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Microvascular regulatory role and increased expression of vascular endothelial growth factor receptor type 2 in experimental gingivitis

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Objective: The aim of the present study was to investigate the possible microvascular regulatory role of vascular endothelial growth factor receptor type 2 (VEGFR2) in experimental gingivitis in rats.

Background: Our previous results demonstrated that functionally active VEGFR2s are located in the venules of rat gingiva. While there is no remarkable endogenous gingival VEGF production under normal circumstances, exogenous VEGF, via VEGFR2, shows venodilatory effects. We assumed that VEGF plays an important role in vasoregulatory processes (vasodilation, increased permeability, angiogenesis) of gingival inflammation.

Methods: Gingivitis was induced by placing ligatures and composite material around and between the lower incisors of anesthetized Wistar rats next to the gingival margin. Seven days later, VEGFR2 antagonist (ZM323881), was dripped upon the labial gingiva next to the lower incisors. Diameter changes of the selected gingival venules were measured by vital microscopy. Animals with healthy gingiva served as controls. Venule diameter changes were compared to the baseline and to control groups (no ligature). Immunohistochemical and Western blot analysis for VEGFR2 were utilized.

Results: After 15, 30 and 60 min of local application of ZM323881, there was a significant venoconstriction in the inflamed gingiva compared to the baseline, while no change was recorded in controls. Endothelium, smooth muscle cells and pericytes of the gingivitis group showed increased VEGFR2 expression.

Conclusion: Our findings suggest that there is an increased VEGF production in gingivitis, which may play an important role in vasodilation of rat gingival venules.

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Key words: experimental gingivitis; rats; VEGF receptor type 2; venule; ZM323881

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VEGF is synthesized and released by vascular smooth muscle cells (SMCs), epithelial cells, platelets, leukocytes, and macrophages (1). VEGF expression is influenced by cytokines, or nitric oxide (NO), and is strongly upregulated in conditions characterized by increased microvascular permeability and angiogenesis, such as physiologic wound healing, ischemia, tumor growth or acute inflammation caused by endotoxins (1–4). It is well-documented that *in vitro* VEGF is able to promote growth of vascular endothelial cells, where its name came from (3). Moreover, VEGF can increase vascular permeability to fluid and proteins 50,000 times to that of histamine (3, 5–14). VEGF induces vasodilatation partially via NO synthesis (7, 15–17). There is now agreement that among the three tyrosine kinase receptor subtypes of VEGF, VEGF receptor 2 (VEGFR2), is the major mediator of the mitogenic, angiogenic, permeability-enhancing and vasodilatory effects of VEGF (3, 17, 18). VEGFR2 is also the major player in inflammation, as its increased levels were shown in inflammatory diseases (19, 20). VEGF's role in the healthy or inflamed periodontal tissues is less known. Most studies investigated only VEGF levels, but not its receptor's expression or its direct effects (5, 6, 9, 21, 22). Higher concentrations of VEGF have been noted in inflamed periodontal tissues and in the healing stage of chronic inflammatory periodontal disease (5, 9, 23). In case of periodontitis the gingival crevicular fluid and serum VEGF levels correlate positively with clinical periodontal parameters (23). This can be in connection with the observation that angiogenesis seems to be important for the maintenance of periodontal tissue (22, 24), in which VEGF is one of the most potent regulatory agents (21). Furthermore, VEGF may play a role in the initiation of gingivitis and its progression to chronic periodontitis, possibly by promoting expansion of the local vascular network (5, 21, 25). These findings support the important role of VEGF in the regulation of periodontal microcirculation under pathological states.

Previously, we have shown that while there is no remarkable endogenous gingival VEGF production under normal circumstances, exogenous VEGF through VEGFR2 is able to dilate the venules, thus decreasing gingival vascular resistance (26). Furthermore, our data supported that the venodilatory effect of VEGF is mediated via endothelium derived NO production (26). The role of VEGF in the regulation of small gingival venules of oral mucosa is of special importance, because leukocyte adherence, rolling, migration and the exudation responses take place in postcapillary venules, where resistance regulation of the microcirculation also happens (27, 28). Furthermore, postcapillary venules seem to have a key role in postnatal angiogenesis in collaboration with the preformed microvasculature pericytes (29).

