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THREE DIMENSIONAL ARCHITECTURE OF LYMPHATIC VESSELS IN THE TONGUE

A. Fujimura, S. Seki, M-Y. Liao, X. Hu, M. Onodera, Y. Nozaka

First Department of Oral Anatomy (AF,MYL,XH,MO,YN) and First Department of Oral and Maxillofacial Surgery (SS), School of Dentistry, Iwate Medical University, Morioka, Japan

ABSTRACT

We have three-dimensionally reconstructed the lymphatic architecture of the rabbit tongue using a computer graphic three-dimensional reconstruction method together with histochemically stained serial cryo-sections.

Three collecting lymphatic vessels lying in an anteroposterior orientation were identified in the tongue body. A superior longitudinal muscle accompanying collecting lymphatic (SLCL) that lies in the border between superior longitudinal muscle and transverse muscle was identified in this study, in addition to collecting lymphatics in the lingual septum (LSCL) and deep lingual artery accompanying collecting lymphatics (DLCL) that we reported previously. The vertical muscle accompanying collecting lymphatics (VCL) and the transverse muscle accompanying collecting lymphatics (TCL) were also identified as collecting lymphatics that joined the above three collecting lymphatics.

Specific regional lymph flow was identified for each collecting lymphatic. A transverse right/left cross flow was identified for the LSCL, while the SLCL served the upper 1/3 of the tongue and the DLCL served the lower 2/3 of the tongue. Each collecting lymphatic that accompanied the internal lingual muscles joined to the SLCL, DLCL and LSCL, and were derived from blind-ended lymphatic capillaries that ran in the endomysium. Internal lingual muscle accompanying collecting lymphatics joined each other in the endomysium, and their blind-ended lymphatic capillaries had no branches.

Various studies describe the lymphatic architecture of different internal organs (1-15). Others describe the lymphatic architecture in the oral region, including the dental pulp (16-18), the periodontal ligament (19-22), the periodontal tissues (23,24), and our previous studies on the tongue, periodontal tissues, submandibular node and jaw (25-32).

A two-dimensional analysis of the various internal organs that have a three-dimensional blood vessel system structure is inadequate since the lymphatic architecture is similar to that of the arterial and venous systems. The characteristic analysis of each internal organ relies on pigment injection and resin cast specimens that result in a three-dimensional reconstruction of the blood vessel system. However, these methods cannot be reliably used for lymphatic vessels. Previously, we reported the lymphatic architecture of various oral organs (26,27,30-32), the meninges (12), esophagus (15) and anus (14) of the rodent fetus using a three-dimensional computer graphic reconstruction method for serial sections (25). We were able to follow the course of the lymphatic vessels from the regional lymph node and the venous angle to the periphery in serial sections of these tissues.

In addition, we have examined collecting lymphatic vessels in the tongue of the golden hamster using two-dimensional images and enzyme histochemical staining (30,31). However, a three-dimensional observation has not been performed.

In the present study, we have examined the tongue of a rabbit, which was used previously in examining an increase of tumor size and a metastasis of an experimentally explanted tumor (33-36), and reconstructed a lymphatic capillary and the course of collecting lymphatic vessels in the body of the tongue. We used a combination of serial cryo-sectioning (37) and 5'-nucleotidase staining, which is a reliable method to detect lymphatic vessels.

MATERIALS AND METHODS

Five Japanese white male rabbits, weighing 2.0-2.5 kg, were purchased from Clea Japan Incorporated. The rabbits were confirmed to have no abnormalities in general or intraoral conditions after one week of preparatory keeping (room temperature: 22 ± 2°C, humidity: 55 ± 5%) in the vivarium of Iwate Medical University. Rabbits received a standard pellet diet with water ad libitum.

Rabbits were killed by an overdose of Nembutal intravenous anesthesia (120 mg/kg). The tongue was removed and trimmed for serial cryosectioning preparation. Briefly, the trimmed tongue was frozen in hexane cooled by dry ice (-80°C), and then embedded in 5% carboxymethyl cellulose (CMC) in the same hexane. The embedded sample was placed in the cryostat and 10 μm serial sections were made using the film transfer method (37) (Cryo-film transfer kit, Finetec Co., Japan). The sections were stained by 5'-nucleotidase staining (5'-Nase staining), as described previously (39,40), and two-dimensional (2D-) images of these sections were input into the computer (Macintosh G4, Apple, USA) using a light-microscope (E800M, NIKON, Japan) equipped with a color chilled 3CCD camera (C5810, Hamamatsuphotonics, Japan).

