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ORIGINAL ARTICLE

Vascular endothelial growth factor (VEGF) levels of gingiva and gingival crevicular fluid in diabetic and systemically healthy periodontitis patients

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Abstract It has been demonstrated that diabetes mellitus (DM) may have an inductive effect on the vascular endothelial growth factor (VEGF) levels of periodontium during periodontal disease. The aim of this study is to confirm this phenomenon, investigating whether it is also valid for diabetic periodontitis patients under good metabolic control. Sixteen type II DM patients, all with a glycosylated hemoglobin (HbA1c) value less than 7 (test), and 15 systemically healthy (control) chronic periodontitis patients were included in the study. The VEGF concentrations in the gingival supernatants and gingival crevicular fluid (GCF) samples of the study groups were measured by enzyme-linked immunosorbent assay. The data were analyzed by Student's *t* test in statistical means. The VEGF levels were significantly higher in the gingival supernatants of the test group (55.89±8.11 pg/ml) than that of the control group (24.81±2.04 pg/ml; $p<0.01$). However, there was no statistically significant difference in the VEGF levels of GCF between the study groups (38.96±4.89 pg/ml in the test and 32.20±4.02 pg/ml in the control group; $p>0.05$). Our study confirms that DM affects the VEGF levels of periodontal soft tissues in periodontal disease, and our results also suggest that this effect may not be influenced by the metabolic control of DM.

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Introduction

Vascular endothelial growth factor (VEGF) is a 45-kd homodimeric glycoprotein with potent vascular permeability and angiogenic effects [28, 30, 43, 44]. VEGF mainly causes these effects by endothelial cell proliferation, secretion of proteolytic enzymes, chemotaxis, and migration [15, 16]. It induces the permeability of fluids and proteins 50,000 times more than histamine [12]. VEGF has been isolated from many tissues, healing wounds, and pathological conditions such as hypersensitivity reactions and rheumatoid arthritis [7–9]; therefore, it has been regarded as a potent regulator of inflammation.

Studies about periodontitis revealed that there was a relationship between an increased number of blood vessels and progression of the disease with evidence of marked thickening of vascular basement membranes, especially affecting capillaries and venules [40, 50, 51]. The increased transport of inflammatory cells, nutrients and oxygen through inflammation caused by angiogenesis in periodontitis, can enhance the severity of the inflammation [24]. In other words, increased endothelial surface can cause an increased transition of different cytokines, adhesion molecules, and other factors of inflammation. The increased concentration of VEGF in periodontitis [4, 10, 22, 24, 49] may be one of the reasons of the increased vascularization and permeability, and it may be regarded as a marker for severity of periodontitis.

There are numerous reports that have investigated the relationship between diabetes mellitus (DM) and periodon-

titis [17, 19, 26, 27, 29, 34–38]. These investigations revealed the contribution of DM to periodontitis and to the severity of periodontitis via its effects on vasculature, inflammatory and immune response, alterations in collagen synthesis, and genetic predisposition. It has been demonstrated that DM results in increased expression of VEGF in numerous tissues as a response to both hyperglycemia and tissue ischemia [31]. Studies about the mechanism and molecular involvement in the pathogenesis of diabetic microvasculopathy have suggested that VEGF has a major role in microangiopathy and causes an increased angiogenic response of DM [1, 14, 18, 23, 32]. Therefore, important diabetic microvascular complications related to VEGF include tissue ischemia, angiogenesis and permeability in many organs, and alteration in blood glucose levels [1, 2, 11, 21, 31, 39, 45].

Although the DM–VEGF relationship in periodontitis has been demonstrated in various investigations, there are limited studies that have investigated the role of DM on theVEGF concentration in periodontitis patients [10, 49]. Moreover, in these studies, quantification of VEGF levels in gingiva were done nonparametrically and somewhat subjectively, and only one study also utilized the VEGF concentration in gingival crevicular fluid (GCF) as an assessment parameter in the study groups. The common feature of these studies is a test group composed of diabetic patients under poor metabolic control. Hence, the objectives of our study were: (1) to confirm the effect of DM on the VEGF concentrations of both gingiva and GCF in periodontitis and (2) to investigate whether this phenomenon is also valid for diabetic periodontitis patients under good metabolic control.

