Analysis of vascular distribution and growth factors in human gingival tissue associated with periodontal probing depth

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Summary: Vascular endothelial growth factor (VEGF) is a key regulator of blood vessel endothelium. Tissue levels of this angiogenesis marker are unknown in human gingival tissue, as is the correlation between vascular growth factors and hypoxia-inducible factor. We examined the expression of VEGF, type III tyrosine kinase receptors (VEGF-R2), platelet-endothelial cell adhesion molecule (CD31) and hypoxia-inducible factor (HIF) mRNA from human gingival tissue of the oral cavity. Tissue samples were from a small quantity of gingival sample biopsy with gingival sulcular depth (GSD) < 2 mm (Group 1), 2 to 4 mm (Group 2), and > 4 mm (Group 3). We found that the levels of VEGF-R2, CD31 and HIF mRNA were higher in the gingival tissue of Group 2 than that of Group 1, and VEGF in the Group 3 was also higher than that of Group 1. The different mRNA levels of these markers may reflect the mRNA levels reflect the vasculature state of gingival tissue based on GSD. VEGF-R2 and HIF also indicate the presence of an elongated blood vessel in the gingival tissue. In the early stage of angiogenesis, VEGF-R2 leads to expression of VEGF, and HIF-1 mediates increased VEGF expression in response to hypoxia in swollen tissues or during the expansion of periodontal tissues, which is useful in the early diagnosis of periodontal diseases.

Introduction

The density of blood vessels increases with increasing pocket depth of the gingival tissue (Chapple et al., 2000). The vessels’ endothelial permeability and increases in size and volume are accomplished by vasculogenesis and angiogenesis. Angiogenesis is preceded by periodontal conditioning, i.e., enlargement of the vessels in the gingival sulcus may reflect vascular remodeling with endothelial cells in capillary proliferation. However, in previous reports, it remains unknown whether differences in remodeling reflect the gingival sulcular depth (GSD). Vascular endothelial growth factor (VEGF) protein affects the vascular network during the progression of periodontal condition (Booth et al., 1998; Johnson et al., 1999). VEGF expression and VEGF binding to the tyrosine kinase receptor VEGF-R are regulated by vasculogenesis and angiogenesis (Brown et al., 1992; Peters et al., 1993). VEGF acts as a paracrine factor whose signals are mediated by two type III tyrosine kinase receptors on the surface of endothelial cells: VEGFR-1 and VEGFR-2. VEGFR-2 is a major mediator of the mitogenic and angiogenic effects of VEGF (Grando et al., 2007). The platelet-endothelial cell adhesion molecule (CD31) is also an important marker of vascular endothelial cells in periodontal tissue (Gemmell et al., 1994).

The mRNA for hypoxia-inducible factor (HIF)-1alpha is increased in the presence of interleukin-1 (IL-1) (Thorton et al., 2000). IL-1 protein is more prevalent in diseased gingival tissue (Lester et al., 2009). HIF-1alpha also coordinates vessel development (Ramirez-Bergeron et al., 2006), which regulates VEGF expression (Daikoku et al., 2003), an essential mediator of angiogenesis. The relationship between VEGF, VEGF-R2 and HIF-1 in human periodontal tissue is unknown. During increased of GSD, these markers provide important information about the vessel’s endothelial permeability as well as increases in vessel size and volume at the biopsy level. Therefore, we sought to examine these factors in GSD-associated human gingival tissue. We used a quantitative real-time PCR analysis, which quantified the expression levels of VEGF and VEGF-R2 mRNA and compared CD31 and HIF from small biopsies. We also analyzed the immunohistochemical localization of VEGFR-2, VEGF and HIF in human gingival tissue to undertake a comparative evaluation using mRNA analysis.
Materials and Methods

Samples
Participants (129) in this study included 61 males and 68 females (mean age 33.4 years, range 20–75 years), who all provided informed consent. Before biopsy, the GSD was measured with a single calibrated periodontal probe by a periodontist. The investigator assessed and classified gingival samples at each site and, according to criteria of following sentence, divided participants into three groups. Gingival samples were defined as clinically healthy when the GSD was <2 mm (Group 1), and other samples were classified with GSD from 2 to 4 mm (Group 2) and a GSD > 4 mm (Group 3). Tissue samples were obtained from the gingival tissue adjacent to the second molar tooth in the mandible.

Ethics
The study was approved by the Human Research Committee (approved no. 05-08) of Nippon Dental University. The study was also performed with written informed consent from the patients.

Quantitative real time PCR analysis
Biopsied tissues were soaked in RNA later (AMBION, Texas, USA) for 24 h at room temperature and stored at −80°C until use. To isolate the RNA, each individual periodontal tissue specimen was thawed and stored on ice to prevent RNA degradation by RNAase. Rnasy® (QIA-GEN, CA, USA) was used to isolate RNA by following the manufacturer’s protocol. The RNA concentration (mg/ml) was determined for each sample with a spectrophotometer (260 nm; Biowave S2100 diode array spectrophotometer; WPA, Cambridge UK). One milligram of total RNA was reverse transcribed into cDNA using AMV Reverse transcriptase XL (Takara, Tokyo, Japan). Each amplification reaction (50 μL) contained 100 ng cDNA, 900 nM forward primer, 900 nM reverse primer, 250 nM fluorogenic probe, and 25 μl Universal Master Mix (Applied Biosystems). Human VEGF and VEGFR-2 primers and probes were obtained from Applied Biosystems ‘Assays-On-Demand’: Assay ID CD31; Hs00169777_m1, VEGF; Hs00173626_m1 and VEGFR-2; and Hs00176676_ml. HIF-beta primers and probes were obtained from the gingival tissue adjacent to the second molar tooth in the mandible.

