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Regular Article

Localization of Both CD31- and Endomucin-Expressing Vessels in Mouse Dental Pulp

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We investigated the localization of both CD31- and endomucin-expressing vessels in mouse dental pulp to elucidate their relationship with dentin formation. The maxillae of C57BL/6 male mice (1, 4, 8, 12, and 56 weeks old) were fixed with 4% paraformaldehyde solution, and cryosections (12-μm-thick) were prepared. Immunofluorescence was performed using anti-CD31 and anti-endomucin antibodies, and calcein labeling was conducted to elucidate relationships with dentin formation. At 1 week, many CD31-expressing (CD31 (+)) and endomucin-expressing (endomucin (+)) vessels were observed throughout the dental papilla. At 4 weeks, CD31 (+) and endomucin (+) vessels decreased in the crown and increased in the root of dental pulp. At 12 weeks, CD31 (+) and endomucin (+) vessels were detected at the root apex, but not in coronal pulp. At 56 weeks, few CD31 (+) and endomucin (+) vessels were observed in dental pulp. Both CD31(+) and endomucin (+) vessels were detected directly beneath calcein-labeled dentin at all sites. These results suggest the presence of CD31 (+) and endomucin (+) vessels in dental pulp and their contribution to dentin formation.

Key words: CD31, endomucin, dental pulp, type H vessel

I. Introduction

Mesenchymal stem cells (MSCs) have been identified in various tissues, including bone marrow [21, 25, 26] and dental pulp [8, 9, 18, 24]. Previous studies indicated that MSCs reside in a perivascular niche and differentiate into osteoblasts, chondrocytes, and adipocytes [2, 19]. Furthermore, MSCs express pericyte markers, such as α-smooth muscle actin and CD146, but not endothelial markers, including von Willebrand factor [26], indicating that they originate from pericytes [5]. Therefore, the perivascular niche is an important area of hard tissue formation.

A vessel network comprising arteries, veins, and capillaries is constructed in bone marrow and is involved not only in the supply of oxygen and nutrients, but also in bone growth and homeostasis. Cross-talk between endothelial cells and surrounding cells, such as perivascular cells, was recently suggested to maintain the niche for osteogenesis [14].

CD31 is known as platelet endothelial cell adhesion molecule 1 (PECAM-1), and expresses in cell surface of vascular endothelial cells. CD31 is localized at cell membranes between adjacent endothelial cells, and associated with cell functions such as signaling and angiogenesis. Endomucin is an endothelial transmembrane sialomucin closely related to CD34, and marks solely on the haematopoietic stem cells [16] and the surface of the capillary and venous, but not arterial endothelium [6]. Recently, niche for osteogenesis forms near specialized blood vessels that highly express both CD31, which is cell surface marker of endothelial cell, and endomucin [14, 31].

Dental pulp is a blood vessel-rich connective tissue that receives a rich vascular supply via blood vessels that enter from the apical foramen. The vessel network is

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present throughout dental pulp tissue and plays an important role at the odontoblast and subodontoblastic layers in mineralization and hard tissue formation [13, 27]. A previous study demonstrated that α-smooth muscle actin-expressing perivascular cells were dental pulp progenitor cells [28]. Furthermore, the localization of MSCs expressing CD90, an MSC marker, was observed at the subodontoblastic layer and CD90-expressing cells were associated with pulp regeneration after stimuli [10, 23]. Due to similarities in bone marrow and dental pulp, blood vessels that highly express both CD31 and endomucin in endothelial cells exist and MSCs or odontoblast progenitor cells localize near to these vessels and form a perivascular niche in dental pulp [17]. However, the relationship between the localization of both CD31- and endomucin-expressing vessels and hard tissue formation in dental pulp has not yet been clarified.

Therefore, we herein investigated the localization of both CD31- and endomucin-expressing vessels in young and aged mouse dental pulp, and discussed their relationship with dentin formation.

