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Lymphatic architecture of human periodontal tissue beneath the oral epithelium of the free gingiva

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Abstract

Several studies have described the lymphatic architecture of periodontal tissues, including gingival tissues, using laboratory animals; however, no study has investigated human samples. In the present study, morphological characteristics and architecture of human gingival lymphatic vessels were investigated using clinically healthy gingival tissue with the aim of developing a drug delivery route through lymphatic vessels for clinical application. We collected free gingival tissue excised for therapeutic purposes during periodontal surgery from six patients, and produced frozen serial sections using the film-transfer method. The sections were then stained with 5'-nucleotidase to expose the lymphatic vessels, and three-dimensional images of lymphatic vessels were reconstructed using 3D visualization software. Lymphatic vessels formed networks in the lamina propria on the oral epithelial side in free gingiva, presenting as a layer of rough irregular hexagonal meshes. Blind-ended vessels were observed entering connective tissue papillae through these meshes. The blind-ended vessels did not branch between the base of connective tissue papilla and rod-like structure at the tip, and were positioned at the center of each papilla. The lymphatic architecture of human free gingiva showed a similar distribution to that of blood vessels, although the lymphatic vessels were fewer in number than blood vessels. Activation of drainage through lymphatic vessels in gingiva is considered to be an important therapeutic measure in terms of improvement in edema. [MVR 3(1): 17-24, 2009]

Key words: lymphatic architecture, gingiva, oral epithelium, 5'-nucleotidase, microcirculation

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Introduction

Periodontitis is a chronic inflammatory disease of periodontal tissue caused by inflammatory agents from dental plaque. The early stages of periodontitis feature hyperemia of microcirculation due to vasodilation, edema caused by effusion of a fluid component due to vascular hyperpermeability by chemical mediators from immunocytes, and cell infiltration¹⁻³). A majority of the fluid component from blood vessels is collected by the venous system; lymphatic vessels are another drainage system collecting 10% of the fluid component containing high levels of molecular protein⁴). Permeability of fluid from blood vessels increases during inflammation, but the ability of the lymphatic vessels to collect the fluid component is maintained at the same level or decreases. Therefore, edema occurs locally.

The lymphatic system is related to the cause of edematous diseases. Edema occurs in cases of hyperpermeability of blood vessels or dysfunction in lymphatic flow. Recovery and promotion of the absorptive function in lymphatic vessels are crucial for alleviation of edema. In periodontitis, inflammation of the gingival tissue causes swelling. Lymphatic vessels play a major role in alleviating the edematous swelling of periodontitis during the healing process. It is known that acute gingival symptoms such as swelling, pain and bleeding together with swelling of the regional lymph nodes, are remitted after local application of antibiotic ointment. This reduction in lymph node swelling suggests that part of the drug is absorbed by the regional lymph nodes through the lymphatic vessels in the gingival tissue, and is unlikely to be caused by absorption of locally applied drugs by blood vessels, since the dosage of the drug is extremely low. This assumption has led researchers to advocate application of drugs via the lymphatic vessels to target regional lymph nodes⁵⁻⁷).

Previous studies have described the lymphatic architecture in periodontal tissue, including gingiva, using laboratory animals⁸⁻²¹); however, none of the studies has investigated human gingiva. In the present study, we observed the morphological characteristics and architecture of clinically healthy gingival tissue obtained by excision during periodontal surgery with the aim of investigating the development of a drug delivery route through the lymphatic vessels for clinical application.

Materials and Methods

Human gingival samples excised from the free gingiva, and including the lamina propria, were collected from six patients undergoing periodontal surgery (Table 1) at the Iwate Medical University Hospital Dental Center, Conservative Dentistry Department Periodontal Clinic. We obtained informed consent from all subjects participating in this study. This study was initiated upon approval of the Iwate Medical University School of Dentistry Ethics Committee (approval number 01065). When pathological areas are excised from gingival tissue, the incision line is determined taking into account a prescribed margin of safety. In the present study, we defined clinically healthy gingiva as the area located on the far side of the pathological areas in the excised gingival tissue. This tissue is considered to be clinically healthy and within the margin of safety. Although the sulcular epithelial side of the samples remained after exci-

Table 1. Clinical diagnosis

Patients	Sex	Age	Teeth	Part	Probing depth (mm)
1	F	26	46	Buccal	4
2	M	50	27	Distal	6
3	M	51	17	Distal	7
4	M	71	11	Mesial	7
5	F	48	16	Distal	4
6	M	71	26	Distal	7

All of them are chronic periodontitis.