Statistical analyses
The statistical significance of differences in the percentage of cases with different reactivity levels in our study was analyzed by one-way Kruskal-Wallis ANOVA and by Dunn’s Multiple Comparison Test for three groups. P-values < 0.05 were considered to be statistically significant.

Results
Sample distributions according to the GSD
The average probing depth of the examined samples is shown in Figure 1 (male: 3.22 ± 0.84 mm, female: 2.77 ± 0.81 mm, and the percentage of Groups 1, 2 and 3 out of the total number of participants are 27.9, 65.1 and 7.0%, respectively) (Fig. 1).
Real time PCR

Comparing the three groups of gingival tissue, the VEGF mRNA expression in Group 3 was higher than that of Group 1. The level in Group 2 was slightly higher than Group 1, but a significant difference was found between Group 3 and Group 1 \((p < 0.05)\). However, the mRNA levels of VEGF-R2 \((p < 0.01)\), CD31 \((p < 0.05)\), and HIF \((p < 0.01)\) in Group 2 were also higher than that of Group 1. However, VEGF-R2, CD31, and HIF \((p < 0.01)\) mRNA levels in Group 3 were higher than that of Group 1 (Figs. 2A–D).

Immunohistochemical localization of VEGF, VEGFR-2 and CD31

The vessels of Group 2 and 3 samples were swollen compared to those of Group 1. The immunoreactions to VEGF, VEGF-R2 and CD31 were positive throughout the vessels in the three groups, but the reaction of HIF was slightly increased in Group 1 and Group 2 (Figs. 3–6). Positive reactions to VEGF in all three groups and to VEGF-R2 in Group 1 vessel endothelial cells were found.
in the gingival tissue (Figs. 3, 4). The intensity of the reaction to VEGF was higher than that of VEGF-R2 (Figs. 3, 4). The immunohistochemical reactions to VEGF, VEGF-R2 and CD31 were scattered among the fibroblasts in the connective tissue surrounding the vessels (Fig. 3). The reaction of VEGF and VEGF-R2 in Group 1 was intense, unlike the other two groups (Figs. 3–6).

Discussion

VEGF is a key regulator of blood vessel growth and is required for both organ development and differentiation during embryogenesis, as well as for angiogenesis, wound healing, and in malignant or certain inflammatory diseases in adults (Brown et al., 1992; Breier et al., 1992; Dvorak et al., 1995A; Dvorak et al., 1995B; Risau, 1997; Nicosia, 1998; Carmeliet et al., 1999; Hiratsuka et al., 2002; Autiero et al., 2003; Brown et al., 1995; Hanahan et al., 1996). Angiogenesis is an important characteristic of inflammation and healing. It can influence the severity of periodontal inflammation because increases in new vasculature facilitate the transport of more inflammatory cells, chemical mediators and cytokines via inflammation. There are several reports concerning the VEGF content of periodontal tissues, and this may reflect permeability and

Fig. 3. Distribution of blood vessels and other positive cells with anti-VEGF antibody (A, B, C) and control (D, E, F) sections in the human periodontal tissue from three groups (A, D: Group 1; B, E: Group 2; and C, F: Group 3). Positive cells (arrows) are detected in these samples. Bar = 100 µm.
proliferation in vascular endothelial cells. In the gingival crevicular fluid, the VEGF level is higher at diseased sites than healthy sites (Booth, 1998). In contrast, the intensity of VEGF is lower in biopsy homogenate samples with peri-implantitis (Cornelini et al., 2001). However, we used enzyme-linked immunosorbent assays of biopsy homogenate and found that VEGF levels are higher in diseased sites adjacent to a 4 to 6 mm GSD than in healthy sites. These results agree with those measured by Johanson’s protocols (Johnson et al., 1999) for sampling from periodontal diseased tissues adjacent to oral sulcular epithelium. Inflammation sites in peri-implantitis mainly occur in bones around implants. Therefore, we hypothesized that VEGF may specifically increase in periodontal disease with inflammation of the periodontal ligament lesion. At the transcriptional level, our results show that VEGF mRNA occurs at higher levels in Group 3 subjects (>4 mm GSD). VEGF regulates microvascular permeability and endothelial fenestration in vascular features (Gregory et al., 1995). We assumed that vascular endothelial hyperpermeability and inflamed angiogenesis increased in Group 3 subjects (>4 mm GSD) because VEGF transcription levels increased from 4 mm GSD.

