TRANSFORMING GROWTH FACTOR BETA 1 AND VASCULAR ENDOTHELIAL GROWTH FACTOR LEVELS IN THE PATHOGENESIS OF PERIODONTAL DISEASE

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Received March 21, 2012 – Accepted April 10, 2013

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 immune pathology of periodontal disease. Cytokines play crucial roles in the maintenance of tissue homeostasis, a process which requires a delicate balance between anabolic and catabolic activities. In particular, two families of growth factors-such as transforming growth factor-β1 (TGF- β1) and vascular endothelial growth factor (VEGF) are thought to play important roles in modulating the proliferation and/or migration of structural cells involved in inflammation and regulation of immune responses. The aim of this work was to analyze gingival samples and periodontal tissue specimens collected from thirty-eight patients with chronic periodontal disease and from forty healthy individuals, in order to detect the expression and distribution of TGF-β1 and VEGF between the two groups. TGF-β1 and VEGF expression levels were detected using immunohistochemical analysis and computer-assisted morphometric analysis. The findings presented here suggest that biomarker such as TGF-β1 and VEGF have an important regulating role in the orchestration of the immune response, which in turn influence the outcome of disease establishment and evolution.

Human periodontal diseases (i.e., gingivitis, periodontitis) result from the interaction of heterogeneous etiologic factors, including the formation of a complex biofilm in the subgingival microenvironment, social and behavioural modulations, and genetic or epigenetic traits of the host, each of which is influenced and/or modulated by the host's immune and inflammatory response.

Key words: periodontal disease, immunohistochemical analysis, inflammation, TGF-beta 1, VEGF

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1721-727X (2013)

Although periodontal bacteria are the causative agents in periodontitis, subsequent progression and disease severity are thought to be determined by the host immune response raised in response to those pathogens (1). It can affect up to 90% of the world population (2) and there are close connections between periodontal inflammation and major chronic conditions, such as diabetes, heart disease and chronic autoimmune disease (3). The underlying mechanisms causing these pathological changes are still unclear. Strong evidence exists that different genes may play a role in the predisposition to and in the progression of periodontal diseases (4). A number of genetic deficiencies or genetic variations in the host's tissue makeup, such as epithelial and fibroblast defects, may contribute to genetic susceptibility to aggressive periodontitis (5). Recently, cytokines attracted much attention as potentially crucial variants influencing the host response in periodontitis (6).

Host-derived cytokines released upon microbial challenge have significant effects on the immune and inflammatory responses in periodontal disease (7, 8). A prominent factor in connective tissue remodelling and inflammatory diseases is transforming growth factor-β (TGF-β). TGF-β possess multiple and sometimes contradicting biological activity, and is involved in regulation of cell function, proliferation, differentiation, and migration (9). One of its isoforms, TGF-β1, is a multifunctional cytokine that regulates cell growth, differentiation and matrix production. TGF-β1 has a central role in wound healing, it is a key mediator of tissue fibrosis and may lead to extracellular matrix accumulation in pathological states (10). It also has potent immunosuppressive activity and down-regulates the transcription of other pro-inflammatory cytokines, including interleukin-1, tumor necrosis factor-alpha and several metalloproteinases (11, 12). Moreover, it was demonstrated that this growth factor mediates fibroblast activation, proliferation and signalling in cells cultures (13).

Pleiotropy of the TGF-β superfamily has been demonstrated by the tissue induction and transformation into osteoblast-like cells secreting bone matrix with collagenous and non-collagenous glycoproteins (14). Bone morphogenetic and osteogenic proteins (BMPs/OPs), pleiotropic members of the TGF-β superfamily, induce de novo endochondral bone formation and act as soluble signals of tissue morphogenesis sculpting the multicellular mineralized structures of the bone-bone marrow organ (15).

