The distribution of lymphatic vessels in the periodontal ligament during tooth root formation

Munenori Kikuchi1), * Yoshinori Ando2) and Akira Fujimura2)
1) Department of Developmental Oral Health Science, Division of Orthodontics, School of Dentistry, Iwate Medical University
2) Department of Oral Biology, Division of Oral Anatomy, School of Dentistry, Iwate Medical University

Abstract

Introduction: Remodeling of tissues frequently occurs in the periodontal ligament during orthodontic tooth movement, including various changes in the vascular system. Although studies have investigated lymphatic vessels in the periodontal tissues, only a few studies have observed lymphatic vessels in the periodontal ligament, leaving many unclear aspects.

Materials and Methods: Using mice (PN-0, 7 and 14 days old) with unerupted mandibular first molars, lymphatic distribution and the existence of lymphatic vessels in the periodontal tissues including the periodontal ligament were immunohistochemically (LYVE-1) observed.

Results: Lymphatic vessels were observed beneath the oral epithelium, beneath the epithelium of the attached gingiva and inside the mandibular canal. Some lymphatic vessels beneath the epithelium of the attached gingiva were present along the alveolus. Although LYVE-1 positive structures distributed irregularly at each age in the areas of periodontal ligament at the future sites of crown formation, root formation and root apical region of root formation, no lymphatic vessels were identified.

Discussion: In the periodontal ligament of adult mice, it was supposed that lymphatic vessels in the periodontal ligament except the apex of root were not distributed from initial stage, and lymphatic vessels were observed near the apex in adult mice was distributed during the apex of root completion stage. This might be connected to the presence of the Hertwig’s epithelial sheath. [MVRC 4(1): 18-25, 2011]

Key words: lymphatic vessel, periodontal ligament, tooth germ, LYVE-1, mouse
Introduction

The recent discovery of a molecular marker for lymphatic vessels has made possible the immunostaining of lymphatic vessels. Since then, lymphatic research has advanced rapidly in fields such as lymphatic metastasis of malignant tumors and lymphedema. Many researchers have investigated lymphatic vessels in the oral region. Orthodontic tooth movement often results in remodeling of the alveolar bone by cells of periodontal ligament and other periodontal tissues accompanied by significant changes in the vascular system. Our previous study reported that observed lymphatic vessels in the periodontal ligament of adult mice revealed that most LYVE-1 positive lymphatic vessels were found in the periodontal ligament at the tooth root apex and were rarely observed in the periodontal ligament at the middle third region of the tooth root. Another study reported that the lymphatic vessels in the gingiva did not enter the periodontal ligament. These studies used adult mice and observed lymphatic vessels during constant occlusal force on the periodontal ligament.

We aimed to eliminate the effects of occlusal forces on the tissues by examining samples from unerupted teeth during the early and middle stages of root formation. By examining the distribution of lymphatic vessels in the periodontal ligament, we expected not only a tissue changes during the process of growth in tooth germs, but also further development process of lymphatic vessels in periodontal tissues.

The aim of this study was to immunohistochemically examine lymphatic distribution around the tooth germs of unerupted mandibular first molars using 0-14 day old mice.

Materials and Methods

Experimental animals and breeding condition: A total of 10 mice (5 males, 5 females, C57BL/6) were purchased from Clea Japan (Osaka, Japan). After feeding and breeding, newborn mice were used for our experiment. Three groups of five mice were selected (total 15) at 0 days (PN-0), 7 days (PN-7) and 14 days (PN-14) after birth. The newborn mice were housed with their mothers and provided with free access to breast milk and water at the Institute for Experimental Animal Sciences Iwate Medical University (air temperature: 23±1°C, humidity: 55±5%) during the experimental period. We confirmed non-eruption of mandibular first molars before each examination. This study was approved by the Committee on Animal Experiments of Iwate Medical University (Approval number 19-011). All experiments were conducted in accordance with the Guidelines for Animal Experiments at Iwate Medical University based on the guidelines for proper conduct of animal experiments from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Breeding: Breeding was conducted at the Institute for Experimental Animal Sciences Iwate Medical University (air temperature: 23±1°C, humidity: 55±5%). Five male mice were housed in one cage, and five female mice were kept in five separate cages. At the time of breeding, male mice were moved to the cages of each female mouse, making one-for-one pairs and each pair was left for one night. Breeding was attempted once a week. We checked for the birth of baby mice every morning at 9:00, and the day we found newborn mice was nominated as PN-0.