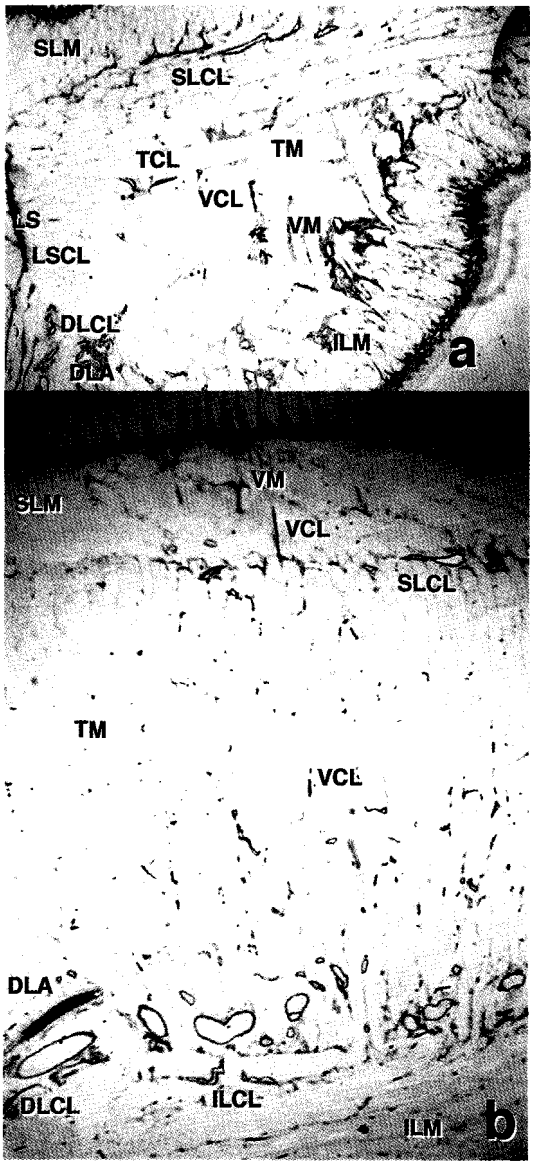

Fig. 1. Two dimensional images of 5' -Nase stained 10μm sections. a: frontal section; b: sagittal section. Internal muscles of the tongue body are seen with blackish brown stained collecting lymphatic vessels in the endomysium. SLM, superior longitudinal muscle; TM, transverse muscle; VM, vertical muscle; ILM, inferior longitudinal muscle; LS, lingual septum; DLA, deep lingual artery. SLCL=collecting lymphatic vessel beneath superior longitudinal muscle; TCL=transverse muscle accompanying collecting lymphatic vessel; VCL=vertical muscle accompanying collecting lymphatic vessel; DLCL= deep lingual artery accompanying collecting lymphatic vessel; ILCL=inferior longitudinal muscle accompanying collecting lymphatic vessel.