In the development of chronic inflammatory disease, an important role is played also by the Vascular Endothelial Growth Factor (VEGF), a 45-kd homodimeric proinflammatory glycoprotein that potently increases microvascular permeability, promotes angiogenesis, and stimulates endothelial cell proliferation, migration, and survival (16, 17); this protein seems to be involved in the onset and progression of gingivitis and periodontitis, mainly promoting the vascular network expansion generally observed during the orthodontic tooth movement (18) and in inflammation (19). Several studies intended to associate the action of VEGF with the pathogenesis of periodontal disease have revealed controversial results. Nevertheless, VEGF expression is more strongly related to the healing stage of periodontal disease than to the destruction stage of the lesion (20, 21).

Therefore, the objective of the present study was to determine, by immunohistochemical and computer-assisted morphometric analysis, the expression and distribution of TGF-β1 and VEGF in the gingival tissues and periodontal ligament of patients with chronic periodontitis compared to a control group of healthy individuals.

MATERIALS AND METHODS

One hundred and forty-two patients were initially enrolled in this prospective clinical study carried out from October 2011 to January 2012 at the University of Messina, Italy. The protocol was approved by the Ethics and Research Committee of University of Messina, and ethical approval was obtained for the experimental procedures applied on 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. The inclusion criteria were: age over 18 years, good general health (excluding the case definition), and presence of more than 18 erupted teeth. The exclusion criteria were: diabetes mellitus, liver, kidney, or salivary gland dysfunction, history of alcoholism, recent history or the presence of other acute or chronic infection, systemic antibiotic treatment or immunosuppressant medication within the previous three months, pregnancy and intense

physical activity, smoking history or the presence of an oral mucosal inflammatory condition. Seventy-seven patients were excluded as they were not in line with the selection criteria of the study. So the final number of the sample analyzed in the study was reduced to a total of seventy-eight patients.

The selected patients were divided into two groups according to the presence or not of periodontal disease: thirty-eight patients with adult Chronic Periodontitis (CP group) (15 male, 23 female, mean age 47.8, SD±9.2) based on the criteria defined by the American Academy of Periodontology (22) and forty healthy individuals, as Control group (CO group) (18 male, 22 female, mean age 43.9, SD±8.7) were enrolled in this study.

Plaque Index (PI) (23), Periodontal Probing Depth (PD), Clinical Attachment Level (CAL), Community Periodontal Index of Treatment Needs (CPITN) (24) and presence of bleeding on probing (BOP) were measured at 6 sites for each tooth, except third molars, and recorded. Every clinical periodontal measurement was performed at baseline by the same examiner (Table I).

Subjects in 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) >2mm. The healthy controls 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 (Endoral X-rays).

All the clinical measurements and the surgical procedures were performed by one surgeon (GI). The gingival biopsies in the CP group were obtained, after local anesthesia, from the interdental papilla between the mandibular canine and first premolar, and the collection of periodontal ligament (PDL) specimens was carried out from the root surface during extraction of compromised teeth. As for the CO group, gingival tissue and periodontal samples were carried out during routine erupted third molar extractions, advanced caries and orthodontic indications. The gingival specimens dissected were in close contact with the surface of the tooth extracted and comprised the entire zone of gingiva above the level of the alveolar bone crest, therefore including the oral gingival epithelium, the junctional/pocket epithelium and the supracrestal part of the gingival connective tissue.

The collected tissues, 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

Twenty-five sections were prepared from each biopsy,. The samples were snap-frozen in liquid nitrogen and 20-

Table I. *Clinical characteristics of the patients at baseline (78 patients). The data presented as mean ± SD.*

	Control group (Mean ±SD)	Test group (Mean ± SD)	P
Number of patients	40	38	
Years	43.9 ± (8.7)	47.8 ± (9.2)	NS
Gender			
Male	18	15	
Female	22	23	
PD	2.75 (0.5)	5.9 (1.6)	**
CAL	2.77 (0.53)	6.1 (1.5)	**
BOP	0.4 (0.7)	2.7 (0.9)	**
PI	0.6 (0.44)	2.3 (1.1)	**
CPITN	0.2 (0.4)	2.1 (1.3)	**

*PD: Probing Depth; CAL: Clinical Attachment Level; PI: Plaque Index; BOP: Bleeding On Probing; CPITN: Community Periodontal Index of Treatment Needs; SD: Standard Deviation; P, Significance; *, significant (p<0.05); **, highly significant (p<0.001).*

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 (sc 146, Santa Cruz Laboratories inc, Heidelberg), anti VEGF diluted 1:50 (VG1, Novus biologicals, Littleton, CO). 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 carried out on 2,250 images by a pathologist who performed the analysis in a blinded manner. The sections were then analyzed and images 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 2048 × 2048 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-thick 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).

