

Immunocytochemical characterization of ectopic enamel deposits and cementicles in human teeth

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Despite the relative frequency and clinical relevance of radicular enamel deposits and cementicles, their etiology and nature are unknown. The purpose of the present study was therefore to evaluate the presence and distribution of mineralization-associated non-collagenous matrix proteins (NCPs) in various types of root-associated ectopic mineralizations. Human teeth were processed for embedding in epoxy or acrylic resins. Tissue sections were incubated with antibodies to amelogenins (AMEL), bone sialoprotein (BSP), and osteopontin (OPN). Radicular enamel deposits contained residual organic matrix that labeled for AMEL. In contrast, BSP and OPN were not detected in the residual enamel matrix, they were found in the cementum deposited on its surface as well as in collagen-free cementicle-like structures in the adjacent periodontal ligament. True cementicles consisted of a collagenous matrix intermixed with a non-collagenous ground substance. Labeling for BSP and OPN was mainly associated with the interfibrillar ground substance. No immunoreactivity for AMEL was detected in cementicles. These data indicate that ectopic enamel deposits on the root retain a high amount of AMEL, whereas cementicles contain BSP and OPN, two NCPs typically found in bone and cementum. These NCPs may, like in their normal tissue counterparts, play a role in the mineralization process.

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Ectopic enamel deposits on the root and cementicles occur frequently enough to be of clinical importance (1, 2). The enamel deposits take the form of projections or isolated drops or pearls, and exhibit a more irregular structure than crown enamel (2–6). Cementicles are mineralized structures that either freely reside in the periodontal ligament or adhere to the root surface (7). Enamel projections or pearls have been associated with periodontal destruction (2, 6, 8–14). The presence of cementicles and ectopic enamel deposits on the root may compromise plaque and calculus removal (9–11). Most studies have therefore concentrated on their prevalence and distribution in human teeth (2).

Despite the clinical implications, little information is available on the etiology and development of cementicles and ectopic enamel. A localized ameloblast-like activity of portions of Hertwig's epithelial root sheath (HERS) that remain adherent to the dentin surface is believed to be responsible for ectopic enamel formation. The characterization of the cells that initially are involved in cementicle formation is poor. Epithelial rests of Malassez have been implicated in the formation of cementicles (7). Conversely, the close structural resemblance of cementicles with root cementum suggests an involvement of cementoblasts in the formation of cementicles. Although ectopic enamel deposits and cementicles morphologically resemble their normal tissue counterparts, their biochemical compositions are entirely unknown. Non-collagenous matrix

proteins (NCPs) involved in the formation of crown enamel and root cementum include amelogenins (AMEL), other enamel-related matrix proteins (EMPs) (15), bone sialoprotein (BSP), and osteopontin (OPN), respectively (16, 17). These NCPs have been proposed to function in cell attachment, cell differentiation, and regulation of mineralization.

The purpose of the present study was to characterize the nature and to evaluate the fine distribution of typical enamel- and cementum-related NCPs in ectopic enamel deposits on the root compared with cementicles using colloidal gold immunocytochemistry.

Material and methods

Tissue sampling and processing

A large collection of human teeth provided 23 tissue samples demonstrating enamel deposits or cementicles on the root surface. The teeth had been extracted for orthodontic or other reasons and were processed for histological and immunocytochemical analyses. Immediately following extraction, teeth were fixed for 24 h at 4°C in 1% glutaraldehyde and 1% formaldehyde, buffered with 0.08 M sodium cacodylate (pH 7.3). After washing twice in 0.1 M sodium cacodylate containing 5% sucrose and 0.05% CaCl₂, pH 7.3, the teeth were decalcified in 4.13% ethylenediaminetetraacetic acid (EDTA) for 6 wk (18) at 4°C, and washed extensively again in the wash-buffer. The mesial and distal surfaces of the partially

decalcified roots were subdivided in a corono-apical direction into numerous thin segments as described elsewhere (19). Some tissue samples were then postfixed with potassium ferrocyanide-reduced osmium tetroxide (20) and processed for embedding in Taab 812 epoxy resin (Marivac, Halifax, Nova Scotia, Canada). Osmicated and non osmicated tooth samples were also processed for embedding in LR White resin (Mecalab, Montreal, Quebec, Canada).

Light- and transmission electron microscopy

Semithin survey sections (1 μm thick) were cut with glass or diamond knives on a Reichert Ultracut E microtome (Reichert-Jung, Optische Werke, Wien, Austria), stained with toluidine blue and observed by light microscopy. For transmission electron microscopy, selected areas were trimmed, cut (80–100 nm thick) with a diamond knife, mounted on formvar- and carbon-coated nickel grids, and contrasted with uranyl acetate and lead citrate. Examination of the sections was performed in a JEOL TEM 2000FX-II transmission electron microscope (JEOL, Tokyo, Japan) operated at an accelerating voltage of 80 kV.

Immunocytochemistry

The high-resolution protein A-gold technique (21) was used for the immunocytochemical localization of AMEL, BSP, and OPN. All incubations were performed at room temperature. Thin sections of LR White-embedded tissues were mounted on formvar- and carbon-coated nickel grids. Osmicated tissue sections were first treated with a saturated solution of sodium metaperiodate (22) for 15 min and washed with distilled water. They were then floated on a drop of 0.01 M phosphate buffered saline (PBS), pH 7.3, containing 1% ovalbumin (Sigma, St Louis, MO, USA) in order to saturate non-specific binding sites. Sections were transferred and incubated for 1 h on a drop of one of the following antibodies: rabbit antihuman bone sialoprotein (LF-6), rabbit antihuman osteopontin (LF-7), each diluted 1 : 20 with PBS (courtesy of Dr L. W. Fisher, National Institutes of Health, Bethesda, MD, USA) (23), sheep antiporcine Bio-Gel peak C affinity purified amelogenin diluted 1 : 100 with PBS (courtesy of Dr H. Limeback, Faculty of Dentistry, University of Toronto, ON, Canada) (24), or egg yolk chicken antirat 24 kDa amelogenin diluted 1 : 150 with PBS (25). For the immunodetection of amelogenins, sections were incubated for 1 h on a drop of the corresponding polyclonal rabbit antish sheep or antichick IgGs (Cappel, Scarborough, ON, Canada). Following incubations with primary or secondary antibodies, the grids were rinsed with PBS, floated on PBS–1% ovalbumin for 10 min, and incubated for 30 min with protein A-gold complex prepared with gold particles of approximately 8 nm or 12 nm (26). As controls, sections were incubated with protein A-gold alone, non-immune serum, or unrelated anti-IgG antibodies. The grids were washed with PBS and distilled water, and contrasted as described above for transmission electron microscopy analysis.

