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Evaluation of GCF MMP-1, MMP-8, TGF- β 1, PDGF-AB, and VEGF levels in periodontally healthy smokers

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Background/aim: The effect of smoking on inflammatory biomarkers in gingival crevicular fluid (GCF) is well established in the presence of periodontal inflammation. However, it is not clear if smoking has an influence on matrix metalloproteinase (MMP) and growth factor levels in the GCF of periodontally healthy subjects. The aim of this study was to investigate GCF levels of MMP-1, MMP-8, transforming growth factor (TGF)- β 1, platelet-derived growth factor (PDGF)-AB, and vascular endothelial growth factor (VEGF) in smoking versus nonsmoking periodontally healthy subjects.

Materials and methods: Thirty-two periodontally healthy subjects were included in this study. Probing depths, bleeding on probing, and plaque index was assessed. GCF levels of MMP-1, MMP-8, TGF- β 1, PDGF-AB, and VEGF were analyzed by enzyme-linked immunosorbent assay.

Results: No significant differences were observed in the distribution of demographic data between study groups. GCF total amount of PDGF-AB was significantly lower in smokers compared to nonsmokers ($P = 0.014$). Total amount of GCF MMP-1, MMP-8, TGF- β 1, and VEGF levels were similar in both study groups ($P = 0.022$).

Conclusion: Smoking has the effect of decreasing GCF PDGF-AB while it does not affect GCF MMP-1, MMP-8, TGF- β 1, and VEGF in periodontally healthy subjects. Since increased levels of these molecules are involved in periodontal breakdown, our findings may emphasize the importance for maintenance of periodontal health in smokers.

Key words: Smoking, gingival crevicular fluid, matrix metalloproteinases, cytokines

1. Introduction

Smoking is one of the risk factors for periodontal disease, and it affects host defense mechanisms by impairing neutrophil function, antibody production, fibroblast activities, and inflammatory mediator production (1–7). Several studies have demonstrated that smokers are more likely to have a 2- to 8-fold increased risk for periodontitis than nonsmokers (2,8,9). Cross-sectional studies have shown increased prevalence and severity of periodontitis, greater marginal bone loss, deeper periodontal pockets, and more severe attachment loss among smokers (10,11). Recently it has been stated that smoking is considered an important risk factor for periodontitis not only in adults but also in younger subjects (12).

Matrix metalloproteinases (MMPs) are a superfamily of proteases, and they have an important role in the degradation and remodeling of the extracellular matrix (13–16). MMP activity plays important roles in the pathogenic mechanism associated with periodontal soft

and hard tissue destruction (16). MMP-1 is expressed from various cells depending on the status of the tissue turnover cycle, through healing by keratinocytes, fibroblasts, and inflammation caused by defense cells. MMP-8 can be activated by bacterial products or host inflammatory mediators, and it is mainly secreted by neutrophils (17,18). Toxic components of cigarette smoke, especially nicotine, have negative effects on both the periodontal and systemic health status (19). It has been shown that MMP-1 expression increases in gingival fibroblasts cultured with nicotine (20). Nicotine treatment in human periodontal ligament cells also led to the upregulation of MMP-1 and MMP-8 (21).

Growth factors mediate many events associated with the turnover, repair, and regeneration of periodontal tissues (22). Transforming growth factor (TGF)- β 1 plays a role in wound healing, and it has both antiinflammatory and proinflammatory functions that might change the local microbial environment (23,24). TGF- β 1 acts as an