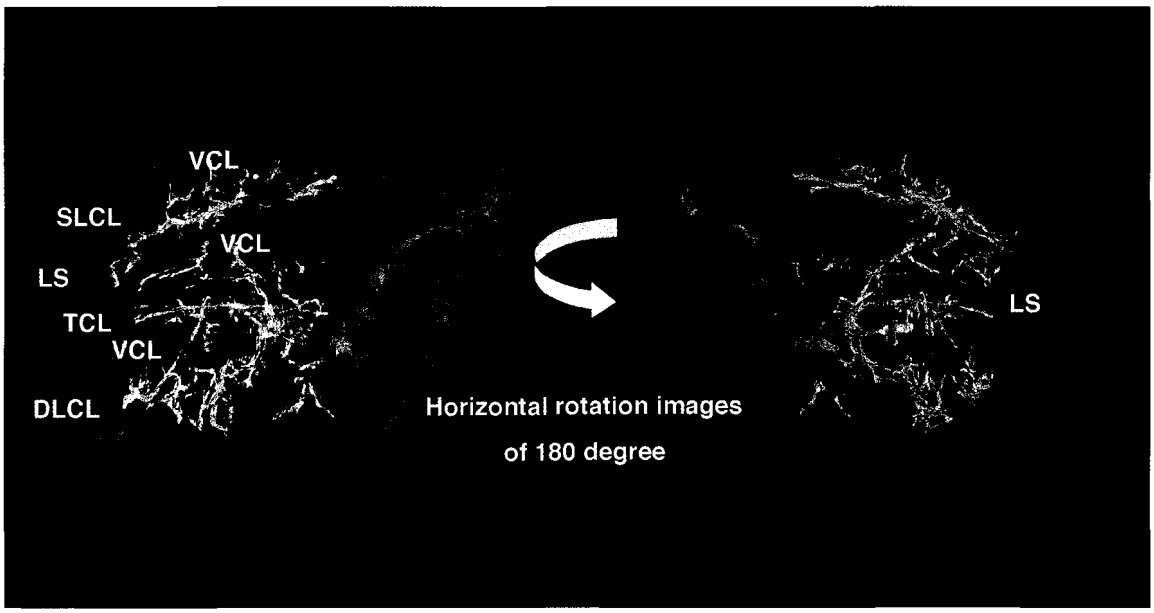

Fig. 2. Three dimensional reconstruction images from a hundred serial sections stained with 5'-Nase staining. See Fig. 1 for acronyms.

5'-Nase positive lymphatic vessels were extracted from these images using a computer application and threshold treatment (Photoshop, ver. 7.0 Adobe, USA). Three-dimensional (3D-) reconstructed images from serial sections were made by the volume rendering method (Boxblast, ver. 2.3.3., Vaytec. Inc., USA), and then observed from various directions by rotating animations (12,25).

This study strictly followed the guidelines of the animal care committee of Iwate Med. Univ., which adheres to governmental legislation in Japan. Handling of animals, including surgery, postoperative care, tissue sampling and euthanasia was monitored by officially qualified animal care personnel.

RESULTS

Collecting Lymphatic Vessels

Intrinsic muscles, which compose the tongue body, are the bilateral superior and inferior longitudinal muscles (SLM and ILM), the transverse muscles (TM) and the vertical (VM) lingual muscles *(Fig. 1)*. There are 30-50 μm in diameter collecting lymphatic vessels in the endomysium of the ILM, TM and VM, but no accompanying collecting lymphatics in SLM. However, 100 μm in diameter collecting lymphatic vessels run in an anteroposterior direction of tongue at the boundary of the SLM and TM. We named these collecting lymphatic vessels the inferior longitudinal muscle accompanying collecting lymphatic vessels (ILCL), the transverse muscle accompanying collecting lymphatic vessels (TCL), the vertical muscle accompanying collecting lymphatic vessels (VCL) and the collecting lymphatic vessels beneath the superior longitudinal muscle (SLCL). The deep lingual artery accompanying collecting lymphatic vessels (DLCL) and the collecting lymphatic vessel in the lingual septum (LSCL) were observed and have been reported previously (30) *(Figs. 2-4)*.

We identified two routes of the VCL joining the SLCL and one route of the VCL

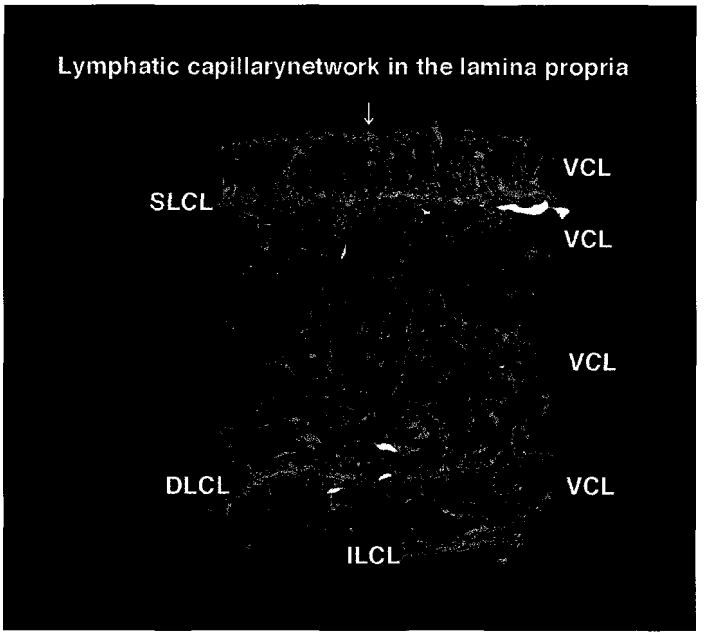

Fig. 3. Collecting lymphatic vessels in the tongue body are shown on the three dimensional reconstruction image from sagittal serial sections stained for 5'-Nase. See Fig. 1 for acronyms.