Results

Light microscopy

All enamel deposits observed were exclusively found in cervical or furcational root regions. They varied in

thickness and shape from very flat to ovoid. Residual organic matrix was detected in larger enamel deposits (Fig. 1A,B) and a structural organization was frequently observed (Fig. 1B). In the periodontal ligament and directly adjacent to the ectopic enamel deposits, conspicuous round matrix structures were consistently observed (Fig. 1A,B). Two types of such structures were identified. The first type was characterized by cementicle-like bodies that stained intensely with toluidine blue and revealed a concentric lamellation at their periphery and a light-staining core. The second type was more numerous and was characterized by a lighter staining and a less prominent concentric lamellation. Their morphological appearances varied greatly. They appeared to fuse with one another and/or with the ectopic enamel surface. Some of them had an intensely stained surface layer, while others had not. True cementicles were observed in teeth lacking signs of enamel matrix deposition on the root (Fig. 1C,D). They were either freely dispersed in the periodontal ligament or partly or totally embedded in the cementum matrix.

Ultrastructure and immunocytochemistry

In ectopic enamel deposits rich in organic matrix, alternating layers containing variable amounts of organic matrix (Fig. 2A) and areas displaying a radial orientation (Fig. 2B) were observed. Epithelial and/or mesenchymal cells covered the surface of the ectopic enamel deposits. All ectopic enamel deposits were immunoreactive for AMEL (Fig. 3A). Immunolabeling for AMEL was often found in association with dentinal tubules in the peripheral root dentin subjacent to the enamel deposits (Fig. 3B). A collagen-free matrix layer resembling acellular afibrillar cementum frequently covered the enamel deposits on the root. This layer was continuous with the acellular extrinsic fiber cementum laid down more coronally and directly onto the dentin (Fig. 1A) and also labeled for BSP (Fig. 3C) and OPN (Fig. 3D).

The cementicle-like structures in the periodontal ligament adjacent to the enamel deposits that were designated as the first type revealed concentrically arranged lamellae with alternating layers varying in electron-density at their periphery and an electron-lucent core (Fig. 4A). An apparently homogeneous gold labeling for BSP (Fig. 4B) and OPN (not shown) was confined to the electron-dense, lamellated periphery. Collagen fibrils and immunolabeling for AMEL could not be detected in these deposits. The morphological appearance of the second type of round matrix structures consistently associated with ectopic enamel deposits varied greatly. Lamellation was less prominent and the organic content less electron-dense and more homogeneous than in the first type (Fig. 4C). Some of these round structures consisted of an extremely homogeneous and almost amorphous matrix, while others occasionally revealed ultrastructural details reminiscent of cellular elements (not shown). For all antibodies used, there was no labeling discernible in the major matrix portions of these structures. A peripheral electron-dense matrix layer was