Image analysis

Each image was analysed with the ImageJ software (National Institute of Health). Briefly, a standard was created by selecting an area of 0.5 cm × 1.0 cm from a tissue section that had desired green colour from TGF-β1 and VEGF 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 colour 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 green colour, that is similar to the standard on an image being determined, were highlighted. The quantification was made 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.

Statistical analysis

Frequency distributions, media and standard deviation (SD) were determined at baseline in each group to describe the clinical parameters (PD, CAL, CPITN, PI and BOP). The Kruskal–Wallis and the Mann–Whitney U tests were carried out when comparing the clinical parameters (PD, CAL and CPITN) in the three independent groups, and the Wilcoxon Singed Rank Test was used when comparing two matched-pair groups. The differences were considered statistically significant when the test description levels (p) were lower than 0.05 (or 5%). The data were analyzed with software Prism (GraphPad Instat, version 5.00 - GraphPad Software, San Diego, CA, USA). The Student *t*-test was used to compare test and control sample staining. A *P* value of 0.05 was considered to indicate statistical significance.

RESULTS

The mean ± SD age between two groups was 43.9±8.7 in the CO group and 47.8±9.2 in the CP group, respectively. No significant difference in mean age was found between the two groups (Table I).

The ratios of female/male in were 22/18 (1.3) in the CO group, and 23/15 (1.66) in the CP group, respectively. The proportion of females did not differ significantly among the groups (p = 0.11). The means of PD from the patients in a CO and CP group were 2.75±0.5 and 5.9±1.6 mm, respectively. The means of CAL from the sampled sites in the two study groups were 2.77±0.53 for the CO and 6.1±1.5 mm for the CP group, respectively. A higher CAL (≥ 4mm), PD site (≥ 5mm) and PD values (< 1), was observed in the CP group compared with the controls (P<0.001); additionally, the BOP values (< 1) were significantly higher in the CP group than the controls (P< 0.001). Results are presented with media and Standard Deviation in Table I. Higher levels of PI were found in the CP group compared to the CO group. BOP, as a measure of acute periodontal inflammation, was elevated in patients with CP compared to CO group.

The immunohistochemical TGF-β1 and VEGF expression was observed in all gingival samples at the cellular cytoplasmic level and observed in epithelial, dermal, endothelial cells; in the periodontal samples, the same analysis was observed in the periodontal ligament cells. First of all, we performed a negative control both on gingival samples with TGF-β1 (Fig. 1A) and VEGF (Fig. 1C) and periodontal ligament samples with TGF-β1 (Fig. 1B) and VEGF (Fig. 1D) using the secondary antibody only.

