A morphological study of the blood vessels associated with periodontal probing depth in human gingival tissue

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Summary: Gingival tissues in human cadavers were examined the blood vessel diameter in the depths of the gingival pockets such as three groups: gingiva adjacent to a sulcus of 2 mm (Group 1); gingiva adjacent to a 2–4-mm sulcus (Group 2); and gingiva adjacent to a sulcus of > 4 mm (Group 3). A meaningful significant difference was seen observed in gingival pocket side, intermediate and outer layer side regions of the gingiva. A meaningful significant difference was seen found in intermediate part and the outer layer of the gingiva in Group 3. Other gingival biopsies were performed on a human body donation specimen to examine CD-31 positive endothelial cells of blood vessels by an immunohistochemical method. Our results suggest that the periodontal probing depth reflect the blood vessel organization of human gingival tissue.

Introduction

A periodontal probing depth (PPD) is an important information for the evaluation of oral tissue conditions in periodontal maintenance. The PPD is a measurement of the space caused by the resorption and migration of the periodontal ligament attachment from the root of the tooth (Armitage, 1999). The expansion and remodeling of the gingival blood vessels results are related to the diameter of the blood vessels in gingival biopsies of human gingival tissues (Zoellner and Hunter, 1991). The number of blood vessels is related to the PPD. The platelet endothelial cell adhesion molecule (PECAM or CD-31) is an important endothelial cell marker in normal tissues (Pusztaszeri *et al.*, 2006) and is strongly expressed in the endothelial cells of blood vessels (Osawa *et al.*, 1997; Shaw *et al.*, 2001; Ilan and Madri, 2003; Pusztaszeri *et al.*, 2006). Vascular endothelial growth factor (VEGF) is also a novel growth factor for vascular endothelium (Ferrara *et al.*, 2003). However, the VEGF concentrations are high, and there is an increase in the gingiva associated with periodontal diseases; they are not correlated with sulcular depth (Johnson *et al.*, 1999). Therefore, we measured the diameter (μm) of the blood vessels in human cadavers with hematoxylin and

eosin staining (HE) staining, after CD-31 positive endothelial cells of blood vessels from gingival biopsies on a human body donation specimen were examined by an immunohistochemical method. Our study may provide useful information for the diagnosis of oral periodontal tissue conditions.

Materials and Methods

Sample selection

The investigator assessed and classified gingival samples at each site and divided the participants into three groups; gingiva adjacent to a sulcus of < 2 mm (Group 1); gingiva adjacent to a 2–4 mm sulcus (Group 2); and gingiva adjacent to a sulcus of > 4 mm (Group 3). Gingival samples were defined as clinically healthy when the periodontal probing depth according to the method of Johnson *et al.* (1999). Samples of periodontal tissue were obtained from the gingival tissue adjacent to the molar tooth. Four biopsy samples (mean age 47.8 years) were used for immunohistochemical or general configurations of gingiva observations. Samples of human cadaver ($n = 3$, mean age 75.0 years, each three portions) were

used for the measurement of the diameter of the blood vessels.

Measurement of Blood Vessels

All cadavers had been donated for human dissection. Samples were injected with 10% formalin with return perfusion through the femoral artery. After anatomical dissection, the gingival tissues were removed from the samples. We devided three parts of the gingival groove: gingival pocket side (GPS), intermediate part (IP), and outer layer part (OP) (see fig. 1). These three parallel lines were defined by a tooth axis, and the gingival sulcus was cut at a right angle for the gingival aspect to surface at the buccolingual section of the tooth. We measured the diameter ($\phi = \mu\text{m}$) of blood vessel in three areas. The measurement areas were traced from blood vessel to paper via a camera lucida (drawing device) using a microscope from each section.

Ethics

The study was approved by the Human Research Committee (approved no. 05–08) for the Nippon Dental University. The study was performed after obtaining written informed consent from the patients. The human cadavers were obtained from donation system using the guidelines from the Law Concerning Body Donation for Medical and Dental Education (the Body Donation Law) and the Law Concerning Cadaver Dissection and Preservation (LCCDP).

