Abstract. Periodontal lesions are associated with activation of pathological angiogenesis and a high number of newly-formed blood vessels. Most angiogenic growth factors have been studied in the crevicular fluid or serum, but tissue correlations with vascular density or endothelial proliferation, are very rare, even inexistent. We assessed the VEGF/VEGFR2 axis expression in a multimodal fashion, in both epithelial and stromal compartments, with emphasis to endothelial proliferation and severity of periodontal lesions. Compared to normal gingiva, negative for VEGF/VEGFR2, periodontal lesions had a progressive increase for these markers from low to severe periodontal lesions. The transition from low to moderate periodontal lesions represents the milestone in disease progression and implies an active angiogenesis based on the highest angiogenic parameter variability observed for these lesions. Epithelial vascularization was firstly observed in moderate periodontal lesions and persists during severe periodontal disease. All the parameters used to quantify angiogenesis in periodontal lesions, were significantly increased in severe periodontal lesions dependent on VEGF expression in both the epithelial and stromal compartment. Our results support the use of anti-VEGF/VEGFR2-targeted therapy as adjuvant treatment for severe periodontal lesions.

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Key Words: Periodontal disease, VEGF, VEGFR2, microvessel density, endothelial proliferation.

VEGF/VEGFR2 Axis in Periodontal Disease Progression and Angiogenesis: Basic Approach for a New Therapeutic Strategy

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Periodontal lesions induce tissue changes both inside the gum and the alveolar bone. Continuous action of etiological factors that induce and sustain pathological changes in periodontal disease may induce irreversible changes in the affected tissues, representing a solid motivation for the application of guided tissue re-generation that precedes the use of dental implants. Cellular polymorphism involved in the pathogenesis of periodontal lesions have a direct impact on the heterogeneity of factors that determine disease progression.

Inflammation, one of the main mechanisms for periodontal lesions is associated with the activation of pathological angiogenesis and a high number of newly-formed blood vessels quantified as microvessel density (MVD) (1). Several angiogenic factors are responsible for the angiogenesis process activation, among them, VEGF (2, 3), FGF2 (4) and recently PDGF (5), are the ones most intensely studied. Most growth factors have been studied in the crevicular fluid or in serum (6, 7), but tissue correlations with vascular microdensity and moreover, with endothelial proliferation, are very rare or even inexistent. Existing studies regarding angiogenesis evaluation in periodontal disease have been limited to MVD quantification (8) without considering the endothelial cells’ proliferative ability. Moreover, VEGF expression have been mostly appreciated in the gum epithelium, with its’ expression in the endothelium or in stromal cells being usually neglected. All this information interpreted separately does not present a significant prognostic and therapeutic impact in periodontal lesions.

Our study was designed to assess the VEGF/VEGFR2 axis in a multimodal fashion, taking into account their expression heterogeneity inside epithelial and stromal compartments of gingiva with periodontal disease and also at the endothelial level, with all these aspects being evaluated in close relationship with the degree of the periodontal lesion.
VEGF/VEGFR2 expression was further evaluated regarding its impact on microvessel density and periodontal lesions’ progression.

Materials and Methods

Patients’ data. We selected biopsies from 51 patients with different-severity grades of periodontal lesions and without any significant adjacent pathologies. In the present study, 12 patients without significant changes of the oral mucosa, 15 patients with mild, 16 with moderate and 8 with severe inflammatory lesions of the gingiva, as found by the clinical examination, were included. Punch biopsies were obtained from each patient and washed with saline buffer and then fixed in formalin. The local research Ethics Committee approved the study protocol and informed consent was obtained from all subjects, according to the World Medical Association Declaration of Helsinki.

Primary processing. After 24 h fixation in 10% buffered formalin specimens were paraffin embedded, using standard histological techniques. Three-micrometer-thick sections were performed for each case and stained with the routine haematoxylin-eosin method to analyze the morphological changes of the epithelium and to evaluate the severity of periodontal lesions. Based on morphologic evaluation, additional slides were selected for immunohistochemistry.

Immunohistochemistry. Detection of blood vessels’ endothelium was performed by monoclonal mouse anti-human CD34. Identification of proliferative endothelial cells used a double immunostain procedure able to colocalize CD34 antigen (cytoplasmic) and Ki-67 nuclear proliferative marker. VEGF and receptor 2 for VEGF (VEGFR2, concentration 500 μg/ml, recognizing the activated VEGFR2) completed the protocol proposed for the present study. All steps of the immunohistochemical procedures followed the standardized protocols of fully-automated Bond Max Automated System for Immunohistochemistry (Leica Microsystem, Nussloch, Germany) and the slides were mounted with permanent mounting media (Leica Mount, Leica Microsystem, Nussloch, Germany). Details on antibodies and working systems are summarized in Table I.

