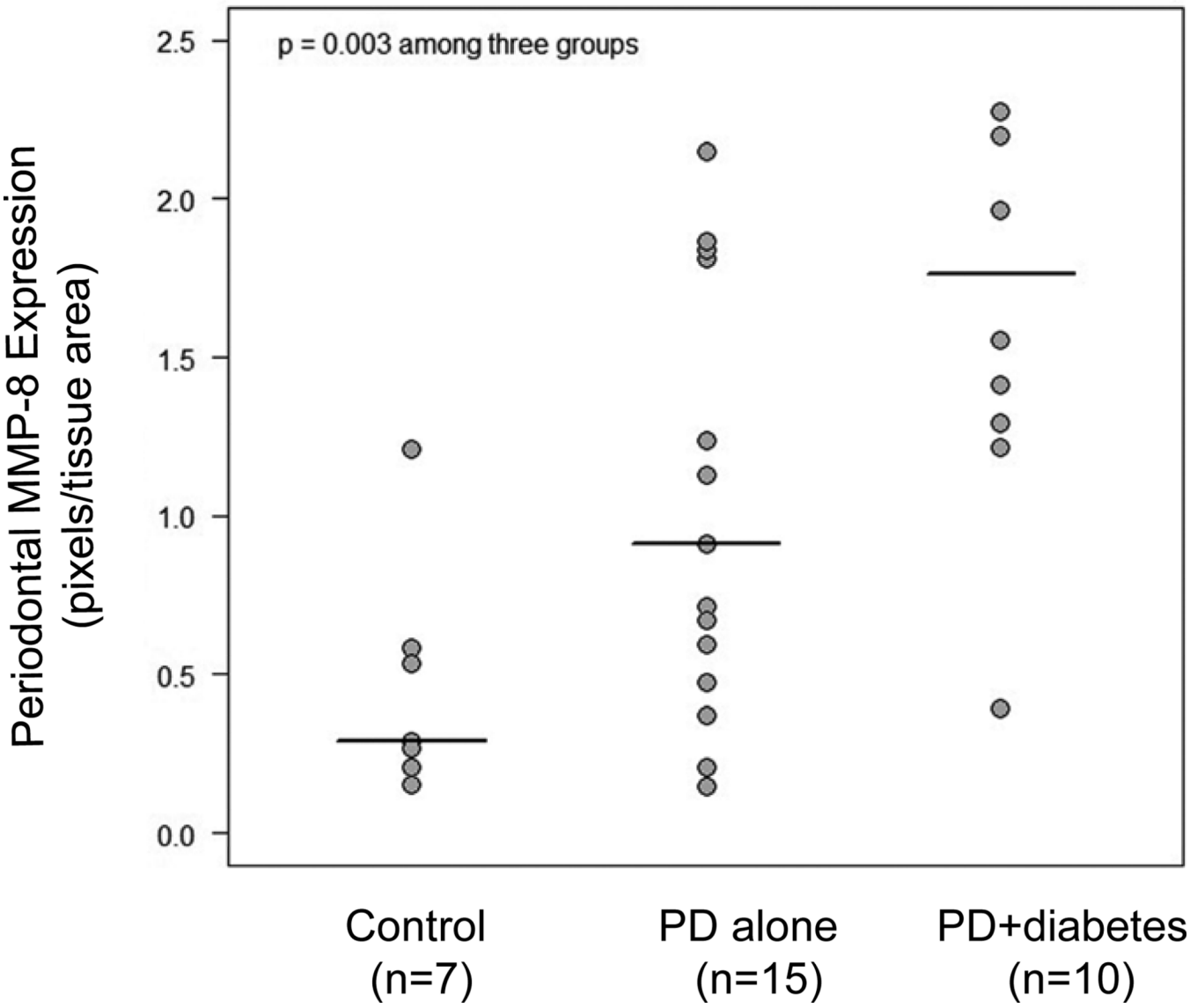

Figure 1.

Statistical analysis of periodontal MMP-8 protein expression among Group 1 (patients with neither periodontal disease nor diabetes), Group 2 (patients with periodontal disease alone), and Group 3 (patients with both diseases). **MMP-8** protein expression was detected in periodontal tissue and quantified as described in Materials and Methods. The Kruskal-Wallis test was performed to analyze the difference of MMP-8 expression levels among the three groups. Horizontal lines indicate the group median levels. PD, periodontal disease.