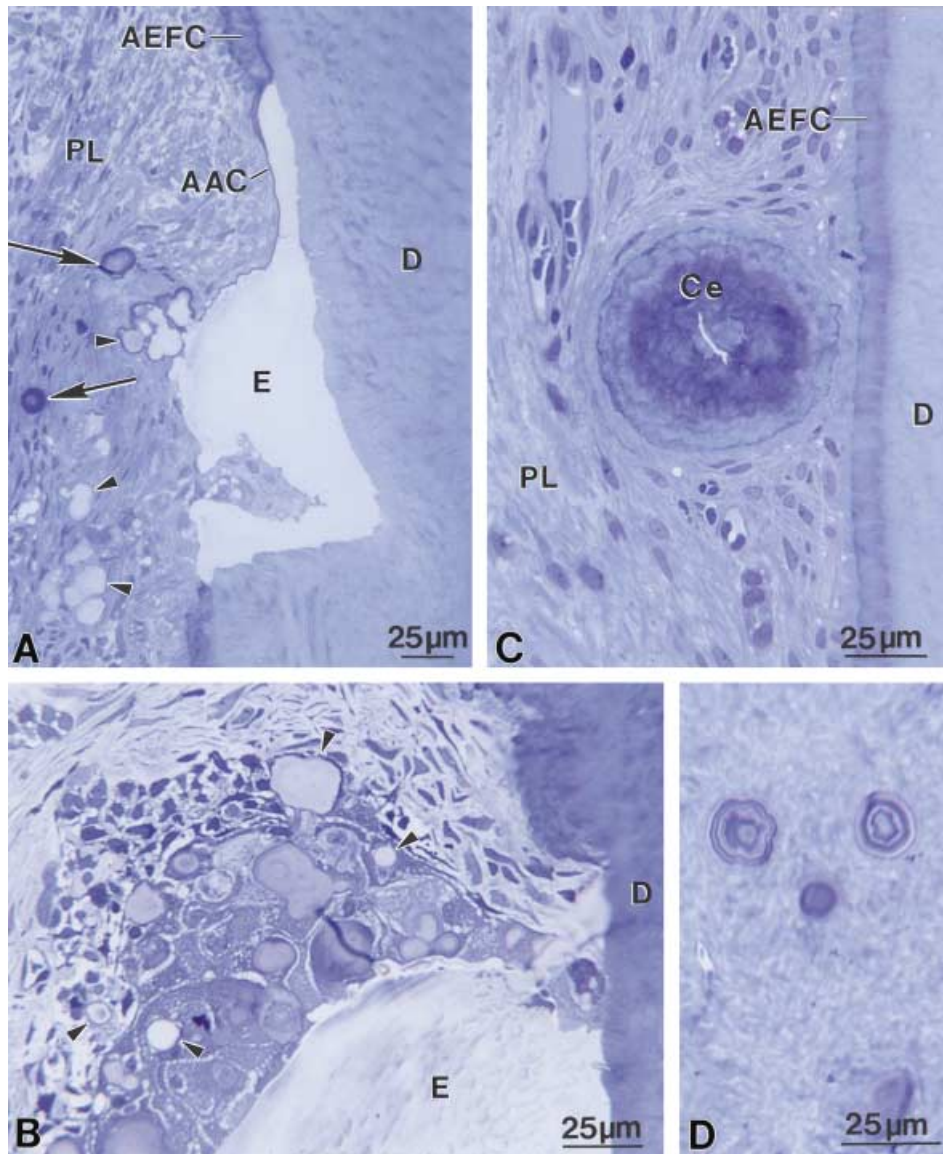


Fig. 1. Light photomicrographs showing: (A,B) two enamel (E) deposits on the root dentin (D); (C) a cementicle (Ce) in the periodontal ligament (PL); (D) cementicle-like structures in the root cementum matrix. Residual enamel matrix was usually present (A,B) and structural organization frequently observed (B). Note the presence of two types of round (globular) bodies (arrows for cementicle-like and arrowheads for enamel-like) in the portion of the periodontal ligament (PL) directly adjacent to the ectopic enamel deposits (A). (C) illustrates a free cementicle with a concentric lamellation and encircled by connective tissue cells in close proximity to the root surface. AAC, acellular afibrillar cementum; AEFC, acellular extrinsic fiber cementum.

either absent (not shown) or present (Fig. 4C,D). Peripheral labeling for BSP or OPN was only detected if such a peripheral matrix layer was clearly visible (not shown).

True cementicles had an electron-dense core devoid of collagen fibrils but presented with a peripheral collagen-rich matrix (Fig. 5A,B). Concentric lamellation was particularly evident in their core (Fig. 5A). Free and attached cementicles showed a similar labeling pattern for both BSP and OPN. Gold particles were concentrated over the electron-dense matrix compartments that were also poor in collagen (Fig. 5A,B). The cells on the surface of the cementicles resembled fibroblasts (Fig. 5B).

Discussion

This study, analysed for the first time the ultrastructure of the organic matrices of small ectopic enamel deposits on the root, associated cementicle-like structures, and true cementicles. In addition, the nature and fine distribution of some of their NCPs were characterized. All antibodies used in the present study have been previously characterized and extensively applied in studies on bone, cementum, dentin, and enamel (27). Nevertheless, as a control, the labeling pattern of the ectopic mineralizations was compared with that of the various cementum types and the cervical-most crown enamel, which has recently been described (17, 28).

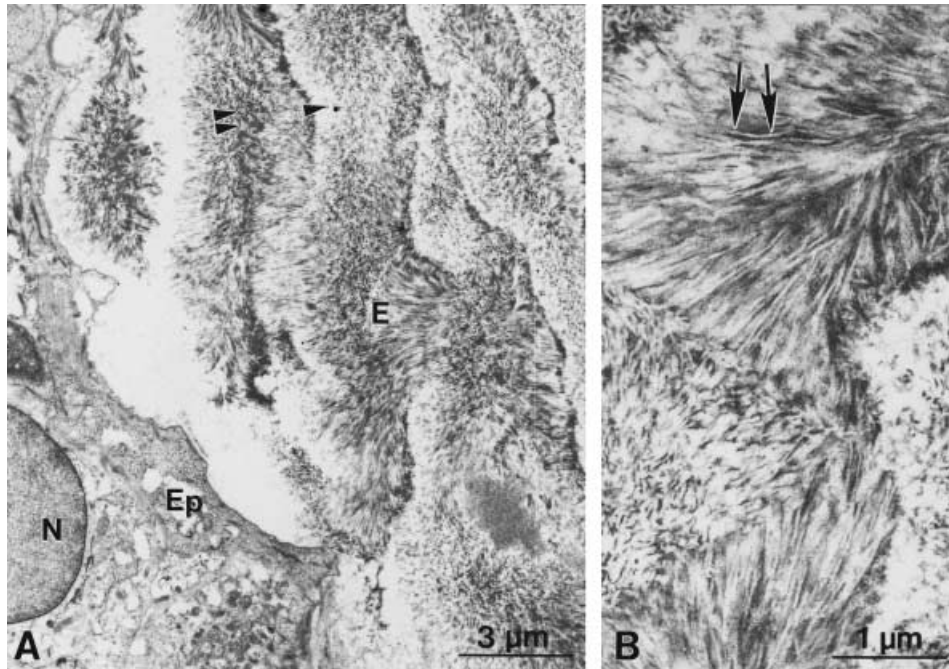


Fig. 2. Transmission electron photomicrographs showing an enamel (E) deposit on the root dentin. The peripheral portion of this enamel deposit contains large amounts of residual organic matrix that is structurally organized in alternating layers of low (arrowhead) and high (double arrowhead) organic contents (A). At higher magnification, organic enamel crystallite profiles ('crystal ghosts', arrows) are seen (B). Ep, epithelial cell; N, nucleus.

The observed high content of residual organic matrix and the structural irregularity are characteristic of small ectopic enamel deposits. It also reflects the immature nature and lower mineral content of small ectopic enamel deposits compared with both larger enamel pearls and the bulk of crown enamel (2, 4, 5). It is interesting to note that there is a similarity between the small ectopic enamel deposits and the cervical-most portion of crown enamel. At this site, ameloblasts produce a thin enamel layer that retains more organic material in its mature stage than the rest of crown enamel (27) and exhibits an irregular shape and arrangement of rods (3–5, 29–32). Because AMEL were detected in all enamel deposits on the root, they appear to be an essential matrix component. In crown enamel formation, amelogenins constitute the bulk of the enamel matrix and apparently assemble into quaternary supramolecular structures, called nanospheres, which may regulate the organization of crystal pattern and thickness (15, 33, 34).