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inhibitor of MMP-1 activity, and this antiinflammatory function of TGF- β 1 proves that it mediates tight control of the MMP/tissue inhibitor of MMP equilibrium (25). Platelet-derived growth factor (PDGF) supports healing in the periodontal soft tissues, due to its chemotactic effect on fibroblasts. PDGF not only induces collagen synthesis but also stimulates fibroblasts to synthesize the proteoglycans (25). PDGF is also an important growth factor for maturation and remodeling of newly formed blood vessels (26). Vascular endothelial growth factor (VEGF) has been detected within vascular endothelial cells, neutrophils, plasma cells, and junctional, pocket, and gingival epithelium. VEGF initiates endothelial cell proliferation and blood vessel formation (27). Nicotine has the ability of changing gingival fibroblast function and growth factor production, and it has a negative impact upon the wound healing cascade (23). It has been demonstrated that nicotine induces a significant increase in the release of the PDGF form PDGF-BB from endothelial cells and the exposure to cigarette smoke inhibits VEGF-induced cellular migration in endothelial cells (28,29).

Tissue-degrading enzymes, cytokines, and growth factors have been assessed in clinical studies in an attempt to investigate the effect of smoking on inflammatory markers in GCF (23,30,31). Smoking subjects with chronic periodontitis exhibited higher levels of TGF- β 1 in GCF compared to nonsmoking patients with chronic periodontitis (23). It was demonstrated that inflamed periodontal tissues from smokers with chronic periodontitis had increased MMP-8 expression compared to those of nonsmokers (32). To the best of our knowledge, no studies have investigated the effect of smoking on these molecules in the GCF of periodontally healthy subjects. Since it is known that smoking has an impact on immune cells during inflammatory response, it might be hypothesized that smoking affects the levels of GCF MMPs and growth factors in subjects with healthy periodontium. In order to investigate if smoking has an effect on products of immune cells in periodontal healthy subjects exposed to smoking, the present study aimed to evaluate GCF levels of MMP-1, MMP-8, TGF- β 1, PDGF-AB, and VEGF in periodontally healthy smoking subjects and to compare these MMP and growth factor levels in the GCF between periodontally healthy smokers and nonsmokers.

2. Materials and methods

2.1. Study population

A total of 32 periodontally healthy subjects were recruited for the present cross-sectional study. These subjects were divided into 2 groups as smokers ($n = 16$, 6 females and 10 males, age range: 19 to 25 years) and nonsmokers ($n = 16$, 8 females and 8 males, age range: 19 to 27 years). The

subjects were undergraduate students of the Ege University School of Dentistry and they were included in the study during the period between May 2013 and September 2013 in the Ege University School of Dentistry, Department of Periodontology. The purpose and procedures were fully explained to all participants prior to participation and written informed consent was obtained from all those wishing to participate, in accordance with the Helsinki Declaration. This study was reviewed and approved by the Ethics Committee of the Ege University School of Medicine.

2.2. Clinical examination

Measurements of probing depth (PD) and clinical attachment levels were assessed at 6 sites per tooth with a calibrated Williams periodontal probe (Hu-Friedy, Chicago, IL, USA) in the whole mouth, excluding third molars. Bleeding on probing (33) and the amount of plaque accumulation (34) were also evaluated. All clinical parameters were measured 1 day before GCF sampling. Clinical examinations were performed by an experienced and calibrated examiner (GE). The intraexaminer reproducibility for PD measurements was evaluated, and the interclass correlation coefficient was 88%.

2.3. Inclusion and exclusion criteria

The following criteria were used to include the subjects in the study: presence of at least 20 teeth and no presence of sites with PD of >3 mm, clinical attachment level of >2 mm, $>20\%$ of sites presenting bleeding on probing, and no radiographic evidence of alveolar bone loss.

For all subjects, the exclusion criteria were as follows: presence of any health condition affecting their systemic status such as diabetes mellitus, immunologic disorders, human immunodeficiency virus infections, and hepatitis; presence of pregnancy or lactation; medication with oral contraceptive drugs; or history of any antibiotics within the last 3 months and any periodontal treatment within 6 months.

2.4. Smoking status

Smoking status of the subjects was determined in accordance with their self-reports. Subjects who had >10 cigarettes/day for at least 5 years were determined as smokers (35). Former smokers were excluded. Subjects who had never smoked constituted the nonsmoker group.