How VEGF regulates microcirculation in gingivitis is still incompletely understood. We hypothesize that VEGF might play a role in the maintenance of microcirculatory vasodilation in gingivitis through the activation of VEGFR2s. Furthermore, apart from endothelial cells, it might influence other cell types as well, which could be important in microcirculatory regulation. The present study prompted the investigation of the localization, amount and effects of VEGFR2 of postcapillary venules in inflamed rat gingiva.

Material and methods

Induction of experimental gingivitis

The experimental design and conditions of the animals were approved by the Animal Ethical Committee of Semmelweis University, Budapest, Hungary (22.1/4268/003/2009).

Fourteen adult male Wistar rats (300 ± 37 g) were randomly divided into two study groups. Complete anesthesia was achieved by intraperitoneal pentobarbital sodium injection (Nembutal, 0.5 mL/kg, 6 m/m %, Sigma-Aldrich, St. Louis, MO, USA). In the first group ($n = 7$) gingivitis was provoked by placing ligatures around and between the lower incisors next to the gingiva and coating the ligatures with light-cured resin composite (SureFil, Dentsply, York, PA, USA) to create plaque retention. Care was taken to avoid tongue damage by the apparatus and not to alter the bite. Ligatures were fixed in place by stitching them around the mandible at the symphysis. Animals in the second group ($n = 7$) served as controls, received no plaque retentive element.

Measurement of venular diameter

After awakening the first group from anesthesia, both groups were housed separately and received food and water *ad libitum*. Seven days later the animals were reanesthetized. Tracheostomy was performed to maintain the free airway. The left femoral artery was cannulized to monitor the systemic blood pressure by a computerized data-acquisition system (Haemosys, Experimetria Ltd., Budapest, Hungary). Rectal temperature was recorded by a digital electronic thermometer, which kept the value stable (38 ± 0.3°C) by means of a feedback mechanism. The lower lip was turned inside out and fixed to the cheek using sutures to provide adequate access to the lower labial gingival area of the mandible. Vital microscopy was used to observe the labial gingiva next to the lower incisors (Nikon SMZ-1B, Tokyo, Japan) (26). Cold light illumination was applied to enhance visibility (Intralux 5000, VOLPI AG, CH-8952 Schlieren, Schwitzerland).

Direction of vascular blood flow was determined and a test postcapillary venule next to the lower incisor was selected for measurements. We tried consistently to use the same venule leaving the superficial layer, but considering the variability in vascular morphology among animals, this seems difficult to justify (26). This venule could be found halfway between the tip of the papilla incisiva and the fornix in about 60% of the rats. If we were not able to clearly identify the same venule, we used another one with comparable location and size across animals throughout the study. Selective

VEGFR2 antagonist 5-((7-benzyloxyquinazolin-4-yl)amino)-4-fluoro-2-methylphenol-hydrochloride (ZM323881, synthesized at Budapest University of Technology and Economics, Faculty of Chemical and Bioengineering, Hungary) was dripped (10 μL, 20 μg/mL) upon the labial gingiva next to the lower incisors using a Hamilton syringe (Hamilton syringe, Hamilton Company, Reno, NV, USA), which allowed precise dripping of the test substance on to the examined vessel (30). We used the highest concentration of ZM323881, that still did not cross-react with other VEGF-binding receptors (VEGFR1, platelet-derived growth factor receptor β, fibroblast growth factor receptor 1, epidermal growth factor receptor and erbB2; $IC_{50} > 50$ μM in tissue culture) (31) and the experimental solution was applied locally, thereby minimizing systemic side-effects that would have influenced our results indirectly. To avoid saliva contamination and dilution of the investigated materials, the examined area was set to be the highest part of the oral cavity. Diameter changes of the selected gingival venule were observed using a transmission light microscope (Nikon SMZ-1B, Tokyo, Japan, 72× zoom) combined with a digital camera (Nikon Coolpix 950, Tokyo, Japan, 3× optical zoom) and images were recorded before, and 1, 5, 15, 30 and 60 min after test substrate application. Aluminum foil was used to cover the investigated area between snapshots to exclude light and avoid desiccation of the surface. Digital images were coded for each sample to avoid examiner bias. The outer diameters of venules were evaluated with ImageTool software (UTHSCSA Image Tool for Windows v3.00, San Antonio, TX, USA, Fig. 1).