II. Materials and Methods

Animals and tissue preparation

All animal experiments were performed according to the Guidelines for the Treatment of Animals at the Tokyo Dental College (Approval No. 192302, 212302). C57BL/6 male mice (1, 4, 8, 12, and 56 weeks old) were used in the present study. At each time point, mice were anesthetized intraperitoneally using a mixed anesthetic of medetomidine hydrochloride (0.3 mg/kg, Nippon Zenyaku Kogyo, Koriyama, Japan), midazolam (4 mg/kg, Astellas Pharma, Tokyo, Japan), and butorphanol tartrate (5 mg/kg, Meiji Seika Pharma, Tokyo, Japan). After deep anesthesia, perfusion fixation with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) was performed transcardially, and the dissected upper jaws were immersed in the same fixative for 24 hr. After decalcification with Morse's solution (10% sodium citrate and 22.5% formic acid; FUJIFILM Wako Pure Chemical Corporation, Tokyo, Japan) at 4°C for 24 hr, tissues were immersed in 30% sucrose solution, embedded into a gel (Super Cryoembedding Medium, SECTION-LAB Co., Ltd., Yokohama, Japan), and quickly frozen in cold isopentane. Sagittal cryosections (12-μm-thick) were prepared with an adhesive film (Cryofilm Type IIc, SECTION-LAB Co., Ltd.) at −20°C for immunofluorescence.

To visualize new hard tissue formation, calcein labeling was performed. In brief, mice were intraperitoneally administered calcein green (20 mg/kg body weight; DOJINDO, Kumamoto, Japan) one week and one day before sacrifice, and the calcein-treated mice were sacrificed at 4, 8, and 12 weeks of age. The maxillae were removed and embedded in caroxymethyl cellulose (CMC), and were frozen in cold isopentane without decalcification. Frozen undecalcified sections (12-μm-thick) were prepared with an adhesive film (Cryofilm Type IIc, SECTION-LAB Co., Ltd.) according to a previously described method [11, 12].

Immunofluorescence

Cryosections were washed in PBS to remove the compound for 10 min, and non-specific binding was blocked with TNB blocking buffer (containing 0.1 M Tris-HCl, 0.15 M NaCl; Perkin Elmer) at room temperature (RT) for 30 min. Sections of decalcified samples were incubated with an Alexa Fluor 488-conjugated anti-CD31/PECAM-1 goat polyclonal antibody (dilution 1:100, FAB3628G-10, R&D Systems, Minneapolis, MN, USA) and a rat monoclonal antibody against mouse endomucin (dilution 1:200, sc-65495, Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 20 ± 5°C for 8 hr or 4°C overnight in the dark. After the incubation with primary antibodies, the sections were washed in PBS for 5 min three times and then incubated with secondary antibody, an Alexa Fluor 555-conjugated anti-rat IgG (ThermoFisher Scientific Inc., Waltham, MA, USA) to detect endomucin at 20 ± 5°C for 2 hr in the dark.

To detect CD31 on the undecalcified sections, hamster anti-PECAM-1 monoclonal antibody (2H8, Merck KGaA, Darmstat, Germany) was used as primary antibody, and Alexa Fluor 647-conjugated goat anti-hamster IgG (ThermoFisher Scientific Inc.) was applied as secondary antibody, instead of the above ones. After washing in PBS, counterstaining with 4',6-diamino-2-phenylidole dihydrochloride (ThermoFisher Scientific Inc.) was performed in the dark and all specimens were photographed using a confocal laser scanning microscope (LSM880 Airy NLO, Carl Zeiss, Oberkochen, Germany).

Histological evaluation

In the density analysis of CD31-expressing (CD31 (+)) and endomucin-expressing (endomucin (+)) vessels, we measured the areas of CD31 (+), endomucin (+), and both CD31 (+) and endomucin (+) in the whole dental pulp of the first molars using ImageJ (National Institutes of Health, Bethesda, MD, USA). In brief, the merged images were split into green and red channels, and an optimal threshold (CD31: 0–115, endomucin: 0–160) that distinguished between positive reaction and background was set. Area of green-colored (A), and red-colored (B) vessels was measured separately. Yellow-colored area indicating merged colored (C) vessels were displayed after using image calculator, and measured. The ratio of both CD31 and endomucin double-expressing areas (C) was calculated as follows;