2.5. GCF sampling

GCF samples were obtained from buccal aspects of interproximal sites in single-rooted teeth in each subject. Paper strips (Periopaper, ProFlow Inc., Amityville, NY, USA) were carefully inserted 1 mm into the crevice for 30 s (36). Care was taken to avoid mechanical trauma. The absorbed GCF volume of each strip was estimated by an electronic device (Periotron 8000; Oraflow Inc., Plainview, NY, USA.). Each strip was then placed into one

polypropylene tube before freezing at -40°C . The readings from the electronic device were converted to an actual volume (μL) by reference to the standard curve. All GCF samples were stored at -40°C until the laboratory analysis.

2.6. Analysis of GCF levels of MMPs and growth factors

GCF samples were analyzed by appropriate enzyme-linked immunosorbent assay (ELISA) kits (Boster Biological Technology, Ltd., Fremont, CA, USA). Before the quantitation of MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB, the GCF samples were eluted from the strips by placing them in 2 mL of phosphate buffered saline and shaking the tubes on an ELISA plate shaker for 45 min. GCF MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB levels were assayed with commercially available ELISA kits according to the manufacturers' instructions. The minimum detectable limits of MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB were 156 pg/mL, 156 pg/mL, 15.6 pg/mL, 31.2 pg/mL, and 31.2 pg/mL, respectively. The levels of MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB in each sample were calculated based on the dilutions, and the results were expressed as the total amount and the concentration in GCF sample. A calculation of the concentration data for each growth factor and enzyme was performed by dividing the amount of each mediator by the volume of the sample.

2.7. Statistical analysis

Considering a difference of 50% in mean GCF levels of the biochemical markers and assuming standard deviations to be a maximum 80% of the mean values and accepting a power of 90%, with a P-value of 5% in smoker and nonsmoker groups, the minimum sample size was calculated. All data analyses were performed using a statistical package (SPSS 14.0, SPSS Inc., Chicago, IL, USA). A chi-square test was used to determine differences in sex and smoking state between the study groups. Independent sample Student t-tests were applied for comparison of age between smokers and nonsmokers. A normality test was performed to evaluate the distribution

of clinical periodontal parameters and levels of growth factors, MMP-1, and MMP-8. Growth factor and MMP levels were compared by Mann-Whitney U test between study groups.

3. Results

3.1. Demographic and clinical findings

Power calculation analyses revealed that the minimum required sample size was 16 subjects for each group. Demographic data, PD values, and smoking status of the subjects are presented in Table 1. No significant differences were found in the distribution of age and sex between smokers and nonsmokers ($P = 0.628$). The mean PD scores in sampling sites were not statistically different between smoker and nonsmoker groups ($P = 0.559$) (Table 1).

3.2. Laboratory findings

Table 2 represents the distribution of GCF volume and total amounts of GCF MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB in the study groups. In the present study, a certain amount of GCF volume was observed from all samples of smoking and nonsmoking subjects. The median value of GCF volume in all sites for smokers was $0.14 \pm 0.6 \mu\text{L}$ and $0.16 \pm 0.7 \mu\text{L}$ for nonsmokers. No statistically significant differences were noted in the GCF volumes obtained from smokers and nonsmokers ($P = 0.734$). There were no significant differences in GCF total amounts of MMP-1 and MMP-8 between smoking and nonsmoking groups ($P = 0.426$, $P = 0.160$, respectively). The GCF TGF- β 1 and VEGF total amounts were also similar in both study groups ($P = 0.221$, $P = 0.169$, respectively). GCF PDGF-AB total amounts in smokers were significantly lower than those of the nonsmokers ($P = 0.014$).

When the data were expressed as concentrations, smoking participants had lower concentrations of GCF MMP-1 and MMP-8 compared to nonsmoking participants, but the differences were not statistically significant ($P = 0.128$, $P = 0.069$ respectively). GCF TGF- β 1 and VEGF concentrations were not statistically different

Table 1. Demographic data of participants and clinical periodontal parameters of sampling sites.