The presence of immunolabeling for AMEL in the dentinal tubules of root dentin directly adjacent to enamel pearls parallels findings from crown development. It is suggestive of a diffusion of AMEL from the ameloblast layer towards the pulp (35–38). While it has long been believed that odontoblasts do not synthesize enamel proteins (38–40), very recent findings do support an odontoblastic origin (41, 42). Transient mRNA expression in pre-odontoblasts has also been reported for amelin (43) and ameloblastin (44). Thus, EMPs in dentin can theoretically originate from odontoblasts and/or ameloblasts. The relatively low amelogenin expression in odontoblasts (42), and the fact that amelogenins are

readily detectable by immunocytochemical methods, favors an ameloblast origin of EMPs in the dentin matrix. This conclusion does not interfere with the concept that EMPs are synthesized by both epithelial and mesenchymal cells and have a role in cell signaling events between differentiating ameloblasts and odontoblasts.

Another association has been suggested between EMPs and the induction of cementogenesis (46). HERS cells are assumed to secrete EMPs that aggregate on the root dentin into a matrix layer (47, 48) and induce the formation of acellular extrinsic fiber cementum (49–52). In the present study, amelogenin immunoreactivity could only be detected in association with ectopic enamel deposits (i.e. the dentin, dentino-cemental junction and cementum layer in regions apical and coronal to the enamel deposits, did not reveal any gold particle labeling). Thus, this restricted distribution pattern, the lack or sporadic occurrence of gene and protein expression of EMPs along forming tooth roots (28, 40, 53–58), and our observations that a layer of acellular afibrillar cementum frequently covered the ectopic enamel deposits (discussed below) do not support the view of a causal link between EMPs and the induction of acellular extrinsic fiber cementum formation. A histological study using human teeth treated with EMPs as an adjunct to promote periodontal regeneration has also reached the same conclusion (59). In this context, it must be noted that periodontal ligament cells are triggered to produce acellular extrinsic fiber cementum, whenever they come in close proximity to a calcified surface, be it a calcifying collagen implant, Bio-Oss particles (Geistlich, Wolhusen, Switzerland) or denuded enamel (60).

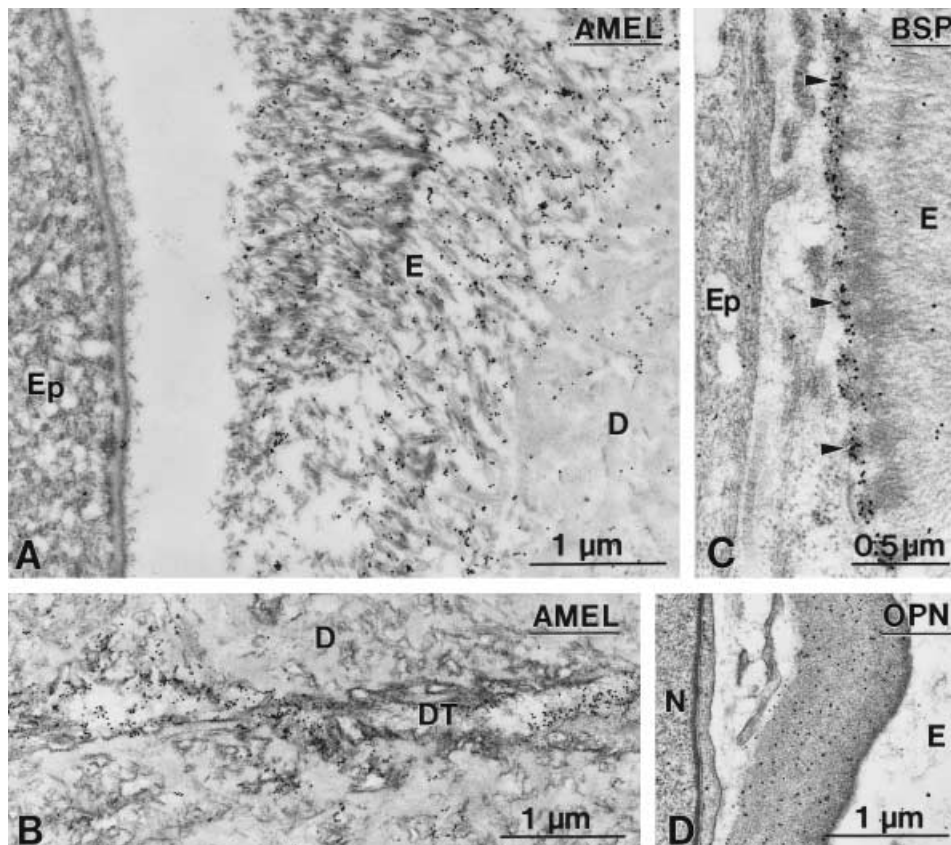


Fig. 3. Transmission electron photomicrographs showing (A, C, D) enamel matrix (E) deposited onto the root dentin (D) and (B) the peripheral dentin matrix (D) underneath an enamel deposit on the root. Incubations with an antiamelogenin (AMEL) antibody reveal gold particle labeling over the enamel deposit (E) in association with residual organic matrix (A) and over dentin (D) in association with a dentinal tubule (DT) (B). A thin matrix layer (arrowheads) immunoreactive for bone sialoprotein (BSP) covers the surface of this enamel deposit (C). Less numerous gold particles are dispersed in the enamel matrix itself. The matrix layer over this enamel deposit has a quite homogeneous texture and labels for osteopontin (OPN) (D). Ep, epithelial cell; N, nucleus.

The presence of a cementum-like matrix layer over ectopic enamel deposits on the root has been reported previously (2, 61, 62). Our results confirm the occasional presence of such a matrix layer over portions of the surface of ectopic enamel deposits and assign it to an acellular afibrillar type of cementum (17, 27). The two NCPs, BSP and OPN, may play a role in the mineralization process of this collagen-free matrix layer. Similarly, the labeling pattern observed in the true cementicles parallels what was found in acellular extrinsic fiber cementum and is suggestive of a participation of BSP and OPN in the interfibrillar mineralization process (17). A comparison with the region of the cemento-enamel junction, where the acellular afibrillar cementum frequently covers a thin enamel layer (63), suggests that cementum formation associated with ectopic enamel deposition recapitulates normal tooth developmental events.