Flap technique: modified Widman flap surgery

sion in two out of six cases, most of the junctional epithelium was destroyed. Therefore, the area from the gingival crest to the oral epithelium was the focus of this study.

Observation of histological structure: Each tissue sample was divided into two parts. One part was immersed in 4% phosphate paraformaldehyde for a week for fixation; then dehydrated with an ascending series of alcohol solutions. After penetration with xylene, the samples were embedded in paraffin in the conventional method. Buccolingual serial sections (5 µm thick) were cut using a sliding microtome Sledge Microtome (IVS-400®, Sakura, Tokyo, Japan) and stained with conventional hematoxylin-eosin stain to observe the histological structure using a light microscope (E1000M®, Nikon, Tokyo, Japan).

Preparation for serial cryosections: The remaining part of the unfixed gingival tissue was cryo-embedded in 4% carboxymethyl cellulose (CMC) in liquid nitrogen-cooled hexane. The specimens were then placed in a cryostat (CM3050S®, Leica, Bensheim, Germany : cutting edge angle: 5°, CT -22°C, OT -17 °C) with a steel blade (Leica 818 High Profile Microtome Blades, Leica) and 10 µm buccolingual serial sections were cut using the film-transfer (Kawamoto) method (Cryofilm TYPE I-B®, Fintec, Tokyo, Japan)²²).

Staining of lymphatic and blood vessels: The serial sections were immersion fixated in 100% alcohol, then washed with a tris-maleate buffer solution (pH 7.2) and immersed in a 5'-nucleotidase substrate solution (5'-Nase: 0.2 M tris-maleate buffer (pH7.2) 20 ml, 5'-AMP sodium salt 25 mg, 0.1 M MgSO₄ 5 ml, sucrose 3-4 g, distilled water 22 ml, 2% Pb (NO₃)₂ 3 ml, L-tetramisole 20 mg) at 37°C for 30 minutes for reaction. After washing with the same buffer, the sections were immersed in 1% ammonium sulfide solution for 2 minutes at room temperature to color the lymphatic vessels. After washing with distilled water, the sections were mounted in 30% glycerin for observation and capture of images using a light microscope. To identify blood vessels, alkaline phosphatase stain (ALPase: Naphtol AS-MX phosphate disodium salt 40 mg, N,N-dimethylformamide 2 ml, 0.1 M Tris-HCl buffer (pH 9.2) 40 ml, Fast blue BB 40 mg) was applied to some of the serial sections, which were then remounted in 30% glycerin for observation and capturing of images²³⁻²⁶). The rest of the serial sections were counterstained with hematoxylin, and remounted with 30% glycerin for observation and image capturing.

Three-dimensional reconstruction: The serial sections with lymphatic staining were photographed using a light microscope (E1000M®, Nikon, Tokyo, Japan) with a color chilled 3CCD camera (C5810®, Hamamatsu photonics, Hamamatsu, Japan). The resulting two-dimensional images were directly input to a computer (Fig. 1 A,B,C).

Fig. 1. Image processing methods for three-dimensional reconstructed images (Patient3)
 A-C. Two-dimensional serial section images imported to the computer. D-F. Images after coordination of axes (A→D, B→E, C→F). G-I. Dichotomized images after extraction of lymphatic vessels from D-F; white: lymphatic vessels, grey: lamina propria (D→G, E→H, F→I).

Photoshop® CS3 (Adobe, San Jose, USA) was used to coordinate the axes of the two-dimensional images (Fig. 1 D,E,F) extracted for 5'-Nase positive lymphatic vessels and process (dichotomize) the images (Fig. 1 G,H,I). Three-dimensional images of lymphatic vessels were then reconstructed using 3D visualization software (ZedView®DB, Ver.5.0, LEXI, Tokyo, Japan)^{16,17}. This structure was made by about 100 images. Based on the complete three-dimensional images, rotating animations were constructed to observe the distribution of the lymphatic vessels beneath the oral epithelium of the free gingiva.

Results

Histological structure: Considerable inflammatory cell infiltration and dilation of vessels (Fig. 2 A,B) were observed in the lamina propria near the base of the sulcus in clinically healthy gingival tissue. Notable hyperplasia of the epithelium, elongated and tortuous connective tissue papillae (length: approximately 280 μ m, thickness: approximately 45 μ m), and blunted epithelial legs were observed in the gingival tissue. Additionally, in the lamina propria of the free gingiva, collagen fibers (dentogingival fibers, alveo-gingival fibers) were linearly distributed (Fig. 2 A). Finely branched connective tissue papillae were irregularly and thinly elongated (length: approximately 200 μ m, thickness: approximately 40 μ m). Epithelial legs were sharpened and collagen fibers ran radially (Fig. 2 B).