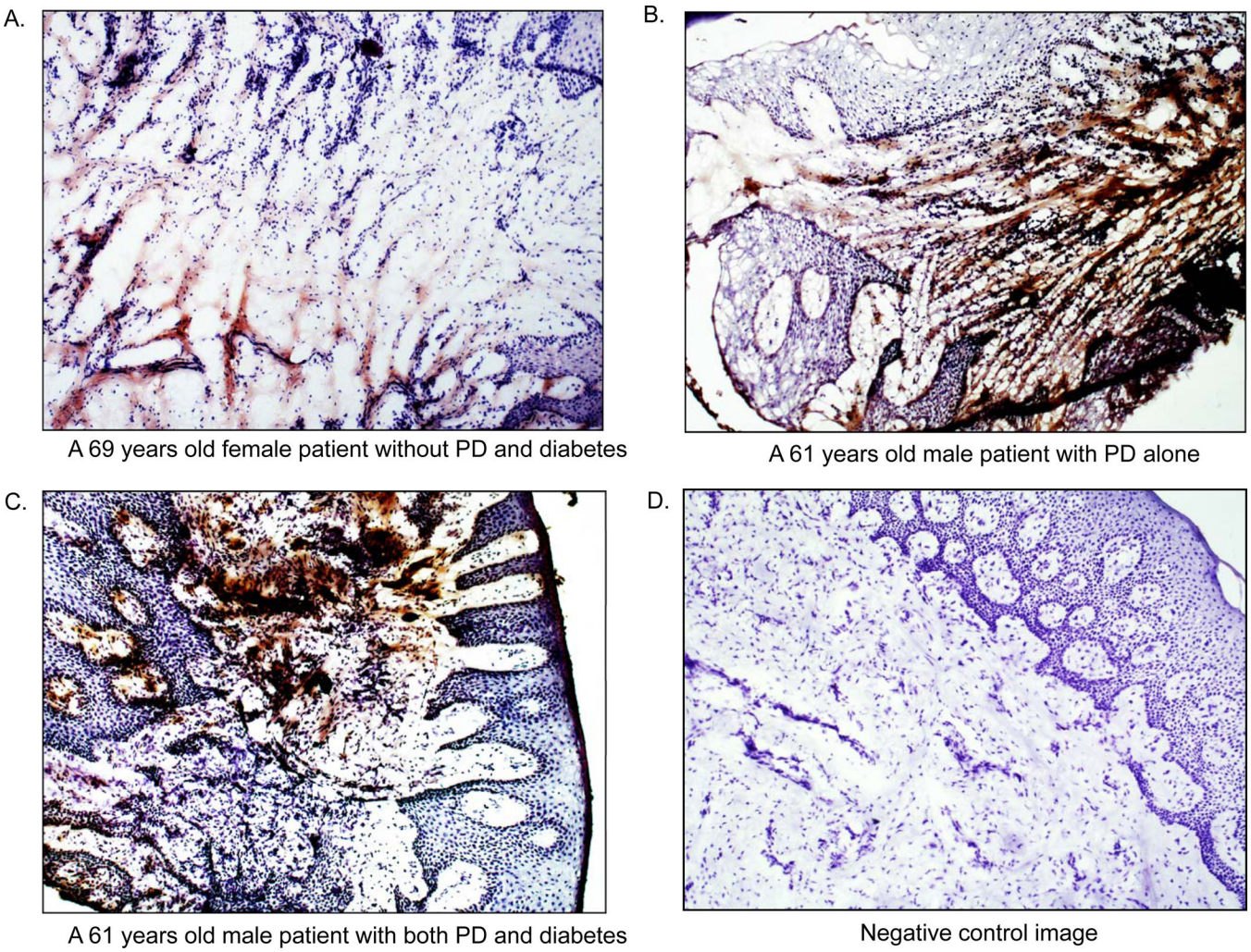

Figure 2.

Representative images of periodontal MMP-8 protein immunostaining. A: Immunostaining of MMP-8 in periodontal tissue collected from a 69 years old female without periodontal disease (PD) and diabetes. B: Immunostaining of MMP-8 in periodontal tissue collected from a 61 years old male with PD alone. C: Immunostaining of **MMP-8** in periodontal tissue collected from a 61 years old male with both PD and diabetes. D: Negative control for MMP-8 immunostaining using normal mouse IgG as control primary antibody.