joining the DLCL. The first route came down from the lamina propria and crossed with the SLM *(Fig. 3)*. The second route came up from the upper one third of the tongue body and the third route came down from the lower two thirds of the tongue body *(Figs. 2-4)*. The former two routes of the VCL faced each other *(Fig. 4)*, because they were accompanied by the VM. A part of the TCL in the upper one third of the TM in the tongue body joined the second route of the VCL. The remainder of TCL at this site ran to the lingual septum and joined the LSCL. The bilateral TCL joined the SLCL, and the LSCL, which was 30 μm in diameter, and ran in the lingual septum. On the other hand, the third route of the VCL, to which the TCL joined in the lower two thirds of the tongue body, joined the DLCL. This VCL formed sac-like lymphatics which were not connected by large lymphatics with the surrounding lymphatics. There were ILCL, 50-80 μm in diameter, in the ILM that ran both in the front and back of the tongue. These ILCL did not join the fourth route of the VCL *(Figs. 3,4)*.

Lymphatic Capillary

Three types of lymphatic capillaries were observed: a lymphatic capillary network of 10 μm in diameter in the lamina propria, a blind end in the papilla of the lamina propria that arises from former network, and a blind end in the endomysium of the internal tongue muscle that arises from collecting lymphatic vessels *(Fig. 1)*. Lymphatic capillaries from the dorsal side joined the SLCL and those of the sublingual side joined the DLCL *(Figs. 2,3)*. The blind end of the lymphatic capillaries from the VCL infiltrated the VM and SLM on the dorsal side. On the other hand, the blind end of lymphatic capillaries from the VCL invaded the VM, but not the ILM on the sublingual side. The blind end of lymphatic capillaries from the ILCL invaded the ILM *(Figs. 3,4)*. The blind-ended lymphatic capillaries branched out from each collecting lymphatic vessel, which showed no branches.

