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Immunolocalization of Calbindin D28k and Vitamin D Receptor During Root Formation of Murine Molar Teeth

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ABSTRACT

Cells in the epithelial rest of Malassez (ERM cells) express calbindin D28k (CB); however, the hormonal regulation of CB in ERM cells remains to be elucidated. We investigated the immunohistochemical localization of CB and 1,25-dihydroxyvitamin D3 receptor (VDR) during root formation of mouse molar teeth in order to clarify whether the expression of CB in ERM cells is dependent on vitamin D. At the early stage of root formation (postnatal (PN) days 10–14), both CB- and VDR-immunoreactive cells were observed intermittently along the root surface. In the apical portion, almost all CB-immunoreactive cells showed VDR immunoreactivity; however, VDR-immunoreactive cells in the most apical portion were immunonegative for CB. In the middle and cervical portions, the distributions of the two proteins were completely different. At the late stage of root formation (PN28d) and in adult animals, CB immunoreactivity was distributed in cells found along the acellular cementum at the bifurcation region, as well as between the dentin and cellular cementum in the apical portion (although these lacked immunoreactivity for VDR). The present results indicate that CB expression in newly disrupted cells from Hertwig's epithelial root sheath occurs in a vitamin-D dependent manner, whereas the expression of CB in mature ERM cells may be independent of vitamin D. Anat Rec Part A 273A:700–704, 2003.

Key words: calbindin D28k; epithelial rest of Malassez; immunohistochemistry; vitamin D receptor

Calbindin D28k (CB), an intracellular soluble vitamin D-dependent calcium-binding protein, is a member of a family of proteins with a high affinity for Ca^{2+} (Andressen et al., 1993). It was initially detected in chick intestines (Wasserman and Taylor, 1966) and has also been found in several mammalian tissues, including kidney (Roth et al., 1982; Taylor et al., 1982; Schreiner et al., 1983), nervous system (Baimbridge et al., 1982; Sans et al., 1986), and cartilage (Balmain et al., 1986). Using oral tissue specimens, many studies have demonstrated the presence of CB in ameloblasts and cells of the enamel-free area during tooth formation in rats (Taylor, 1984; Berdal et al., 1989, 1991, 1993, 1996; Hotton et al., 1995; Onishi et al., 1999, 2000a, b), as well as in odontoblasts (Berdal et al., 1993, 1996; Onishi et al., 1999). Further, cells in Hertwig's epithelial root sheath (HERS) lack CB immunoreaction, whereas fragmented cells from HERS express CB during root formation (Onishi et al., 1999). In adult rats, fibroblasts in the periodontal ligament and cells of the epithelial rest of Malassez (ERM cells) show CB immunoreactivity (Onishi et al., 1999). In calcium-transporting tissues, such as intestine, kidney, and ameloblasts, CB is involved in transcellular calcium transport (Christakos et

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al., 1989; Hubbard, 1995, 1996). In addition, CB is also considered to have cytoprotective effects in non-calcium-transporting tissues, such as neurons (Andressen et al., 1993). In a previous study, we proposed that CB may play an important role in the survival of ERM cells, because ERM cells remain in an inactive state for years (Onishi et al., 1999). It has also been suggested that CB plays a role in the responses of periodontal fibroblasts against mechanical forces caused by the occlusion (Onishi et al., 2000b).

The dependency of CB expression on vitamin D differs among organs, as CB is expressed in a vitamin D-dependent manner in intestines and kidneys (Varghese et al., 1988), whereas in neurons, its expression is unresponsive to vitamin D (Varghese et al., 1988; de Viragh et al., 1989). Berdal et al. (1989) revealed that ameloblasts from vitamin D-deficient rats lack CB immunoreactivity. A single injection of $1,25(OH)_2$ D_3 into vitamin D-deficient rats resulted in an increase of CB mRNA in ameloblasts and odontoblasts from rat incisors (Berdal et al., 1993). Moreover, immunoreactivity for 1,25-dihydroxyvitamin D3 receptor (VDR) is present in all progenitor cells in rat incisors, and progressively decreases during the differentiation process (Berdal et al., 1993). This line of evidence indicates that the expression of CB in ameloblasts and odontoblasts may be regulated by vitamin D. Although ERM cells are known to express CB immunoreactivity, the hormonal regulation of CB in ERM cells remains to be elucidated. Furthermore, since ERM cells are non-calcium-transporting cells, the dependency of vitamin D by ERM cells for expressing CB may be different from calcium-transporting cells such as ameloblasts and odontoblasts. In the present study, we analyzed the distribution of CB and VDR immunoreactivity during root formation in order to clarify whether the expression of CB in ERM cells is dependent on vitamin D.