	Smokers (N = 16)	Nonsmokers (N = 16)
Age, years, mean \pm SD	22.4 \pm 1.3	22.7 \pm 1.9
Sex, M:F	8:8	6:10
Cigarettes per day, N		
10–20	5	
\geq 20	11	
Smoking duration, years, mean \pm SD	6.5 \pm 1.41	
PD, mm, mean \pm SD	1.94 \pm 0.29	1.89 \pm 0.18

Table 2. The distribution of GCF volume and total amounts of GCF MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB in the study groups.

	Smokers	Nonsmokers
GCF volume, μ L	0.14 \pm 0.6	0.16 \pm 0.7
MMP-1, pg/sample	214.31 (192.14–364.58)	216.78 (192.14–256.19)
MMP-8, ng/sample	4.45 (2.66–5.81)	4.96 (4.23–6.59)
TGF- β 1, ng/sample	0.53 (0.26–0.97)	0.52 (0.27–1.07)
VEGF, ng/sample	0.91 (0.1–1.68)	0.91 (0.47–1.85)
PDGF-AB, pg/sample	205.91* (165.69–468.34)	280.14 (223.15–469.29)

*Significant difference between groups, $P = 0.014$. Data are given as median (range) or mean \pm SD.

between study groups ($P = 0.187$, $P = 0.132$, respectively). However, the mean PDGF-AB concentration in the smoker group was significantly lower than that of the nonsmoker group ($P = 0.022$; Table 3).

4. Discussion

This cross-sectional study evaluated GCF MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB levels in smoking and nonsmoking periodontally healthy subjects. Our results demonstrated that the total amounts of GCF PDGF-AB were significantly lower in periodontally healthy smokers than those of nonsmokers. However, the total amounts of MMP-1, MMP-8, TGF- β 1, and VEGF in GCF did not differ between study groups.

The volume and flow rate of GCF depends on the degree of inflammation at the sampling sites (37). It has been stated that GCF fluid flow rate is lower in healthy sites than in inflamed areas (38,39). Since GCF volume is very small, especially in healthy sites, reporting GCF data by total amount is more appropriate than expressing them in concentration (36,40). Lamster (40) reported that the total amount of GCF data is more sensitive than expressing it as

a concentration. Therefore, the results were considered as total amount instead of concentration in the present study.

Previous studies have shown that GCF levels of MMP-8 were similar in both smoking and nonsmoking chronic periodontitis patients (31,41,42). However, to our knowledge, there are no studies examining GCF levels of MMP-1 in smoking and nonsmoking periodontitis subjects. On the other hand, there are few studies that evaluated MMP-1 and MMP-8 levels in serum or gingival tissues of periodontally healthy smoking and nonsmoking subjects (35,43). Mouzakiti et al. (43) investigated the expression of MMP-1 and MMP-8 in gingival tissues of subjects with healthy periodontium. They found that the expression of MMP-1 and MMP-8 did not differ between smokers and nonsmokers (39). Özçaka et al. (35) reported that there was no significant difference between serum MMP-8 concentrations of periodontally healthy smokers and nonsmokers. No significant differences were found in GCF levels of MMP-1 and MMP-8 between smoking and nonsmoking periodontally healthy subjects in the present study, which was consistent with previous studies (35–43). Taken together, GCF levels of MMP-1 and MMP-8 do not

Table 3. The distribution of concentrations of GCF MMP-1, MMP-8, TGF- β 1, VEGF, and PDGF-AB in the study groups.

