Immunohistochemical analysis of TGF-β1 and VEGF in gingival and periodontal tissues: A role of these biomarkers in the pathogenesis of scleroderma and periodontal disease

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Abstract. Periodontal disease is characterized by inflammation and bone loss. The balance between inflammatory mediators and their counter-regulatory molecules may be fundamental for determining the outcome of the immune pathology of periodontal disease. Transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) represent a family of polypeptide proteins involved in the inflammation and regulation of immune responses, especially in rheumatic disease. The relationship between these growth factors and periodontitis has resulted in a new field of osteoimmunology and provides a context for better understanding the pathogenesis of periodontal disease. Therefore, the aim of this study was to compare the protein expression profile of these inflammatory mediators in 90 patients divided in three groups: healthy control, chronic periodontitis and in rheumatic disease, scleroderma. The findings presented here highlight that biomarkers, such as TGF-β1 and VEGF, play a key role in the evolution of the immune response, which in turn influences the outcome of disease establishment.

Introduction

Chronic periodontal disease is an inflammatory condition characterized by a shift in the microbial ecology of subgingival plaque biofilms and the progressive host-mediated destruction of tooth supporting structures (1). It can affect up to 90% of the world wide population: there are close connections between periodontal inflammation and major chronic condition, such as diabetes, heart disease and chronic autoimmune disease (2). The underlying mechanisms causing these pathological conditions are still unclear. Two mechanisms were proposed to explain this progression: bacteria acquire the ability to penetrate the deeper tissues (3), and/or the host response is degraded (4). In particular resident periodontal ligament and gingival fibroblasts have been reported to secrete matrix metalloproteinases (MMPs) and chemoattractants for epithelial cells (5). An important role in the inflammation during periodontal disease seems to also be played from different proteoglycans and glycosaminoglycans, such as syndecan 1 (6,7). The progression of inflammation and periodontal destruction requires a response to a bacterial insult that includes resident cells that sustain signals to trigger the immune response. Host-derived cytokines released upon microbial challenge have significant effects on the immune and inflammatory responses in periodontal disease (8,9); indeed, many cross-sectional studies have explored the production of Th1 and Th2 cytokines in human periodontitis (10,11). A prominent factor in connective tissue remodeling, and inflammatory diseases, is transforming growth factor-β (TGF-β): one of these isoforms, TGF-β1 is a multifunctional cytokine that regulates cell growth, differentiation and matrix production (12). It has potent immunosuppressive activity and downregulates the transcription of other pro-inflammatory cytokines, including interleukin-1, tumor necrosis factor-α and several metalloproteinases (13). TGF-β is a key mediator of tissue fibrosis and may lead to ECM accumulation in pathological states (14), moreover it mediates fibroblast activation, proliferation and signaling in cells cultures (15). The gene polymorphisms of this cytokine have been associated with risk for systemic diseases, including cardiovascular diseases and rheumatoid arthritis which are related to periodontitis (16).

In the development of chronic inflammatory disease, an important role is also played by the vascular endothelial growth

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factor (VEGF), a 45-kDa a homodimeric pro-inflammatory glycoprotein that potently increases microvascular permeability, promotes angiogenesis, and stimulates endothelial cell proliferation, migration, and survival (17); this protein seems to be involved in the onset and progression of gingivitis and periodontitis, mainly promoting the vascular network expansion generally observed in inflammation (18). Studies intended to associate the action of VEGF with the pathogenesis of periodontal disease have reported controversial findings. Nevertheless, VEGF expression is more strongly related to the healing stage of periodontal disease than to the destruction stage of the lesion (19).

In recent years, it has become apparent that patients with rheumatic diseases and periodontitis share common pathogenetic characteristics, such as a pro-inflammatory trait (20). Many reports have shown that periodontal disease is more common in rheumatoid arthritis (21), and that periodontal therapy reduces the severity of rheumatoid arthritis (22).