VEGF receptors are a key regulator of blood vessel growth and are expressed for organ development and differentiation, as well as for angiogenesis during

Fig. 4. Distribution of blood vessels and other positive cells with anti-VEGF-R antibody (A, B, C) and control (D, E, F) sections in the human periodontal tissue from three groups (A, D: Group 1; B, E: Group 2; and C, F: Group 3). Positive cells (arrows) are detected in these samples. Bar = 100 µm.
VEGF-R1 regulates vasculogenesis and angiogenesis during early embryogenesis. VEGF-R2 plays a role in physiological and pathological angiogenesis. In contrast, VEGF-R3 is a receptor for lymphatic growth factors (VEGF-C or VEGF-D) and regulates vascular and lymphatic endothelial cell function during embryogenesis (Shibuya et al., 2006). VEGF-R2 exists in the endothelial cell membrane and requires many receptor binding cells. Purine nucleotide receptors (P2YRs) promote endothelial cell tubulogenesis in breast cancer, and the P2YR-VEGF-R2 interaction and resultant signal transduction are important in angiogenesis (Rumjahn et al., 2009). VEGF-R2 inhibitory activity also affects tumor progression (Heckman et al., 2008). In contrast, VEGF-R2 proteins impact capillary growth in rat extensor digitorum longus muscles (Milkiewicz et al., 2005). In contrast to the expression of VEGF, our results demonstrate that elevated expression of VEGF-R2 mRNA is found in early stage gingivitis in Group 2 (corresponding to a 2 to 4 mm GSD,) and decreased expression was noted in Group 3. The VEGF transcription level remained low in Groups 1 and 2, but the level of VEGF-R2 in Group 2 was significantly higher than that in Group 1. The mechanisms by which VEGF-R2 traffics VEGF, transmits the

Fig. 5. Distribution of blood vessels and other positive cells with anti-CD31 antibody (A, B, C) and control (D, E, F) sections in the human periodontal tissue from three groups (A, D: Group 1; B, E: Group 2; and C, F: Group 3). Positive cells (arrows) are detected in these samples. Bar = 100 µm.
signals to the vascular endothelial, and induces cell proliferation are not clear (Shibuya et al., 2006; Jopling et al., 2009). Therefore, our data reveal that increased VEGF-R2 is correlated with the initiation of gingivitis and periodontal tissue disease. We hypothesized that the VEGF signals in Group 3 might be amplified in the pathways of vasculogenesis in its cells because the level of VEGF-R2 is slightly decreased in Group 3, but the endothelial cells proliferate both in gingivitis (Group 2) and in diseased periodontal tissues (Group 3) in terms of immunohistochemical staining and CD31 transcription levels. Recently, CD31 (platelet endothelial cell adhesion molecule-1 (PECAM-1)) was noted as a component of a mechanosensory complex that mediates endothelial cell responses to shear stress (Chen et al., 2009). Here, we found that CD31 transcript levels in Groups 2 and 3 were higher than in Group 1. Swollen periodontal tissues or their expansion in elongated GSD may lead to mechanical stress, which can also increase vessel volume or remodeling. Expression of VEGF-R2 is an initial step in signal transduction, receptor tyrosine kinase (RTK) phosphorylation, and throughout all of the numerous pathways that activate proliferation, survival, migration, and the permeability of vascular endothelium (Gemmell et al., 1994). We found that the

Fig. 6. Distribution of blood vessels and other positive cells with anti-HIF antibody (A, B, C) and control (D, E, F) sections in the human periodontal tissue from three groups (A, D: Group 1; B, E: Group 2; and C, F: Group 3). Positive cells (arrows) are detected in these samples. Bar = 100 µm.
VEGF-R2 transcription level in Group 2 was significantly higher than in Group 3. The first step in angiogenesis commences with the expression of VEGF-R2 mRNA, and then VEGF is required for revascularization of the mRNA indicates that it is expressed, VEGF-R2 shows the importance of timing of expression. Therefore, VEGF-R2 is one of the most important factors in angiogenesis. HIF-1α acts as a transcriptional activator in metabolic processes, including glucose control, amino acid, nucleotide and pH regulation, drug resistance, cellular proliferation, apoptosis and survival, transcriptional regulation, extracellular matrix metabolism, cytoskeletal structure, cell-cell adhesion, angiogenesis and vascular tone control (Quintero et al., 2004; Brennan et al., 2005). Therefore, HIF-1α is an essential component in altering the transcriptional response to angiogenesis. HIF mRNA expression is induced in response to gingivitis in Group 2 (corresponding to a 2 to 4 mm GSD), whereas induction is observed in response to mechanical stress, indicating that HIF-1α mediates increased VEGF expression specifically in response to hypoxia. VEGF and HIF-1α mRNA are significantly elevated by frequencies of electrical stimulation, and capillary density is significantly increased in the ischecnic skeletal muscle of rabbit (Shen et al., 2009). In contrast, a prompt increased in HIF activity and VEGF expression occurs in the presence of acute coronary artery occlusion (Lee et al., 2000). HIF-dependent gene/protein expression leads to vascular remodeling following ischemic insults. Therefore, the early stages of swollen periodontal tissues or their expansion may also lead to a VEGF expression-orchestrated angiogenesis program.

References