Scoring. Scoring was applied for the stratification of periodontal lesions as low, moderate and severe and also, to quantify VEGF and VEGFR2 expression and microvessel density (MVD) of proliferating and non-proliferating blood vessels. Severity of periodontal lesions was based on the presence of inflammatory infiltrate scored as 0 (absent), +1 (low grade, isolated inflammatory cells, less than 10/×400 magnification field), +2 (moderate severity characterized by aggregates of inflammatory cells in the lamina propria only), and +3 (severe lesions with aggregates of inflammatory cells in the lamina propria associated with intraepithelial lymphocytes). VEGF was differentially assessed as follows: VEGFep expression in the gingival epithelium from periodontal lesions; VEGFen expression in the endothelium from stromal compartment blood vessels. VEGFR2 noted the expression of receptor 2 for VEGF inside endothelium of newly-formed blood vessels from the stromal compartment. Proliferative endothelial cells (noted as ENDO Ki-67) were counted in a semi-automated manner using the protocol previously described by Suciu et al. (9). For microvessel density assessment, a modified Weidner method (10) was applied based on the “hot spot” analysis of global MVD (MVD-G, including both proliferative and non-proliferative blood vessels), proliferative vessels MVD from the stromal (MVD-P) and epithelial (MVDepP) compartment.

Table I. Antibody details and workflow description for immunohistochemical assessment of the specimens.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type, clone, manufacturer, dilution</th>
<th>Incubation time</th>
<th>Working system</th>
<th>Cromogen</th>
<th>Expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>Mouse monoclonal, QBEnd 10, Novocastra, UK, 1:200</td>
<td>30 min, RT</td>
<td>Novolink Max Polymer (Novocastra)</td>
<td>Vina Green</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Mouse monoclonal, MIB1, DAKO, Carpinteria, USA, 1:300</td>
<td>30 min, RT</td>
<td>Novolink Max Polymer (Novocastra)</td>
<td>3,3’diaminobenzidina</td>
<td>Nuclear</td>
</tr>
<tr>
<td>VEGF</td>
<td>Mouse monoclonal, VG1, DAKO, Carpinteria, USA, 1:50</td>
<td>30 min, RT</td>
<td>Novolink Max Polymer (Novocastra)</td>
<td>3,3’diaminobenzidina</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Rabbit polyclonal, Reliatech, Germany, 1:100</td>
<td>30 min, RT</td>
<td>Novolink Max Polymer (Novocastra)</td>
<td>3,3’diaminobenzidina</td>
<td>Membranar and cytoplasmic</td>
</tr>
</tbody>
</table>

Results

VEGF and VEGFR2 quantification. In periodontal lesions VEGF and VEGFR2 quantification was performed comparatively in the normal gingiva and in the tissues with periodontal lesions. VEGF expression was assessed in gingival epithelium and the connective tissue compartment (stromal cells as well as in the vascular endothelium). VEGFR2 was exclusively quantified in the vascular endothelium.

In the normal gingiva, we noticed the absence of immunohistochemical expression of both VEGF and its’
corresponding receptor VEGFR2. A particular aspect was represented by the low expression of VEGF in smooth muscle cells of the specialized vascular structures such as the ‘pillow’ arterioles, their endothelium also being negative. Two other components positive for VEGF in the normal gingiva were macrophages and mast cells that intensely expressed this marker.

From the total number of cases included in the study, 53% presented VEGF expression in the gingival epithelium (noted as VEGFep), 64% in the vascular endothelium (VEGFen) and 30% of cases presented VEGF co-expression in both the gingival epithelium and vascular endothelium (VEGFep/en). VEGFR2 was observed in 50% of cases, co-expression of VEGFep/VEGFR2 as well as VEGFen/VEGFR2 being present in 30% of the cases. Considering the grade of periodontal lesions, VEGFep was positive in 33.3% of the cases with low-grade inflammatory lesions, 72.72% of cases with moderate inflammatory lesions and 72% of cases with periodontal lesions with severe inflammation and gingival epithelium. VEGFen was present in the vascular endothelium in 64.7% of cases with low-grade periodontal lesions, 54.54% of the moderate periodontal lesions and 83.33% of the severe periodontal lesions. Co-expression of VEGFep/en was made in 29.41% of the low periodontal lesions, 45.45% of the moderate periodontal lesions and 66.6% of the severe periodontal lesions. VEGFep/VEGFR2 co-expression in case of low periodontal lesions was found in 64.7% of cases. In cases of moderate periodontal lesions, VEGFep/VEGFR2 correlation was present in 72.72% of cases. The severe periodontal lesions presented VEGFep/VEGFR2 in 61.1% of cases.