Fabrication of serial cryosections: The mice were killed by an overdose of an intraperitoneal injection of Somnopentyl®, and the heads were removed. The heads were then cryo-embedded in 5% carboxymethyl cellulose (CMC) in hexane cooled by liquid nitrogen without fixation and decalcification. The samples were placed in a cryostat (CM3050S, Leica, Bensheim, Germany; cutting edge angle: 7-10°, CT -22°C, OT -22°C) and 3-µm frontal serial cryosections were cut with a tungsten carbide blade (TC-658®, Leica, Germany) using the film-transfer method (Kawamoto’s method) (Cryofilm TYPE. I-B®, Leica, Tokyo, Japan). Lymphatic staining: After immersion fixation with 100% alcohol, cryosections produced by the film transfer method were immersed in PBS solution (0.01 mol/l, pH 7.4, Mitsubishi Chemical Medience Corp., Tokyo, Japan) for 5 minutes at room temperature. The sections were washed three times, and immersed in 3% H2O2 in PBS for 15 minutes at room temperature to remove endogenous peroxidase. After washing further three times, the sections were blocked with 1% BSA in PBS in a moist chamber for 30 minutes at room temperature. The sections were then reacted with goat anti-mouse LYVE-1 antibodies (R&D Systems Inc., Minneapolis, USA) diluted 1 : 20 in 1% BSA in PBS as primary antibodies in a moist chamber for 1 hour at room temperature. Thereafter, the sections were washed with PBS solution again and reacted with Histofine® Simple Stain MAX-PO(G) (Nichirei Co., Tokyo, Japan) as secondary antibodies in a moist chamber for 30 minutes at room temperature. After washing with PBS solution, the color of the sections was developed with a DAB substrate kit (Vector Laboratories, Peterborough, UK) for 5 minutes at room temperature. After washing with distilled water, the sections were counterstained with hematoxylin, and then the sections were embedded in 30% glycerin.

After observing the stained sections using an optical microscope (E-1000, Nikon, Tokyo, Japan) with a color chilled 3CCD camera (DS-5Mc®, Nikon), two-dimensional images were obtained for identification of lymphatic vessels, and observation of the tooth germs and surrounding tissues.

Observation of LYVE-1 positive lymphatic vessels: Lymphatic vessels were identified as tube-like structures exhibiting a dark brown LYVE-1 positive reaction on the two-dimensional images. Entities exhibiting a LYVE-1 positive reaction without tube-like structures were defined as LYVE-1 positive structures. LYVE-1 positive structures that existed at the same location in the previous or following multiple serial sections were also identified as lymphatic vessels.

The six areas for observation of the tooth germs and surrounding tissues are presented in Figure 1 and consist of the area beneath the oral epithelium (Fig. 1 area 1); the area beneath the epithelium of the attached gingiva (Fig. 1 area 2); between the dental follicle and alveolus corresponding to the future periodontal ligament including the crown formation area (Fig. 1 area 3); the root formation area (Fig. 1 area 4); the apical region of root formation area (Fig. 1 area 5); and the inside of the mandibular canal (Fig. 1 area 6) communicating with the area corresponding to the periodontal ligament.

Measuring method: The size of tooth germ

The size of tooth germs were measured in the following way. The dental lamina side was regarded as the top of the frontally sectioned sample and a line was drawn along the
maximum buccolingual length of the tooth germ. Lines were then drawn along the long axis perpendicular to the first line and the maximum length was measured. Measurements in a buccolingual direction included the areas between both sides of the outer enamel epithelium and the reduced enamel epithelium. Measurements drawn along axis included the area between the oral side of the oral epithelium and the dental follicle of the apex of the root formation area.

Lymphatic vessels
To clarify the position of the distribution of lymphatic vessels in the area beneath the oral epithelium, the shortest distance was measured between the point immediately below the oral epithelium and the inner wall of the lymphatic vessels. The measurements performed also on the size of lymphatic vessels distributed in the observation area. The widest diameter was considered to be a major axis, and the line perpendicular to the long axis was set as a minor axis.

Results
Status of tooth germ formation: We observed the status of mice tooth germ formation used in this study. At PN-0, some formation of enamel and dentin was observed, providing a connection of the tooth germ and the oral epithelium at the dental lamina. The tooth germs were in the late bell stage (Fig. 2a). At PN-7, enamel and dentin were clearly formed. In addition, the Hertwig’s epithelial sheath was curved toward the dental papilla at the apex of the root formation area (Fig. 3a). At PN-14, further progress of enamel and dentin formation and calcification could be observed and the odontoblastic zone, cell free and cell rich zones in the dental papilla were clearly visible (Fig. 4a).

