

Vascular Endothelial Growth Factor and Microvessel Density Around Healthy and Failing Dental Implants

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Inflammatory infiltrate may be important in the evolution of inflammatory processes involving peri-implant tissues. Angiogenesis is an important feature of inflammation and healing, but its role in the development and progression or in the healing of periodontal lesions has not been elucidated. Vascular endothelial growth factor (VEGF) is a potent inducer of endothelial cell proliferation. The aim of the present study was to conduct a comparative immunohistochemical evaluation of VEGF and microvessel density (MVD) in normal keratinized gingiva and in peri-implant soft tissues surrounding failing implants. Fifteen patients participated in this study. Ten biopsies were taken from healthy keratinized gingiva, and 10 were taken from peri-implant soft tissues surrounding failing non-submerged implants. In healthy sites, the endothelial lining cells of the vessels always tested positive for VEGF; also, VEGF intensity was high in most cases. Stromal cells were positive for VEGF in 70% to 90% of samples. The MVD was 60.250 ± 5.123 . In peri-implantitis samples, the cells of the inflammatory infiltrate were positive for VEGF in 80% to 100% of cases, and the VEGF intensity was low in all cases. The stromal cells were positive for VEGF in 90% to 100% of cases, and in most cases the intensity was low. The MVD was 101.800 ± 11.256 . The difference in MVD between healthy sites and peri-implantitis was statistically significant ($P = .0158$). Expression of VEGF was lower in peri-implantitis samples, and this difference was statistically significant ($P = .0373$). Because of its extensive presence, VEGF is probably a factor in both the maintenance of periodontal physiology and in the progression of peri-implant inflammatory disease. (INT J ORAL MAXILLOFAC IMPLANTS 2001;16:389–393)

Key words: implant failure, microvessel density, periodontal disease, peri-implantitis, vascular endothelial growth factor

Many complex inflammatory and immune responses are implicated in the etiopathogenesis and progression of periodontitis,¹ and the com-

position of the inflammatory infiltrate may be important in the evolution of inflammatory processes of peri-implant tissues.² In a recent report, no statistically significant differences were found in inflammatory cell subsets associated with periodontitis and peri-implantitis.³ The inflammatory process that remains confined to the soft tissues surrounding the implant is termed *peri-implant mucositis*, while the presence of progressive peri-implant bone loss occurring together with soft tissue inflammation around a functioning osseointegrated implant is called *peri-implantitis*.^{4,5} Esposito and coworkers⁶ defined peri-implantitis as a site-specific, plaque-induced infection with progressive loss of the bone supporting a functioning implant.

Signs of inflammation of peri-implant tissues are pocket formation, radiographic bone destruction, suppuration, swelling, color changes, and bleeding

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Table 1 Comparison of Healthy Keratinized Gingiva (Control Samples) Versus Soft Tissues with Peri-implantitis

Patient	Sex	Age	Peri-implantitis samples		Control samples	
			Stromal VEGF	MVD	Stromal VEGF	MVD
1	M	50	90%	97	75%	53
2	M	46	95%	105	—	—
3	F	32	94%	93	—	—
4	F	54	91%	110	77%	60
5	M	56	93%	92	—	—
6	M	55	98%	95	80%	65
7	M	47	99%	121	—	—
8	F	45	94%	96	70%	57
9	M	52	97%	108	—	—
10	F	43	95%	103	90%	64
11	M	41	—	—	77%	55
12	M	59	—	—	83%	60
13	F	39	—	—	85%	63
14	M	38	—	—	78%	58
15	M	48	—	—	71%	65

Stromal VEGF (vascular endothelial growth factor): $P = .0373$; MVD (microvessel density): $P = .0150$.

on gentle probing.^{4,5,7} Multiple factors can contribute to implant failure, and a cause-and-effect relationship between the accumulation of bacterial plaque and the development of peri-implant mucositis has been demonstrated.⁸ Angiogenesis is an important characteristic of inflammation and healing, but its role in the development and progression, or in the healing, of periodontal lesions has not been elucidated.⁹ Vascular endothelial growth factor (VEGF) is a peptide and a potent inducer of microvascular permeability.^{10,11} It has been shown to stimulate endothelial proliferation in vitro and also to have angiogenic activity in vivo.^{10,11} A correlation has been found between VEGF expression and neovascularization, assessed by Factor VIII immunostaining evaluated by microvessel density (MVD).¹¹ Moreover, MVD is correlated to poor clinical outcome in several malignant tumors.¹¹

The aim of the present study was to conduct a comparative immunohistochemical evaluation of VEGF and MVD in normal keratinized gingiva and in peri-implant soft tissues surrounding failing non-submerged implants.