MATERIALS AND METHODS

All of the animal experiments were reviewed and approved by the Osaka University Graduate School of Dentistry Intramural Animal Use and Care Committee prior to the study.

Animals and Tissue Preparation

ICR mice (10, 14, and 28 postnatal (PN) days old) and adults (10–15 weeks old) were purchased from CLEA Japan (Tokyo, Japan). The animals were deeply anesthetized with chloral hydrate (500 mg/kg b.w., intraperitoneally) and perfused transcardically with 0.02 M phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The maxilla were dissected out, postfixed in 4% paraformaldehyde in 0.1 M PB at 4°C for an additional 2–3 days, and then decalcified with 7.5% ethylene diaminetetraacetic acid (EDTA) for 7–14 days at 4°C under gentle agitation. After decalcification was completed, the specimens were embedded in OCT compound, sectioned at a thickness of 14 μm with a cryostat, and mounted onto aminopropylsilane-subbed glass slides.

Immunohistochemistry

The specimens were incubated with PBS containing 3% normal swine serum (NSS; Dako, Glostrup, Denmark) and 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) for 30 min, and then some sections were incubated with monoclonal rat anti-VDR antiserum (1:500; Chemicon, Temecula, CA) overnight at room temperature. After the sections were rinsed in PBS, they were incubated with biotinylated anti-rat IgG (1:500; Vector, Burlingame, CA) for 90 min at room temperature. The other sections were incubated with polyclonal rabbit anti-CB antiserum (1:20,000; SWant, Bellinzona, Switzerland) and then with biotinylated anti-rabbit IgG (1:500; Dako). These sections were then incubated with avidin-biotin complex (Vector) for 90 min at room temperature. Horseradish peroxidase (HRP) activity was visualized by incubation with 0.05 M Tris-HCl buffer (pH 7.5) containing 0.04% 3,3′-diaminobenzidine (DAB) and 0.003% H_2O_2. Immunohistochemical controls were performed by replacing the primary antibody with non-immune serum. The specificity of the primary antibody for CB had been determined by preabsorption in a previous study (Ochi et al., 1997).

