Immunolocalization of podoplanin/E11/gp38, CD44, and endomucin in the odontoblastic cell layer of murine tooth germs

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ABSTRACT

In this study, we attempted to localize the immunoreactivities of podoplanin/E11/gp38 and CD44, a counterpart possessing a high affinity to podoplanin/E11/gp38, as well as endomucin-immunoreactive blood vessels in the regions of odontoblast layers and the underlying sub-odontoblastic layers in murine tooth germs. Endomucin-reactive small blood vessels were scattered throughout the dental papillae of the tooth germs at postnatal day 1 but came to be localized close to the odontoblast/sub-odontoblastic layers until day 3. After postnatal day 5, small blood vessels were seen in odontoblast cell layers, while blood vessels with relatively larger diameters were seen forming in sub-odontoblastic layers. Immunoreactivities of podoplanin/E11/gp38 and CD44 were not detectable in the cells of dental papillae facing the inner enamel epithelium at postnatal day 1. However, at around postnatal days 3–5, podoplanin/E11/gp38 was localized in the odontoblast layer but not in the sub-odontoblastic layer, whereas CD44 was observed in the sub-odontoblastic layer but not in the odontoblast layer. The exclusive immunolocalization of podoplanin/E11/gp38 and CD44 in the odontoblast layers and sub-odontoblastic layers was seen after postnatal day 3 of the tooth germs, when the mesenchymal cells of dental papillae have already differentiated into mature odontoblasts at the cusp tip. Taken together, it seems likely that endomucin-reactive small blood vessels extended to the podoplanin/E11/gp38-positive odontoblast layer, whereas endomucin-reactive large blood vessels were already present in CD44-immunopositive sub-odontoblastic layer, indicating the cellular regulation on the vascularization of endomucin-reactive endothelial cells during odontogenesis of the tooth germs.

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Secretory odontoblasts are fully differentiated polarized cells containing numerous organelles, such as endoplasmic reticulum and Golgi complex. Odontoblasts synthesize abundant collagen fibrils and non-collagenous proteins, as well as matrix vesicles to form unmineralized predentin that is subsequently mineralized to become mature dentin (29). During
odontogenesis in tooth germs, pre-odontoblasts differentiate from dental papillae beneath the inner enamel epithelium and increase the height of the cell bodies, thus extending their cytoplasmic processes toward the enamel organs. The differentiated odontoblasts move away toward the underlying dental pulp, formerly termed dental papillae, leaving their cytoplasmic processes within the dentinal tubules (20). Such mature odontoblasts are regularly-arranged beneath the dentin, while sub-odontoblastic layers come to be developed close to the pulp side of the mature odontoblasts (7). Meanwhile, the distribution patterns of peripheral blood vessels seem to be associated with the odontoblast layer and sub-odontoblastic layers. Yoshida and Ohshima (27, 41) have described that when dentin deposition begins, capillaries begin to invade into the odontoblast layer and finally settle close to the predentin while continuous capillaries form a coarse vascular network distant from the odontoblast layers, e.g., sub-odontoblastic layers. Therefore, they have suggested that the changes in the peripheral capillaries are closely related to the secretory activity of odontoblasts (27, 35, 41). Thus, the distribution patterns of peripheral blood vessels appear to be related to the cellular activities of mature odontoblasts and cells in sub-odontoblastic layers. In addition, recent studies have suggested the presence of bone-specific blood vessels which strongly showed not only CD31 but also endomucin (14, 30); although it is well known that CD31 is a general hallmark of blood vessels in many tissues, they provided a new insight that endomucin, which had been shown as an endothelial sialomucin closely related to CD34 (22) and a marker of hematopoietic stem cells (21), was intensely positive to blood vessels in bone. However, few studies have examined if tooth germs would include blood vessels immunopositive for endomucin as could be seen in bone (14, 30). Therefore, it might be of interest to clarify if the tooth germs would show endomucin-positive blood vessels.