MATERIALS AND METHODS

Fifteen patients, 10 males and 5 females with a mean age of 47 years (range 32 to 59 years), participated in this study (Table 1). All the patients were nonsmokers and gave their informed consent. Biopsies from marginal keratinized mucosa surrounding transmucosal

osseointegrated implants (ITI, Straumann, Waldenburg, Switzerland) were obtained under local anesthesia; 10 were taken from healthy keratinized gingiva, and 10 were taken from the peri-implant soft tissues surrounding failing implants. The biopsies included oral, sulcular, and junctional epithelium plus the underlying and supracrestal connective tissue. All implants had been in place for at least 1 year.

The biopsy specimens for the healthy sites were chosen according to the following criteria¹²:

- No clinically visible plaque accumulation
- A probing depth of no more than 2 mm
- No bleeding on probing
- No gingival erythema

In all cases of peri-implantitis, the following signs and symptoms were present⁵:

- Radiologic evidence of bone loss
- Presence of a peri-implant pocket of more than 5 mm
- Bleeding on probing
- Suppuration
- Swelling of tissues

Radiographic and clinical signs of osseointegration were present in all functioning implants.

All biopsies from selected patients were fixed in 10% neutral buffered formalin and embedded in paraffin. All the hematoxylin and eosin-stained sections were reviewed, the quality of the slides was

checked, and slides were selected for quantitative evaluation. Immunostaining for VEGF and Factor VIII was performed using the alkaline phosphatase/anti-alkaline phosphatase method (APAAP) with a rabbit polyclonal antibody (Anti-VEGF Santa Cruz, Santa Cruz, CA). Sections of 4 μm were cut and mounted on poly-L-lysine-coated slides. Paraffin sections were dewaxed by xylene, rehydrated, and finally washed in tris-buffer (pH 7.6) for 10 minutes. The VEGF required proteinase-K predigestion in a working solution of 0.4 mg/mL (Dako, Glostrup, Denmark) for 10 minutes at room temperature.

The following steps were optimized by automatic staining (Dako, TechMate 500). Sections were incubated with primary antibody solution for VEGF at a dilution of 1:400 and for factor VIII at a dilution of 1:30,000 for 25 minutes at room temperature. Slides were rinsed in buffer (Buffer Kit, Dako) and immunoreaction was completed with the APAAP kit (Dako). The secondary antibody was an alkaline phosphatase-labeled monoclonal calf antibody, and the detection antibody was a monoclonal anti-calf mouse antibody. After incubation with a chromogen alkaline phosphatase substrate (Fast Red, Dako), specimens were counterstained with Mayer's hematoxylin and coverslipped.

Levels of VEGF were evaluated in the vessels; in the cells of the inflammatory infiltrate (mainly lymphocytes, plasma cells, and neutrophils); in the stromal cells (fibroblasts); in Langerhans and Merkel cells present in the stratified squamous epithelium; and in the stratified squamous epithelium. Inflammation was graded as mild (+) or severe (++) . VEGF expression was determined by evaluating the percentage of VEGF-positive elements. The value was indicated by (-) when less than 10% of the cells were positive for VEGF, by (+) when the percentage of cells positive for VEGF was between 10% and 50%, and by (++) when the percentage of positive cells was more than 50%. Moreover, the intensity of the stained cells was also graded, and the positive cases were classified as low intensity (+) when the cells were lightly stained with brown and high intensity (++) when the cells were strongly stained with brown.

A quantitative histologic analysis of vascularization was performed. The antibody against human factor VIII-related antigen was used to highlight the blood microvessels; all the morphologic structures with a lumen surrounded by factor VIII-positive endothelial cells were considered as blood microvessels. The microvessel count was performed by 2 pathologists in a blind and independent way using an IBAS-AT image analyzer (Kontron,

Munich, Germany). For the evaluation, 400 \times magnification was used, and the individual microvessel profiles were circled to prevent the duplication or omission of microvessel count. For each sample, 10 high-power fields (HPF), corresponding to 1.1 mm^2 , were measured. The values were expressed as the number of microvessels per square millimeter of healthy keratinized gingiva and the peri-implant soft tissues surrounding failing implants (ie, MVD).