Ratio of both CD31 (+) and endomucin (+) vessel area = $C/(A + B - C)$

Statistical analysis

Data were expressed as means and standard deviations (SD) and statistically analyzed using a one-way analysis of

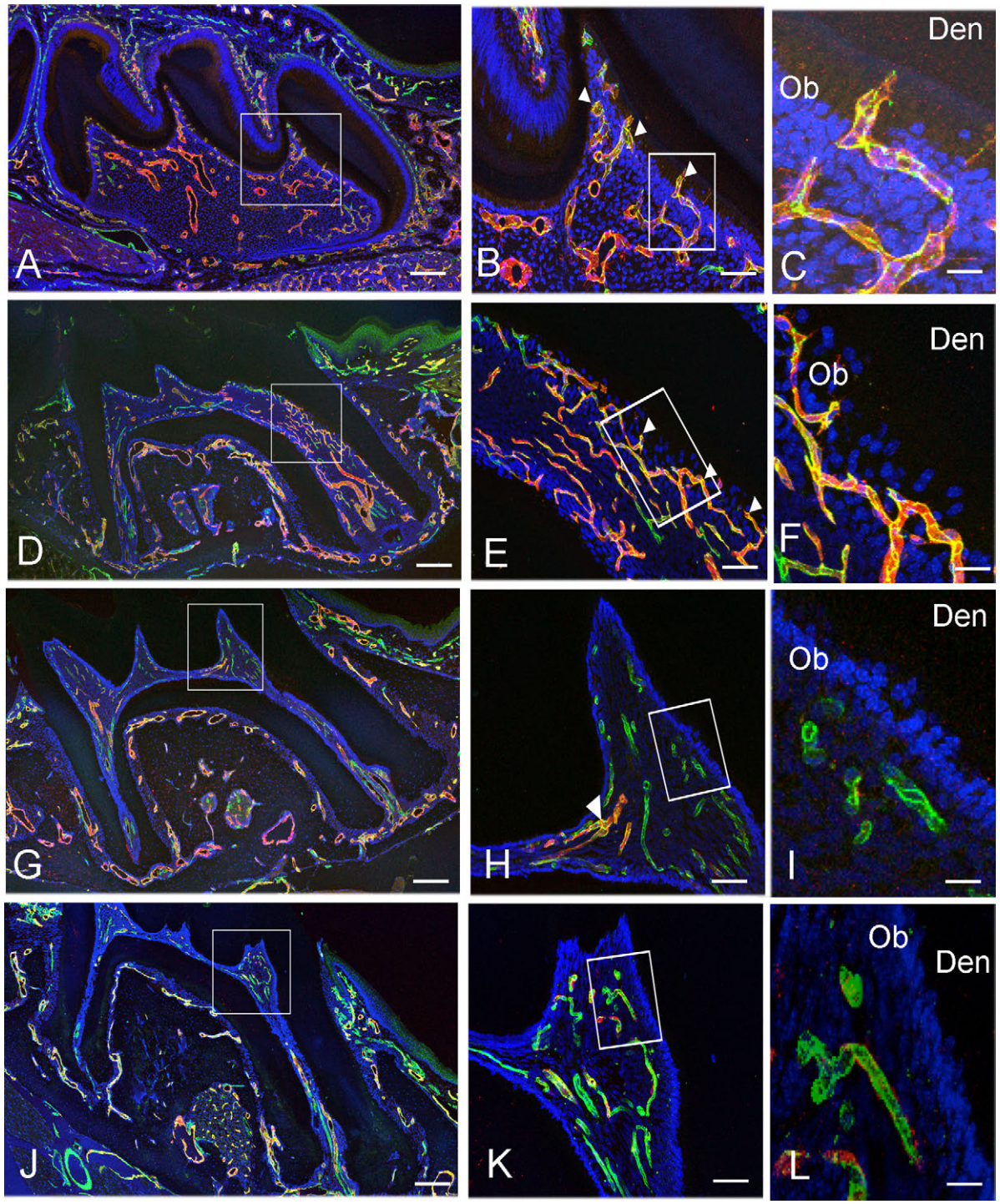

Fig. 1. Localization of CD31 (+) and endomucin (+) vessels. (**A**) Stage of tooth crown formation at 1 week. Endomucin (+) vessels (red) mainly exist in the dental pulp. (**B**) Higher magnifications of the boxed regions in **A**. Both CD31(+) and endomucin (+) vessels (yellow) are narrow and located beneath the coronal dentin. (**C**) Higher magnifications of the boxed regions in **B**. Merged immunoreaction (yellow) is localized at thin vessels in the odontoblast layer. (**D**) Dental pulp at 4 weeks. Both CD31 (+) and endomucin (+) vessels decreased in the crown and increased in the root of dental pulp at 4 weeks. (**E**) Higher magnifications of the boxed regions in **D**. Thin vessels are colored in yellow, and express both CD31 (+) and endomucin (+) and located near the radicular dentin (arrowheads). (**F**) Higher magnifications of the boxed regions in **E**. Note the merged immunoreaction (yellow) shows mesh-like pattern. (**G**) Dental pulp at 12 weeks. Root canals at 12 week are narrower than those at 4 week. (**H**) Higher magnifications of the boxed regions in **G**. CD31 (+)-immunoreaction is mainly seen, and Both CD31 (+) and endomucin (+) vessels are faintly seen in dental pulp (arrowhead). (**I**) Higher magnifications of the boxed regions in **H**. CD31 (+) vessels are seen beneath the odontoblast layer. (**J**) Dental pulp at 56 weeks. Few CD31 (+) and endomucin (+) vessels were detected in dental pulp. (**K**) Higher magnifications of the boxed regions in **J**. Main vessels are positive for CD31 in the coronal pulp. (**L**) Higher magnifications of the boxed regions in **K**. Immuno-reaction for endomucin was separately seen at the CD31(+) vessels. Green: CD31 (+), Red: Endomucin (+), Yellow: CD31 (+) and endomucin (+). Arrowheads: CD31 (+) and endomucin (+) vessels. Den: dentin; Ob: odontoblast layer. Bars = 200 μm (**A**, **D**, **G**, **J**), 50 μm (**B**, **E**, **H**, **K**) and 15 μm (**C**, **F**, **I**, **L**).