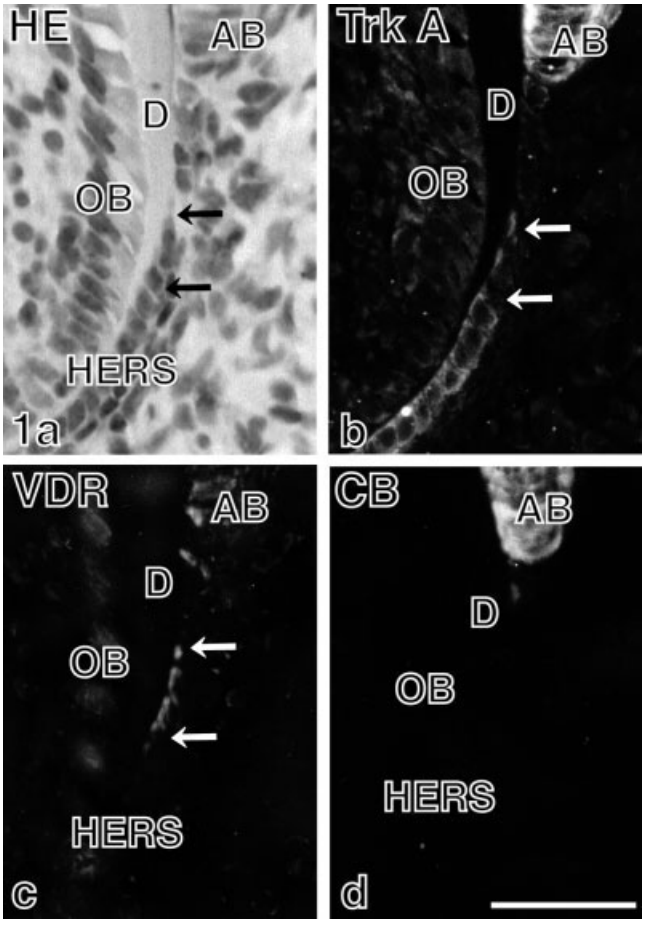

Fig. 1. Photomicrographs of the mesial root of the upper first molar in a PN10d mouse. **a:** HE staining. HERS has already begun to fragment (as indicated between the two arrows). **b:** Cell membranes of ameloblasts (AB), cells of HERS, and the disrupted cells from HERS (as indicated between the two arrows) exhibit Trk-A immunoreactivity. **c:** Ameloblasts, odontoblasts (OB), and newly disrupted cells from HERS show VDR immunoreactivity. **d:** Ameloblasts express strong immunoreactivity for CB. Odontoblasts and cells of HERS lack immunoreaction. Images a–c were taken from the same section, and d was taken from the section adjacent to that shown in a–c. D, dentin. Scale bar = (a–d) 50 μm.