An intriguing observation was the consistent presence of two different types of small round (i.e. globular) matrix bodies in the periodontal ligament tissue directly adjacent to the enamel deposits on the root. A coexistence of enamel pearls and such bodies has been observed in other studies (61, 64), and similar structures have been reported in Pindborg tumors (65). Some of these bodies

had the same radiodensity as the enamel of associated pearls, while others had a concentric structure and resembled cementicles (61). Our data show that the cementicle-like structures, designated as the first matrix type, are devoid of collagen fibrils, have a concentric lamellation and, like the true cementicles, label for both BSP and OPN. The second and more abundantly observed type, which was also devoid of collagen fibrils, had a more homogeneous matrix structure that did not label for BSP and OPN. It is possible that this second type corresponds to the calcified bodies that exhibit the same radiodensity as the ectopic enamel deposits and that are considered as an enamel-like calcification (61). However, it is surprising that these structures did not label for AMEL. A possible explanation for the lack of AMEL labeling is that AMEL were present at the beginning of their formation but were completely removed as the matrix matured. The presence of residual organic matrix in these structures, however, suggests retention of some NCPs. Possible candidates are amelogenin fragments that are not recognized by our antibodies, other EMPs such as ameloblastin/amelin or other, not yet identified matrix constituents.

Enamel deposits on the root have been associated with periodontal destruction (2, 6, 8-14). The presence of a

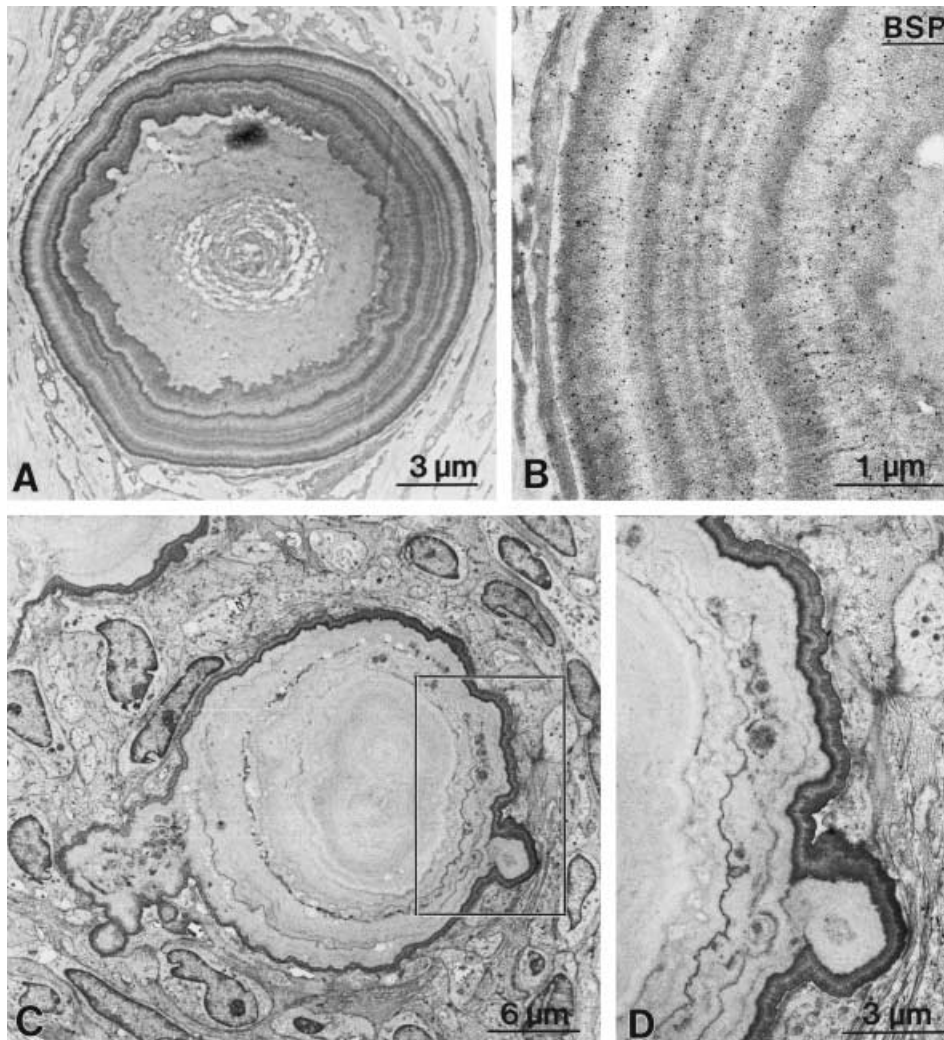


Fig. 4. Transmission electron photomicrographs illustrating (A,B) a cementicle-like and (C,D) a more homogeneously structured matrix deposit in the connective tissue adjacent to an enamel deposit on the root. The area outlined in (C) corresponds to (D). The cementicle-like structure is round and distinctly displays concentrically arranged lamellae (A,B) that label for BSP (B) and osteopontin (OPN, not shown). In contrast, lamellation in the other matrix deposit is less prominent and smaller coalesced matrix subunits are seen (C). An electron-dense matrix like the one seen in (C) and (D) and that labeled for both BSP and OPN was not always seen at the periphery of these matrices. Common to both matrix structures is that they lack collagen fibrils (B,C). However, the surrounding matrix is collagenous in nature (D).

'long junctional epithelium' and a possible plaque retention function are common explanations for this association. Thus far, a possible link between a localized altered structure and function of the periodontal ligament and the development of a localized periodontal lesion has not been postulated. The coexistence of ectopic enamel deposits, cementicle-like and enamel-like structures suggests that aberrant behavior of HERS is not restricted to ectopic enamel formation alone, but affects also the development of the adjacent periodontal ligament. While there is general agreement that during normal root development some HERS cells form the epithelial rests of Malassez, other cell fates are disputed. For example, apoptosis (66, 67) and epithelial-mesenchymal transformation (17, 27, 28, 46, 57, 68–72) have also been suggested to occur. In view of the latter cell differentiation process, it is of interest to note that the

local hindrance of cementoblast differentiation consistently resulted in the formation of both mesenchymal and epithelial calcifications. Whether HERS-derived cells alone or together with dental follicle-derived cells are involved in this process is presently unknown. Islands of HERS have indeed been shown to be associated with mineralized tissue formation (73) and epithelial rests of Malassez have been implicated in the formation of cementicles (7). However, it was not possible to determine the source of BSP and OPN in the present study. In addition to a local cell synthesis, an origin from the blood plasma must also be considered, at least for OPN (74).