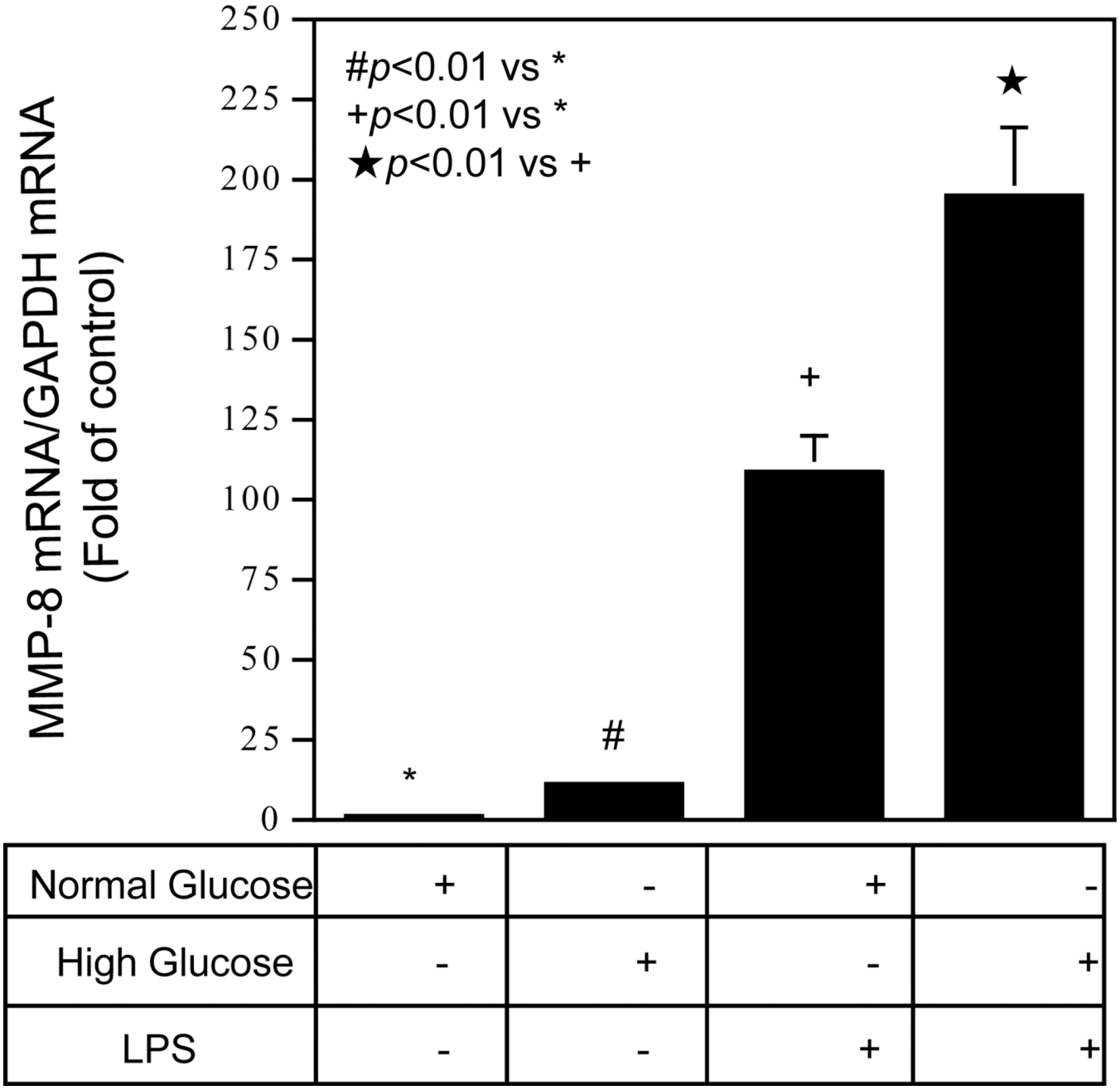

Figure 3.
The effect of high glucose on MMP-8 expression by U937 mononuclear cells exposed to LPS. U937 cells pre-exposed to normal (5 mM) or high (25 mM) of glucose for one week before treatment with or without 100 ng/ml of LPS for 24 h. After the treatment, RNA was isolated from cells and subjected to real-time PCR to detect MMP-8 mRNA.

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 (periodontal sites from which samples were collected)		7 (7)	15 (15)	10 (10)
Age (mean±SD)		53 ± 19	48 ± 13	55 ± 13
Gender	Female	6	6	4
	Male	1	9	6
	Female/Male	6.0	0.67	0.67
Race & Ethnicity	White (non-Hispanic)	7	9	6
	Black (non-Hispanic)	0	5	4
	Hispanic	0	1	0
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

NE: not examined. The data presented are mean ± SD.