variance (ANOVA), followed by Tukey's honestly significant difference test with significance set at a *P*-value of 0.05. All statistical analyses were performed using a statistical program (IBM SPSS Version 27 for Windows, SPSS, Chicago, IL, USA).

III. Results

Changes in the localization of CD31 (+) and endomucin (+) vessels

At 1 week, teeth had not yet erupted and were in the stage of tooth crown formation (Fig. 1A). Many CD31 (+) and endomucin (+) vessels were observed throughout the dental pulp (Fig. 1B). The CD31 (+) and endomucin (+) vessels were thin at the odontoblast layer and immunoreaction was seen in mesh pattern (Fig 1C). At 4 weeks, teeth were in the stage of root formation (Fig. 1D), and CD31 (+) and endomucin (+) vessel numbers increased in the root of dental pulp (Fig. 1E, F). The pattern of the immunoreaction was observed in mesh pattern in the odontoblast layer (Fig. 1F). At 12 weeks, which is the stage of root maturation, the CD31 (+) and endomucin (+) vessels were slightly observed, but were reduced at the coronal pulp (Fig. 1G, H). Immunoreaction of CD31(+) was mainly seen in the subodontoblast layer of the dental pulp of the coronal dentin (Fig. 1I). At 56 weeks, which is the senile stage, CD31 (+) and endomucin (+) vessel numbers were low in dental pulp (Fig. 1J, 1K), and CD31(+) or endomucin (+) vessels were solely seen in the coronal pulp (Fig. 1L).

The densities of CD31 (+) and endomucin (+) vessels in dental pulp tissue are shown in Fig. 2. The results obtained showed that the ratios of CD31 (+) and endomucin (+) vessels 1 and 4 weeks after birth were 3.5 and 2.9%, respectively, with a slight decrease being observed. On the other hand, these ratios 12 and 56 weeks after birth were 0.8 and 0.5%, respectively, which were significantly lower than those at 1 and 4 weeks after birth ($P < 0.05$).