Odontoblasts have been reported to express podoplanin (1, 11, 34), which has been shown to be identical to the molecules called E11 (39), gp38 (5), OTS-8 (25), and T1a (40) (Although Schwab et al. have originally employed the term of E11 expressed in odontoblasts (34), we here describe this molecule as “podoplanin/E11/gp38” for the sake of better understanding of readers in various research fields). Podoplanin/E11/gp38 is a 38-kDa type I transmembrane glycoprotein (1, 5, 34) and has been demonstrated to play pivotal roles in cell motility (13), cell adhesion (37), elongation of cytoplasmic processes (2) and organogenesis (16, 31, 33). Most of these roles are presumably mediated with the rearrangement of the actin cytoskeleton (17, 19). When odontoblasts are differentiated enough to synthesize the dentin matrix, they may dynamically change the assembly of cytoskeletons that mediate podoplanin/E11/gp38 signaling. Meanwhile, CD44, a hyaluronan receptor of the cell membrane (4), is reportedly expressed in tooth germs, including secretory ameloblasts and the inner enamel epithelium, as well as stratum intermedium (6, 15, 23). CD44 has been postulated to interact with hyaluronan and regulate the vascularization of endothelial cells by affecting the expression of the chemokines CXCL9 and CXCL12 and their receptors (28). CD44 also reportedly interacts with podoplanin/E11/gp38 to promote directional cell migration, suggesting its co-operation with podoplanin/E11/gp38 (18). Ohizumi et al. suggested that the interaction between CD44 and podoplanin/E11/gp38 regulates endothelial cell growth and/or migration in tumor (26).

Thus, the interplay of CD44 and podoplanin/E11/gp38 appears significant for cell activities of odontoblasts, pre-odontoblastic cells, and vascular endothelial cells during the development of tooth germs. Herein, we aimed to examine the immunolocalization of podoplanin/E11/gp38 and CD44, as well as endomucin-positive vascular endothelial cells in the regions of odontoblast layers and sub-odontoblastic layers in the coronal tooth germs, which may provide some clues for better understanding of the cellular interaction between odontoblasts/pre-odontoblasts and vascular endothelial cells.

MATERIALS AND METHODS

Animals and tissue preparation. Postnatal mice at 1, 3, 5 and 7 days, and 2 and 3 weeks after birth (CLEA Japan Co. Ltd, Tokyo) were used in this study (n = 4–6 for each), and the animal experiments were conducted under the Hokkaido University guidelines for animal experimentation (approval No. 15-0041). Mice were euthanized with an intraperitoneal injection of sodium pentobarbital and were immediately extracted heads. The samples were then immersed in 4% paraformaldehyde solution diluted in a 0.067 M phosphate buffer (pH 7.4) for 24 h at 4°C (9, 10). After fixation, the specimens were decalcified with 10% ethylenediamine tetraaetatic disodium salt (EDTA-2Na) solution between 3 day and 3 weeks before paraffin-embedding. Five-micrometer-thick paraffin sections parallel to the longitudinal
Podoplanin, CD44 and endomucin in odontoblast layers

axis of the line of upper molars were made, and all
the paraffin sections subjected to the following im-
nunohistochemistry were photographed under a
Nikon Eclipse E800 microscope (Nikon Instruments
Inc. Tokyo, Japan), and light microscopic images
were acquired with a digital camera (Nikon DX-
M1200C, Nikon).