Finally, a statistical descriptive analysis was performed, and the Mann-Whitney *U* test was used to evaluate the presence of statistically significant differences.

RESULTS

Healthy Samples

The mucosa was covered by a stratified squamous epithelium supported by a layer of vascular fibrous connective tissue (the lamina propria). No inflammatory infiltrate was present in the lamina propria. In the stratified squamous epithelium, the rarely present lymphocytes tested negative for VEGF. Langerhans and Merkel cells were negative. The stratified squamous epithelium was VEGF positive, with values ranging from 60% to 80% (Fig 1a). The basal layer was more strongly positive than the superficial layer, and the positivity was always cytoplasmatic. The VEGF positivity was strong in most cases. The vessels had a regular distribution. The endothelial lining cells of the vessels were VEGF positive in 100% of the specimens (Fig 1b), and the positivity was always cytoplasmatic. In most cases, the VEGF intensity was strong. Stromal cells (fibroblasts) were VEGF positive, with values ranging from 70% to 90% (Fig 1c). The positivity was located in the cytoplasm and in most cases, the intensity was strong. The MVD was 60.250 ± 5.123 (range, 53 to 65).

Peri-implantitis Samples

In all cases, the inflammatory infiltrate was present in the lamina propria. Langerhans and Merkel cells present in the stratified squamous epithelium were negative. The stratified squamous epithelium was VEGF positive, with values ranging from 50% to 70%. The basal layer was more positive than the superficial layer, and the positivity was always cytoplasmatic. The VEGF intensity was weak in most cases. The inflammatory infiltrate was, in most cases, severe and composed mainly of lymphocytes and plasma cells, while only rarely was it possible to observe neutrophils. The cells of the inflammatory infiltrate (lymphocytes and neutrophils) were

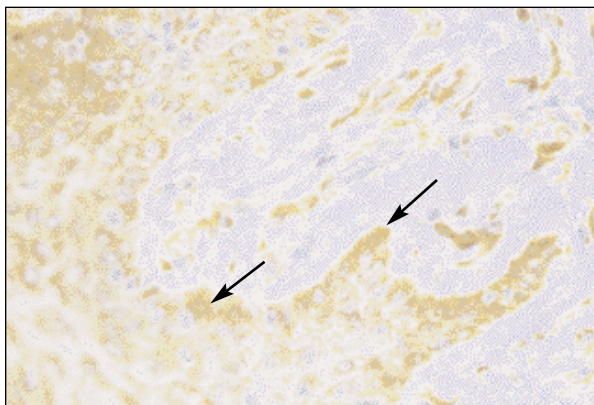


Fig 1a Healthy mucosa showing positivity to VEGF of the epithelium. There is a stronger positivity of the cells of the basal layer (arrows) (APAAP; original magnification $\times 40$).

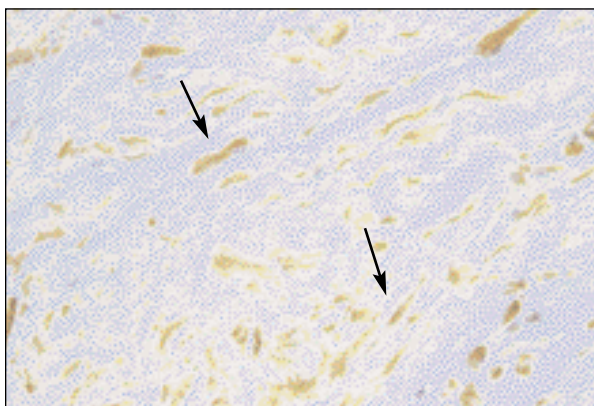


Fig 1c Healthy mucosa showing positivity to VEGF of the stromal cells (arrows) (APAAP; original magnification $\times 40$).

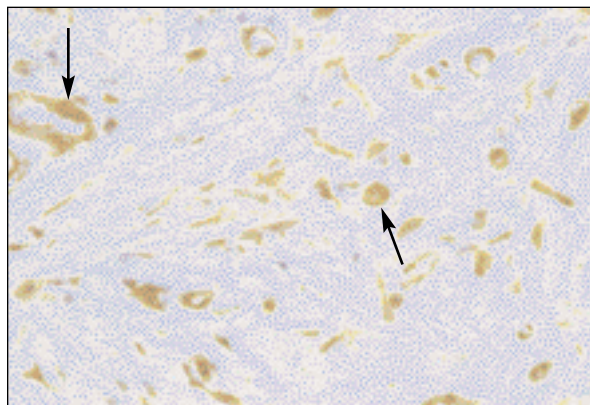


Fig 1b Healthy mucosa showing positivity to VEGF of the endothelial cells (arrows) (APAAP; original magnification $\times 40$).