Dentin formation and CD31(+) endomucin (+) vessels

Calcein-injected mice were used to investigate the relationship between localization of endomucin (+) vessels and dentin formation. In calcein-injected mice, two lines were observed in dentin, cementum, and alveolar bone with calcein injected one week and one day before sacrifice. The presence of hard tissue in the two lines indicated newly formed hard tissue in the last week. The distance between the two lines reflected the amount of newly formed hard tissue.

At 4 weeks, dentin with a large distance between the two calcein-lines was observed in the crown and root dentin (Fig. 3A). CD31(+) vessels colored in cyan and endomucin (+) vessels colored in red were present in the dental pulp. Both CD31(+) and endomucin(+) vessels colored in white were mainly localized at the dental pulp directly beneath calcein-labeled dentin (Fig. 3B). The distance between the two lines became narrower at 8 weeks than at 4 weeks (Fig. 3C), and the lines were mainly present in root dentin (Fig. 3C). Both CD31(+) and endomucin(+) vessels were located directly in the odontoblast layer in root pulp (Fig. 3D), while CD31(+) vessels were seen in center of the dental pulp. At 12 weeks, calcein-labeled areas were only present in newly formed apical cementum (Fig. 3E, F). Both CD31(+) and endomucin (+) vessels were detected in the periapical tissue, that was adjacent to the apical cementum (Fig. 3F), and CD31(+) vessel was rare in the dental pulp. Localization of CD31(+) and endomucin (+) vessels at 56 weeks were similar to that at 12 weeks (data not shown).

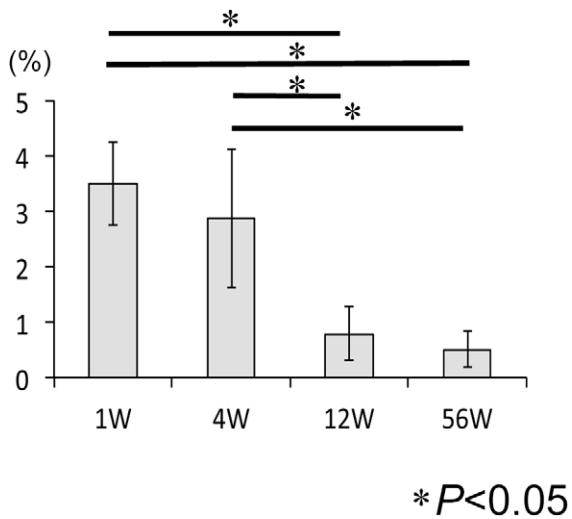

Fig. 2. Ratio of CD31 (+) and endomucin (+) vessels. Data represent means ± standard deviations (SD). Four mice were used for statistical analysis in each group. These ratios 12 and 56 weeks were significantly lower than those 1 and 4 weeks. * Statistical significance ($P < 0.05$)

IV. Discussion

In the present study, we showed that CD31 (+) and endomucin (+) blood vessels were present in dental pulp and that their localization and quantity changed with aging. Previous studies reported the localization of CD31 (+) and endomucin (+) in blood vessels in bone marrow [13]. In recent years, CD31 (+) and endomucin (+) blood vessels have been reported in the dental papilla [1], periodontal ligament and alveolar bone [3, 30], and dental pulp [17]. In bone marrow, age-related changes were observed in the number and location of CD31 (+) and endomucin (+) blood vessels [4]. However, age-related changes in the number and localization of CD31 (+) and endomucin (+) blood vessels have not been demonstrated in dental pulp; therefore, to the best of our knowledge, we are the first to report this result.

Bone formation is active at the epiphyses of long bones, at which there are many CD31 (+) and endomucin (+) blood vessels, and osteoprogenitor cells that express