**Immunolocalization of podoplanin/E11/gp38, endomucin, and CD44 in the developing tooth germs.** Dewaxed paraffin sections were subjected to immu-
nodetection of podoplanin/E11/gp38, endomucin, and
CD44 as previously described (8). The sections were
immersed in PBS containing 0.3% H₂O₂ for 30 min
to block endogenous peroxidase. To reduce non-spe-
cific binding, 1% bovine serum albumin (BSA; Bio-
logicals Proteins Inc. Kankakee, IL) in PBS (1% BSA-PBS) was applied to the sections for 20 min.
Then, they were incubated with polyclonal goat IgG
against mouse podoplanin (R&D systems Inc., Min-
neapolis, MN) at a dilution of 1 : 100 with 1% BSA-
PBS at room temperature (RT) for 2 h. Following
several washings in PBS, they were incubated with
horseradish peroxidase (HRP)-conjugated rabbit anti-
body against endomucin (Santa Cruz Biotechnology,
Inc., San Clemente, CA) for 1 h at a dilution of 1 : 100 or with rat anti-
CD44 monoclonal antibody (BD Biosciences, Systems & Reagents Inc., San Jose,
CA) for 1 h at a dilution of 1 : 100 or with rat anti-
body against endomucin (Santa Cruz Biotechnology,
Inc., Dallas, TX) at a dilution of 1 : 100 overnight
at 4°C. After washing with PBS, these sections were
incubated with HRP-conjugated anti-rat IgG (Zymed
Laboratories Inc., South San Francisco, CA) at a di-
lution of 1 : 100. Immune complexes were visual-
ized using 3, 3′-diaminobenzidine tetrahydrochloride
(Dojindo Laboratories, Kumamoto, Japan).

**RESULTS**

**The distribution of endomucin-immunoreactive blood vessels in odontoblasts/sub-odontoblastic layers of the coronal areas of developing tooth germs.** Endomucin-immunoreactive blood vessels were uni-
formly scattered throughout dental papillae at post-
natal day 1; at this time, tooth germs did not yet show abundant enamel and dentin (Figs. 1A, 2A). Endomucin-reactive small blood vessels reached the region of mesenchymal cells adjacent to the inner enamel epithelium (Fig. 2D). On postnatal day 3, when tooth germs exhibited apparent enamel and
dentin (Fig. 1B), the endomucin-reactive blood ves-
sels tended to be localized in the peripheral end of
dental papillae (dental pulp at this development
stage), i.e., close to or along odontoblasts/sub-odon-
toblastic layers (Fig. 2B). At a higher magnification,
small blood vessels were found to have extended to
the odontoblast layers, while some larger blood ves-
sels appeared, at least in part, to be present in
sub-odontoblastic layers (Fig. 2E). By postnatal days
5–7, abundant enamel and dentin had formed
(Figs. 1C, D), and enlarged endomucin-reactive
blood vessels predominantly existing in sub-odonto-
blastic layers were localized slightly distant from
the odontoblast layers, whereas the smaller blood
vessels were evenly scattered in the odontoblast lay-
ers (Figs. 2C, F, G, J). At postnatal weeks 2 and 3,
when tooth germs were about to erupt or had erupt-
ed (Figs. 1E, F), a tendency of small endomucin-
positive blood vessels forming in the odontoblast
layers and reaching close to the predentin was ob-
served, whereas the enlarged endomucin-reactive
blood vessels were positioned in sub-odontoblastic
layers (Figs. 2H, I, K, L).

**Immunolocalization of podoplanin/E11/gp38 and**
**CD44 in the coronal odontoblasts/sub-odontoblastic layers of developing tooth germs.**