Representative images of gingival samples

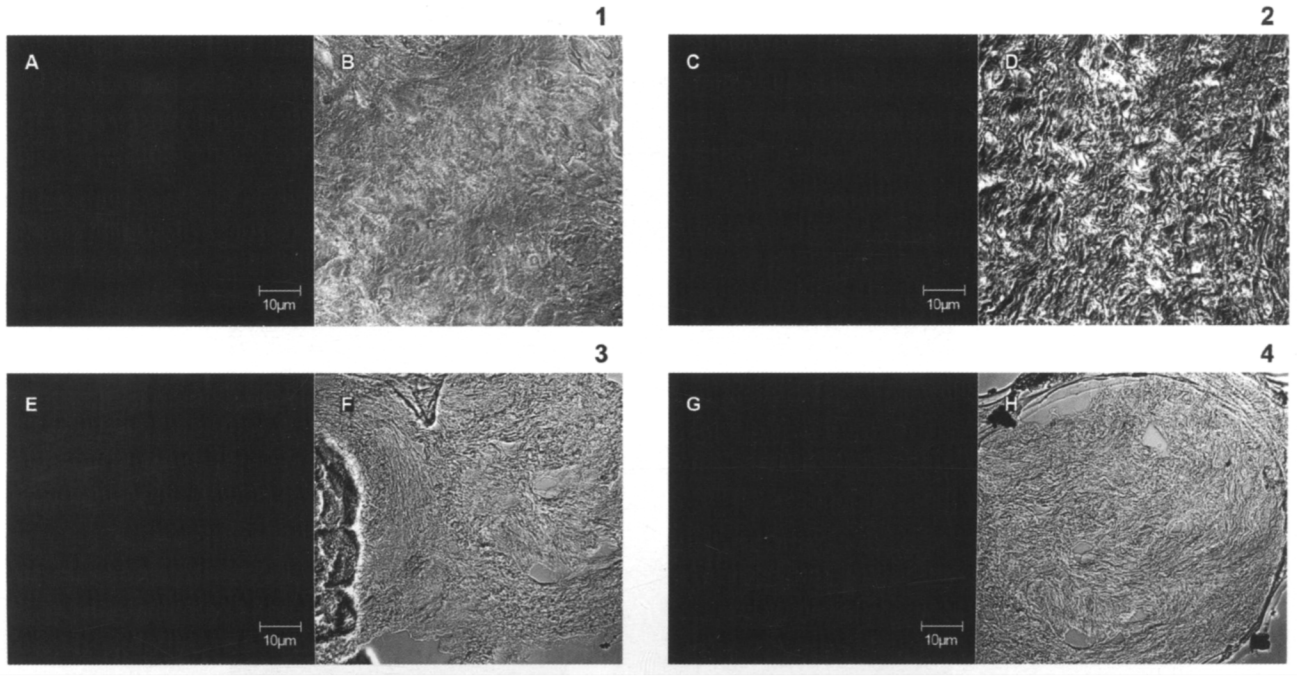

Fig. 1. *Negative control of a longitudinal section of gingival and periodontal samples immunolabeled with the secondary antibody only (**A, C, E, G**), and corresponding transmitted light (**B, D, F, H**).*

sections with TGF-β1 and VEGF immunostaining are presented in Figs. 2 and 3, respectively. These images show that the intensity and area of TGF-β1 immunostaining was decreased in the CP group (9.24±5.41; P <0.001) compared to the CO group (24.78±4.56). Most TGF-β1 immunostaining appeared to be associated with cells (Fig. 2).

Immunostaining analysis in the gingival samples showed also a decreased expression of the VEGF in the CO group (35.44±8.61) than in the CP group (42.25±6.34; P <0.05). TGF-β1 and VEGF immunostaining analysis in periodontal samples showed the same results observed in the gingival samples (Fig. 3).

The TGF-β1 expression was decreased more in the CO group (5.26±2.39) than in the CP group (27.35±2.84; P <0.001; Fig. 3C). The mean percentage of VEGF expression in the periodontal samples was higher in the CP group (39.22±3.35) compared to the CO group (32.14±4.57) (Fig. 4).

VEGF expressions and TGF-β1 of the control site had a high correlation with the difference on the clinical parameters: there was a higher statistically difference (p<0.001) in percentage of sites with CAL between CP and CO group (Table I).

DISCUSSION

The study of new genetic polymorphisms has become a field for new insights in periodontology, as there are several inflammatory cytokines and growth factors involved with the complex mechanisms of periodontal disease.