Differentiation between lymphatic and blood vessels: Several sections were double-stained with 5'-Nase-ALPase to distinguish lymphatic and blood vessels. The 5'-Nase positive vessels stained a brown-black color (Fig. 3 A) and the ALPase positive vessels stained a blue color (Fig. 3 B).

5'-Nase positive lymphatic vessels distributed near blood vessels showed an irregular beaded tube structure in the connective tissue papillae (Fig. 3 C). Immediately beneath the epithelium, the blood vessel networks were distributed closer to the epithelial side than the lymphatic vessel networks. Similarly, in the connective tissue papillae, the blood vessel loops were distributed closer to the epithelium than the lymphatic vessels (Fig. 3 C, insert).

Reconstructed images of lymphatic vessels: Lymphatic vessel networks were observed from the gingival crest to the free gingival sulcus in the lamina propria on the oral epithelial side (Fig. 4 A,B,C). The networks were present approximately 40 μ m below the epithelium, presenting as one layer of rough irregular hexagonal mesh (minor axis: 152.35 ± 18.46 μ m, major axis: 303.30 ± 54.50 μ m) (Fig. 4 B). The diameter of the lymphatic vessels forming the network was 17.35 ± 5.78 μ m. From this network, precollecting lymphatic vessels joined collecting lymphatic vessels on the alveolar bone periosteum, and then threaded through spaces among the collagen fibers in a complex alignment (Fig. 4B*). Blind-ended vessels 10.56 ± 3.45 μ m in diameter extended from the lymphatic vessel networks beneath the epithelium through the length of the connective tissue papillae, with no branching occurring between the base of the connective tissue papillae and their tips (Fig. 5 A,B,C). Most of the blind-ended tips took the form of a rod-like structure (Fig. 5 A,C). We also observed that the lymphatic vessels were positioned at the center of each papilla (Fig. 5C).

Discussion

Periodontal disease is lifestyle-related disease characterized by chronic inflammation. Acute episodes may also oc-

Fig. 2. Histological images of buccolingual sections of human clinically healthy gingival tissue

A. Patient 1, B. Patient 5. Hyperplasia of the epithelium, and elongated and tortuous connective tissue papillae (arrow: cross-sectional image) can be seen. In the lamina propria at the base of the sulcus, inflammatory cell infiltration (lymphocytes, plasma cells) (□) and irregular alignment of collagen fibers can be seen. OE: oral epithelium, OSE: oral sulcular epithelium, LP: lamina propria. Original magnification $\times 4$ (A), $\times 2$ (B), hematoxylin and eosin staining.

Fig. 3. Differentiation between lymphatic and blood vessels in histological sections (Patient 3)

A. 5'-Nase single staining image. B. 5'-Nase-ALPase double staining images. C. Magnified images of 5'-Nase-ALPase double staining, showing distribution of blood and lymphatic vessels in the connective tissue papilla. 5'-Nase positive vessels: brown-black (thin arrow: $\rightarrow$), ALPase positive vessels: blue (thick arrow: $\rightarrow$). C insert. Three-dimensional reconstructed images of blood and lymphatic vessels in the connective tissue papilla. In the connective tissue papilla, lymphatic vessels are distributed near blood vessels; loops of blood vessels are formed closer to the epithelium than the lymphatic vessels. Red: blood vessels, green: lymphatic vessels. D. 5'-Nase-hematoxylin counter stained images. Penetration of blind-ended lymphatic vessels into the connective tissue papillae (thin arrow: $\rightarrow$) can be seen. OE: oral epithelium, LP: lamina propria, CTP: connective tissue papilla. Original magnification $\times 20$ (A,B), $\times 40$ (C), $\times 10$ (D).

cur during exacerbation of symptoms. In Japan, periodontal diseases were clinically classified in 2006 into seven pathological categories²⁷⁾ based on the classification system for periodontitis formulated by the American Academy of Periodontology (AAP) in 1999²⁸⁾. Surgical resection generally takes place during the chronic stage. The tissue used in this

study was obtained from chronic inflammatory gingival tissue (Table 1); therefore, we investigated lymphatic vessels in the tissue during the recovery phase.