In summary, we have analysed the ultrastructure and partial biochemical composition of various root-related ectopic calcifications. Ectopic enamel deposits were enriched in residual organic matrix containing, at least,

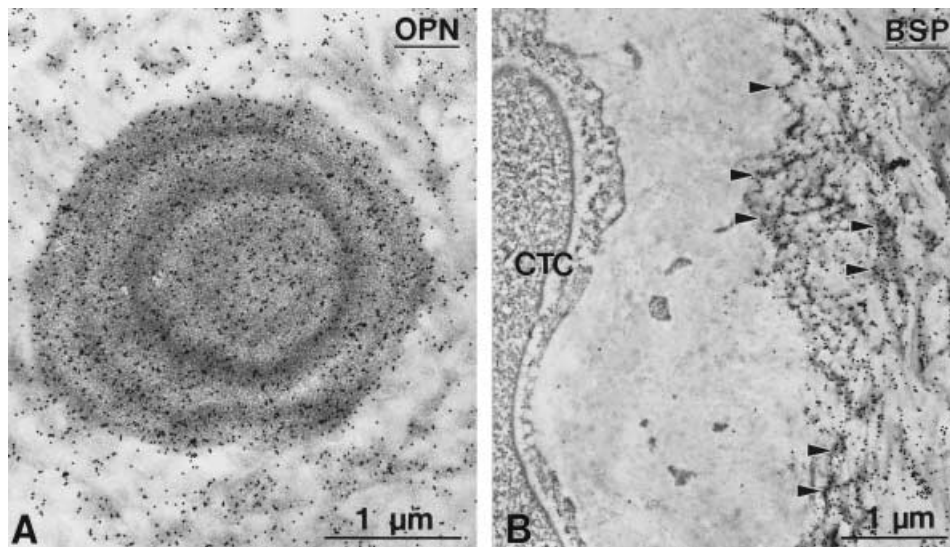


Fig. 5. Immunocytochemistry of cementicles with antibodies to (A) osteopontin (OPN) and (B) bone sialoprotein (BSP). Gold particles are particularly concentrated over the electron-dense, collagen-deficient core that displays a concentric lamellation (A). Immunogold labeling is also strong over electron-dense matrix compartments (arrowheads) at the periphery of this cementicle (B). CTC, connective tissue cell.

AMEL. Collagen-free cementum-like and enamel-like calcifications were consistently present in the periodontal ligament adjacent to ectopic enamel deposits. Their coexistence suggests a causal connection. True cementicles occurred independently of ectopic enamel formation and resembled acellular extrinsic fiber cementum in structure and composition. While AMEL appear to be involved in the mineralization process of enamel, BSP and OPN are thought to play a role in connective tissue mineralization. Similarly, these NCPs may play similar roles in the formation of ectopic calcification along the tooth root. Because enamel deposits on the root are highly associated with periodontal destruction, the observed tissue alterations in the periodontal ligament adjacent to ectopic enamel deposits require further investigation.

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References

- HOLTON WL, HANCOCK EB, PELLEU GB. Prevalence and distribution of attached cementicles on human root surfaces. *J Periodontol* 1985; **67**: 321–324.
- MOSKOW BS, CANUT PM. Studies on root enamel. (2) Enamel pearls. A review of their morphology, nomenclature, occurrence, classification, histogenesis and incidence. *J Clin Periodontol* 1990; **17**: 275–281.
- RISNES S. Ectopic tooth enamel. An SEM study of the structure of enamel in enamel pearls. *Adv Dent Res* 1989; **3**: 258–264.
- GASPERIC D. Histogenetic aspects of the composition and structure of human ectopic enamel, studied by scanning electron microscopy. *Arch Oral Biol* 1992; **37**: 603–611.
- GASPERIC D. Enamel microhardness and histological features of composite enamel pearls of different size. *J Oral Pathol Med* 1995; **24**: 153–158.
- RISNES S, SEGURA JJ, CASADO A, JIMÉNEZ-RUBIO A. Enamel pearls and cervical enamel projections on 2 maxillary molars with localized periodontal disease. Case report and histologic study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000; **89**: 493–497.
- SCHROEDER HE. The periodontium. In: OKSCHE A, VOLLRATH L, eds. *Handbook of microscopic anatomy*, Vol. V/5. Berlin: Springer, 1986, pp 1–411.
- CROFT LK. Periodontal abscess from enamel pearl. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1971; **32**: 154.
- GOLDSTEIN AR. Enamel pearls as contributing factors in periodontal breakdown. *J Am Dent Assoc* 1979; **99**: 210–211.
- SKINNER MA, SHILOAH J. The role of enamel pearls in localized severe periodontitis. *Quintessence Int* 1989; **20**: 181–183.
- ASKENAS BG, FRY HR, DAVIS JW. Cervical enamel projections with gingival fenestration in a maxillary central incisor: report of a case. *Quintessence Int* 1992; **23**: 103–107.
- LIMA AFM, HEBLING E. Cervical enamel projection related to furcation involvement. *Braz Dent J* 1994; **5**: 121–127.
- HOU GL, TSAI CC. Cervical enamel projection and intermediate bifurcational ridge correlated with molar furcation involvements. *J Periodontol* 1997; **68**: 687–693.
- DARWAZEH A, HAMASHA AA. Radiographic evidence of enamel pearls in Jordanian dental patients. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000; **89**: 255–258.
- SMITH CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Medical* 1998; **9**: 128–161.
- BUTLER WT, RITCHIE H. The nature and functional significance of dentin extracellular matrix proteins. *Int J Dev Biol* 1995; **39**: 169–179.
- BOSSHARDT DD, ZALZAL S, MCKEE MD, NANJI A. Developmental appearance of bone sialoprotein and osteopontin in human and rat cementum. *Anat Rec* 1998; **250**: 13–33.
- WARSHAWSKY H, MOORE G. A technique for the fixation and decalcification of rat incisors for electron microscopy. *J Histochem Cytochem* 1967; **15**: 542–549.