However the dental lamina appeared to be receding at PN-14, it did not completely disappear.

Observation of LYVE-1 positive lymphatic vessels:
1) PN-0 group
The lymphatic vessels appearing as tube-like structures were observed in the area beneath the oral epithelium (area 1) (Fig. 2). These distances ranged from approximately 4 μm to 40 μm. Most of the observed lymphatic vessels were distributed near the oral epithelium.

Lymphatic vessels were also found in the area beneath the epithelium of the attached gingiva (area 2), where they were sparsely but broadly distributed, parallel to the alveolus and maintaining a relatively fixed distance of approximately 20 μm from the outer surface of the alveolus.

Lymphatic vessels with a lumen diameter of approximately 3 μm and 5 μm were frequently observed beneath the oral epithelia and the attached gingiva, respectively (Fig. 2c).

2) PN-7 group
Lymphatic vessels appearing as tube-like structures were found beneath the oral epithelium (area 1) in the PN-7 group (Fig. 3), at distances ranging from 5 μm to 75 μm from the epithelium.

Beneath the epithelium of the attached gingiva (area 2), lymphatic vessels were observed in a distribution parallel to the outer surface of the alveolus, maintaining an almost fixed distance of approximately 15 μm from the outer surface of the alveolus (Fig. 5 thick arrows). Connective tissue papillae beneath the epithelium also exhibited lymphatic distribution (Fig. 5 arrowheads).

Most of the lymphatic vessels observed beneath the oral epithelium and the epithelium of the attached gingiva had lumen diameters of approximately 5 μm and 4 μm, respectively (Fig. 3c).

In this group, LYVE-1 positive structures with no tube-like structures were also observed inside the mandibular canal. These structures were found between the inner wall of the mandibular canal and the inferior alveolar nerve in a scattered pattern surrounding the inferior alveolar nerve (Fig. 6a,b,c). If these structures were observed at the same position in more than ten sections, they were identified as lymphatic vessels (Fig. 6d,c).

3) PN-14 group
At PN-14, lymphatic vessels with tube-like structures were observed beneath the oral epithelium (area 1) (Fig. 4). The closest lymphatic vessels to the oral epithelium were observed immediately below the epithelium.

Lymphatic vessels were observed beneath the epithelium of the attached gingiva (area 2). In this group as in the other groups, the lymphatic vessels maintained a parallel distribution about 10 μm from the outer surface of the alveolus. The lymphatic vessels in this area could also be seen branching into the subepithelial connective tissue papillae.

Most lymphatic vessels beneath the oral epithelium had a lumen diameter of approximately 5 μm, with some relatively large vessels with lumens up to 7 μm in diameter. Smaller diameter lymphatic vessels (approximately 3 μm) were found beneath the epithelium of the attached gingiva. In both areas, the number of lymphatic vessels clearly increased from the younger groups.

Lymphatic vessels were observed inside the mandibular canal (area 6) in the PN-14 group. As in the PN-7 group, LYVE-1 positive entities without tube-like structures were observed, and where the same structures were observed at the same position in more than ten sections, the entities were
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identified as lymphatic vessels.

Observation of the area corresponding to the periodontal ligament

The areas corresponding to the future periodontal ligament consisted of the crown formation area (area 3), the root formation area (area 4), and the apical region of root formation area (area 5). We observed these areas at each age.

LYVE-1 positive structures without tube-like were observed in areas 3 and 4 at all observed ages (Fig. 7). In this area, LYVE-1 positive structures were observed irregularly throughout the entire areas of 3 and 4 (Fig. 7a,b,c). Similar structures were not observed in the same position in adjacent serial sections (Fig. 7d,e), so the structures were not identified as lymphatic vessels.

LYVE-1 positive structures were observed in all the groups in area 5 (Fig. 8). As in areas 3 and 4, LYVE-1 positive structures were present irregularly throughout the entire area (Fig. 8a,b,c). Adjacent serial sections did not reveal the same structures at the same position, as in areas 3 and 4 (Fig. 8d,e).

Discussion

Previous studies of lymphatic vessels in the oral cavity were conducted using animals such as mice and golden hamsters. In the oral cavity of mice, lymphatic distribution of the tongue, palatal mucosa, buccal mucosa, the inside of the mandibular canal, gingiva and periodontal ligament has been investigated.

Our study, based on the experimental method used to in...
vestigate the periodontal ligament, looked at the lymphatic distribution in the periodontal ligament and periodontal tissues without the effect of occlusal force.