A 100 μm marking graticule was used to enable conversion from pixels to micrometers. Mean ± SEM were calculated after measuring the distance between the two given scales 10 times. The analysis was considered to be calibrated if SEM was < 0.5. The analyzing person was not aware as the images being measured which experimental group belonged to.

At the end of the experiment, the lower interincisal gingival tissue of both groups was excised for immunohistochemical and Western blot analysis.

Vascular endothelial growth factor receptor type 2 immunohistochemical localization

The excised tissue blocks harvested from both ligated ($n = 3$) and control groups ($n = 3$) were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h and embedded in paraffin. Several series of 12 μm sections were cut and mounted on gelatin-coated glass slides. The sections were then rehydrated and processed for preembedding immunocytochemistry. The slides were reacted overnight at room temperature with a VEGFR2 primary antibody raised in rabbit [dilution 1 : 20 in 0.01 M (PBS), Cell Signaling, Danvers, MA, USA]. Following brief rinses in PBS, the sections were exposed to a secondary antiserum (1 : 50, Immpress, Vector, Burlingame, CA, USA) and the immunoprecipitate was visualized by the brown color of diaminobenzidine (Sigma-Aldrich). As controls, the primary antibody or the secondary antiserum were omitted to verify specificity. The slides were then counterstained with hematoxylin and covered in mounting medium (DePeX, Electron Microscopy Sciences, Fort Washington, PA, USA). Photomicrographs were taken using a transmission light microscope (Olympus Vanox, Tokyo, Japan). In an effort to quantify our data, microphotographs were taken from sections in both the ligated and control groups. Areas corresponding to 5000 pixels were randomly selected on colored photomicrographs from both the control and ligature groups, and the color density was recorded and analyzed with the help of Image-J software. The saturation of pixels within the red channel was taken as a representative of the color density of diaminobenzidine (brownish red) resulting from VEGFR2 immunocytochemistry. The scale was arbitrarily set between 0 and 255, from white to dark red. To exclude false positivity, the blue component was filtered out and the redness of selected areas was measured instead of their absolute darkness, as we have employed hematoxylin counterstaining following immunolabeling to aid identification of cells/nuclei.

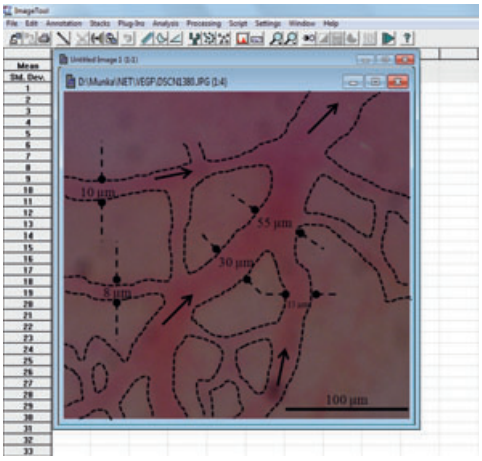

Fig. 1. Representative micrograph of venule outer diameter measurement. Image Tool software was used to measure the distance in pixels. To convert the pixels to micrometers a calibration was performed. Note the confluence of the smaller venules (diameters shown at the dotted lines). Arrows represent the direction of blood flow.

Vascular endothelial growth factor receptor type 2 protein analysis by Western blot

For Western blot analysis, the excised specimens (control group $n = 4$, ligated group $n = 4$) were immediately