Systemic sclerosis (SSc) or scleroderma is a rheumatic acquired disorder that typically results in the fibrosis of the skin and internal organs (23). Previous findings indicate that the pathogenesis of this disorder includes inflammation, autoimmune attack, and vascular damage, leading to fibroblast activation (24,25). However, cultured SSc fibroblasts, which are free from such environmental factors, continue to produce the excessive amount of extracellular matrix (ECM) proteins, suggesting that SSc fibroblasts establish a constitutive self-activation system once activated (26).

The etiology of SSc remains uncertain, but one of the major cytokines that may be involved in this process is TGF-β1 (27), and the principal effect of this cytokine on mesenchymal cells is the stimulation of ECM deposition and angiogenesis alterations. We have already studied the distribution, by immunohistochemical analysis, of the major integrin and sarcoglycans subcomplex in bisphosphonate-induced osteonecrosis of the jaw (28), and the distribution of collagen I and IV into the periodontal ligament during orthodontic tooth movement (29) and for the first time, in the literature, we investigated the role of TGF-β1 and VEGF in the gingival tissues in the pathogenesis of rheumatic disease, SSc.

Therefore, the objective of the present study was to immunohistochemically determine the expression and distribution of TGF-β1 and VEGF in the gingival tissues and periodontal ligament of patients with chronic periodontitis (CP) and SSc compared to the healthy control group (CO).

Materials and methods

Patient selection. Ninety patients were enrolled in this cross-sectional study performed from August 2010 and July 2011 at the University of Messina, Messina, Italy.

Thirty patients (5 male, 25 female, mean age 52.4, SD ±8.5), as defined by the American College of Rheumatology classification criteria for SSc were classified as diffuse SSc or limited SSc based on the extent of skin involvement (30). Disease onset was determined by patients' recall of the first non-Raynaud symptom clearly attributable to scleroderma (31). Thirty patients with chronic adult periodontitis (11 male, 19 female, mean age 54, SD ±9.2) based on the criteria defined by the American Academy of Periodontology (32) and 30 healthy control subjects (16 male, 14 female, mean age 48.9, SD ±8.2) were enrolled. Therefore, the patients were divided in: CP, SSc and CO groups.

The protocol was approved by the Ethics and Research Committee of University of Messina, and ethical approval was obtained for the experimental procedures applied in humans, in accordance with the provisions of the World Medical Association's Declaration of Helsinki of 1975, as revised in 2000. All patients included in the study signed an informed consent form. Patients with: diabetes mellitus, liver, kidney, or salivary gland dysfunction, history of alcoholism, a recent history or the presence of other acute or chronic infection, systemic antibiotic treatment or immunosuppressant medication (non SSc groups only) within previous three months, pregnancy and intense physical activity, smoking history or the presence of an oral mucosal inflammatory condition, were excluded from the study. Inclusion criteria included: ≥18 years of age who were in good general health (excluding the case definition) and had ≥18 erupted teeth.

The CP group had >30% of sites with bleeding on probing (BOP), >20% of sites with probing depth (PD) >4 mm, >10% of sites with interproximal clinical attachment level (CAL) >2 mm. The healthy control subjects had <10% sites with BOP <2% of sites with PD >5 mm, no sites with PD >6 mm, <1% of sites with CAL >2 mm and no radiographic bone loss (evident in posterior vertical bitewings films).

Clinical measurements. On each subject, plaque index (PI) (33), PD, clinical attachment level (CAL), community periodontal index of treatment needs (CPITN) (34) and presence of BOP were measured at 6 sites and recorded on each tooth. Every clinical periodontal measurement was performed by one calibrated examiner.

Gingival tissue biopsies. Gingival tissue and periodontal samples were carried out during routine erupted third molar extractions, advanced caries and orthodontic indications for the PD, SSc and healthy control groups. The collection of tissues of 2x2 mm in size were washed with saline solution and fixed in 10% neutral-buffered formalin, transported at 4˚C and processed for immunohistochemistry.

