

LYMPHATIC ARCHITECTURE OF THE HUMAN GINGIVAL INTERDENTAL PAPILLA

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ABSTRACT

Many studies have investigated the lymphatic architecture of head and neck using experimental animals, confirming the existence of lymphatic networks beneath the epithelium in gingival tissue. In this study, we investigated the use of these lymphatics as a drug delivery route by studying the architecture of lymphatic vessels in human interdental papilla. Serial cryosections were cut using the film-transfer method. To identify lymphatics, the sections were stained using enzyme histochemical and immunohistochemical techniques and three-dimensional images of lymphatics were reconstructed using 3D visualization software. Capillary lymphatic networks were observed in the lamina propria beneath the epithelium in human interdental papilla, and they joined with lymphatic networks beneath the epithelium in free gingiva. The networks consisted of a single layer of large irregular, hexagonal meshes and precollecting lymphatic vessels heading toward collecting lymphatic vessels that exited on the periosteum of the alveolar crest. These findings suggest that lymphatic flow from the interdental papilla drains into collecting lymphatic vessels running buccolingually on the alveolar crest of the interdental papilla.

This may be an important anatomical feature during inflammation throughout the oral cavity in that the drainage function is maintained by part of lymphatic flow that is not impaired during the healing process.

Keywords: lymphatic architecture, interdental papilla, human gingiva, 5'-nucleotidase, D2-40

The majority of lymphatic vessel research in periodontal tissues has involved the use of experimental animals (1-8), and the existence of lymphatic networks beneath the epithelium in gingival tissues has been confirmed. Some investigators have suggested that these networks could be utilized for clinical treatment (4,7,8). Generally, gingival tissues are divided into the free gingiva, the attached gingiva, and the interdental papilla (gingival tissue between the teeth). The tip of the pyramid-shaped interdental papilla is positioned immediately below the proximal contacts, and the gingival col, a V-shaped depression connecting the buccal and lingual interdental papillae, is adapted to the shape of proximal contact area of the tooth (9-11). This area has a suitable shape for retention of ointment medicine in dental treatment. Moreover, the attached epithelium, the

bottom of the gingival sulcus, has a high substance permeability because of the wide intercellular space (12). Medication entering into the lamina propria just beneath the epithelium is absorbed by the blood and lymphatic vessels. A recent study summarized the existence of lymphatic vessels in various human organs (13), but few studies have investigated the distribution of lymphatic vessels in the head and neck region focusing on the separate gingival tissues. Although we have been investigating human lymphatic architecture in the buccal and lingual free gingiva (14), no studies have conducted a detailed investigation of lymphatic distribution in the interdental papilla connecting the lingual and buccal tissues. To use lymphatic vessels as a drug delivery route based on the knowledge of lymphatic architecture and distribution, investigation using normal human samples is essential since there may be morphological differences between humans and animals. The purpose of this study is to reveal the lymphatic architecture beneath the epithelium in the human interdental papilla using three-dimensional reconstructed images with enzyme histochemical and immunohistochemical staining.

MATERIALS AND METHODS

Sample Preparation

Human gingival samples were collected from seven patients who visited the periodontal clinic at the Iwate Medical University Hospital. Informed consent was obtained from all subjects who participated in this study. This research was initiated upon approval of the Iwate Medical University School of Dentistry Ethics Committee (approval number 01065). Samples used in this study consisted of human interdental papilla including the lamina propria obtained from excision for therapeutic purposes during periodontal surgery. Since gingival tissues are usually excised with a margin of safety consisting of normal tissue, we defined

clinically healthy gingiva as the area located near the normal tissues without severe inflammation. The incised gingival tissue was cryo-embedded in 5% carboxymethyl cellulose (Kanto Chemical Co., Inc., Tokyo, Japan) in hexane cooled by liquid nitrogen without fixation. The specimen was then placed in a cryostat (CM3050S, Leica, Bensheim, Germany: cutting edge angle: 5°, CT: -22°C, OT: -18°C) and 10 µm buccolingual or mesiodistal serial cryosections were produced using the film-transfer (Kawamoto) method (15).