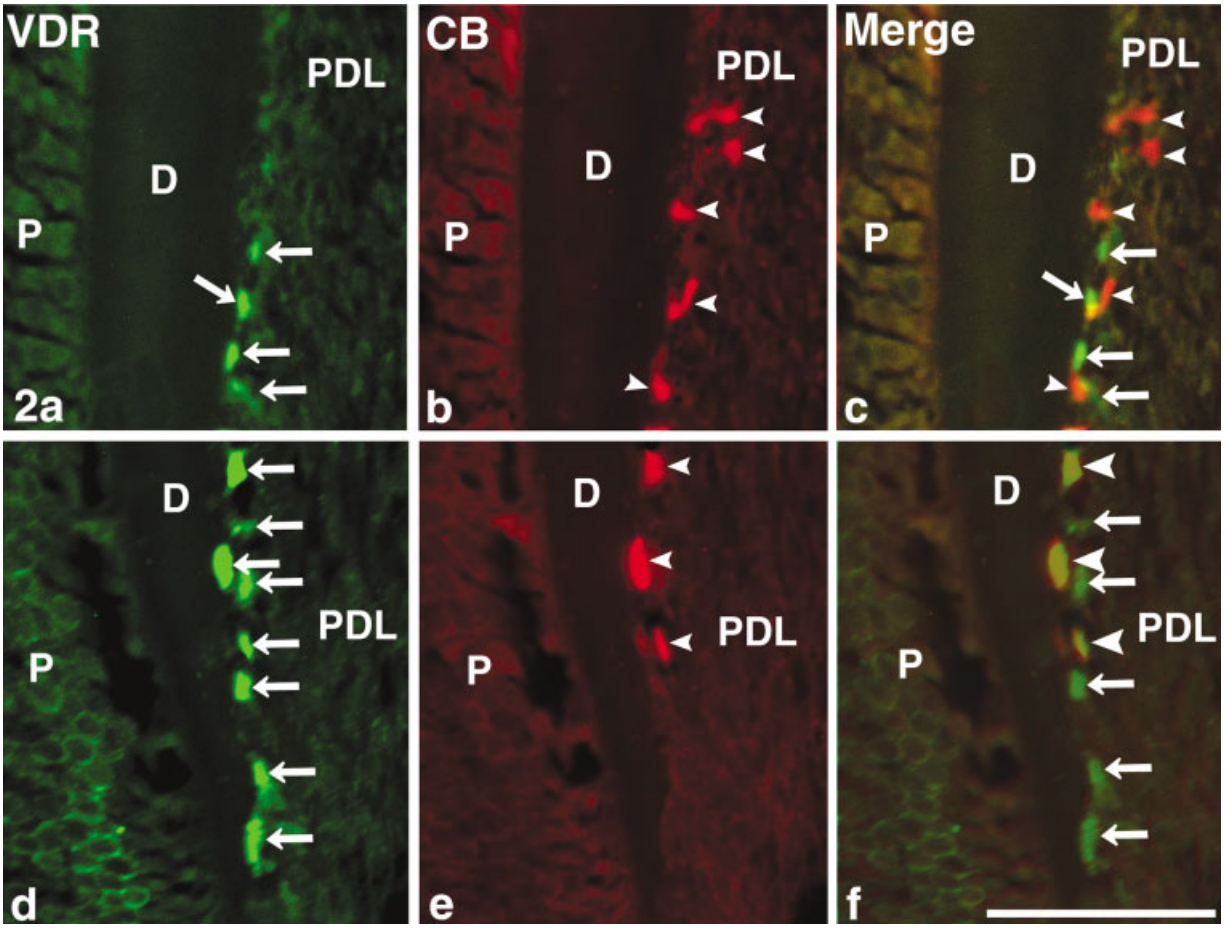

Fig. 2. Photomicrographs of the mesial root of the upper first molar in a PN14d mouse. **a:** In the middle and cervical portions, VDR-immunoreactive cells are intermittently localized along the root surface (arrows). **b:** CB-immunoreactive cells are also intermittently observed along the root surface (small arrowheads). **c:** Merged image of a and b. The distribution of the immunoreactions for CB along the acellular cementum is completely different from that of VDR. **d:** In the apical portion, VDR-immunoreactive cells are seen intermittently along the root surface (arrows). **e:** CB-immunoreactive cells occur intermittently along the root surface (small arrowheads). **f:** Merged image of d and e. CB-immunoreactive cells express VDR immunoreactivity (large arrowheads). VDR-immunoreactive cells in the most apical portion are immunonegative for CB (arrows). The photographs in each row were taken from the same section. D, dentin; P, pulp; PDL, periodontal ligament. Scale bar: (a–f) 50 μm.

To examine the correlation of distributions between VDR and CB, or VDR and Trk A, which is a protein marker for epithelial cells (Yamashiro et al., 2000), a double-immunofluorescence method was applied to the other sections. The sections were incubated with a mixture of polyclonal rabbit anti-CB antiserum (1:5,000) and monoclonal rat anti-VDR anti serum (1:100), or polyclonal rabbit anti-Trk A antiserum (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) and monoclonal rat anti-VDR antiserum (1:100) overnight at room temperature. After the sections were rinsed in PBS, they were labeled with Cy3-conjugated anti-rabbit IgG (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) and then fluorescein isothiocyanate (FITC)-conjugated anti-rat IgG (1:500), each for 90 min. They were coverslipped with PermaFluor (Shandon, Pittsburgh, PA) and examined with a Carl Zeiss fluorescence microscope (Carl Zeiss, Hallbergmoos, Germany). The images were captured by a CCD camera (Axio Cam) and processed in Adobe Photoshop. After observation, the coverslips were carefully removed, and then stained with hematoxylin and eosin for general histological observation.

RESULTS

In PN10d mice, root formation was found to be initiated and HERS had already begun to fragment (Fig. 1a). Trk A immunoreactivity was observed in the cytoplasm of ameloblasts and the cells of HERS, as well as in some disrupted cells from HERS (Fig. 1b). VDR immunoreactivity was found in some ameloblasts, odontoblasts, and cells that had just disrupted from HERS (Fig. 1c). Although strong immunoreactivity for CB was detected in the ameloblasts, the cells of HERS and disrupted cells from HERS were immunonegative for CB at this stage (Fig. 1d).