Materials and methods

Study population

Thirty-one male, nonsmoking chronic periodontitis patients (with a mean age of 50.58±1.54 years) referred to our clinic for treatment participated in the study. The test group patients included 16 of these individuals who were prediagnosed as type II DM (without additional systemic disorders) with a disease history of 2–5 years and good metabolic control, demonstrating a glycosylated hemoglobin (HbA1c) value less than 7 (mean value of 6.59±0.23) since the DM treatment period. The metabolic control regimen of all patients was being performed by physicians with diet or oral hypoglycemics and without insulin replacement. The other 15 systemically healthy patients were used as the positive control group of the study population. All patients were preinformed about the clinical trial, and their consents were collected before the study procedures. The study was carried out according to "Helsinki Declaration of 1975," as revised in 2000.

Clinical procedures

Full-mouth Silness-Löe Plaque Index (PI), Löe-Silness Gingival Index (GI), pocket depth (PD), gingival recession (GR), and clinical attachment loss (AL) scores were measured in the patients. PI and GI measurements were performed at four sites (mesio-/disto-buccal/lingual) per tooth whereas PD, GR, and AL calculations were performed at six sites (mesio-/mid-/disto-buccal/lingual) per tooth. Both gingivally and periodontally active teeth were included in the study design. The greater than or equal to 2 GI score was utilized for the standardization of the gingivally active sites, and the sites demonstrating greater than 5 mm PD with bleeding on probing were accepted to have active periodontitis. Only one tooth among these teeth and one site in the teeth were selected per patient. The site with greater than 5 mm AL and greater than or equal to 2 GI score in the selected tooth was utilized for the test procedures. In the case of several sites fulfilling these criteria for a tooth, the region demonstrating the deepest AL value was chosen for the sample collection. Only the measurements of the selected site were recorded for the later procedures. All of the measurements were done by one investigator. PD, GR, and AL were recorded by an automated periodontal probe (Florida Probe Version 3.0, Florida Probe, Gainsville, FL, USA) applying constant force (20 g) during the examinations. Open-flap debridment was planned for the study regions, and the gingival and GCF samples were collected before the flap operations.

Sample collection

Sixteen gingival and GCF samples in the test group and 15 gingival and GCF samples in the control group were used in the study. Biopsies including the whole gingival structures approximately in 2×2 mm^2 dimensions were taken from the predetermined sites in the teeth, and they were stored in 0.1 M sucrose solution at −70°C until the laboratory procedures. GCF samples were obtained from the same sites before the gingival biopsy collection. The teeth were isolated by cotton rolls, gently removing any supragingival depositions, and the sampling sites were dried with cotton pledgets. Afterwards, two paper strips (Periopaper®, Ora Flow, New York, USA) were placed into the gingival crevicular orifice of each measurement site for 30 s, and GCF was collected via these strips. After volume calculation by Periotron® 8000 (Pro Flow, Amityville, NY, USA), the strips (two papers for each measured site) were stored at −70°C for the laboratory procedures.

Laboratory procedures

Biochemical analysis of the gingival biopsies

Gingival samples were blotted, weighed in a microbalance, and then placed into a sufficient volume of phosphate-buffered solution (PBS; 4°C, pH 7.0) containing a protease inhibitor (5 μg/ml aprotinin and 1 mM ethylenediamine tetraacetic acid) to assure a dilution of 10 mg tissue/ml PBS+protease inhibitor solution. First, the samples were homogenized four times at 8,500 rpm for 30 s with 10 s intervals and then the homogenate was processed twice with freeze–thawing procedures, followed by sonication three times at 4–5 μm for 30 s with 10 s intervals. After centrifugation at 15,000 rpm for 16 min, supernatants were collected for VEGF analysis. All these supernatant preparation processes were carried out on ice-medium approximately at 0–4°C. Enzyme-linked immunosorbent assay (ELISA) was utilized for the detection of VEGF concentrations using an AC 300 VEGF Kit (Accucyte VEGF EIA Lot # AV211-DAC4302AQ, Cyt Immune Sciences, Maryland, USA).

Biochemical analysis of the GCF samples

GCF elution from the periopapers was performed by a modification of the protocol described by Curtis et al. [13]. Briefly, the strip pairs sampled from each site were put into a 400 μl Eppendorf tube containing 100 μl of 2% bovine serum albumin in PBS and then incubated for 60 min at 4°C. This tube was placed into a 1.5 ml microcentrifuge tube, and centrifugation was carried out in 10,000 g for 5 min at 4°C, after creating a hole on the bottom of the Eppendorf tube to provide the elution of GCF into the microcentrifuge tube. The procedure was repeated twice, and the collected 200 μl samples were stored at −70°C. VEGF detection was performed by ELISA with the same Kit used for the GCF samples.