Histological Staining

Enzyme histochemical staining

After immersion fixation of the produced serial sections in 100% ethanol, sections were rinsed with a tris-maleate buffer solution (pH 7.2) and immersed in 5'-nucleotidase (5'-Nase) substrate solution at 37°C for 30 minutes. After rinsing again with a tris-maleate buffer solution, sections were immersed in a 1% ammonium sulfide solution for 2 minutes to color the lymphatic vessels (16). After rinsing with distilled water, sections were mounted in 30% glycerin for observation and image capturing. After confirming distinguishing lymphatic and blood vessels by alkaline phosphatase staining in some sections, counter staining was conducted on the remaining sections using hematoxylin. Sections were observed and photographed after remounting with 30% glycerin.

Immunohistochemical staining

After immersion fixation of the produced serial sections in 100% ethanol, serial sections were rinsed for 5 minutes with PBS solution (0.01 mol/l, pH 7.4, Mitsubishi Chemical Medience Inc., Tokyo, Japan) at room temperature. Repeating this procedure three times, endogenous peroxidase activity was blocked with 3% H₂O₂ in PBS and sections

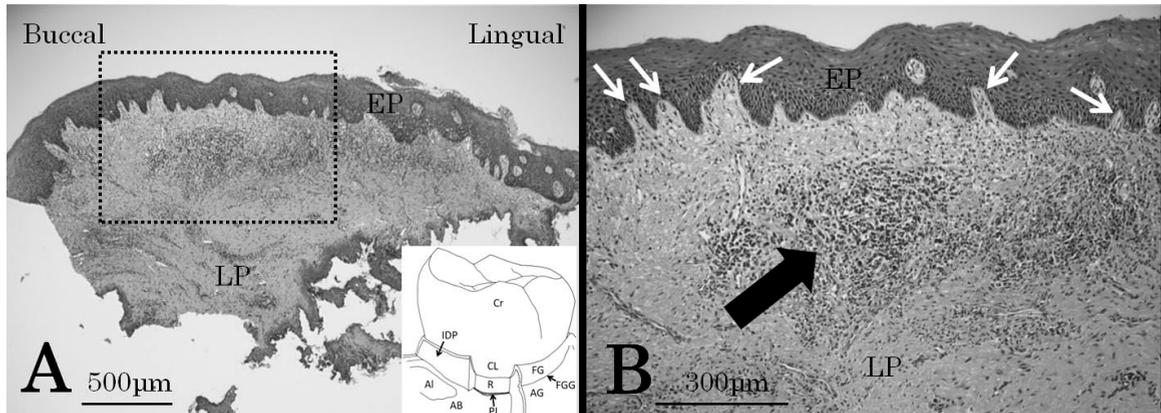


Fig. 1. Histological images of the interdental papilla (hematoxylin and eosin staining). **A:** Overview image (4x). **B:** Magnified image of the square in Fig. 1 (10x). Inflammatory cell infiltration can be seen near the upper layer of the lamina propria beneath the epithelium (thick arrow). Connective tissue papillae are shown extending towards the epithelium (thin arrow). EP: epithelium, LP: lamina propria. Fig. 1A) Insert: Schema of the gingival tissue. Cr: Cervical line, R: Root, Cr: Crown, IDP: Interdental Papilla, FG: Free gingiva, AG: Attached gingiva, FGG: Free gingival groove, Al: Alveolus, AB: Alveolar bone, PL: Periodontal ligament.

were rinsed again with PBS. In a moist chamber, Special Block A (ACUITY Biotin Free Mouse-on-Mouse Polymer Detection System, Covance, California, USA) was applied onto sections and a 30 minute reaction time was allowed at room temperature. Sections were immersed in a 1:40 dilution of D2-40 mouse monoclonal antibody (Covance) with 1% BSA in PBS in a moist chamber for 1 hour at room temperature. After rinsing with PBS solution for 5 minutes three times, Special Block B was applied in a moist chamber. After 10 minutes of reaction at room temperature, sections were rinsed for 5 minutes three times. After rinsing, the same operation using MoM Polymer Link. Color was then developed with a DAB substrate kit (Vector Laboratories, Burlingame, USA) for 5 minutes at room temperature. Following rinsing with distilled water, sections were counterstained with hematoxylin, and embedded in 30% glycerin.

Reconstruction of three-dimensional images

Serial sections with lymphatic staining were observed and photographed using an optical microscope (E1000M[®], Nikon, Tokyo,

Japan) with a color, chilled 3CCD camera (C5810[®], Hamamatsu Photonics, Tokyo, Japan). The obtained two-dimensional images were directly input to a computer. Coordinating the axes of the two-dimensional images, 5'-Nase positive and D2-40 positive lymphatic vessels were extracted and image processing (dichotomizing) was manually conducted using Photoshop[®] CS4 (Adobe, San Jose, USA). Three-dimensional images of lymphatic vessels were then reconstructed using 3D visualization software (ZedView[®]DB, Ver.6.0, LEXI, Tokyo, Japan) (14,17). Rotating animations were constructed to observe distribution of the lymphatic vessels beneath the epithelium from all directions.