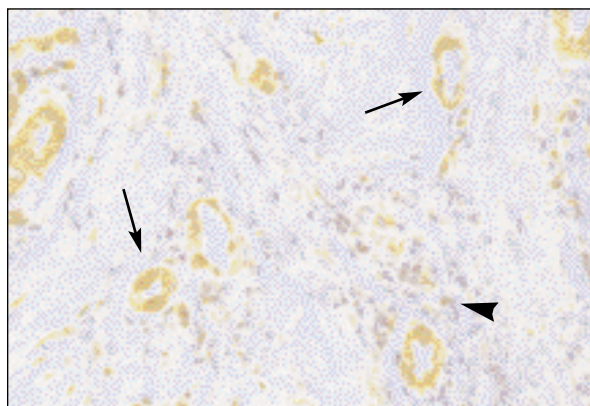


Fig 2 Peri-implantitis sample displaying positivity to VEGF of some inflammatory cells (arrowhead) and of the endothelial cells (arrows) (APAAP; original magnification $\times 40$).

VEGF positive in a percentage ranging from 80% to 100% (Fig 2). The positivity was always located in the cytoplasm of these cells. The intensity was strong in 100% of the specimens. The vessels were always VEGF positive and the positivity was located in the endothelial lining cells. In most of the specimens the intensity of the positivity was strong. The stromal cells were positive in a percentage ranging from 90% to 100%. The positivity was located in the cytoplasm and the intensity was strong in 80% of the specimens. The MVD was 101.800 ± 11.256 (range, 93 to 121).

Differences Between Healthy and Peri-implantitis Sites

With respect to the MVD, the difference between healthy sites and peri-implantitis was statistically significant ($P = .0158$). The difference between VEGF expression in the stromal cells of the healthy

sites and of the peri-implantitis sites was also statistically significant ($P = .0373$). No statistically significant difference was detected between the 2 groups in the VEGF expression of the vessels.

DISCUSSION

Previous studies have shown that the soft tissues surrounding failed implants contain a large number of macrophages, human leukocyte antigen D-related-positive cells, lymphocytes, and plasma cells.¹³ Moreover, a relationship between periodontal disease and peri-implantitis has been established, based on the findings of increased gram-anaerobic flora with high levels of spirochetes associated with failing or failed implants.¹⁴

Angiogenesis is defined as the process by which new blood vessels are produced by sprouting from

established vessels.¹⁵ Inflamed tissues show enhanced expression of inflammatory mediators, many of which can promote angiogenesis.¹⁵ Angiogenesis can also contribute to the severity of the inflammation as the result of the ability of new blood vessels to transport proinflammatory cells and supply nutrients and oxygen to the inflamed tissues.¹⁵ The periodontal vasculature is profoundly affected during the progression of periodontal disease.¹⁵

The results of the present investigation confirm the data reported by Booth and coworkers⁹ and by Johnson and associates¹⁵ that VEGF plays a role both in periodontal health and as a likely factor in the etiology of gingivitis and its progression to periodontitis, possibly by expansion of the vascular network. Moreover, Johnson and associates¹⁵ found that the number of blood vessels increased with increasing pocket depth. The present data also show that there was a statistically significant increase in the MVD in the peri-implantitis sites as compared to healthy sites. As for VEGF expression, it was found that vessels were always positive in both healthy sites and peri-implantitis, while the cells of the inflammatory infiltrate were positive in a percentage ranging from 80% to 100%. Contrary to the findings of Johnson and associates¹⁵ of a lower VEGF concentration in normal gingiva, the present authors found a decreased VEGF expression in the stromal cells of the peri-implantitis specimens. This could be the result of different stages in the progression of the pathology of peri-implant disease. Moreover, the importance of VEGF presence in the progression of the pathologic processes or the healing response is not yet clear.⁹ In the future, more studies will certainly be necessary to evaluate and elucidate this question.

ACKNOWLEDGMENTS

This work was partially supported by the National Research Council (C.N.R.), Rome, Italy, and by the Ministry of the University, Research, Science and Technology (M.U.R.S.T.), Rome, Italy.

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