Regarding VEGFep/VEGFR2 in case of low, moderate and severe periodontal lesions, the correlations were 53%, 36% and 61% respectively. The variation of VEGF and VEGFR2 values, dependant on the grade of periodontal lesions, are summarized in Table II.

The global graphical analysis of VEGF and VEGFR2 expression in the 50 periodontal lesions demonstrated a relatively homogenous distribution of the two markers’ expression (Figure 1a). However, through graphical analysis of VEGF and VEGFR2, made on the types of periodontal lesions dependant on their lesional grade, significant differences between the expression and/ or coexpression of VEGF/VEGFR2 have been registered in case of low (Figure 1b), moderate (Figure 1c) and severe periodontal lesions (Figure 1d). The gradual increase in the VEGFep and VEGFen expression was accompanied by a decrease in the VEGFR2 expression, this aspect being most evident in the cases with severe periodontal lesions.

Particular attention was paid to severe periodontal disease. This group presented VEGF expression in the epithelium and in the connective tissues below the epithelium, where a high number of positive endothelial cells was also identified.
Inside the epithelium, VEGF expression was observed in dendritic cells (intensely positive) and also, epithelial cells presented a particular distribution, being disposed in small groups of positive cells giving the appearance of a heterogeneous distribution (Figure 2a).

VEGFen was found intensely and heterogeneously positive in the endothelium of blood vessels, located deeply inside gingival stroma (Figure 2 b). Along the same vessel, VEGFen was intensely expressed in the sprouting zones. In the pre-existing vessels with a large lumen its’ expression was either

Table II. Co-expression variability of proposed markers for angiogenesis evaluation in periodontal disease. Note the high number of associations between markers and also the polymorphism of correlations for severe periodontal lesions.

<table>
<thead>
<tr>
<th>Severity grade</th>
<th>Low</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameters correlation (p-value)</strong></td>
<td>VEGFep</td>
<td>VEGFR2</td>
<td>VEGFep</td>
</tr>
<tr>
<td>MVD-G</td>
<td>-</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>ENDO-Ki67</td>
<td>-</td>
<td>-</td>
<td>0.07</td>
</tr>
<tr>
<td>MVD-P</td>
<td>-</td>
<td>-</td>
<td>0.04</td>
</tr>
<tr>
<td>MVD Gep</td>
<td>0.036</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>MVDepP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 2. VEGF expression in the gum epithelium and in the vascular endothelium from periodontal stromal lesions. Note the intense expression in the gum epithelium in cases with severe lesions (a) and the absent expression in the normal gum mucosa (a, inset). The vessels belonging to severe lesions were intensely and heterogeneously positive for VEGF (b). The focal expression in epithelial cells from around the papillae characterized the moderate lesions (c) and the restriction of VEGF expression only in case of the stroma for the low lesions (d) with the highest intensity in the macrophages.
low or absent (Figure 2b inset). In cases of moderate periodontal lesions, the epithelial area situated around the papillae intensely expressed VEGF (Figure 2c) with positive fibroblast-like stromal elements inside the connective tissue, close to the epithelium. Low periodontal lesions presented VEGF expression only in the stromal compartment, being positive in the vascular endothelium from the stroma and negative in case of vessels from the papillae. Also, stromal macrophages were intensely positive for VEGF (Figure 2d).

VEGFR2 presented a weakly positive expression in the stromal component and had focally positive areas in the epithelium, restricted to the superficial layer of the gingival epithelium and lacking expression in the newly-formed vessels.