dropped into liquid nitrogen. The frozen rat gingiva tissues were then homogenized with glass homogenizer in an ice-cold buffer containing 25 mM Tris (pH 7,4), 1% NP-40, 100 mM NaCl, 4 mM EDTA, 1 mM $NaVO_4$, 10 mM NaF, 1 mM DTT (all chemicals were purchased from Sigma) and supplemented with 50xPIC protease inhibitor cocktail (BD Biosciences Pharmingen, San Diego, CA, USA). Protein concentrations were determined by using the bicinchoninic acid method (Pierce, Rockford, IL, USA). Tissue lysates were mixed 1 : 1 with 2× Laemmli sample buffer and boiled for 5 min at 95°C. Equal amounts of protein for each sample were loaded onto 8% polyacrylamide gels (Biorad, Hercules, CA, USA), separated by SDS-PAGE, and transferred to nitrocellulose membranes (Biorad). The membranes were blocked with 5% non-fat dry milk and incubated with anti-VEGFR2 rabbit monoclonal antibody (Cell Signaling, Danvers, MA, USA) and anti-rabbit IgG-HRP secondary antibody (Cell Signaling). Specific bands were visualized with the Amersham enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK) using the GeneGnome Chemiluminescent detection system (GeneGnome Chemiluminescent detection system, Syngene, Frederik, MD, USA).

Statistics

Venule diameter changes for the animals of the ligated (i.e. experimental gingivitis group) were compared both to baseline and to values of the control group.

At pre-analysis, raw data were evaluated and described as mean ± SEM, and their normality was checked by Shapiro–Wilk's W test. Further statistical analysis was performed using two-way (treatment × time) repeated measures analysis of variance (ANOVA) with contrast values, followed by Fisher LSD *post hoc* test when a significant difference among means was detected. To evaluate quantification of immunohistochemistry data, Student's *t*-test was used. Differences were considered statistically significant at $p < 0.05$. Each analysis was performed using statistical software package (SAS/STAT, Software Release 9.1.3., SAS Institute Inc., Cary, NC, USA).

Results

The baseline blood pressure measured both in the control (123/82 ± 6/5 mmHg), as well as in the ligated group (125/85 ± 8/7 mmHg) did not change significantly after the local application of VEGF or ZM323881.

With respect to mean baseline venule diameter, there was a significant difference between the two groups (27 ± 2 and 51 ± 8 μm, for control and gingivitis group, respectively, Fig. 2). Within 15, 30 and 60 min after the local application of ZM323881 there was a significant vasoconstriction in the venules of the inflamed gingiva, as compared to baseline (84.8 ± 6%, 81.8 ± 6.4%, 82.5 ± 4.8%, respectively, Fig. 3). There was no change of venule diameter in the control group (Fig. 3).

In gingivitis, VEGFR2 expression in gingival vessels was markedly increased as revealed by VEGFR2 immunohistochemistry, whereas in the vascular elements of the control group no (Fig. 4A and 4B), or only little (Fig. 4C and 4D) immunopositivity was observed. However, occasionally, in these control sections, the vascular endothelium elicited a weak labeling and a few pericytes were also VEGFR2 immunoreactive (Fig. 4C and 4D).

In specimens from the ligated group, there was a high level of specific immunoreactivity within the wall and neighboring areas of gingival vessels. The intramural components of arterioles and venules elicited specific VEGFR2 immunopositivity, including the

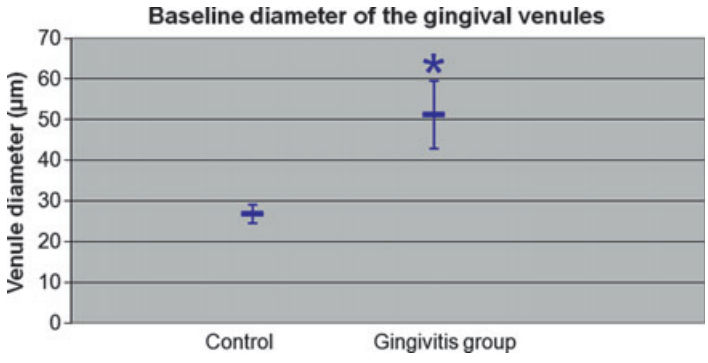

Fig. 2. Baseline diameter of gingival venules. The mean baseline diameter of the gingivitis group is significantly larger, than that of control (mean ± SEM, $*p < 0.05$)