19. BOSSHARDT DD, SCHROEDER HE. Attempts to label matrix synthesis of human root cementum *in vitro*. *Cell Tissue Res* 1993; **274**: 343–352.
20. NEISS WF. Electron staining of the cell surface coat by osmium-low ferrocyanide. *Histochemistry* 1984; **80**: 231–242.
21. BENDAYAN M. Colloidal gold post-embedding immunocytochemistry. *Progr Histochem Cytochem* 1995; **29**: 1–159.
22. BENDAYAN M, ZOLLINGER M. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. *J Histochem Cytochem* 1983; **31**: 101–109.
23. FISHER LW, STUBBS JT, YOUNG MF. Antisera and cDNA probes to human and certain animal model bone matrix non-collagenous proteins. *Acta Orthop Scand* 1995; **66**: 61–65.
24. LIMEBACK H, SIMIC A. Biochemical characterization of stable high molecular-weight aggregates of amelogenins formed during porcine enamel development. *Arch Oral Biol* 1990; **35**: 459–468.
25. CHEN WY, NANJI A, SMITH CE. Immunoblotting studies on artifactual contamination of enamel homogenates by albumin and other proteins. *Calcif Tissue Int* 1995; **57**: 145–151.
26. FRENS G. Controlled nucleation for the regulation of particle size in monodispersed gold suspensions. *Nature Phys Sci* 1973; **241**: 20–22.
27. BOSSHARDT DD, NANJI A. Immunodetection of enamel- and cementum-related (bone) proteins at the enamel-free area and cervical portion of the tooth in rat molars. *J Bone Miner Res* 1997; **12**: 367–379.
28. BOSSHARDT DD, NANJI A. Immunolocalization of epithelial and mesenchymal matrix constituents in association with inner enamel epithelial cells. *J Histochem Cytochem* 1998; **46**: 135–142.
29. DAVIDSON CL, HOEKSTRA IJ, ARENDS J. Microhardness of sound, decalcified and etched tooth enamel related to calcium content. *Caries Res* 1974; **8**: 135–144.
30. FEATHERSTONE JDB, TEN CATE JM, SHARIATI M, ARENDS J. Comparison of artificial caries-like lesions by quantitative microradiography and microhardness profiles. *Caries Res* 1983; **17**: 385–391.
31. THEUNIS HM, VAN DIJK JWE, JONGEBLOED WL, GROENEVELD A. The mineral content of human enamel studied by polarizing microscopy, microradiography and scanning electron microscopy. *Arch Oral Biol* 1983; **28**: 797–804.
32. SCHEMEL W, HUMMEL K, KREKELER G. Härteprüfungen an Schmelz, Dentin und Zement rezenter menschlicher Zähne. *Schweiz Monatsschr Zahnmed* 1984; **94**: 1029–1041.
33. FINCHAM AG, MORADIAN-OLDAK J, DIEKWISCH TG, LYARUU DM, WRIGHT JT, BRINGAS P JR, SLAVKIN HC. Evidence for amelogenin 'nanospheres' as functional components of secretory-stage enamel matrix. *J Struct Biol* 1995; **115**: 50–59.
34. GIBSON CW, YUAN ZA, HALL B, LONGENECKER G, CHEN E, THYAGARAJAN T, SREENATH T, WRIGHT JT, DECKER S, PIDDINGTON R, HARRISON G, KULKARNI AB. Amelogenin-deficient mice display an amelogenesis imperfecta phenotype. *J Biol Chem* 2001; **276**: 31871–31875.
35. INAI T, KUKITA T, OHSAKI Y, NAGATA K, KUKITA A, KURISU K. Immunohistochemical demonstration of amelogenin penetration toward the dental pulp in the early stages of ameloblast development in rat molar tooth germs. *Anat Rec* 1991; **229**: 259–270.
36. NANJI A, SMITH CE. Development and calcification of enamel. In: BONUCCI E, ed. *Calcification in biological systems*. Boca Raton: CRC Press, 1992; 313–343.
37. NAKAMURA M, BRINGAS P JR, NANJI A, ZEICHNER-DAVID M, ASHDOWN B, SLAVKIN HC. Translocation of enamel proteins from inner enamel epithelia to odontoblasts during mouse tooth development. *Anat Rec* 1994; **238**: 383–396.
38. KARG HA, BURGER EH, LYARUU DM, WOLTGENS JH, BRONCKERS AL. Gene expression and immunolocalisation of amelogenins in developing embryonic and neonatal hamster teeth. *Cell Tissue Res* 1997; **288**: 545–555.
39. BLEICHER F, COUBLE ML, FARGES JC, COUBLE P, MAGLOIRE H. Sequential expression of matrix protein genes in developing rat teeth. *Matrix Biol* 1999; **18**: 133–143.
40. HU JC, SUN X, ZHANG C, SIMMER JP. A comparison of enamel and amelogenin expression in developing mouse molars. *Eur J Oral Sci* 2001; **109**: 125–132.
41. VEIS A, TOMPKINS K, ALVARES K, KUIRU W, WANG L, WANG XS, BROWNELL AG, JENGH SM, HEALY K. Specific amelogenin gene splice products have signaling effects on cells in culture and in implants *in vivo*. *J Biol Chem* 2000; **275**: 41263–41272.
42. OIDA S, NAGANO T, YAMAKOSHI Y, ANDO H, YAMADA M, FUKAE M. Amelogenin gene expression in porcine odontoblasts. *J Dent Res* 2002; **81**: 103–108.
43. FONG CD, CERNY R, HAMMARSTRÖM L, SLABY I. Sequential expression of an amelogenin gene in mesenchymal and epithelial cells during odontogenesis in rats. *Eur J Oral Sci* 1998; **106**: 324–330.
44. BÈGUE-KIRN C, KREBSBACH PH, BARTLETT JD, BUTLER WT. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. *Eur J Oral Sci* 1998; **106**: 963–970.
45. ZEICHNER-DAVID M, DIEKWISCH T, FINCHAM A, LAU E, MACDOUGALL M, MORADIAN-OLDAK J, SIMMER J, SNEAD M, SLAVKIN HC. Control of ameloblast differentiation. *Int J Dev Biol* 1995; **39**: 69–92.
46. BOSSHARDT DD, SCHROEDER HE. Cementogenesis reviewed: a comparison between human premolars and rodent molars. *Anat Rec* 1996; **245**: 267–292.
47. LINDSKOG S. Formation of intermediate cementum. I. Early mineralization of aprismatic enamel and intermediate cementum in monkey. *J Craniofac Genet Dev Biol* 1982; **2**: 147–160.
48. LINDSKOG S. Formation of intermediate cementum. II. A scanning electron microscopic study of the epithelial root sheath of Hertwig in monkey. *J Craniofac Genet Dev Biol* 1982; **2**: 161–169.
49. SLAVKIN HC. Towards a cellular and molecular understanding of periodontics: Cementogenesis revisited. *J Periodontol* 1976; **47**: 249–255.
50. LINDSKOG S, HAMMARSTRÖM L. Formation of intermediate cementum. III. ³H-tryptophan and ³H-proline uptake into the epithelial root sheath of Hertwig *in vitro*. *J Craniofac Genet Dev Biol* 1982; **2**: 172–177.
51. SASANO Y, KAJI Y, NAKAMURA M, KINDAICHI K, SLAVKIN HC, KAGAYAMA M. Distribution of glycoconjugates localized by peanut and *Maclura pomifera* agglutinins during mouse molar root development. *Acta Anat* 1992; **145**: 149–155.
52. HAMMARSTRÖM L. Enamel matrix, cementum development and regeneration. *J Clin Periodontol* 1997; **24**: 658–668.
53. SLAVKIN HC, BRINGAS P, BESSEM C, SANTOS V, NAKAMURA M, HSU MY, SNEAD ML, ZEICHNER-DAVID M, FINCHAM AM. Hertwig's epithelial root sheath differentiation and initial cementum and bone formation during long-term organ culture of mouse mandibular first molars using serumless, chemically defined medium. *J Periodont Res* 1989; **23**: 28–40.
54. LUO W, SLAVKIN HC, SNEAD ML. Cells from Hertwig's root sheath do not transcribe amelogenin. *J Periodont Res* 1991; **26**: 42–47.
55. FONG CD, SLABY I, HAMMARSTRÖM L. Amelin: an enamel-related protein, transcribed in the cells of epithelial root sheath. *J Bone Miner Res* 1996; **11**: 892–898.
56. THOMAS HF, JIANG H, CHEN J, MACDOUGALL M, KREBSBACH P. Ameloblastin expression by cells of the murine epithelial root sheath. *J Dent Res* 1997; **76**(Spec Iss): 266.
57. BOSSHARDT DD, NANJI A. The pattern of expression of collagen determines the concentrations and distribution of noncollagenous proteins along the forming root. In: GOLDBERG M, BOSKEY A, ROBINSON, C., eds. *Chemistry and biology of mineralized tissues. Proceedings of the 6th international conference*. Rosemont: American Academy of Orthopaedic Surgeons, 2000; 129–136.
58. FONG CD, HAMMARSTRÖM L. Expression of amelogenin and amelogenin in epithelial root sheath remnants of fully formed rat molars. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000; **90**: 218–223.