Immunohistochemistry. From each biopsy, 25 sections were prepared. The samples were snap-frozen in liquid nitrogen and 20 μm sections were prepared in a cryostat for their use in a protocol to perform immunofluorescence. Finally, the sections were incubated with primary antibodies. The following primary antibodies were used: anti-TGF-β1 diluted 1:50 (sc146, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), anti-VEGF diluted 1:50 (VG1, Novus Biologicals, Littleton, CO, USA). Primary antibodies were detected using Texas Red-conjugated IgG (Jackson ImmunoResearch Laboratories, Inc.). Slides were finally washed in PBS and sealed with mounting medium.

In the analysis, each specimen was divided into the following three areas to allow quantification of the distribution of cluster designation (CD) marked cells: i) the sulcular epithelium, ii) the middle area (*lamina propria*), and iii) the oral gingival epithelium. The investigations were conduced on 2,250 images by a blinded pathologist who performed the analysis in a blinded manner. The sections were then analyzed

Table I. Clinical characteristics of the patients at baseline (90 patients).

	Healthy control		Chronic periodontitis		Scleroderma	
Clinical measurement	n	%	n	%	n	%
CAL (mm)						
<2.5	24	80.0	-	-	4	13.3
>2.5 to <3.5	6	20.0	2	6.6	10	33.3
≥3.5 to <4.5	-	-	18	60.0	12	40.0
≥4.5	-	-	10	33.3	4	13.3
Median	3		5		4	
CI (95%)	21		44		55	
L-U	2.83-2.91		4.83-5.26		4.25-4.78	
PD (mm)						
<2.5	22	73.3	-	-	5	16.6
≥2.5 to <3.5	8	26.6	5	16.6	9	30.0
≥3.5 to <4.5	-	-	16	53.3	11	36.6
≥4.5	-	-	9	30.0	5	16.6
Median	3		5		5	
CI (95%)	22		5		47	
(L-U)	2.69-2.91		4.7-5.19		4.53-4.98	
PI (value)						
<1	25	83.3	-	-	5	16.6
>1 to <2	4	13.3	21	70.0	15	50.0
≥2 to <3	-		9	30.0	5	16.6
Median	0		1		2	
CI (95%)	20		37		27	
(L-U)	0.28-0.48		1.01-0.36		1.44-1.7	
BOP (value)						
<1	25	83.3	1	3.7	7	23.3
>1 to <2	5	16.6	21	70.0	17	56.6
≥2 to <3	-	-	7	26.3	3	10.0
Median	0		1		1	
CI (95%)	19		27		37	
L-U	0.25-0.44		1.39-1.65		0.91-1.27	
CPITN (value)						
<1	29	96.6	4	13.3	6	20.0
>1 to <2	1	3.3	11	36.6	17	56.6
≥2 to <3	-	-	12	40.0	4	13.3
≥3 to <4	-	-	3	10.0	3	10.0
Median	0		1		3	
CI (95%)	18		33		29	
L-U	0.06-0.24		1.21-1.54		2.57-2.85	

PD, probing depth; CAL, clinical attachment level; PI, plaque index; BOP, bleeding on probing, CPITN, Community Periodontal Index of Treatment Needs; CI, confidence limit; L, lower quartile; U, upper quartile.

and images were acquired using a Zeiss LSM 5 DUO confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). All images were digitalized at a resolution of 8 bits into an array of 2,048x2,048 pixels. Optical sections of fluorescent specimens were obtained using a HeNe laser (wavelength, 543 nm) and an Argon laser (wavelength, 458 nm) at a 1-min 2-sec scanning speed with up to 8 averages; 1.50-μm sections were obtained using a pinhole of 250. Each image was acquired within 62 sec, in order to minimize photodegradation (Adobe Photoshop 7.0; Adobe Systems, Palo Alto, CA, USA).