RESULTS

Histological Structure

The lamina propria beneath the epithelium of the human interdental papilla samples showed an inflammatory cell infiltration in the upper layer. This infiltration was largely absent from the deeper layers of the lamina propria (Fig. 1A,B). No prominent vasodilatations were observed

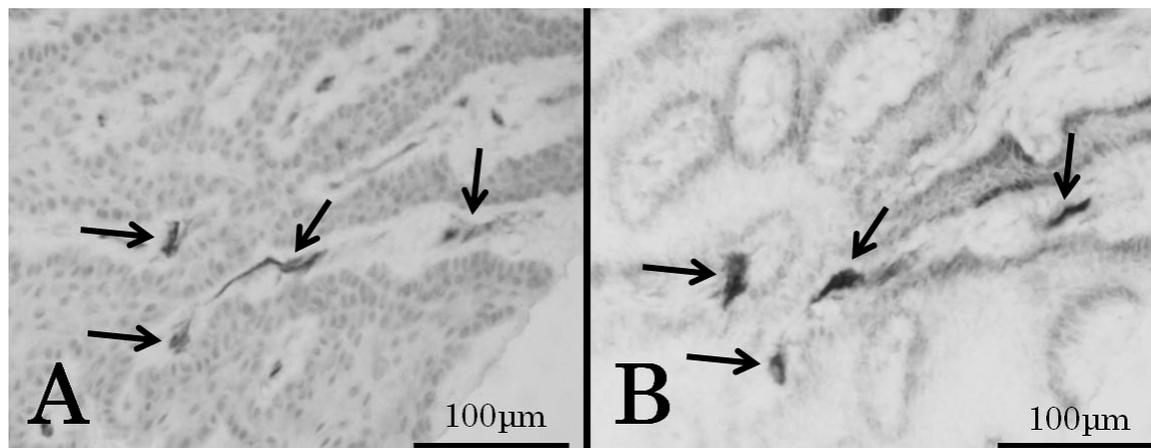


Fig. 2. Exposure of lymphatic vessels by enzyme histochemical (5'-Nase) staining and immunohistochemical (D2-40) staining. A: Image with 5'-Nase staining and hematoxylin counter staining ($\times 20$). B: Image with D2-40 staining only ($\times 20$). Fig. 2A and Fig. 2B are serial sections. 5'-Nase and D2-40 positive lymphatic vessels can be seen in approximately the same area (arrows: lymphatic vessels).

in the lamina propria. Connective tissue papillae were observed extending into the lamina propria towards the epithelium. The connective tissue papillae (height: approximately 150 μm) were irregularly shaped, appearing as either straight or tortuous extensions. No prominent thickening in the epithelium of the interdental papilla and no derangement of collagen fibers in the lamina propria were observed.

Images of Lymphatic Vessels

5'-Nase positive vessels (brown-black staining due to coloration of the lead in the 5'-Nase substrate solution by ammonium sulfide during 5'-Nase enzyme histochemical staining; Fig. 2A) and a D2-40 positive image (brown staining caused by DAB in D2-40 immunohistochemical staining; Fig. 2B) were observed in the lamina propria. In the serial sections, most D2-40 positive images were observed in approximately the same regions as the 5'-Nase positive images.

Reconstruction Images of Lymphatic Vessels

Networks of lymphatic capillaries were

found in the lamina propria beneath the epithelium of human interdental papilla. These networks were located approximately 30 μm below the epithelium and consisted of one layer in a large hexagonal mesh. The diameter of the lymphatic vessels forming these networks was approximately 15 μm (Fig. 3A). Precollecting lymphatic vessels in the lymphatic networks beneath the epithelium could be seen extending toward the deep layers of the lamina propria (Fig. 3A,B: \rightarrow) and most were directed towards the buccal side (Fig. 3B). In the superficial layers of the lamina propria in the interdental papilla, a lymph capillary network is clearly visible (Fig. 3B). This is in contrast with the deep layer of the lamina propria, in which the mesh of the precollecting lymphatic vessels is not clearly visible. Additionally, the precollecting lymphatic vessels are sparsely distributed (Fig. 4-2**) relative to those in the superficial layer (Fig. 4-2*).