Immunohistochemical Staining

Gingival samples were embedded into OCT compound (Tissue Tek II, Sakura Finetechnical Co., Ltd, Tokyo, Japan), frozen in liquid nitrogen and stored at -80°C until use. Frozen tissues were coronally sectioned using a cryostat. The sections were collected serially and

Fig. 1. Measurements region of the human gingival by Hematoxylin and Eosin (H-E) Staining in Group 1. Three parts of the gingival groove: gingival pocket side (GPS), intermediate part (IP), and outer layer part (OP).

Fig. 2. Schema of measurement of blood vessels diameter (Short axis). Numbers (62–79) are blood vessels which are selected from the sagittal section of the human gingival. B, Number 64 is one of the samples in the selected blood vessels from Fig 2A

thaw-mounted on glass slides. After air-drying, the sections were fixed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH7.2) for 5 minutes. After three 10 minute washes in PBS, the sections were immersed in a blocking solution (PBS containing 0.5% Triton-X-100 and 5% normal goat serum) for 60–90 minutes at room temperature, and then incubated for 1 day at room temperature or 2 days at 4°C with rabbit polyclonal anti-CD31 antibody (1:100; Lab vision, Fremont, CA; catalogue number RB10333). Finally, sections were incubated with goat biotinylated anti-rabbit antibody (1:200; biotinylated anti-rabbit goat antibody kit, Vector Laboratories, Burlingame, CA) for 60–90 minutes at room temperature and visualized by ABC (ABC Elite kits, Vector Laboratories, Burlingame, CA) with diaminobenzidine containing 0.03% nickel ammonium sulphate. We performed hematoxylin and eosin staining (HE) to observe general configurations of gingiva and measurement of blood vessels with a light microscopy, and photographs were taken as digital images by digital camera, Leica DFC290 (Leica Microsystems, Germany).

Statistical Analysis

The statistical significance of difference in the percentage of cases with different reactivity levels in our study was analyzed by one-way Kruskal-Wallis ANOVA and

Dunn's Multiple Comparison Test for three groups. P-values less than 0.05 indicated statistical significance.

Results

Measurement of periodontal probing depth

The PPD for males was 3.44 ± 0.84 mm (average), and it was 2.77 ± 0.81 mm (average) for females. The male ($p = 0.0244$) and female ($p = 0.06073$) depths were significantly correlated regarding depth pocket and aging (see fig 3).

Blood Vessel Diameter ($\phi = \mu\text{m}$)

In Group 1, the blood vessel diameter was $33.8 \pm 3.5 \mu\text{m}$ in the gingival pocket side (GPS), $27.5 \pm 5.1 \mu\text{m}$ in the intermediate part (IP), and $21.1 \pm 2.2 \mu\text{m}$ in outer layer part (OP) of the gingival groove. Significant differences were found between the IP and OP ($p < 0.05$) as well as the GPS and OP ($p < 0.05$). The blood vessel diameter had a tendency to decrease from the GSP to the OP.

In Group 2, the blood vessel diameter had various values in the three areas (GPS, $31.6 \pm 3.2 \mu\text{m}$; IP, $30.1 \pm 1.6 \mu\text{m}$; and OP, $37.0 \pm 2.4 \mu\text{m}$) on the gingival groove, but a significant difference was not seen between each area.

Fig. 3. Distribution of blood vessel thickness by pocket depth in three parts of the human gingival tissue in three groups: gingiva adjacent to a sulcus of <2 mm (Group 1, A); gingiva adjacent to a 2–4 mm sulcus (Group 2, B); and gingiva adjacent to a sulcus of >4 mm (Group 3, C). Three parts of the gingival groove: gingival pocket side (GPS), intermediate part (IP), and outer layer part (OP).

In Group 3, the blood vessel diameter was 33.5 ± 3.0 μm in the GPS, 40.0 ± 1.6 μm in the IP, and 25.7 ± 1.8 μm in the OP. A significant difference was found between the OP and IP ($p < 0.01$). The blood vessel diameter in Group 2 and Group 3 had a large value compared to that of Group 1 (ANOVA; $p < 0.05$).