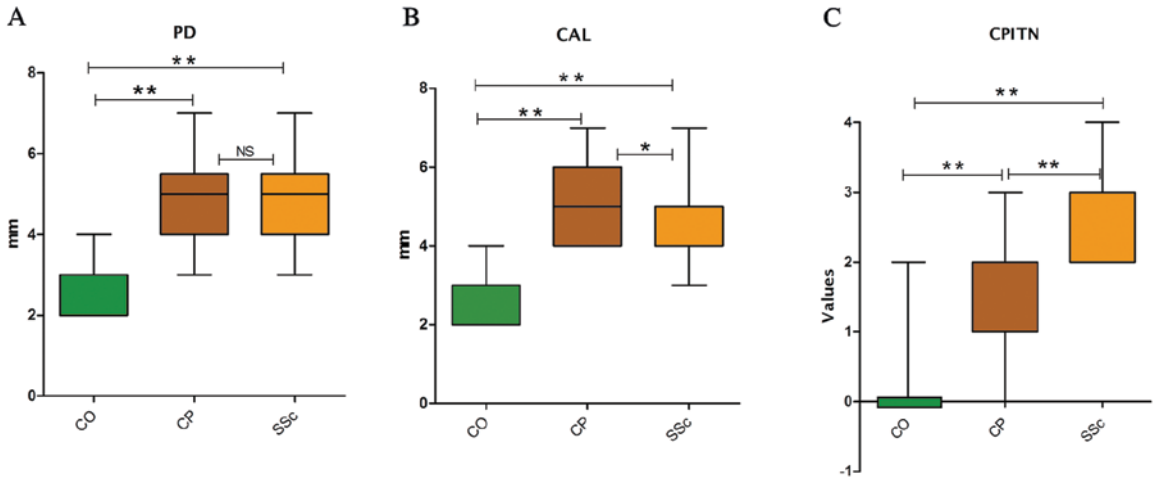

Figure 1. Comparison of clinical parameters between three groups (n=90 patients). Results of (A) probing depth (PD) and (B) clinical attachment level (CAL) are shown as box plots, with median, upper and lower quartiles and max and min values. (C) Community Periodontal Index of Treatment Needs (CPITN) shown as bars with standard deviation (SD) of the values. CO, healthy control group (n=30); CP, chronic periodontitis group (n=30); SSc, scleroderma group (n=30). The medians for PD were 3, 5 and 5 for the CO, CP and SSc groups, respectively. The medians for CAL were 3, 5 and 4 and those for CPITN were 0, 1 and 3 for the CO, CP and SSc groups, respectively. Comparison between the three groups test: $^{*}P\leq0.05$ (two-sided) significant; $^{**}P\leq0.001$ (two-sided) highly significant; NS, not significant. Multiple comparisons: significance is obtained between CP and controls, SSc and controls and CP and SSc. PD, **CP vs. CO; **SSc vs. CO; NS, CP vs. SSc. CAL, **CP vs. CO; **SSc vs. CO; *CP vs. SSc. CPITN, **CP vs. CO; **SSc vs. CO; **CP vs. SSc.

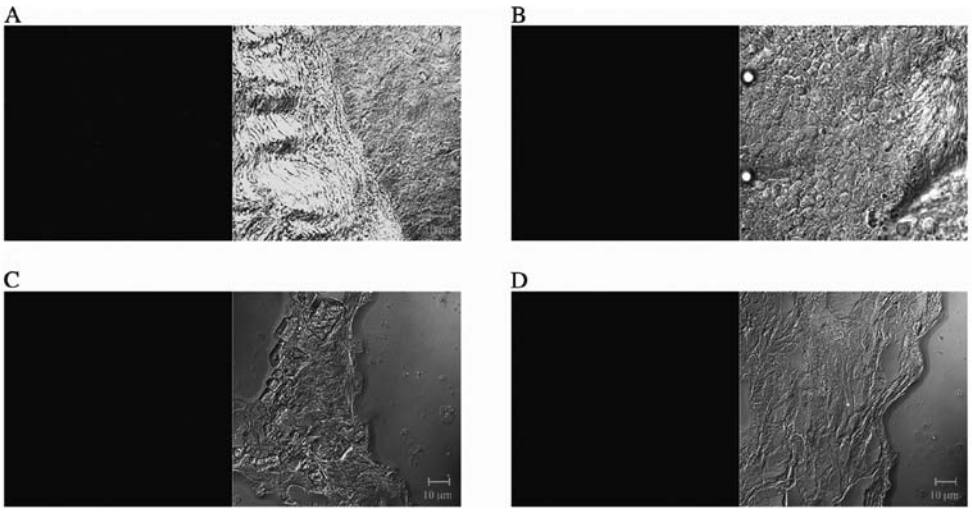

Figure 2. Negative control of gingival and periodontal samples sections and corresponding transmitted (A and C) light immunolabeled with TGF-β1 and (B and D) immunolabeled with VEGF.