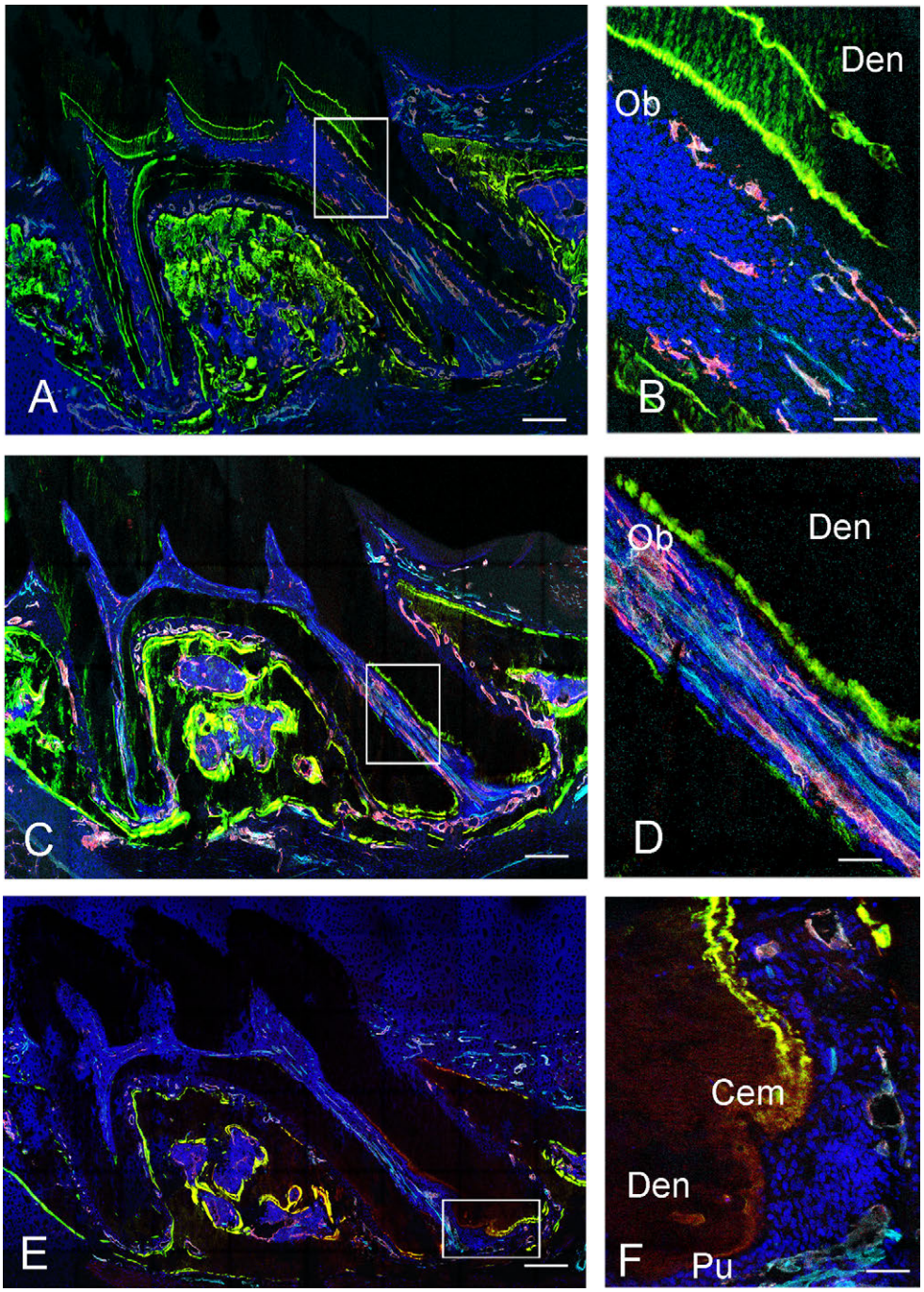

Fig. 3. Calcein labeling and endomucin (+) vessels. (**A**) At 4 weeks after birth. Dentin with a large distance between the two calcein-lines was observed in the crown and root dentin. (**B**) Higher magnification of the boxed regions in **A**. Both CD31(+) and endomucin (+) vessels colored in white were thin and present directly beneath calcein-labeled dentin at all sites. (**C**) At 8 weeks after birth. Although the lines were present in root dentin, they were negligible in coronal dentin. The distance between the two lines at 8 weeks were narrower than that at 4 weeks. Both CD31 and endomucin (+) vessels were scattered in coronal dental pulp. (**D**) Higher magnification of the boxed regions in **C**. Both CD31 and endomucin (+) vessels were located at the odontoblast layer in root pulp. (**E**) At 12 weeks after birth. Calcein-labeled areas were only present in newly formed apical cementum, and were faint or not seen at coronal and root dentin. (**F**) Higher magnification of the boxed regions in **E**. CD31(+) and ndomucin (+) vessels were seen neat the newly formed cementum and faintly detected in dental pulp. Den: dentin, Cem: cementum, Ob: odontoblast layer, Pu: dental pulp. Bars = 200 μm (**A**, **C**, **E**), 30 μm (**B**, **D**, **F**). Green: calcein labeling, Cyan: CD31(+) vessel, Red: endomucin (+) vessel, White: both CD31(+) and endomucin(+) vessel, Blue: nuclear.

osterix are present around blood vessels, indicating a niche for progenitor cells and stem cells [14]. Regarding teeth, a previous study showed that CD31 (+) and endomucin (+) blood vessels were abundant in dental papillae [1], suggesting that CD31 and endomucin are strongly expressed in vascular endothelial cells during the period when hard tissue formation is active. Based on these findings, we hypothesized that many CD31 (+) and endomucin (+) blood vessels are present during active dentin formation and, thus, investigated their localization during crown formation, root formation, and thereafter in this study. The present results showed that CD31 (+) and endomucin (+) blood vessels were abundant at the hard tissue formation site near the odontoblast layer of crown pulp in 1-week-old mice and near the odontoblast layer of root pulp in 4-week-old mice. Furthermore, endomucin (+) blood vessels were seen in the pulp at 4- and 8-week-old mice, that actively produce dentin with calcein labeling. CD31 (+) and endomucin (+) blood vessels were found to gather in the layer directly below odontoblasts [17]. Therefore, the results obtained herein suggest that CD31 (+) and endomucin (+) blood vessels are related to active dentin formation in the dental pulp.