In the area between the interdental papilla and the free gingival margins on the buccal and lingual sides, the lymphatic capillary networks beneath the epithelium joined with the precollecting lymphatic

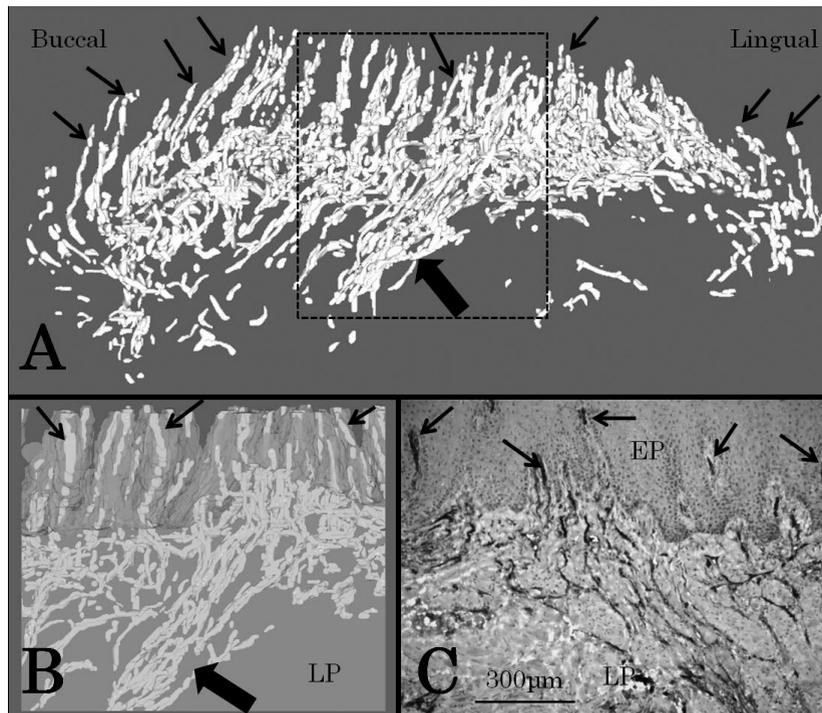


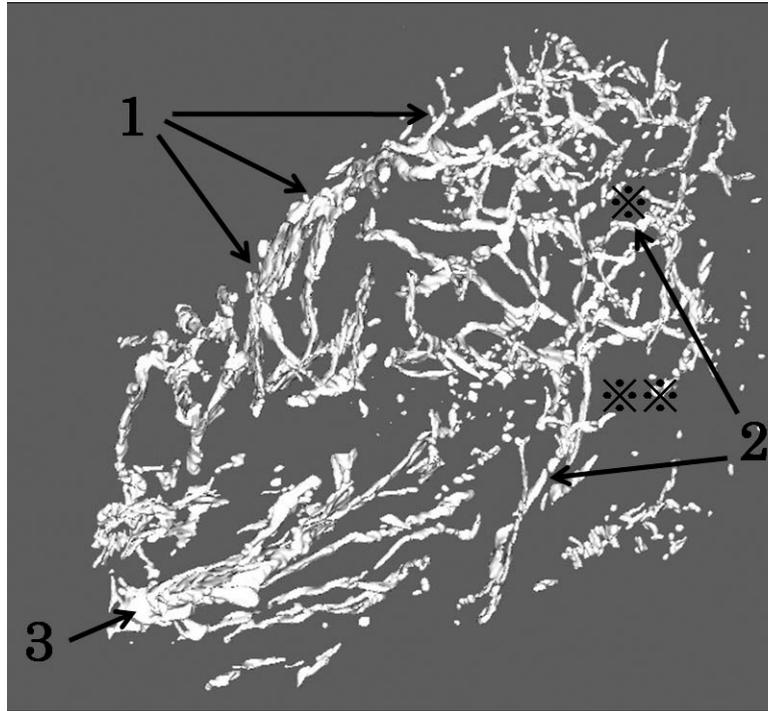
Fig. 3. Reconstructed images of lymphatic vessels in the interdental papilla. **A:** Overall image of the interdental papilla (buccolingual direction). **B:** Magnified image of the square in Fig. 3A, three-dimensional reconstructed image merging lymphatic vessels and the lamina propria **C:** Sectioned image of Fig. 3B (5'-Nase-hematoxylin counter staining). Lymphatic capillary networks are present in the lamina propria beneath the epithelium. From these networks, precollecting lymphatic vessels can be seen heading toward the deep layers of the lamina propria (Fig. 3A,B: thick arrow), with one blind-end of the lymphatic vessel entering each connective tissue papilla (Fig. 3A,B,C: thin arrow). thick arrow=precollecting lymphatic vessel, thin arrow=blind-ended vessel

vessels running through collagen fibers in the connective tissue and extended towards collecting lymphatic vessels exiting on the periosteum of the alveolar crest (Fig. 4-3).