Faint podoplanin/E11/gp38 immunoreactivity was
observed mainly in the inner enamel epithelium at
postnatal day 1 (Fig. 3A). When observing at cervi-
cal loops, podoplanin/E11/gp38 was shown to be lo-
calized in both outer and inner enamel epithelia (See
an inset of Fig. 3A). In contrast, CD44 immunoreac-
tivity was seen in both outer and inner enamel ep-
thelia (Fig. 4A), and intensely observed in dental
follicle but not in dental papillae (See an inset of
Fig. 4A). On postnatal day 3, podoplanin/E11/gp38
was intensely observable in stellate reticulum and in
odontoblasts facing dentin (Fig. 3B). Unlikely, CD44
immunoreactivity was evident in inner enamel ep-
thelium/ameloblasts but not in mature odontoblasts
(Fig. 4B). On postnatal days 5–7, podoplanin/E11/
gp38 immunoreactivity became apparent in odonto-
blast, stratum intermedium and stellate reticulum,
but not in ameloblasts (Figs. 3C, D). CD44 showed
its intense localization in ameloblasts and the sur-
rounding stellate reticulum, but its little localization
in the dental pulp (Figs. 4C, D). After 2 weeks,
podoplanin/E11/gp38 was seen in odontoblasts and
in the region of stratum intermedium (Fig. 3E), and
was still observable in the odontoblast layers even
after tooth eruption at 3 weeks (Fig. 3F). In contrast,
CD44 was seen very faintly in dental pulp, but in-
Fig. 1 Histological aspects of the developing tooth germs. At postnatal day 1, the tooth germ does not show enamel and dentin (Fig. 1A). During postnatal days 3–7, the tooth germs continuously show progressive development of enamel (E) and dentin (D) (Figs. 1B–D). The tooth root begins to be developed at 2 and 3 weeks when the tooth germs are about to erupt or had erupted on the oral cavity (Figs. 1E, F). DP: dental papilla (dental pulp), od: odontoblast, am: ameloblast, sr: stellate reticulum, Bars A–F: 200 μm
Fig. 2 The distribution of endomucin in developing tooth germs. The endomucin-positive small blood vessels (brown color) are scattered in the region of dental papilla (DP) at postnatal day 1 (Figs. 2A, D). After postnatal day 3, the endomucin-positive blood vessels tend to be localized to the dental papilla close to odontoblasts (od)/sub-odontoblastic layers (Figs. 2B, C, G–I). From postnatal days 3–5, the blood vessels located in the odontoblast layers show a small vascular diameter (Figs. 2E, F, J–L), whereas the enlarged endomucin-positive blood vessels (asterisks in Figs. 2F, J–L) exist in sub-odontoblastic layers (arrows in Figs. 2F, J–L). D: dentin, am: ameloblast, sr: stellate reticulum, Bars A–C, G–I: 200 μm; D–F, J–L: 30 μm
Fig. 3  The distribution of podoplanin/E11/gp38 in the developing tooth germs. The immunoreactivity of podoplanin/E11/gp38 (brown color) is faintly observed in the inner enamel epithelium (IEE) and the outer enamel epithelium (OEE) of the tooth germs of days 1 and 3 (See the insets of Figs. 3A, B). On the specimens of postnatal days 3–7, podoplanin/E11/gp38 is intensely observed in odontoblasts (od) facing on dentin, stratum intermedium (si) and stellate reticulum (sr, Figs. 3B–D). After 2 weeks, podoplanin/E11/gp38 is seen in odontoblasts and in the region of stratum intermedium (Fig. 3E), and even after tooth eruption at 3 weeks it was still detectable in the odontoblast layers (Fig. 3F). Note that ameloblasts (am) do not show podoplanin/E11/gp38 immunoreactivity in all the experimental periods (Figs. 3B–D). DP: dental papilla (dental pulp), D: dentin, Bars A–F: 200 μm
Fig. 4 The distribution of CD44 in the developing tooth germs. At postnatal days 1 and 3, there seems an immunoreactivity of CD44 (brown color) in outer enamel epithelia (OEE), inner enamel epithelia (IEE) and dental follicle (DF) (See insets of Figs. 4A and 4B). Note inner enamel epithelium and ameloblasts (am) are positive for CD44 (an inset of Fig. 4B). At around days 5–7, CD44 continuously appears on the ameloblasts and stellate reticulum (sr) (Figs. 4C, D). Note little immunoreactivity of CD44 in dental pulp (DP). At 2 weeks, CD44 is shown to be weakly localized in dental pulp, but intensely observed in the region of stratum intermedium (si) until tooth eruption at postnatal week 3 (Fig. 4E). After tooth eruption, CD44 immunoreactivity is detectable in the periodontal ligament (PDL) (Fig. 4F). od: odontoblast, DP: dental papilla, D: dentin

Bars: A–F: 200 μm
tensely observed in the region of stratum intermedium at 2 weeks (Fig. 4E). At 3 weeks, CD44 was observed mainly in periodontal ligaments after tooth eruption when the enamel organs including ameloblasts and stratum intermedium have been peeled out (Fig. 4F).