To study the role and the production of autocrine TGF-β1 and VEGF signalling, the production of these growth factors in the CO and CP groups were examined, analyzing gingival and PDL biopsies obtained from each patient. Contrast and brightness were established by examining the most brightly labelled 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 had major severe periodontal disease in respect to the CO group. These biomarkers contribute to sustaining the destructive inflammatory cascades seen in

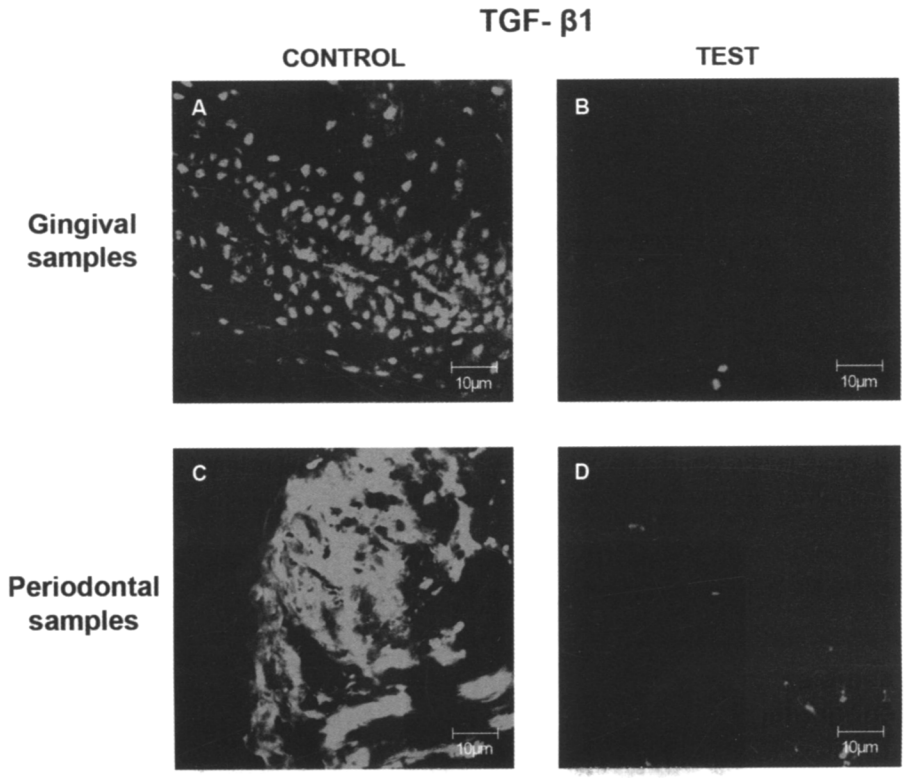

Fig. 2. *Confocal scanning laser microscope observation: gingival samples immunolabeled with TGF- β1 in a control (**A**) and test group (**B**); the same image showed periodontal ligament samples immunolabeled with TGF-β1 in a control (**C**) and test group (**D**).*

chronic periodontitis. The TGF-β1 expression was found in a higher frequency in the severe than in the control and moderate groups. We also showed a ratio between the different clinical parameters of the patients and the expression of TGF-β1 and VEGF. In fact, the patients of the CP group showed a reduced immunohistochemical expression of the TGF-β1 in respect to the CO group. In contrast, the patients of the CP group expressed higher levels of the VEGF expression in respect to the CO group, and, also for this protein, the levels of the expression were directly correlated to the worsening of the clinical parameters, especially PD and CAL.

One of the main concerns in chronic periodontitis is the definition of active periodontal lesions where tissue destruction is occurring. Transforming Growth Factor β-1 (TGF-β1) is known as the one of major anti-inflammatory cytokines (25). 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. (26) found elevated TGF-β1 levels in gingival crevicular fluid samples from sites with deeper periodontal pockets.

Our results suggest that high TGF-β1 production might be a protective factor for periodontitis: potentially, this growth factor accelerates also connective tissue remodelling and angiogenesis (27): its biologic activity results in insufficient remodelling and perfusion of tooth-supporting tissues contributing to periodontal destruction. However, previous reports rendered contradictory findings on the role of this growth factor (28). Up-regulation of TGF-β1 in patients with adult periodontitis may counterbalance for destructive gingival inflammatory responses in acute phase of periodontitis (29).