We observed that each lymphatic vessel (approximately 10 µm in diameter) in the connective tissues of the lamina propria entered a connective tissue papilla and terminated in a blind end without branching from the basal area to the tip (Fig. 3A,B,C). These blind-ends of the lymphatic capillaries extended from the lymphatic capillary networks beneath the epithelium (Fig. 3B). In addition, the blind-ends of the lymphatic capillaries entering the connective tissue papillae followed the outline of the papillae.

DISCUSSION

Periodontitis is an inflammatory disease caused by oral bacteria in dental plaque (18). It presents clinically with symptoms such as redness, swelling, pain, bleeding, proliferation and recession of gingival tissues, periodontal pocket formation, and swelling of regional lymph nodes. The progress of periodontal diseases accompanied with various phenomena such as vasodilation and edema of blood vessels, and inflammatory cell infiltration in the early stage of inflammation, has a strong impact on the microcirculation of gingival tissues. These clinical symptoms are resolved after periodontal treatment, but it has been confirmed that they leave morphological changes in gingival tissues. In humans especially, all gingival tissues retain a certain level of inflammation; therefore, we



*Fig. 4. Three-dimensional reconstructed images from the interdental papilla to the free gingival margin on the buccal side. Lymphatic capillary networks beneath the epithelium (Fig. 4-1) join the precollecting lymphatic vessels (Fig. 4-2) and head towards the collecting lymphatic vessels (Fig. 4-3). The precollecting lymphatic vessels in the deeper layer (Fig. 4-2**) are sparsely distributed relative to those in the superficial layer (Fig. 4-2*). 1=lymphatic vessel network, 2=precollecting lymphatic vessel, 3=collecting lymphatic vessel*

may never see truly normal gingival tissues. Thus, in the present study, it is appropriate to say that we examined clinically normal gingival tissues. Previous studies have examined morphological characteristics of blood vessels in experimental animals through the microcirculation of gingival tissues (19-22). However, few studies have investigated lymphatic vessels. Fujimura et al studied lymphatic routes in the head and neck region and organs in the oral region using experimental animals (1,4,8,17,23-26). Recently, our research group investigated the lymphatic architecture of the human free gingiva (14). In the present study, we observed lymphatic vessels beneath the epithelium in the interdental papilla.

Histological Findings

Gingiva around the teeth consists of three regions: the free gingiva, the attached gingiva, and the interdental papilla. The free gingiva can be distinguished from the attached gingiva by its lack of attachment to the tooth surface.

The attached gingiva extends continuously from the free gingiva and attaches strongly to the periosteum of the alveolar bone. The attached gingiva transitions into alveolar mucosa at the mucogingival junction. The interdental papilla is gingival tissue occupying the proximal area below the contact point. The epithelium of the col in the interdental papilla is normally non-keratinized stratified squamous epithelium (9). As can be seen, each region of the gingiva presents its own characteristics.

Examination of the samples in this study

confirmed the formation of connective tissue papillae in the epithelium and parakeratinized epithelium in the interdental papilla. The samples were obtained from tissues that had undergone periodontal treatment, and were therefore in the process of recovering from inflammation and symptoms. Therefore, it is likely that these gingival tissues had a history of some level of inflammation. We considered the abovementioned morphology was a consequence of the acute symptoms of inflammation.

Because of the gingival morphology, the interdental papilla in particular tends to accumulate food residue and plaque which develops into a bacterial reservoir. In addition, repetitive inflammation occurs due to the difficulty in cleaning the interdental papilla. The morphology of the interdental papilla and the epithelium may be greatly influenced by the existence of adjacent teeth, proximal contact conditions, and gingival anatomy and recession.