CD31 (+) and endomucin (+) blood vessel numbers significantly decreased in the long bones of aged mice and humans in parallel with age-related declines in bone formation and osteoporosis [29, 32]. This reduction was also detected in aged murine alveolar bone [3, 30]. However, age-related changes in the number of CD31 (+) and endomucin (+) blood vessels in dental pulp remain unclear. In the present study, a large number of CD31 (+) and endomucin (+) blood vessels were observed from 1 week (tooth crown formation stage) to 4 weeks (tooth root formation stage) after birth. Therefore, the decline in the number of CD31 (+) and endomucin (+) blood vessels was attributed to a decrease in hard tissue formation with aging. On the other hand, although the number of these blood vessels decreased at 12 weeks when formation of the tooth root was complete, their number at 56 weeks was the same as that at 12 weeks (data not shown). These results reflect the completion of dentin formation, as shown by the change in the amount of dentin formation labeled with calcein at one week. In the present study, CD31 (+) and endomucin (+) blood vessels were evaluated by immunofluorescence. There are two types of CD31 (+) and endomucin (+) blood vessels: type H blood vessels that strongly express both and type L blood vessels that express low levels of both [3, 15, 20, 29]. The former is involved in hard tissue formation. Although previous studies investigated CD31 (+) and endomucin (+) blood vessels in dental pulp, the difference between type H and type L vessels has not yet been clarified. Since type H and type L vessels are distinguished by a flow cytometric analysis [14, 22], there is a limit to the ability of morphological and immunohistochemical analyses to strictly distinguish between them, as in the present study. The endothelial cells of the venous and capillary express CD31 and endomucin, but those of artery do not express [6]. Furthermore, type L vessels are not related to hard tissue formation [7]. Therefore, thin and narrow CD31 (+) and endomucin (+) vessels at the odontoblast layer beneath the active dentin formation would be type H vessels. Other factors or methods need to be applied in order to confirm that CD31 (+) and endomucin (+) blood vessels are the type H blood vessels in the dental pulp.

In conclusion, we herein demonstrated age-related decreases in CD31 (+) and endomucin (+) blood vessels in dental pulp, suggesting that these blood vessels contribute to dentin formation.

V. Conflicts of Interest

The authors declare that there are no competing interests.

VI. Acknowledgments

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VII. References

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