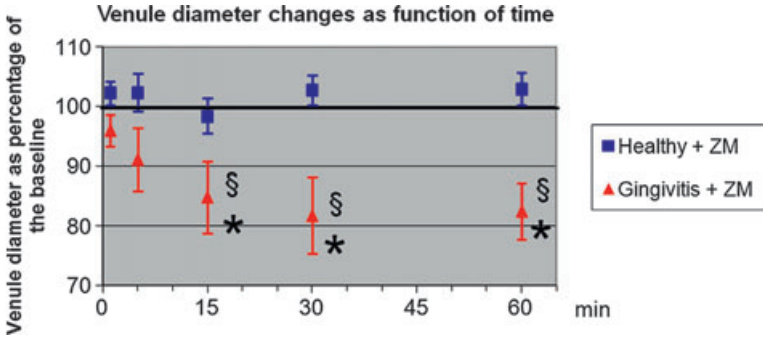

Fig. 3. Gingival venule diameter response after vascular endothelial growth factor receptor type 2 (VEGFR2) inhibition as function of time. VEGFR2 blocker ZM323881 (20 μg/mL) was dripped locally. Squares and triangles denote the control group and gingivitis group, respectively. ($^{§}p < 0.05$ to the start-up, $*p < 0.05$ between groups, mean ± SEM).

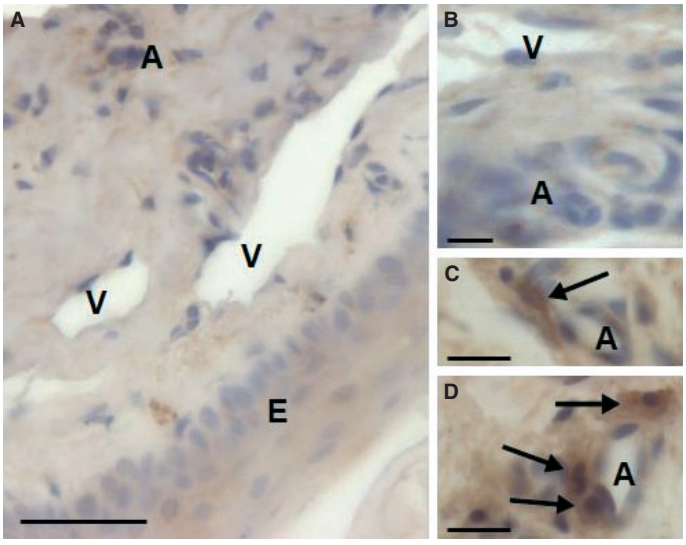

Fig. 4. Vascular endothelial growth factor receptor type 2 immunohistochemistry in control sections. Generally, small blood vessels exhibit no, or very little specific immunostaining within their endothelia or smooth muscle cells as shown in (A, B) or (C, D). However, infrequently, a weak endothelial reactivity is apparent accompanied by the presence of a few immunopositive pericytes (arrows in C, D). A, small artery/arteriole; V, small vein/venule; E, epithelium. Scale bar: (A) 50 μm; (B, C, D) 10 μm.

innermost lining layer of the vascular wall, endothelium and middle layer, representing SMCs (Fig. 5A and 5B). It is worth noting that besides pericytes, located adjacent to the vascular wall, we found numerous VEGFR2 immunoreactive fibroblasts in the vicinity of highly reactive vessels (Fig. 5C).