Figure 3. Angiogenesis in periodontal lesions compared to normal gum mucosa (a). Isolated, proliferative endothelial cells (b) associated with 'cord'-type structures with the tendency to form a lumen (c) and vessels with a patent lumen lined by intensely proliferative endothelial cells (d). The sprouting (e) was occasionally associated with intussusception (f), characterized by intrapillar endothelial cells, that were also proliferative.
Microvessel density (MVD) and endothelial proliferation in periodontal disease. Co-localization of CD34 with ki-67 allowed us to assess MVD in close relationship with endothelial proliferation. For the normal gingiva, the blood vessels had a well-defined lumen, endothelial proliferation, being sporadically and inconstantly observed (Figure 3a). Since the evolution of periodontal lesions, the morphology of vessels and proliferation rate of endothelial cells have been significantly modified. A high angiogenic process was suggested by the presence of several small blood vessels, isolated cells with endothelial morphology, distributed in the stromal compartment of periodontal lesions (Figure 3b); they were mixed with vascular structures with a ‘cord-like’ aspect and with the tendency of forming a lumen (Figure 3c) or small blood vessels with a lumen lining by proliferating endothelial cells (Figure 3d). All the above described aspects have sustained the activation of angiogenesis in periodontal lesions through the sprouting mechanism, which was certified due to existence of several vascular branches observed in moderate and severe periodontal lesions (Figure 3e). In addition to this mechanism that predominates periodontal lesions, we noticed a particular aspect though the presence of the intussusception phenomenon associated with intravascular endothelial proliferation and intravascular pillars, respectively (Figure 3f).

Due to the fact that microvessel density (MVD) quantified as a unique parameter does no longer represent a prognostic and therapeutic factor unanimously accepted, we studied MVD associated with endothelial proliferation. Four main parameters were assessed: the number of CD34-positive vessels present in the epithelium and in the stroma, the number of proliferative vessels for each separate compartment as well as, separately, the number of Ki67-positive nuclei within endothelial cells that lined the quantified vessels. The average of MVD value for each case and parameter is separately described in Table III. Also, these parameters were differentially quantified in low, moderate and severe lesions.

MVD in the stromal and intraepithelial compartment, as well as the endothelial proliferation presented significant variations in periodontal lesions, dependent on the lesions’ degree. All evaluated parameters varied according to the severity of the periodontal lesion.

The highest values were observed, as expected, in severe periodontal lesions, but, the greatest value differences of all parameters between lesional severity groups were registered for transition from low to medium periodontal lesions. In the stromal compartment, the number of proliferative endothelial cells (ENDO-Ki-67) as well as the number of proliferative vessels (MVD-P), significantly increased, having a value about 3.5-times higher in moderate periodontal lesions compared low-grade periodontal lesions. For the intraepithelial compartment, a particular aspect was that of the increase in the number of proliferative vessels of about 8-times in case of moderate periodontal lesions, compared to low ones, this being the greatest variation observed in the MVD study of periodontal lesions.

Statistical analysis of the correlation between VEGF, VEGFR2 expression with the vascular microdensity and the endothelial proliferative index. VEGF and VEGFR2 over-expression, as well as the great proliferation rate of endothelial cells observed in periodontal lesions have suggested that angiogenesis in case of periodontal lesions is dependent on VEGF expression and its’ corresponding receptor. Due to this fact, a statistical analysis of the correlation between the parameters used in order to quantify angiogenesis in periodontal lesions was necessary.

The severity grade in periodontal lesions was significantly correlated with global MVD, Ki-67 expression in endothelial cells and the number of proliferative vessels from the stromal compartment but not with those from the epithelial compartment. Endothelial proliferation (ENDO Ki-67) from the stromal compartment presented a significant statistical correlation with VEGF and VEGFR2 expression in the blood vessels’ endothelium. On the other hand, only VEGFR2 showed a statistically significant co-expression with MVD-P, but also with the high vascular density in the pathological gingival epithelium. Also, VEGF and VEGFR2 were differentially correlated with the other parameters dependent on the lesion severity grade.
Thus, in low periodontal lesions, VEGFep expression is significantly implicated in the conditioning of MVD \textit{G} at the epithelial level \((p=0.037)\) suggesting a strong involvement in the initiation of epithelial vascularisation. Also, VEGFR2 influences the endothelial proliferation in the stromal compartment \((p=0.05)\), but does not have any influence on the other analyzed parameters. Moderate periodontal lesions were characterized by statistically significant correlations between VEGFep and MVD \textit{G} \((p=0.027)\), and low correlations with ENDO Ki-67 \((p=0.05)\), but was very strong for MVD \textit{P} \((p=0.04)\). VEGFen expression seems not to influence MVD and endothelial proliferation in moderate periodontal lesions. On the other hand, VEGFR2 expression was correlated with the number of proliferative endothelial cells from the stromal compartment \((p=0.035)\) as well as with MVD for the proliferative vessels (MVD-P) from the same compartment \((p=0.008)\) but also from the epithelial ones \((p=0.029)\).