In the periodontal tissues, TGF-β1 suppresses

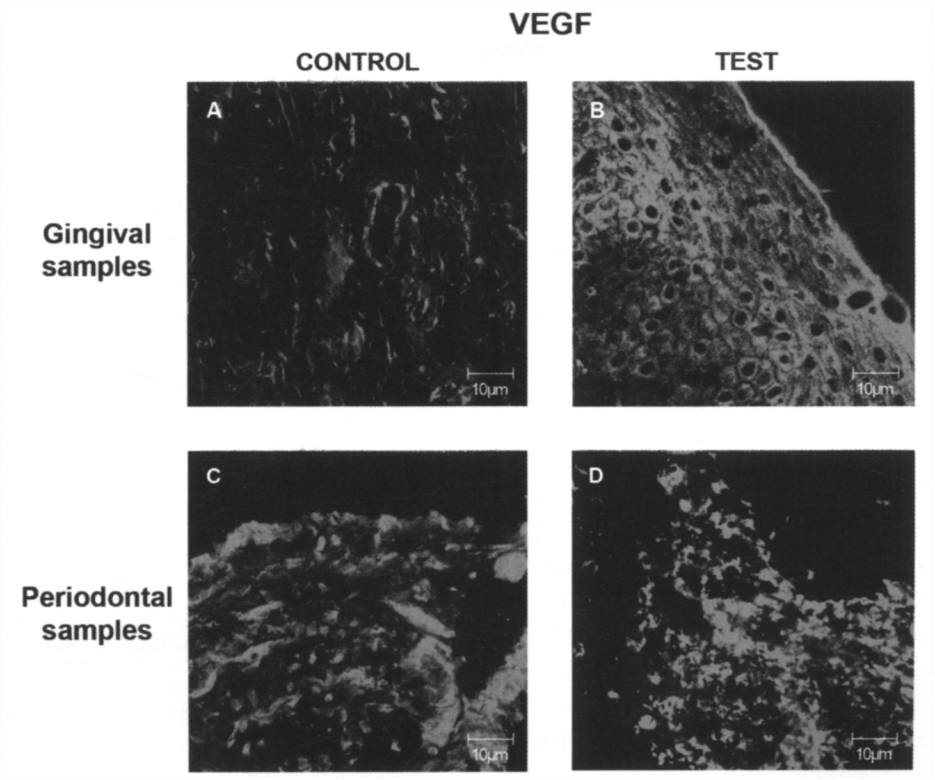

Fig. 3. *Confocal scanning laser microscope observation: gingival samples immunolabeled with VEGF in a control (**A**) and test group (**B**); the same image showed periodontal ligament samples immunolabeled with VEGF in a control (**C**) and test group (**D**).*