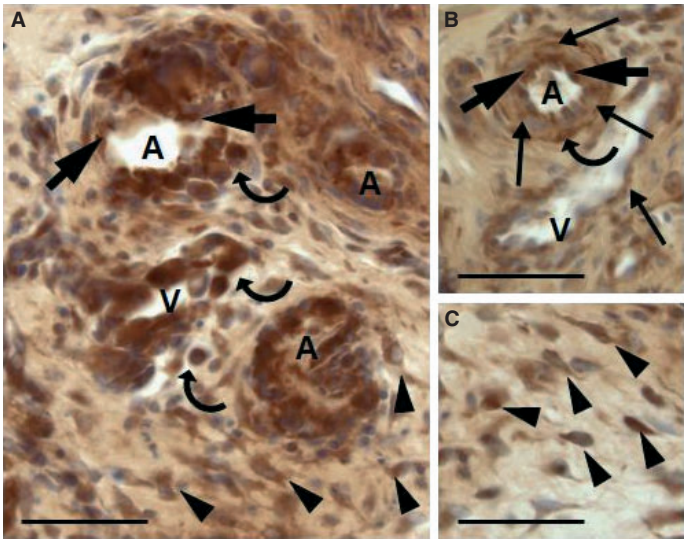

Fig. 5. Localization of vascular endothelial growth factor receptor type 2 immunoreactivity in the ligated gingiva. A massive immunopositivity was observed within the mural layers of the small gingival blood vessels. Densely stained endothelial cells (thick arrows in A, B) compose the innermost layer with their cell nuclei running parallel to the vascular axis. In the middle layer, the smooth muscle cells (thin arrows in B) were represented by punctate immunolabeling. Apart from labeled pericytes (curved arrows in A, B), located external to the muscle layer, several immunopositive fibroblasts have also been observed (arrowheads in A, C). Scale bar: 50 μm in A-C.

In further control slides, where the primary or secondary antisera was omitted, no specific immunolabeling was observed either in arterioles or in venules, differing substantially from the level of background tissue reactivity (not shown).

The finding, that sections from the ligated animals express a darker immunoreaction within or adjacent to the vascular wall has been proven significantly higher than similar areas deriving from control gingivas (Fig. 6). No similar significant difference was observed when the surrounding lamina propria (i.e. background) was analyzed (Fig. 6).

Western blot analysis corroborated this latter result, as there was a significant increase in the amount of proteins typical for VEGR2 (240 and 210 kDa), in the case of gingivitis specimens when compared to the control (Fig. 7).

Discussion

Gingival tissues are often in a state of injury and repair that involve repetitive cycles of production of chemotactic and inflammatory reactions (32). It is well known that VEGF is one of the key regulators in angiogenesis and plays an important role in controlling vascular permeability, particularly in the case of inflammation (1, 13, 14, 19, 33). Although VEGF concentrations of crevicular fluid in different stages of periodontitis were studied in detail (21), the exact mechanisms and participants of the VEGF-derived complex regulatory procedures are hardly investigated in periodontal tissues.

For this reason, the aim of this study was to explore the regulatory role of VEGF in the microcirculation of experimental gingivitis. Therefore, we measured the diameter changes of gingival venules in response to the locally applied VEGFR2 antagonist ZM323881 in inflamed rat gingiva and histologically defined the cell types, which showed immunohistological staining for VEGFR2. We found that the locally applied specific VEGFR2 blocker ZM323881 significantly decreased the diameter of venules

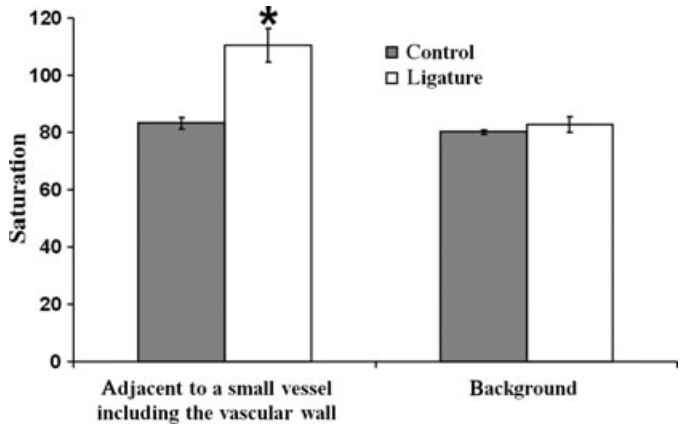

Fig. 6. Saturation of pixels within the red channel as a representative of the diaminobenzidine color density following vascular endothelial growth factor receptor type 2 immunocytochemistry. To exclude false positivity, the blue component was filtered out and the redness of selected areas was measured instead of their absolute darkness, as we have employed hematoxylin counterstaining following immunolabeling to aid identification of cells/nuclei. Gingival sections from ligated animals express an elevated level in color density within the vicinity, and in the wall of, small vessels significantly higher than those of control animals (*$p < 0.01$). No such difference was observed in the case of background areas ($p = 0.477$, not significant).