Statistical analysis

Statistical analysis was performed using a statistical software package (SPSS 12.0 Software Package Programme, Chicago, USA). The results were expressed as means ± standard deviations. Data were firstly analyzed for the normal distribution with *Shapiro-Wilk* test. Mean VEGF levels of GCF and gingival samples between the groups were compared with Student's *t* test.

Results

The mean PI-, GI-, AL-, and GCF-volume values were not statistically different between the test and control groups ($p>0.05$; Table 1). The VEGF concentrations of gingival supernatants in the test group were higher than in the control group (Fig. 1). The mean gingival VEGF level was 55.89±8.11 pg/ml in the test and was 24.81±2.04 pg/ml in the control group, and the difference between the groups was statistically significant ($p<0.01$). The mean VEGF level of GCF in the test group was 38.96±4.89 pg/ml, and the mean VEGF level of GCF in the control group was 32.20±4.02 pg/ml. The difference in the VEGF levels of GCF was not statistically significant between the groups (Fig. 2; $p>0.05$).

Discussion

The role of DM in periodontal disease, particularly in the inflammatory and immune response, has been documented excessively. To date, it is widely accepted that DM aggravates both severity and progression of periodontal disease [14, 20, 27, 42, 47, 48], and poor metabolic control of DM is associated with the severity of periodontitis [25, 33, 37, 41, 47]. It may be stated that microvascular changes because of DM may cause this interaction between DM and periodontal disease, and thus, VEGF may have an essential role in this phenomenon. In the present study, we aimed to assess and compare the VEGF levels of gingival and GCF samples taken from both diabetic periodontitis patients under good metabolic control and nondiabetic periodontitis patients.

One of the differences of our study from the previous similar investigations was the standardization of the test and control group subjects by means of their gender, smoking status, and periodontally active sites. Male patients were selected to eliminate the possible alterations of hormonal conditions seen in women such as in menstruation cycle

Table 1 Clinical measurements in the study groups

	PI (mean ± SD)	GI (mean ± SD)	AL (mm) (mean ± SD)	GCF volume (μl) (mean ± SD)	
Test group (N=16)	1.70±0.39	2.14±0.12	6.10±0.80	51.0±19.50	$p>0.05$[a]
Control group (N=15)	1.93±0.71	2.15±0.14	5.80±0.90	48.90±22.23	

DM diabetes mellitus, *GI* gingival index (Löe and Silness), *PI* plaque index (Silness and Löe), *AL* attachment loss, *GCF* gingival crevicular fluid, *SD* standard deviation

[a] Student's *t* test

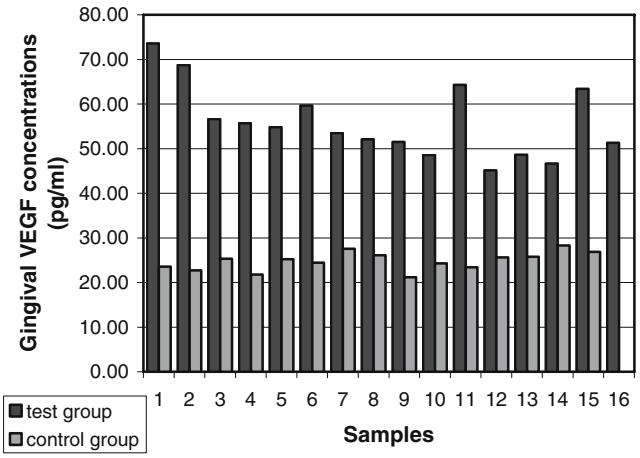

Fig. 1 Gingival VEGF concentrations of the test (diabetic periodontitis) and control (systemically healthy periodontitis) groups

and/or menopausal term. Because of the possible effects of smoking on gingival microcirculation and increased inflammatory cytokine production in GCF, which may aggravate periodontal disease [3, 5, 6], smokers were excluded from the study population. Gingivally active sites were also included in the study regions and therefore, GI (Löe & Silness) was utilized for the standardization of the sites in addition to active periodontitis identification. The other difference was the quantification of VEGF concentrations of gingival tissue samples by ELISA, unlike the previous studies in which immunohistochemistry was used for VEGF detection in gingiva nonparametrically, to establish more objective data for the parametric evaluations.