Statistical analysis. Frequency distributions, median and standard deviation (SD) values were determined at baseline in each group to describe the clinical parameters (CAL, PD, CPITN, PI and BOP). The Kruskal-Wallis and the Mann-Whitney U tests were carried out when comparing the clinical parameters (CAL, PD and CPITN) in the three independent groups and the Wilcoxon singed rank test was used when comparing three matched-pair groups. The differences were considered statistically significant when P<0.05 (or 5%). The data were analyzed with software Prism (Graphpad Instat, version 5.00; GraphPad Software, San Diego, CA, USA).

Results

A higher CAL (≥4 mm), PD site (≥5 mm) and PD values (<1), was observed in the SSc group compared with the controls (P<0.05); additionally, the BOP values (<1) were significantly higher in the SSc group than the healthy controls (P<0.05) (Fig. 1). Results are presented as the medians, confidence levels, lower and upper (L-U) quartiles (Table I). Higher levels of PI were observed in the CP compared to the CO group. BOP, as a measure of acute periodontal inflammation, was elevated in patients with CP compared to the CO group.

In order to investigate the relationship between gingival biomarker levels and clinical parameters of periodontal disease, we performed immunofluorescence reactions on gingival and periodontal ligament samples obtained from CO, CP and SSc, using antibodies against TGF-β1 and VEGF. First of all, we performed a negative control both on gingival samples with TGF-β1 (Fig. 2A) and VEGF (Fig. 2B) and periodontal ligament samples with TGF-β1 (Fig. 2C) and VEGF (Fig. 2D) using the secondary antibody only. Our results on gingival samples

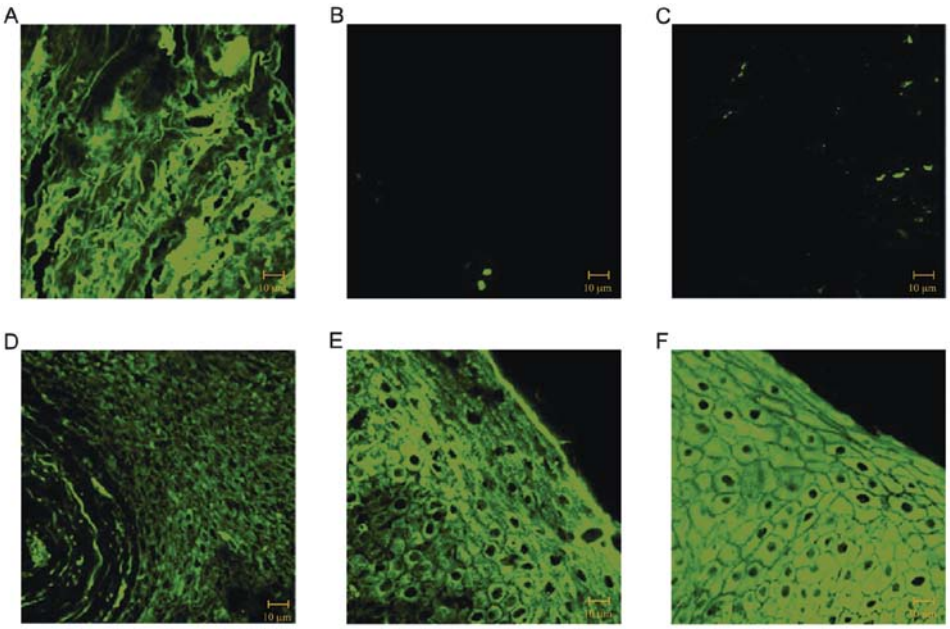

Figure 3. Confocal laser scanning microscopic observations. Gingival samples immunolabeled with (A-C) TGF-β1 and (D-F) VEGF in (A and D) healthy control, (B and E) chronic periodontitis and (C and F) scleroderma. (Scale bar, 10 μm).