Lymphatic Staining

We conducted both enzyme histochemical (5'-Nase) staining and immunohistochemical (D2-40) staining. Since positive images were observed at approximately the same areas in the serial sections, the staining ability of both methods seems to be similar. Approximately 500 serial sections were produced from one sample and about 100 serial sections were used to develop three-dimensional reconstructed images. It was considered that conducting immunohistochemical staining on all sections was cost prohibitive. However, enzyme histochemical staining allowed us to stain all sections at once, providing advantages in terms of cost, time and stainability. Therefore, enzyme histochemical (5'-Nase) staining was used as our main staining method. By conducting immunohistochemical (D2-40) staining on a part of the serial section at the same time, we were able to confirm the accuracy of our identification of lymphatic vessels.

Reconstruction Images of Lymphatic Vessels

There are three sources of blood vessels anastomosing with each other in the gingival tissues: blood vessels in the periodontal ligament, capillaries from the gingival sulcus, and blood vessels running on the alveolar crest through the alveolar bone of the interdental areas (9). Although the distribution of lymphatic vessels is similar to that of blood vessels in human gingival tissues, lymphatic vessels are known to be distributed on the periosteum of bone without running through the inside of the bone (27). In accordance with previous reports, we observed lymphatic vessels in the epithelial area and on the periosteum of the alveolar bone in the interdental papilla. Although detailed distribution of lymphatic vessels from the periodontal ligament has not been elucidated, no studies have reported that lymphatic vessels in the free gingiva enter the periodontal ligament (7). In the present study, tissues in the gingival sulcus were excised at sample collection, and destruction of the tissue could not be avoided. Therefore our observations could not clearly confirm the connection with the periodontal ligament area; however, the existence of lymphatic vessels in the periodontal ligament at the apex has been reported in a previous animal experiment (8). Formation of lymphatic networks has been observed beneath the epithelium of the gingival sulcus and the oral epithelium of the free gingiva of humans and experimental animals (4,14). Similarly, our study confirmed the formation of lymphatic networks beneath the epithelium in buccal and lingual areas in the human interdental papilla and the connection of these lymphatic vessels with the lymphatic networks formed beneath the epithelial areas of the interdental papilla. In addition, lymphatic vessels were observed heading from the lymphatic networks beneath the interdental epithelium to the alveolar crest. These lymphatic vessels were thicker in diameter than the capillary lymphatic networks formed beneath the epithelial area.

It is possible that these vessels were precollecting lymphatic vessels joining with collecting lymphatic vessels on the crest of the alveolar bone. These findings suggest that lymphatic flow in the interdental papilla progresses in buccal and lingual directions below the epithelium, and then travels from the lymphatic networks in the free gingiva to collecting lymphatic vessels above the periosteum of the alveolar bone. Moreover, we suggest that lymphatic flow in the interdental papilla progresses from the collecting lymphatic vessels distributed buccolingually on the alveolar crest to the collecting lymphatic vessels on the periosteum of the buccal and lingual alveolar bone in addition to the lymphatic networks beneath the epithelium. Lymphatic flow could be maintained by these multiple routes. This anatomy may play an important role in maintaining drainage and promoting healing in cases of inflammation. For example, when the buccal lymphatic flow is impaired due to inflammation, lymphatic flow may be still maintained if the lingual route is kept intact.

Clinical Utilization of Lymphatic Vessels

Occlusal force during mastication causes repetitive up and down motions and inclination of the teeth within the physiological range and the flow of food causes movement of gingival tissue. These functional movements stimulate lymphatic flow in the interdental papilla in the same manner as muscle pumping in other body parts. Our results suggest that the lymphatic flow moves both buccally and lingually via lymphatic networks beneath the epithelium. Therefore, mastication is important in terms of lymphatic flow. In addition, lymphatic structures and lymphatic distribution may play an important role in the absorption of antibacterial ointment when applied to the gingival sulcus in cases of acute inflammation. The gingival sulcus provides access to the lymphatic networks and blood vessels existing in the free gingiva and the inter-

dent papilla surrounding the teeth. Drugs applied to the gingival sulcus are mainly absorbed by blood vessel networks to exert their effects. However, a fraction of these drugs is considered to be absorbed by lymphatic vessels and is thought to reduce swelling of regional lymph nodes caused by bacterial infection. Our observation of lymphatic architecture revealed that lymphatic flow existed in gingival tissues without interruption. This result leads to the conclusion that the interdental papilla may be used as an effective location for application of ointment because of its drug retentive anatomy. Therefore, we believe that activating the drainage function of the lymphatic networks in the interdental papilla could be important for the development of drug delivery via the lymphatic route.

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