The VEGF levels in the tissue samples of the diabetic patients were found to be higher than those of nondiabetic subjects. These results were in agreement with the previous investigations. Ünlü et al. [49], in an immunohistochemical study, reported higher VEGF staining in the gingival tissue samples of the diabetic patients with periodontitis. Güneri et al. [22] demonstrated that DM might have an additive effect on the VEGF levels of both healthy and diseased gingival tissues. All of these results suggest that DM may

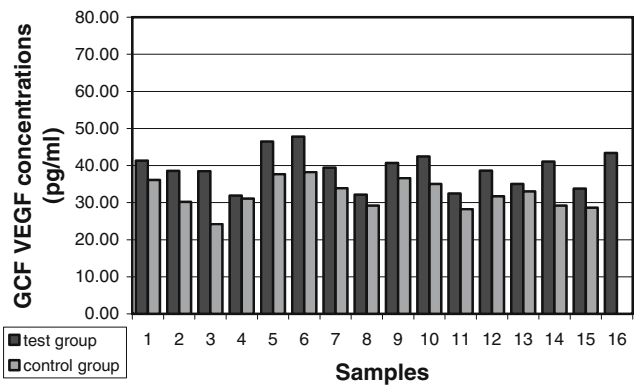

Fig. 2 VEGF concentrations in the GCF of the test (diabetic periodontitis) and control (systemically healthy periodontitis) groups

lead to an increased expression of angiogenic growth factors in periodontal soft tissues, as well as numerous other tissues, because of its microvascular complications. These findings may also propose that the putative effect of DM on VEGF levels may be considered as one of the diabetic factors to designate the severity of periodontal disease with its inflammatory features.

Our test group subjects included metabolically well-controlled patients (HbA1c value less than 7), and our results suggested that DM might have increased the VEGF levels of periodontal tissues even under a good metabolic control condition. In the two previous studies by Ünlü et al. [49] and Güneri et al. [22], the study population of the diabetic group was however composed of metabolically poorly controlled patients (HbA1c value greater than 8). Hence, this finding is one of the first data that reveal that DM may influence the VEGF levels in the tissues, independent of its metabolic control. Despite the consensus that DM may be an important modifying factor in the severity of periodontal disease and particularly in the response to periodontal treatment unless it is metabolically controlled [41, 46, 49–51], this may not be valid for the effect of DM on VEGF levels, at least in periodontal soft tissues. Although statistically insignificant, the severity of periodontitis in our diabetic group in which more AL and GCF volume were observed than that of the periodontitis group alone may also support this conclusion. Again, this finding may be of clinical interest, as it suggests that metabolically well-controlled diabetics and systemically healthy individuals may not have the same susceptibility for periodontal disease.

Although it was not statistically significant, one of the interesting results of our study was the slightly higher VEGF concentration of GCF in the diabetic periodontitis group than that of the periodontitis group alone. This was not in agreement with the study of Güneri et al. [22] in which the mean VEGF level of GCF was higher in the periodontitis group than the diabetic periodontitis group. Their GCF findings suggested that the VEGF levels were primarily affected from the periodontal status rather than the systemic condition. Futhermore, higher VEGF concentration in gingiva was correlated with the enhanced binding of VEGF to the tissue receptors that would cause a lower level in GCF [4]. Hence, we propose that, although it is not statistically significant, the difference in our study may be related to: (1) the severity of periodontitis in our diabetic group (evident with more AL and GCF volume) and (2) the possible degenerative changes in immune system and host response because of DM, which may affect the quantity and function of VEGF receptors in the tissue. Further similar investigations are needed for an appropriate conclusion in this phenomenon. In addition, VEGF and VEGF receptors should also be regarded together in these investigations.

In conclusion; within the limits of this study, our results supported previous studies in that DM may be an important modifying factor for VEGF production in periodontal disease. The results also suggest that this modifying effect during inflammation may not be altered even under good metabolic control of DM. However, there are still not enough data about the destructive and progressive role of VEGF in periodontal disease of diabetic patients, particularly in large populations and in different metabolic control levels. Again, further studies that will investigate the relationship between VEGF, inflammation, and DM in periodontal disease should also match with vascularization, vascular permeability, and fluid dynamics of periodontal tissues.

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