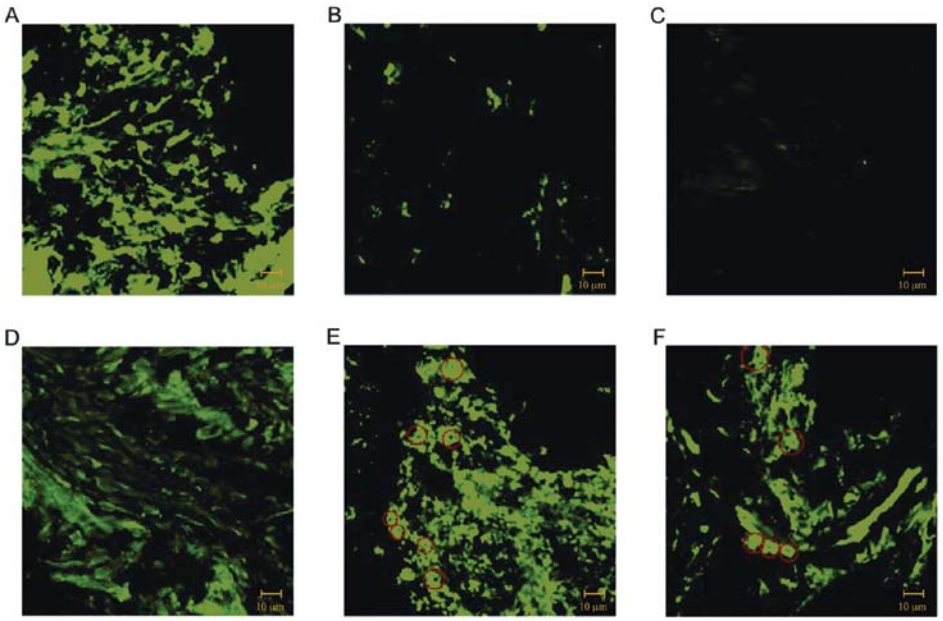

Figure 4. Confocal laser scanning microscopic observation. Periodontal ligament samples immunolabeled with (A-C) TGF-β1 and (D-F) VEGF in (A and D) healthy control, (B and E) chronic periodontitis and (C and F) scleroderma.

clearly showed a normal staining pattern for TGF-β1 in CO (Fig. 3A), whereas the staining was severely reduced in samples of patients with CP (Fig. 3B) and SSc (Fig. 3C). In contrast in immunofluorescence reactions performed using VEGF antibodies, staining patterns showed a higher intensity in CP (Fig. 3E) and SSc (Fig. 3F) than that observed in CO (Fig. 3D).

Similar results were obtained by immunofluorescence analysis of periodontal ligament (PDL) samples. Immunofluorescence reactions performed with TGF-β1, demonstrated that the staining signal was nearly absent in PDL obtained from patients affected by CP (Fig. 4B), and SSc (Fig. 4C) compared to PDL obtained from CO (Fig. 4A). Moreover immunofluorescence performed using the VEGF antibody revealed a higher staining pattern for VEGF in CP (Fig. 4E) and SSc (Fig. 4F) than that observed in CO (Fig. 4D). Furthermore, in periodontal diseases an increase of fluorescence was detected around the blood vessels (red circles in Fig. 4E and F).

Finally comparison of the clinical parameters between the studied groups, by statistical analysis, showed significantly higher differences in PD, CAL and CPITN, between patients with CP and SSc compared to CO.

There was a higher statistically significant difference (P<0.001) in the percentage of sites with CAL among the CP and SSc group and the control group (Fig. 1). There was no statistically significant difference at the PD sites between the CP and SSc group, but a statistically difference (P<0.05) in CAL between the CP and SSc group.