For severe periodontal lesions we registered an increase of statistically significant correlations. VEGFep influences the proliferative rate of endothelial cells, but mostly MVD-P and MVDepP \((p=0.05)\) equally. In comparison to low and moderate lesions, the presence of VEGFen in severe periodontal lesions plays an important role in angiogenesis. VEGFen was strongly correlated with MVD \textit{G} \((p=0.01)\), modestly with ENDO Ki-67 \((p=0.07)\), intensely with MVD-P \((p=0.049)\) in the stromal compartment but also with MVD Gep \((p=0.043)\) and, mostly with MVDepP \((p=0.001)\).

VEGF expression was not restricted in the gingival epithelium and endothelium. In severe periodontal lesions, groups of VEGF-positive macrophages were frequently observed adjacent to the vessels from the stromal compartment. Low periodontal lesions were characterized by the presence of fibroblast-like cells gathered immediately in the sub-epithelial region and by macrophages dispersed amongst them and were also present inside the gingival papillae.

\textbf{Discussion}

Angiogenesis represents an early and continuous process in periodontal lesions, being present in all developmental stages of the disease, as a result of the interaction between the gingival epithelium and stromal compartment \((11)\).

The correlations obtained in our study sustain the heterogeneity of the angiogenic process dependent on the degree of the periodontal lesions. Angiogenesis found in low periodontal lesions may be considered the first stage of a multi-step angiogenic process, when the pathological gum epithelium activates the angiogenic chain reaction in the connective compartment through VEGFR2 \textit{via} VEGF secretion. This aspect is sustained by the significant correlations registered between the two parameters but also by the increase of MVD both in the epithelial compartment and the connective one, located at a certain distance from the epithelium. The double correlation of intraepithelial MVD with both VEGF and VEGFR2 may be considered a particular and specific aspect for low periodontal lesions progressing through moderate ones, that suggests the initiation of the ‘vascularization’ phenomenon that refers to the acquisition of vessels by the affected gingival epithelium. In cases of moderate periodontal lesions, the stromal compartment represented the main site for an extremely active angiogenic process. The endothelial proliferation induced by VEGFep and sustained by a VEGFR2-dependant mechanism produced the increase of proliferative vessels MVD, thus suggesting the fact that in moderate periodontal lesions, angiogenesis is extremely active with the greatest rate of endothelial proliferation. VEGFen presented the most numerous significant statistically correlations with MVD and with endothelial proliferation in severe periodontal lesions. This aspect, along with the lack of correlations for VEGFR2 in these types of lesions, suggests the saturation of receptor 2 due to its’ binding to VEGF of epithelial origin, and the continuing of a significant endothelial proliferation. If in case of moderate periodontal lesions, VEGF expression determined the activation of angiogenesis mostly in the stromal compartment, in case of severe lesions, VEGF action had a strong and similar influence both in the stromal and in the epithelial compartment.

As a paradox, angiogenesis from periodontal disease is incompletely characterized and inconsistent data are found in the literature. Few reports have focused on VEGF expression in normal gingiva compared to aggressive and chronic periodontitis \((2)\) in healthy and diabetic patients \((12)\). But none of these studies appreciated VEGF expression regarding its impact on endothelial proliferation and disease progression, as we performed in the present study. Moreover, different microscopic assessment of VEGF/VEGFR2 expression on paraffin-embedded tissues, as we performed herein, helps for a better understanding over VEGF multiple sources in periodontal lesions and this can act as a strong reason for anti-VEGF treatment association to the conventional therapy of aggressive and severe periodontitits.

\textbf{Conclusion}

The VEGF/VEGFR2 axis represents the major trigger in the initiation and support of periodontal lesions’ angiogenesis. VEGF expression in the gingival epithelium was found to be progressively increased, strongly dependent on lesion severity. VEGF expression is not restricted to the epithelium, being also observed in the macrophages, in mast cells and in fibroblast-like cells of the stromal compartment. In low periodontal lesions, VEGF/VEGFR2 determines the increase of MVD in the connective compartment without influencing endothelial proliferation, most probably through stimulation
of the intussusception mechanism and initiates angiogenesis in the epithelial compartment, most probably through a similar mechanism, a fact that is sustained by the increase of global intraepithelial MVD without modifications of MVDepP. Moderate periodontal lesions showed a significant endothelial proliferation followed by an increase of MVDepP, ENDO Ki-67 and MVD P, in the epithelial and stromal compartments. All parameters used in order to quantify angiogenesis in periodontal lesions, MVDepP, ENDO Ki-67, MVD P, MVD G and MVD Gep, respectively, were significantly increased in severe periodontal lesions dependent on VEGF ep and VEGFen expression. Our results support the use of anti-VEGF/VEGFR2 targeted therapy as adjuvant treatment in severe periodontal lesions.

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