Histological observations

In Group 1, the epithelial layer thickness of the free gingiva was found, and many CD-31 positive blood ves-

sels (minor axis approximately 20 μm) were seen in the lamina propria of the mucous membrane. The epithelium of the GPS is thinner than that of the OP, and compact dense fibers that constitute the lamina propria of the mucous membrane were found. The epithelial layers of the free gingiva of Group 2 and Group 3 were gradually thinner than that of Group 1. The lamina propria of the mucous membrane was also thinner; the blood vessels increased in size (minor axis approximately 30 μm). The infiltrate of the lymphocyte and perivascular hyaline material was also found in the lamina propria of the mucous membrane of Group 3. Perivascular hyaline material (PhyM) was also found in Group 3 by HE staining.

Discussion

The diagnosis of oral tissue conditions for periodontal maintenance was completed by evaluating pocket depth (EPP4 point method), bleeding on probing of the pocket, attachment level, and resorption of the alveolar bone. For this study, we used the classification of probing depth (PPD) described by Johnson *et al.* at 1999. In our results, the thickness of blood vessels in the GPS of Group 2 and 3 was larger than the other areas. Blood vessels were larger from the OP to the GPS. Therefore, capillary permeability may have increased during the periodontal probing of Group 2 and 3. Silveira *et al.* (2008) reported that the blood vessels of human gingiva became larger in size due to physical stress such as laser irradiation. Blood vessels were also found to be large in the gingival connective tissue of long-term, insulin-dependent diabetic patients (Seppälä *et al.*, 1997). The blood vessels of human gingiva may be affected by physical and pathological angiogenesis. The fenestrated capillary networks in dog dental pulp present a loop at the scanning electron microscope level and indicate the existence of slightly large sinusoidal vessels in a part of the capillary blood vessel during angiogenesis after tooth extraction (Matsuo and Takahashi, 2002). The measurement and method are slightly different from this study; the human gingival pocket is more than 4 mm. Regarding the GPS, there are many moderately sized blood vessels (15–25 μm), small blood vessels (10 μm or less), and slightly thin blood vessels (10–15 μm) according to Zoellner and Hunter (1991). These results resemble our results regarding the capillary blood vessel increase of blood vessels in the GPS. Therefore, vascular permeability is becoming high in the GPS and IP. On the other hand, a gingival pocket of less than 3 mm with many thin blood vessels on the OP side suggests that the thickness of the blood vessels increased in the IP when the gingival pocket was more than 4 mm. A similar tendency regarding the deepening of the gingival pocket was seen in our study. Moreover, when a gingival pocket is formed, an accumulation of perivascular hyaline material (PhyM), GPS₄ occurs in the GPS side around the blood vessel. As a

Fig. 4. Immunohistochemical staining using positive CD-31 blood vessels in a human gingival sagittal section. A, Sagittal section of the human gingival (bar = 200 μ m). A-1, Selected square. B, a large magnification of A-1 (bar = 51 μ m). CD-31, positive blood vessel were found in the human gingival. C, The A-1 area is series from other control section (bar = 51 μ m).

Fig. 5. Sagittal section of the human gingiva. A, Small perivascular hyaline material (PhyM) (arrows) was seen near the gingival epithelium by Hematoxylin and Eosin (H-E) Staining in Group 1. A-1, a large magnification of A (scale bar, 20 μ m) (59 years old, female).

B, Large perivascular hyaline material (PhyM) (arrows) was seen around the blood vessel (*) by Hematoxylin and Eosin (H-E) Staining in Group 3. B-1, a large magnification of B (scale bar, 20 μ m) (49 years old, female). P, pocked side; EP, epithelium.

result, inhibition of angiogenesis prevents the movement of polymorphonuclear leukocytes, which leads to an enlargement of the blood vessels and periodontitis. However, PhyM was found in three groups; it does not directly cause gingival pocket depth increase. PhyM is thought to be one of the morphologic characteristics of gingivitis. The gingival tissue presents a durable structure with PhyM for the physical stimulation of specific morphological characteristics, which PhyM effect to decline of tissue fluid by a physical pressure to the blood vessel and decreases gingival crevicular fluid in the blood vessel. It seems that the expansion of gingival tissue and blood vessels in the gingival OP side causes reddening of the

superior region of the gingiva. Therefore, there is an important relationship between the PPD and the blood vessel diameter suggesting that both reflect the organizational state of human gingival tissue.

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