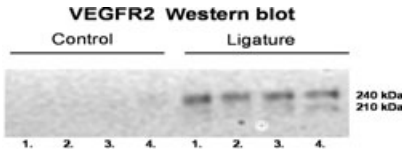

Fig. 7. Vascular endothelial growth factor receptor type 2 (VEGFR2) Western blot analysis. Specific bands were visualized with an enhanced chemiluminescence system (Amersham) using the GeneGnome Chemiluminescent detection system (Syngene). The contrast of the VEGR2-specific 210 and 240 kDa bands was significantly increased in the gingivitis group in comparison to the control.

compared to baseline levels in experimental gingivitis, but not in the healthy control group. Furthermore, VEGFR2 immunoreactivity is increased significantly in all vascular cell types in gingivitis. These results suggest that there is a significant, functionally active VEGF production in gingival tissue in inflammation, which is not characteristic in healthy gingiva and this may have a role in the regulation and remodeling of inflamed gingival microcirculation. Previous studies (33–35) demonstrated quantitative and qualitative changes in the vasculature of periodontal connective tissues in periodontitis. Chapple *et al*. (33) reported a disturbed regulation in the angiogenesis subjacent to periodontal pocket epithelium, which manifested an altered expression of VEGF. Angiogenesis is typically initiated within hypoxic tissues, which need novel blood vessels to maintain adequate oxygenation. When a tissue is hypoxic, cellular oxygen-sensing mechanisms are activated, inducing gene expression of pro-angiogenic proteins.

According to our previous and recent working hypothesis, bacteria induce a protective periodontal host response with the contribution of VEGF by three main pathways (Fig. 8). At first, in the immigrating leukocytes and resident cells there is an enzymatic production of oxidative and nitrosative free radicals, including superoxide, NO and their reaction product, peroxynitrite to kill the invading microorganisms (36). However, their overproduction results in a host defect (e.g. mitochondrial dysfunction with the consequent further shift towards mitochondrial free radical formation from terminal end oxidation and the decrease of O_2 extraction by tissues, both of which seem to be a self-exciting procedure) (36–39). Ultimately, the oxygen consumption and oxidative phosphorylation disconnect. Secondly and thirdly, the cytokine release and the inflammatory tissue hypoxia lead to hypoxia-inducible factor-1 alpha (HIF-1α) stabilization, VEGF production and VEGFR2 upregulation (37, 40–42), which will result in net vasodilation, and angiogenesis to compensate for tissue hypoxia. On the other hand, the inflammatory cell recruitment of VEGF positively stimulates the hypoxia-independent components (i.e. reactive species and/or cytokines) of its own production. The resultant VEGF overproduction can contribute to inflammatory progression. VEGF overproduction can be suppressed by eliminating the cause of inflammation, namely the bacteria or by blocking the different steps of its regulatory loop (e.g. by resveratrol) (5, 23, 43).

The expression of VEGF as an important HIF-responsive fibroblast factor is known to be increased in inflammatory fibroblasts as well (42, 44). In accordance with these findings, we detected VEGFR2 immunopositive fibroblasts around the vessels in the gingivitis group, which also confirms the possible role of fibroblasts in the accompanying vascular changes. The fibroblast changes and HIF-1 stabilization together with VEGFR2 expression may be sufficient to induce inflammatory cell recruitment and angiogenesis as well (40, 44).

The angiogenesis in microcirculation reaches its peak at about the eighth day after an injury and shows correlation with VEGF upregulation (45). In our study, ligature was left around the incisors for 7 d, in consort with the supposed approximate peak time of angiogenesis. The increased concentration of VEGF can modify the contractile response of microvascular pericytes through VEGFRs, and contribute to increased permeability seen in inflammatory states (46). The latter result seems to be supported by our findings, as we detected VEGFR2 immunopositive pericytes located around the inflamed gingival vessels.