In PN14d mice, the first molar teeth were erupting and root formation was advanced. Both CB- and VDR-immunoreactive cells were intermittently observed along the root surface (Fig. 2). In the middle and cervical portions,

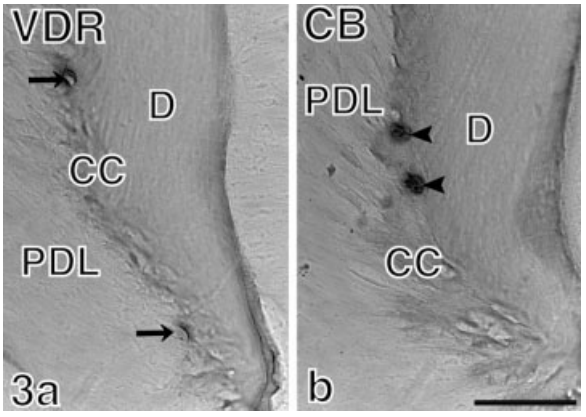

Fig. 3. Photomicrographs of the upper first molar in a PN28d rat. **a:** In the apical portion of the root, VDR immunoreactivity is observed in the cells found along the cellular cementum (CC) (arrows). **b:** CB immunoreactivity is detected in the cells between the dentin (D) and cellular cementum (arrowheads). PDL, periodontal ligament. Scale bar: (a and b) 100 μm.

the distribution of immunoreaction for CB along the acellular cementum was completely different from that of VDR (Fig. 2a–c). In the apical portion, almost all CB-immunoreactive cells showed VDR immunoreaction; however, VDR-immunoreactive cells in the most apical portion were immunonegative for CB (Fig. 2d–f).

In PN28d mice, VDR immunoreactivity was observed in the distal portion in cells along the cellular cementum (Fig. 3a), whereas CB immunoreactivity was observed in cells between the dentin and cellular cementum in the apical portion of the root (Fig. 3b). In the middle and cervical portions, neither CB nor VDRimmunoreactivity was observed (data not shown). In adult animals, no VDR-immunoreactive cells were found in the root and periodontal tissues (Fig. 4a, c, and e). However, cell clusters in the cervical and bifurcation portions, cells between the dentin and cellular cementum in the apical portion of the root, and some fibroblasts in the periodontal ligament exhibited CB immunoreactivity (Fig. 4b, d, and f).

The control sections did not show any specific immunoreactions (data not shown).