DISCUSSION

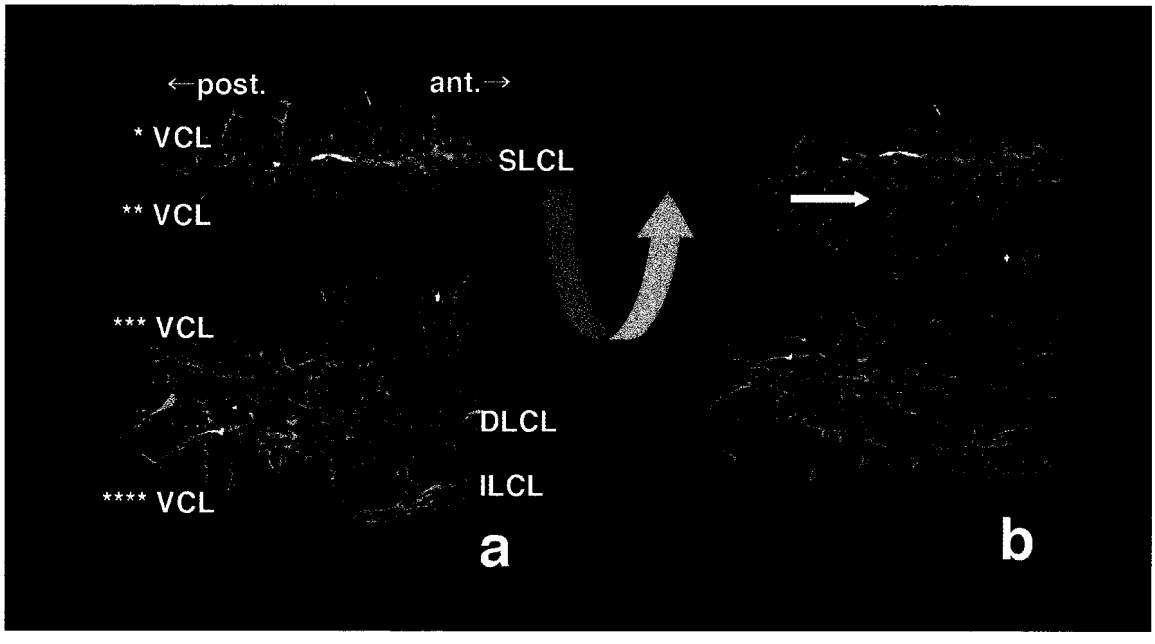

Fig. 4. Three dimensional reconstruction images from a hundred and fifty sagittal sections. a: Notice VCL: the first route comes down from the lamina propria crossing with SLM (*), the second route rises up from the upper one third of the tongue body (**) and the third route comes down from the lower two thirds of the tongue body (***). b: A 45 degree vertical rotation of a. There are a few SLCLs in one side. Notice the connection between SLCLs (arrow). See Fig. 1 for acronyms.

The rabbit was used previously as an experimental model of cancer of the tongue, and showed a high metastatic rate after transplantation of VX2 cancer cells (33-36). We also observed the dynamics of lymphatic construction in the tongue after a VX2 cancer cell transplant (32). Those experiments suggested that lymphatic vessel construction of the cancer circumference had a close relationship with cancer metastasis, but conclusive results required a three dimensional image of lymphatic vessel reconstruction in the normal tongue. Previous attempts to reconstruct the lymphatic vessel organization in organs failed to discriminate between lymphatics and venules. The present method used continuation intercept three-dimensional reconstruction techniques that tracked the lymphatic vessel through serial sections and reduced the analysis time. The efficiency of this method was reduced when using large or medium sized experimental animals due to the time required to produce, analyze, and input the great quantity of serial sections used. Therefore, we applied 5'-Nase staining, which differentiates lymphatics from other vessels (39,40), along with 3D reconstruction. This staining method readily delineates lymphatic vessels in a cost-efficient manner when compared with immunohistochemical staining. However, whereas discrimination between a lymphatic vessel and a blood capillary is relatively easy with these methods, a rare venule may react positively and be characterized as a lymphatic. The "positive" venule can be correctly identified during 3D reconstruction as it joins a vein. In other words, in this study, we identified and extracted 5'-Nase positive vessels as lymphatics using 3D reconstruction methods but only when the vessel did not connect to a blood vessel.

Based on previous studies examining the golden hamster tongue using 5'-Nase staining