Comparative localization of podoplanin/E11/gp38 and CD44 in odontoblasts/sub-odontoblastic layers during tooth development

Next, we compared the immunolocalization of podoplanin/E11/gp38 and CD44 in the regions of odontoblasts and the underlying sub-odontoblastic layers at a higher magnification (Fig. 5). At postnatal day 1, the inner enamel epithelium showed very weak podoplanin/E11/gp38 immunoreactivity and relatively intense CD44 immunopositivity, whereas the adjacent mesenchymal cells of dental papillae did not show immunolocalization of both podoplanin/E11/gp38 and CD44 (Figs. 5A, B). At postnatal day 3, however, the podoplanin/E11/gp38 immunoreactivity was relatively intense along the cell membranes, but faint in the cytoplasmic regions of mature odontoblasts (Fig. 5C). In contrast to podoplanin/E11/gp38, the mature odontoblasts did not reveal CD44, while the sub-odontoblastic layers showed weak and diffusible immunolocalization of CD44 (Fig. 5D). At postnatal day 5, consistent with postnatal day 3, mature odontoblasts featuring a typical cylindrical cell shape demonstrated a strong immunoreactivity of podoplanin/E11/gp38 mainly on the cell membranes, though they did not demonstrate CD44 (Fig. 5E). Conversely, the sub-odontoblastic layers displayed CD44 but not podoplanin/E11/gp38. At this developmental stage, enlarged blood vessels were seen in the CD44-immunoreactive sub-odontoblastic layers (Figs. 5E, F), which is consistent with the observation of endomucin-reactive blood vessels (Compare with Fig. 2F). At postnatal day 7, podoplanin/E11/gp38 reactivity was observed prominently in the cell membranes of odontoblasts and weakly in the cytoplasmic regions of mature odontoblasts, whereas CD44 was shown to be diffusible in sub-odontoblastic layers (Figs. 5G, H). Large blood vessels were still present in the region of sub-odontoblastic layers.

DISCUSSION

To our knowledge, this is the first report that blood vessels in developing tooth germs exhibit intense endomucin immunoreactivity (21), which has been documented to a hallmark of bone-specific blood vessels, being co-expressed with intense CD31 (14). In addition, the distribution patterns of small and large blood vessels were restrictedly related to the odontoblast layers and sub-odontoblastic layers, respectively. In this study, our findings suggest that the size and distribution patterns of endomucin-positive blood vessels are regulated with cell-surfaced molecules expressed in odontoblasts and the cells of sub-odontoblastic layers. Therefore, we believe that this study provides a new insight on the spatial relation of the endomucin-reactive blood vessels against podoplanin/E11/gp38-positive odontoblasts and CD44-reactive sub-odontoblastic layers. However, our preliminary study showed endomucin reactive blood vessels in other organs such as liver and kidney (unpublished data), therefore further examination on the biological function of endomucin in tooth germs appears to be necessary.

Consistent with the previous reports (30, 32, 36, 42), we have successfully localized podoplanin immunoreactivity in odontoblasts as shown in Figs. 3 and 5. The previous reports have demonstrated the presence of CD44 in human mature odontoblasts (6, 15), whereas our immunohistochemistry showed no CD44 in odontoblast layers but its intense presence in the inner enamel epithelium/ameloblasts, stratum intermedium, and outer enamel epithelium, which is in agreement with the report by Nakamura and Ozawa (23). This discrepancy may be due to interspecies differences between human and mouse. Our observation suggests that podoplanin/E11/gp38 and CD44 interact reciprocally at the interface between odontoblasts and cells of sub-odontoblastic layers. When mesenchymal cells of the coronal dental papillae differentiate into podoplanin/E11/gp38-reactive odontoblasts at postnatal day 3, sub-odontoblastic layers appear to express CD44. The extracellular domain of podoplanin/E11/gp38 can reportedly bind to CD44 (18), and thus, it may, at least in part, influence odontoblastic activities, such as cell polarity, adhesion, and elongation of cytoplasmic processes (2, 13, 31, 33, 37). Among a variety of interactions between odontoblasts and the surrounding sub-odontoblastic layers, podoplanin/E11/gp38 and CD44 may be strong candidates that regulate the cellular activities of odontoblasts and sub-odontoblastic layers.