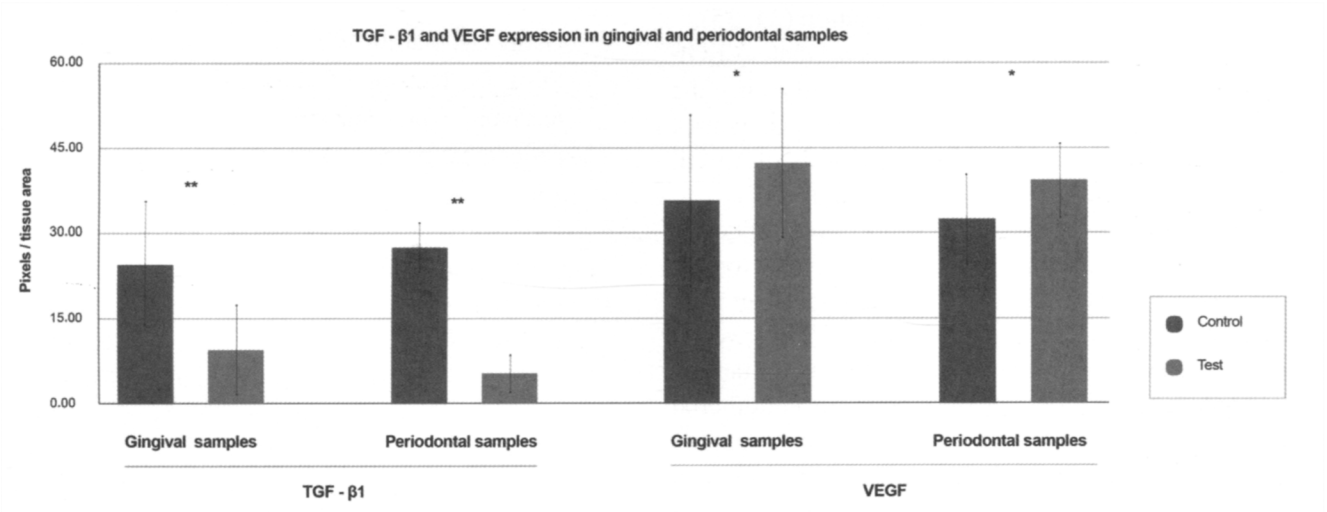

Fig. 4. *Statistical analysis of TGF- β1 and VEGF expression among control and test group. TGF-B1 and VEGF expression was detected in gingival and periodontal sample tissues and quantified as described in Materials and Methods. Student t test was used to compare test and control samples staining: * significant ($p<0.05$), ** highly significant ($p<0.001$).*

proteolytic activity by down-regulating the transcriptional activity of MMPs and activates the expression of tissue inhibitors of MMPs. It can also stimulate the synthesis of connective tissue matrix components by fibroblasts and osteoblasts (30). On the other hand, TGF-β1 was shown to induce the chemotaxis for neutrophils, monocytes, mast cells, and lymphocytes, and it is also an important mediator of T-lymphocyte population. It may also augment the release of leukocyte cytokines, which could contribute to the breakdown of periodontal tissue, especially in the early phases of the inflammatory response (31). Our results could indicate that high TGF-β1 production might be a protective factor for periodontitis. Given the potential for TGF-β1 to increase the rate of wound healing (32) and accelerate connective tissue remodelling (29) and angiogenesis (33), it is tempting to speculate that the lack of indicated biologic activities results in insufficient remodelling and perfusion of tooth-supporting tissues contributing to periodontal destruction. This is supported by the fact that, although TGF-β1 levels are elevated in moderate disease, they declined in fluid samples obtained from the pockets in more advanced experimental periodontitis (26). Also, the role of TGF-β1 was investigated on human gingival fibroblast: in fact, it was shown that TGF-β1 may regulate uPA (urokinase-type plasminogen activator) that involve several MAPK (Mitogen-activated protein kinase), fundamental in the evolution of cancer, wound healing, and inflammation (34, 35).

The present study shows also that VEGF expression was increased significantly in the destruction stage of the lesion, in contrast with a previous report that showed higher levels of VEGF in the healing stage of periodontal disease (17).

VEGF is a mediator of many osteoinductive factors, including transforming growth factor-b1, insulin-like growth factor-1, and fibroblast growth factor-2, which up-regulate VEGF expression in osteoblasts. Furthermore, VEGF influences osteoblasts indirectly via its effects on endothelial cells. VEGF is also an essential factor in the proliferation of the vasculature within inflamed tissues, including gingiva (36, 37). VEGF is induced during Th1 inflammation and increases blood-vessel size, number, and surface area. VEGF can link innate and adaptive immunity at the inflammatory site by augmentation of antigen sensitization, leading to Th2-type cytokine production and the formation of chronic inflammation (38). Thus, VEGF could be a key factor in the severity of gingival inflammation because it links angiogenesis and inflammation (39).

The findings presented here allow to hypothesize that biomarkers such as TGF-β1 and VEGF play an important role in the evolution of the immune response in periodontal disease, which in turn influences the outcome of disease establishment and evolution. It is necessary, therefore, to identify biomarkers related to disease activity, prognosis and response to therapy allowing physicians to accurately identify patients who are likely to respond early and to predict prognosis of the disease.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr. Gianluigi Vaccarino, Dr.ssa Rita Aveni and the staff of the Library of the Faculty of Medicine, University of Messina, Italy, for their technical support and tremendous help in searching the literature.

Giovanni Matarese and Gaetano Isola contributed as senior authors to the research protocol, literature review, discussion of results, drafting the manuscript and share the first authorship.

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