The area beneath the gingival epithelium: We observed lymphatic vessels beneath the oral epithelium and the epithelium of the attached gingiva. In area 1, most lymphatic vessels were found immediately beneath the oral epithelium. As the age of the mice increased, lymphatic vessels with a larger diameter lumen were observed, and the number of lymphatic vessels increased. This finding was considered to be the result of the progress of lymphatic formation and maturation since eruption of the mouse first molar occurs at PN-16.

Beneath the epithelium of the attached gingiva, the lymphatic vessels were distributed almost entirely in area 2, in contrast to the area beneath the oral epithelium. The degree of lymphatic formation in area 2 was examined in reference to the surrounding tissues. We found that the formation of the tissues of the alveolus around the epithelium of the attached gingiva, mandibular canal and inferior alveolar nerve progressed faster relative to the tooth germ around the oral epithelium. Therefore, we suggest that the formation of lymphatic vessels progresses in accordance with the surrounding tissues.

Inside the mandibular canal: In all the groups except the PN-0 group, LYVE-1 positive lymphatic vessels without tube-like structures were observed between the inner wall of the mandibular canal and the inferior alveolar nerve. Since LYVE-1 is specifically expressed in the endothelium of lymphatic vessels without showing a positive reaction for blood vessels, it has recently been used as a molecular marker of lymphatic vessels. Some studies using LYVE-1 antibodies have reported the existence of LYVE-1 positive macrophages. In our study, we identified lymphatic vessels by observing previous and following multiple serial sections. In some cases, LYVE-1 positive structures were found at the same position in a series of more than ten sections. Since the thickness of the sections fabricated in the present study was 3 μm, these structures can be seen inside the mandibular canal (area 6). The LYVE-1 positive structures surround the inferior alveolar nerve in a scattered pattern. Comparison of the serial sections confirm that LYVE-1 positive structures are present at identical positions. These structures were identified as LYVE-positive lymphatic vessels since more than ten serial sections contained the same structures at the same positions.
structures were considered to be more than 30 μm in size. We were therefore able to rule out the possibility of these structures being macrophages, since the macrophages are only approximately 10 μm in size. In addition, the presence of lymphatic vessels in the epineurium of the inferior alveolar nerve in the mouse craniofacial area was confirmed in a study of nerve-related lymphatics by Furukawa et al. using LYVE-1 antibodies.

The irregular epineurium of the inferior alveolar nerve surrounding the inferior alveolar nerve forms ripples between the inner wall of the mandibular canal and the nerve. The lymphatic vessels identified in our study were scattered around the inferior alveolar nerve, located in the same positions observed in Furukawa’s study.

The area corresponding to the periodontal ligament: Lymphatic networks were in the area 2, but they were not observed on the inner surface of the alveolus corresponding to the periodontal ligament. The existence of lymphatic vessels on the periosteum around bone has been previously reported. In the present study, taking the alveolus as a margin, area 2 and the areas 3, 4 and 5 corresponding to the periodontal ligament were considered to be the outer and inner surfaces, respectively. In this situation, the periosteum does not exist on the inner surface, only on the outer surface.
Therefore, we assume that lymphatic vessels were present in area 2 with the peristemum, and none were present in areas 3, 4 and 5. On the other hand, LYVE-1 positive structures were found in areas 3, 4 and 5 in the present study. Some studies have reported lymphatic endothelial cells without tube-like structures scattered in the area corresponding to the mouse tooth pulp by PN-10\(^{23}\), suggesting that lymphatic architecture could be completed in tandem with future completion of root formation.

Research using immunostaining with LYVE-1 as a primary antibody on 9-week-old mice revealed that lymphatic vessels with a weakly-positive reaction were observed at the root apex of first molar\(^{21}\). A tooth root was completed in 9-week-old mice. But at PN-14, the rupture of the Hertwig’s epithelial sheath is not observed and the tooth root was not completion. Therefore, the periodontal ligament formation of the time is not observed\(^{34}\). From these studies, this period does not have periodontal ligament formation, it is considered that the period the lymphatic distribution is also not observed. In addition, other studies observing the lymphatic system in rats (also rodents) observed that lymphatic vessels distributed in the mandibular canal entered the periodontal ligament through the alveolar canals and merged with the lymphatic vessels from the pulp at the root apex\(^{23}\). These findings, including those of the study reporting lymphatic endothelial cells in mouse pulp tissue, taken with the results of our study concerning the lack of lymphatic vessels in the periodontal ligament by PN-14, especially in the area corresponding to the apical region of roots, suggest that lymphatic communication between the mandibular canal and the pulp is formed via the periodontal ligament at the apex by an expansion of the area of lymphatic distribution in these areas in tandem with the completion of root formation.

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