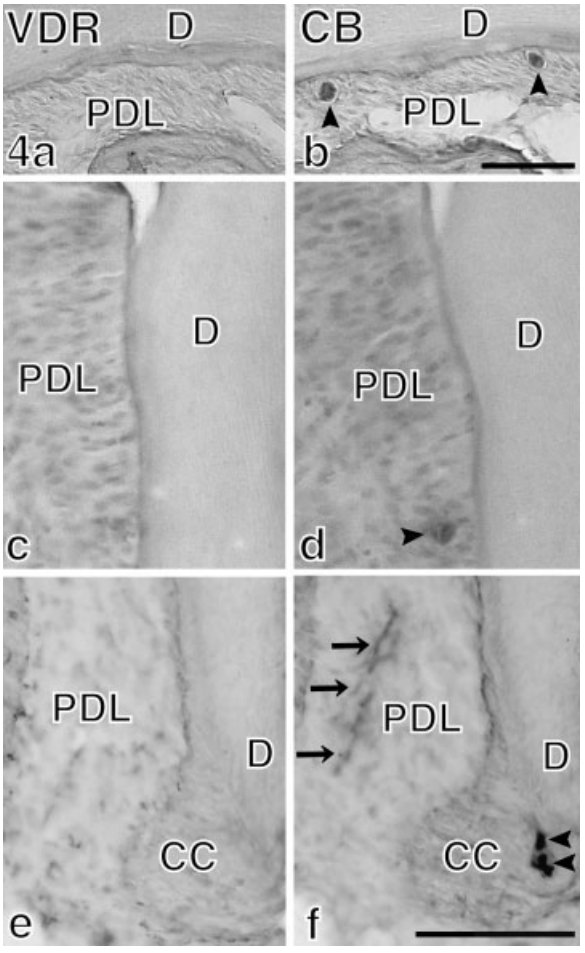

Fig. 4. Photomicrographs of the upper first molar from an adult animal. **a:** In the bifurcation region of the root, no VDR immunoreactivity is observed. **b:** Some cell clusters can be seen displaying CB immunoreactivity (arrowheads). **c:** In the cervical portion, no VDR immunoreactivity is found. **d:** CB-immunoreactive cells (arrowhead) are observed in the cervical region. **e:** In the apical portion of the root, no VDR-immunoreactive cells are observed. **f:** CB immunoreactivity is shown in the cells between the dentin (D) and cellular cementum (CC) (arrowheads), and in fibroblasts in the periodontal ligament (PDL) (arrows). Scale bar: (a and b) 100 μm, and (c–f) 50 μm.

DISCUSSION

We demonstrated that some disrupted cells from HERS in the apical portion of the molar roots showed both CB and VDR immunoreactivity. In contrast, during the more advanced stage of root development and in adults, CB-immunoreactive cells along the root surface did not show immunoreactivity for VDR. These findings suggest that CB expression in newly disrupted cells from HERS may be dependent on vitamin D at the initial stage of root formation, whereas mature ERM cells may express CB independently of vitamin D.

In calcium-transporting tissues, such as intestine, kidney, and ameloblasts, the expression of CB is regulated by vitamin D (Varghese et al., 1988; Berdal et al., 1993). It has been suggested that CB ferries and/or buffers calcium ions in these tissues (Christakos et al., 1989). Amelin mRNA has been detected in cells of HERS (Fong et al., 1996), and it has also been shown that ERM cells have the ability to secrete enamel proteins, such as amelogenin (Hamamoto et al., 1996). Hamamoto et al. (1996) speculated that newly disrupted cells from HERS may be involved in hard tissue formation. The present results on the colocalization of CB and VDR in immature ERM cells supports their hypothesis. However, in neurons CB is expressed independently of vitamin D (Varghese et al., 1988; de Viragh et al., 1989), and it has been proposed that CB has cytoprotective effects in some neurons (Freund et al., 1990; Mattson et al., 1991, 1995). ERM cells are known to remain in an inactive stage for years, and CB-immunoreactive ERM cells are characterized by poor organelles (Onishi et al., 1999). Thus, it is speculated that CB in mature ERM cells may be closely related to the prevention of cell death. The cells between the dentin and the cellular cementum are also derived from HERS and have poor organelles, similar to ERM cells (Onishi et al., 1999),

which suggests that CB may play a role in the cytoprotection of these cells.

In the present study, VDR-immunoreactive cells were observed along the acellular and cellular cementum during root formation. The nature of these VDR-immunoreactive cells is unknown; however, they were shown to be immunonegative for CB. Since ERM cells display CB immunoreactivity (Onishi et al., 1999), these VDR-immunoreactive cells are not ERM cells. It is known that VDR is expressed in cells directly involved in mineralized tissue formation, such as osteoblasts, ameloblasts, and odontoblasts (Bailleul-Forestier et al., 1996; Davideau et al., 1996), and that vitamin D has a role in enamel and dentin mineralization as well as in cytodifferentiation (Berdal et al., 1987). Hence, we speculated that the VDR-immunoreactive cells found along the cementum during root development were cementoblasts; however, further analysis is required on this point.

In conclusion, VDR immunoreaction was observed in disrupted cells from HERS during the initial stage of root development, and perhaps in cementoblasts at the late stage of root development. Further, CB-immunoreactive ERM cells were distributed along the root surface during root formation. At the initial stage, CB-immunoreactive cells showed VDR immunoreactivity; however, CB-immunoreactive cells were immunonegative for VDR at the more advanced stages of development. Hence, ERM cells may be altered in their vitamin D dependence in expressing CB, in that CB expression in newly disrupted cells from HERS may occur in a vitamin D-dependent manner, whereas mature ERM cells may express CB independently of vitamin D.

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