	Smokers	Nonsmokers
MMP-1, ng/ μ L	1.53 (0.64–2.41)	2.17 (0.9–3.84)
MMP-8, ng/ μ L	33.57 (10.92–58.22)	57.65 (18.56–92.57)
TGF- β 1, ng/ μ L	3.49 (1.16–9.24)	4.51 (1.96–9.93)
VEGF, ng/ μ L	5.00 (0.86–16.0)	7.81 (3.39–17.2)
PDGF-AB, ng/ μ L	1.86* (0.57–2.86)	2.38 (1.05–5.23)

*Significant difference between groups, $P = 0.022$. Data are given as median (range).

seem to be affected by smoking, neither in periodontally healthy subjects nor in patients with chronic periodontitis. Further information may be achieved by investigating GCF levels of tissue inhibitor of MMP-1 and MMP-8 in addition to MMP-1 and MMP-8.

Stein et al. (23) investigated GCF TGF- β 1 levels in smoking and nonsmoking chronic periodontitis patients before and after periodontal therapy. At baseline they found that smokers exhibited a statistically higher concentration of GCF TGF- β 1 compared to nonsmokers (23). After initial therapy, elevated levels of GCF TGF- β 1 were found in smokers compared to nonsmokers, but the difference was not statistically significant (23). Similarly, smoking and nonsmoking periodontally healthy subjects had comparable levels of GCF TGF- β 1 in the present study. Collectively, this previous report and the findings of the present study might suggest that GCF TGF- β 1 levels are dynamic depending on local inflammatory conditions and host response traits. Therefore, the results of the present study indicate that smoking does not influence GCF TGF- β 1 levels in periodontally healthy smokers.

Smoking causes hypoxia in the tissues, and it is associated with periodontal tissue breakdown (44). Hypoxia stimulates the expression of several cytokines by various cells (44). For instance, VEGF expression is regulated by the oxygen concentration of the tissues (45). Motohira et al. (46) found significantly higher extracellular VEGF concentrations in a hypoxia group compared to a normoxia group in human periodontal ligament cells (46). However, it was stated that the concentration of VEGF in serum was similar in both smoking and nonsmoking pregnant women (47). Similarly, in our study, there were no significant differences in GCF levels of VEGF between smoker and nonsmoker groups. Contrary to our VEGF data, smokers had significantly lower levels of PDGF-AB than those of nonsmokers in the present study. It is known that VEGF and PDGF have a role in the remodeling of newly formed blood vessels (22,26). To our knowledge,

there is no study that has investigated the GCF VEGF and PDGF-AB levels between smoking and nonsmoking subjects with periodontal health. We suggest that rather than VEGF levels, the lower GCF levels of PDGF-AB in smokers might be caused by the decreased vascularization of the gingival tissues. Further studies are needed to evaluate the relationships between GCF VEGF, PDGF-AB, and smoking in periodontal healthy subjects.

Our study had some limitations. The number of participants enrolled in the study was relatively small to arrange in significant subgroups with respect to smoking habits. To date there is still no consensus on how to best classify smokers and there are no standard definitions of smoking, which has led to variability in the level of smoking considered 'light, moderate, or heavy' in different studies (48). In our study, additionally, the participants were young and their duration of smoking was a short period of time. For this reason, we could not evaluate the effect of low, moderate, or heavy smoking on GCF MMP-1, MMP-8, TGF- β 1, PDGF-AB, and VEGF levels. Furthermore, the smoking habits of our participants were self-reported, and measurements of cotinine in the saliva would be much more reliable. Therefore, further studies including assessment of cotinine levels are required in a larger sample size.

Smoking might contribute to periodontal destruction by altering the GCF levels of these enzymes and growth factors in the presence of periodontal disease. To the best of our knowledge, this is the first study investigating the GCF levels of MMP-1, MMP-8, and growth factors in smoking versus nonsmoking periodontally healthy subjects. Within the limitations of the present study, smoking does not seem to affect the GCF levels of MMP-1, MMP-8, VEGF, and TGF- β 1 in periodontally healthy subjects, excluding PDGF-AB. Since increased levels of these molecules are involved in periodontal breakdown, results of the present study might emphasize the importance of periodontal health in smokers.

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