Previous studies have investigated the system of blood vessels in the head and neck region of human and experimental animals²⁹⁻³³⁾. However, studies of the lymphatic ves-

Fig. 4. Reconstructed images of lymphatic vessels in the free gingival tissue (A. Patient 1, B. C. Patient 6)

The map of the gingiva allows direct observation of the three-dimensional reconstructed images of the lymphatic vessels. A. Overview of the free gingiva. B. Buccolingual view of the image. C. Image observed from the oral epithelial side. Lymphatic vessel networks comprising rough irregular hexagonal meshes are found in the lamina propria beneath the oral epithelium. OE: oral epithelium, OSE: oral sulcular epithelium, AB: alveolar bone, LP: lamina propria, GC: gingival crest

Fig. 5. Three-dimensional reconstructed images of lymphatic vessels on the oral epithelial side of the lamina propria (Patient 3)

A. Three-dimensional reconstructed images of lymphatic vessels. B. Three-dimensional reconstructed images of the lamina propria. C. Three-dimensional reconstructed images combining lymphatic vessels and the lamina propria. Lymphatic vessel networks can be seen in the lamina propria beneath the oral epithelium. One rod-like blind-ended lymphatic vessel with no branches is entering each connective tissue papilla. LVN: lymphatic vessel network, LP: lamina propria, CTP: connective tissue papilla, green: lymphatic vessel, yellow: lamina propria

sels have mainly been limited to experimental animals^{8-21,34}). Recent advances in staining methods for identification of lymphatic vessels include the use of 5'-Nase in enzyme histochemical staining^{23-26,35}), and vascular endothelial growth factor receptor-3 (VEGFR-3), podoplanin, D2-40, prox-1, lymphatic vessel hyaluronan receptor-1 (LYVE-1) and angiopoietin-2 in immunohistochemical staining³⁵⁻³⁸). As a result, identification of lymphatic vessels is increasingly accurate. In addition, clinical lymphography can be used for diagnostic purposes^{39,40}). However, to date, only animal studies have focused on the local architecture of capillary lymphatic vessels⁸⁻²¹). In an attempt to develop drug delivery routes for anticancer agents using lymphatic vessels in the head and neck region, especially beneath the oral

mucosa⁵⁻⁷), Fujimura et al. conducted an investigation of the lymphatic architecture in the head and neck region of experimental animals, and reported the existence of lymphatic vessels in the gingival epithelium¹⁴). However, their presence has not yet been confirmed in human gingival tissue. This study therefore investigates the lymphatic architecture of human gingival tissue focusing on the oral epithelial side of the free gingiva.

Histological findings: The margin between healthy and pathological areas in human gingival tissue is obscure in that even clinically healthy gingival tissue often has a history of gingival or periodontal disease. In the present study, we observed hyperplasia of the epithelium and extension and tortuosity of the connective tissue papillae in clinically

healthy gingiva, as well as inflammatory cell infiltration and derangement of collagen fiber alignment in the lamina propria. These findings suggest that the gingival tissue had previously experienced repeated inflammation and recovery, and was in the recovery phase at the time of excision. We suggest that a certain amount of inflammation may almost always exist in clinically healthy gingival tissue excised during treatment of periodontal disease.

Reconstruction of lymphatic vessels: A number of electron microscope studies have revealed the presence of different morphological characteristics between the areas beneath the oral epithelium and the sulcular epithelium in the blood vessel networks of the gingival tissue of experimental animals^{31,32,41-44}. The blood vessels beneath the sulcular epithelium form a flat hexagonal capillary vessel network, whereas the blood vessels beneath the oral epithelium in the free gingival tissue form relatively flat networks comprising venules and arterioles. In addition, the connective tissue papillae are well developed on the oral epithelial side; small branches of capillary vessels penetrate into the connective tissue papillae from blood vessel networks forming simple ascending and descending loops. Similarly in humans, blood vessel networks exist beneath both the oral epithelium and the sulcular epithelium, showing penetration of loops of capillary vessels into the connective tissue papillae⁴⁵.