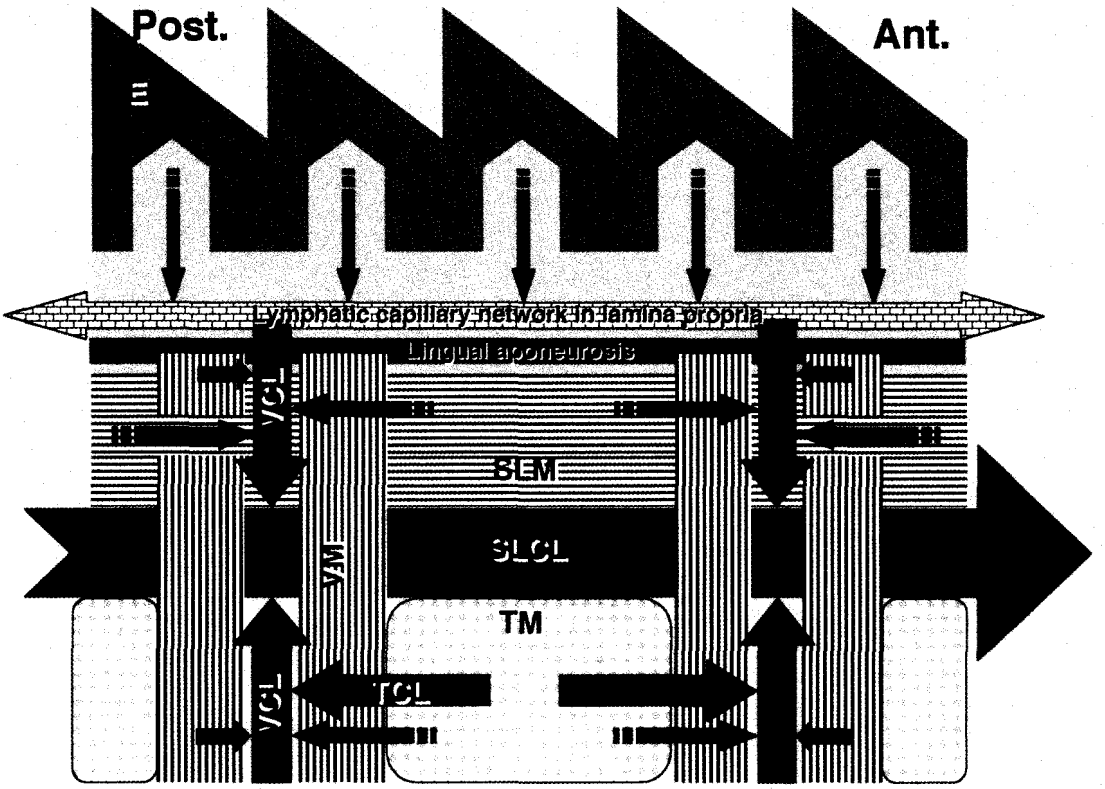

Fig. 5. Schematic drawing of lymphatic drainage in the dorsal side of the tongue body. Direction of arrows indicates lymphatic flow. small arrow, blind end of lymphatic capillary; medial arrow, collecting lymphatic in intrinsic muscle of tongue; large arrow, collecting lymphatic vessel beneath superior longitudinal muscle; arrow of network pattern, lymphatic capillary network in lamina propria. See Fig. 1 for acronyms.

(30,31), lymphatic capillaries in the endomysium of the internal tongue muscle, especially the VM and the TM, joined the VCL and the TCL. These two collecting lymphatics then joined the LSCL and/or the DLCL. Other collecting lymphatic vessels were not examined.

In this study, three types of collecting lymphatic vessels ran in an anteroposterior direction in the tongue body. We confirmed the presence of the SLCL, in addition to the collecting lymphatic vessels in tongue, LSCL and DLCL, that have been reported previously. The SLCL ran with the SLM in an anteroposterior direction at the border between the SLM and the TM *(Fig. 5)*. We recognized two kinds of VCL, which joined the SLCL. The first emanated from the lymphatic capillary network in the lamina propria, VM and SLM, and the second derived from the TM and the VM in the dorsal one third of the tongue. We named the former, the upper superficial VCL, and the latter, the upper profunda VCL. The upper profunda VCL collected lymph from the dorsal one third of the tongue, and the VCL of the sublingual two thirds of the tongue joined to the DLCL. Between these two VCL routes, a large gap was present in the TM. These findings suggested that, in regards to

lymphatic drainage, the LSCL drained across the tongue, the SLCL drained the dorsal one third of the tongue, and the DLCL drained the sublingual two thirds of the tongue. The internal lingual muscle accompanying the collecting lymphatic vessels joined these three collecting lymphatic vessels (LSCL, SLCL and DLCL), and lymphatic capillaries diverged from them. Lymphatic capillaries which diverged from the collecting lymphatics in the endomysium of the internal muscle of the tongue had no branches. The classifications of lymphatic vessels from this study and previous studies include the lymphatic trunk, collecting lymphatics, lymphatic capillaries and blind ends. In this paper, we identified the collecting lymphatics with the terminology of the accompanying anatomical structure. We recommend the following terms for these collecting lymphatic vessels in the tongue: the deep lingual artery accompanying collecting lymphatic vessel in the lower lingual collecting lymphatic (LLCL), the collecting lymphatic vessels in the lingual septum (LSCL), the collecting lymphatic vessels beneath the upper longitudinal muscle in the upper lingual collecting lymphatic (ULCL), the vertical muscle accompanying collecting lymphatics in the vertical lymphatic (VL), the transverse muscle accompanying collecting lymphatic in the transverse lymphatic (TL), and the lower longitudinal muscle accompanying collecting lymphatic in the longitudinal lymphatic (LL).

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Akira Fujimura, DDS, PhD
First Department of Oral Anatomy
School of Dentistry
Iwate Medical University
1-3-27 Chuo-dori, Morioka 020-8505, Japan
Phone: +81-19-621-3661
Fax: +81-19-621-3662
E-mail: akifuji@iwate-med.ac.jp