Though we have shown that all components and neighboring cells of

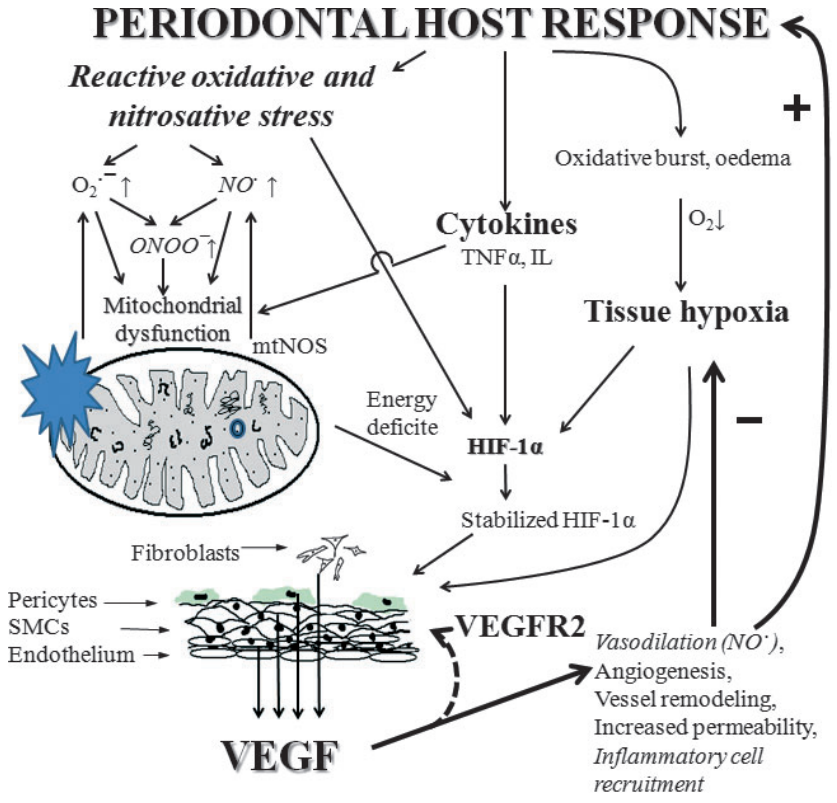

Fig. 8. The proposed main pathways in activation of vascular endothelial growth factor receptor type (VEGFR) 2s in periodontal inflammation. For simplification, only the most significant contributors are illustrated on the figure. In periodontal inflammation the oxidative stress (e.g. superoxide /$O_2^{\bullet-}$/) and the nitrosative stress [e.g. nitric oxide ($NO^{\bullet}$)] and their interaction [peroxinitrite ($ONOO^-$)] are detrimental to the invading bacteria; however, they are toxic for the host tissues as well. This may lead to mitochondrial dysfunction and energy deficits, which together with tissue hypoxia and cytokines [e.g. tumor necrosis factor alpha (TNFα), interleukin (IL)] induce VEGF production through the stabilization of hypoxia-inducible factor-1 alpha (HIF-1α). The short-term (e.g. vasodilation) and long-term (e.g. vessel remodeling) effects of VEGF with the compensation of tissue hypoxia may suppress its own hypoxia-dependent production (negative feedback). However, the VEGF through stimulation of inflammatory cell recruitment may further increase its own production (positive feedback). The overexpression of VEGF may contribute to the periodontal disease. To suppress the hypoxia-independent component of VEGF stimulation, the cause of the periodontal host response, the bacteria have to be eliminated, or elements of the positive feedback loop have to be inhibited. For the development of these effects, it is interesting to note that all mural cells [endothelium, smooth muscle cells (SMCs), pericytes and perivascular fibroblasts] not only bear VEGFR2s, but may also produce VEGF, which elements play in concert in autocrine, paracrine or endocrine regulation. The italic parts of the figure are the recent and earlier contributions to the suggested mechanism of our research group (26, 36).

the vessel wall (endothelium, SMC, pericyte, fibroblast) express VEGFR2 and on the other hand it looks like all these cells are able to produce VEGF as well, it is not clear what kind of complex paracrine, autocrine or endocrine regulation of vessel functions, remodeling and stabilization might be present in the background (47–50). However, the important role of mural cell (pericytes, vascular SMCs and perivascular fibroblasts) derived VEGF, endothelial VEGFR2 signaling and endothelial–mural cell interactions were emphasized in other studies as well (49, 50).

In summary, our findings suggest there is marked VEGF production of gingivitis in rats, which through the expression and activation of VEGFR2s in endothelial cells, SMCs, pericytes and fibroblasts may control the inflammation-induced increase of gingival blood circulation and the remodeling of the local microvascular network.

Acknowledgements

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