It would be interesting to know if the interplay between CD44 and podoplanin/E11/gp38 is associated with the distribution and size of endomucin-immunoreactive blood vessels in odontoblasts/sub-odontoblastic layers. Nandi et al. reported that CD44 anchors hyaluronan to endothelial cell surfaces and that the activation of CD44 is a major regulator of hyaluronan expression on the endothelial
Fig. 5 The comparative localization of podoplanin/E11/gp38 and CD44 immunoreactivity in odontoblasts and sub-odontoblastic layers at postnatal days 1, 3, 5 and 7. At a higher magnification, the inner enamel epithelium (IEE) shows faint podoplanin/E11/gp38 (light brown color in Fig. 5A) and relatively intense CD44 reactivity (brown color in Fig. 5B) at postnatal day 1. Notice an intense reactivity of CD44 in the dental follicles as well (DF in Fig. 5B). At this stage, the mesenchymal cells of dental papillae (DP) (asterisks in Figs. 5A, B) close to the inner enamel epithelium do not show either podoplanin/E11/gp38 or CD44. At postnatal days 3 and 5, mature odontoblasts (od, arrows) reveal an intense podoplanin/E11/gp38 reactivity but not CD44. Unlike ly, sub-odontoblastic layers show weak and diffusible CD44 but no podoplanin/E11/gp38 (asterisks in Figs. 5C–F). Note that the podoplanin/E11/gp38 immunoreactivity is seen mainly along the cell membranes of odontoblasts, rather than in the cytoplasmic region. At postnatal day 7, podoplanin/E11/gp38 is seen intensely in odontoblasts and very weakly in sub-odontoblastic layers (asterisks in Fig. 5G). In contrast, the distribution pattern of CD44 seems to be diffusible as seen in Fig. 5D and F (Fig. 5H). Note that large blood vessels (BV) are located in the region of sub-odontoblastic layers at postnatal days 5 and 7 (Figs. 5E–H). OEE: outer enamel epithelium, Bars: A–H: 30 μm
surface. Further, they reported that the non-covalent interaction between CD44 and hyaluronan is sufficient to provide resistance to shear under physiological conditions (24). Endomucin is a mucin-like sialoglycoprotein that interferes with the assembly of focal adhesion complexes and inhibits the interaction between cells and the extracellular matrix (12). Therefore, it is assumed that endomucin interacts with CD44 or some growth factors/local factors trapped within the complex of CD44/hyaluronan. However, unlike CD44, podoplanin-Fc, a fusion protein comprising the extracellular portion of human podoplanin linked to the Fc region of human IgG1, reduced lymphatic vessel formation in vitro and in vivo (3). Therefore, the podoplanin/E11/gp38 expressed in endodermoblasts possibly inhibits the vascularization of endomucin-reactive blood vessels. In addition, podoplanin/E11/gp38-mediated platelet activation is critically involved in the separation of blood and lymphatic vessels (38), and therefore, it is possible that when large blood vessels in the sub-odontoblastic layer extend to the podoplanin/E11/gp38-reactive odontoblast layers, the large blood vessels branch off into smaller vessels to invade into the odontoblast layers.

However, further examination is necessary to elucidate the cellular mechanism involved in the distribution of endomucin-reactive blood vessels and the interaction between podoplanin/E11/gp38 and CD44 during tooth germ development.

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DISCLOSURES

The authors have no financial conflicts of interest to declare.

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