Discussion

To study the role and the production of autocrine TGF-β1 and VEGF signaling, the production of these growth factors in CO, CP, SSc groups were examined, analyzing gingival and PDL biopsies obtained from each patient. Contrast and brightness were established by examining the most brightly labeled pixels and choosing the settings that allowed clear visualization of the structural details while keeping the pixel intensity at its highest (~200).

This study showed that patients with CP and SSc had major severe periodontal disease in respect to the control group (Fig. 1). These studies also showed a particular relationship between periodontal disease and other chronic inflammatory diseases, such as SSc. These biomarkers contribute to sustaining the destructive inflammatory cascades seen in chronic periodontitis.

Transforming growth factor-β1 (TGF-β1) is known as one of the major anti-inflammatory cytokines (35). The large number of previous reports examining these polymorphisms of the TGF-β1 gene in various diseases reflects the interest in the role of this gene in chronic inflammatory diseases. Skaleric *et al* (36) found elevated TGF-β1 levels in gingival crevicular fluid samples from sites with deeper periodontal pockets.

Our results suggest that high TGF-β1 production may be a protective factor for periodontitis: potentially, this growth factor also accelerates connective tissue remodeling and angiogenesis (37): its biological activities result in insufficient remodeling and perfusion of tooth-supporting tissues contributing to periodontal destruction. However, previous reports rendered contradictory findings about the role of this growth factor (38).

Upregulation of TGF-β1 cytokine in patients with adult periodontitis may counterbalance the destructive gingival inflammatory responses in the acute phase of periodontitis (39). On the other hand, TGF-β1 was shown to induce chemotaxis for neutrophils, monocytes, mast cells and lymphocytes and is also an important mediator of the T-lymphocyte population (40).

Research to date has shown that TGF-β1 is mainly important in the early development stage of SSc (41). The main role in the fibrosis processes could be played by this growth factor, produced in excess by peripheral blood mononuclear cells (PBMC) (42).

TGF-β1 seems to be more significant in the fibrosis process than during the angiokinetic changes. TGF-β1 strongly slows down the PBMC adhesion to the endothelium and the generation of free radicals but can stimulate chemotaxis. Therefore, TGF-β1 may intensify inflammatory infiltrations around the vessels correlating with the beginning of the fibrosis process (43).

In addition, the present study clearly showed that VEGF expression was increased significantly in the destruction stage of the lesion, in contrast with a previous study that showed higher level of VEGF in the healing stage of the periodontal disease (19). Our study also showed that the mean concentrations of VEGF in periodontal tissues increased progressively from healthy to SSc subjects. This growth factor, in concert with TGF-β1, acts to stabilize the vascular wall and its imbalanced expression has been implicated in aberrant angiogenesis, a crucial factor in the pathogenesis of the SSc (44). However, angiogenesis in SSc is somewhat controversial. On one hand, Ozcelik *et al* (45) observed lower percentages of local VEGF expression in gingival biopsies of SSc patients when compared to the control group. On the other hand, Koch and Distler (46) showed, in accordance with our results, an increased production and stimulated neovascularization of VEGF in the blood vessels in the skin of SSc patients.

In conclusion, it is important to determine the exact function of these genetic polymorphisms during periodontitis in order to better understand their importance during disease progression. There is no doubt that we now have the technological basis to generate and analyze large volumes of information in the pursuit of understanding complex diseases such as periodontal disease at the molecular genetic level.

Our results suggest that TGF-β1 may contribute both to inflammatory regulation and remodeling events during periodontal disease. Therefore, we have documented that TGF-β1 downregulation in active periodontitis lesions has a key role in tissue destruction and bone resorption.

The findings presented here make it clear that biomarkers, such as TGF-β1 and VEGF play a key role in the evolution of the immune response in the periodontal and scleroderma disease, which in turn influences the outcome of disease establishment and evolution. It is important, therefore, to identify biomarkers that correlate with disease activity, prognosis and response to therapy allowing physicians to accurately identify patients early, those likely to respond and to predict prognosis of the disease.

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