A study of gingival lymphatic vessels in experimental animals has revealed that lymphatic vessels in the lamina propria run toward collecting lymphatic vessels before merging in the alveolar crest¹⁴. Lymphatic vessel networks have also been observed immediately beneath the epithelium on the oral epithelial side (unpublished observation: Fujimura). However, no studies have provided detailed descriptions of the lymphatic architecture of human gingiva. In the connective tissue papillae on the oral epithelial side, blind-ended vessels branch off from the lymphatic vessel networks beneath the epithelium; their shape varies depending on the region, and may take the form of rod-like or glomerulus-like blind ends¹⁵⁻¹⁷. In this study, lymphatic vessels were observed beneath the oral epithelium and the sulcular epithelium in human free gingival tissue. Rough irregular hexagonal lymphatic vessel networks were observed on the oral epithelial side. No major regional difference was confirmed in these lymphatic vessel networks distributed from gingival margin to the free gingival sulcus; blind-ended vessels were observed extending from the lymphatic vessel networks into the connective tissue papillae. Since the lymphatic vessels beneath the oral epithelium and the lymphatic vessels beneath the sulcular epithelium joined with collecting lymphatic vessels near the alveolar crest, we suggest that drainage occurs in the lamina propria of the free gingiva through the lymphatic vessel networks. In this study, we observed that a majority of the blind-ended vessels in the connective tissue papillae in clinically healthy human gingival tissue terminated in a rod-like structure, unlike those of experimental animals such as mice. The form, thickness and length of lymphatic vessels are thought to be affected by the morphology of the connective tissue papillae. Epithelial legs in the gingival epithelium tend to extend during inflammation, forming long connective tissue papillae. Branching, length and thickness of these connective tissue papillae vary and progress in random directions. Networks of lymphatic vessels form beneath the epithelium, with blind-ended vessels emerging from these networks and penetrating along

with the form of the connective tissue papillae. As a result, the architecture of the lymphatic vessels corresponds to the form of the papillae. The diameter of the lymphatic vessels in the free gingival tissue is approximately 10 μm at the blind ends and 17 μm at the lymphatic vessel network beneath the epithelium, suggesting that these vessels may be capillary lymphatic vessels, respectively.

The blood supply in human gingival tissue consists of three routes: suprapariosteal arterioles (arteries ascending along the outside of alveolar bone); vessels in the periodontal ligament (ascending arteries in the periodontal ligament); and arterioles starting from the interalveolar septum (arteries penetrating through cancellous alveolar bone)^{45,46}. However, previous studies using experimental animals revealed that the lymphatic vessel networks beneath the oral epithelium are connected to collecting lymphatic vessels on the periosteum of the alveolar bone, but none of the lymphatic vessels run toward the periodontal ligament or the interalveolar septum^{18,21,47}. Although the proximal side of the lymphatic vessels was not investigated because we used excised gingival tissue in our study, we were able to observe the collecting lymphatic vessels corresponding to the periosteum of the alveolar crest. In addition, the lymphatic architecture of human free gingival tissue was shown to have a similar distribution to blood vessels although at a lower density. When veins dilate due to inflammation, causing an increase in the permeability of fluid components⁴⁸, the accompanying distribution of lymphatic vessels is essential for the collection of released substances.

Clinical application of lymphatic vessels: Lymphatic fluid flows through collecting lymphatic vessels via lymphatic capillaries and precollecting lymphatic vessels. The lymphatic capillary network terminates in blind-ended vessels in the connective tissue papillae in the gingiva. The blood vessel network forms loops of ascending and descending legs; by contrast, the lymphatic vessels never form loops. Periodontal tissues drain into the cervical regional lymph nodes in the palate and the submental lymph nodes in the anterior mandibular region. Most other areas drain into the submandibular lymph nodes, and then drain into veins at the angulus venosus. In cases where acute inflammation affects lymph nodes as a result of bacterial infection (especially of the oral mucosa), the current treatment is the systemic administration of drugs to relieve symptoms, together with local application of ointment as a local drug delivery system^{49,50}. Systemic drug administration generally requires the drugs to be absorbed through the blood system; and the drugs are metabolized in the liver before effective drug action is obtained in the lymph nodes in the case of bacterial infection. Thus, the dose of drugs administered is determined based on this process. It is highly possible that resolution of swelling of regional lymph nodes by local application of ointment is achieved through lymphatic transport. If drugs are transported via lymphatic vessels, they are not metabolized before reaching the regional lymph nodes, in contrast with absorption and transport through blood vessels. Therefore, highly effective treatment can be achieved in terms of dose and drug level if the drugs directly targeting regional lymph nodes are delivered via the lymphatic vessels. Moreover, we suggest that in cases of infectious periodontal diseases, activation of the drainage systems through the lymphatic vessel networks by massaging the gingiva during tooth brushing is important in resolving edema. The

future development of drugs containing antimicrobial agents with lymphatic tropism may result in a treatment modality delivering antimicrobial action in regional lymph nodes and alleviating edema.

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