59. SCULEAN A, DONOS N, WINDISCH P, BRECX M, GERA I, REICH E, KARRING T. Healing of human intrabony defects following treatment with enamel matrix proteins or guided tissue regeneration. *J Periodont Res* 1999; **34**: 310–322.
60. BEERTSEN W, VAN DEN BOS T, NIEHOF A, EVERTS V. Formation of reparative acellular extrinsic fiber cementum in relation to implant materials installed in rat periodontium. *Eur J Oral Sci* 1998; **106**: 368–375.
61. KEREBEL B, DARD M, LE CABELLEC MT, KEREBEL LM. Les perles d'émail: étude histopathologique. *J Biol Buccale* 1986; **14**: 239–248.
62. FONZI L, BELLI M, GASPARONI A, CAPEZZUOLI L, CARBONCINI S. Enamel pearls: The ultrastructural aspects and morphogenesis hypotheses. *Bull Group Int Rech Sci Stomatol Odontol* 1992; **35**: 85–92.
63. BOSSHARDT DD, SELVIG KA. Dental cementum: The dynamic tissue covering of the root. *Periodontol 2000* 1997; **13**: 41–75.
64. GOTTLIEB B. Zementexostosen, Schmelztropfen und Epithelneester. *Z Stomatol* 1921; **19**: 515–526.
65. EL-LABBAN NG. Cementum-like material in a case of Pindborg tumor. *J Oral Pathol Med* 1990; **19**: 166–169.
66. KANEKO H, HASHIMOTO S, ENOKIYA Y, OGIUCHI H, SHIMONO M. Cell proliferation and death of Hertwig's epithelial root sheath in the rat. *Cell Tissue Res* 1999; **298**: 95–103.
67. CERRI PS, FREYMULLER E, KATCHBURIAN E. Apoptosis in the early developing periodontium of rat molars. *Anat Rec* 2000; **258**: 136–144.
68. THOMAS HF, KOLLAR EJ. Tissue interactions in normal murine root development. In: DAVIDOVITCH Z, ed. *The biological mechanisms of tooth eruption and root resorption*. Birmingham: EBSCO Media, 1988, 145–151.
69. BOSSHARDT DD. *Morphologische, Morphodynamische und Autoradiographische Untersuchung der Zementogenese an Menschlichen Zähnen*. Hamburg: Verlag Dr Kovac, 1993; 1–168.
70. MACNEIL RL, THOMAS HF. Development of the murine periodontium. II. Role of the epithelial root sheath in formation of the periodontal attachment. *J Periodontol* 1993; **64**: 285–291.
71. WEBB PP, MOXHAM BJ, BENJAMIN M, RALPHS JR. Changing expression of intermediate filaments in fibroblasts and cementoblasts of the developing periodontal ligament of the rat molar tooth. *J Anat* 1996; **188**: 529–539.
72. LEZOT F, DAVIDEAU JL, THOMAS B, SHARPE P, FOREST N, BERDAL A. Epithelial Dlx-2 homeogene expression and cementogenesis. *J Histochem Cytochem* 2000; **48**: 277–284.
73. HAMAMOTO Y, NAKAJIMA T, OZAWA H, UCHIDA T. Production of amelogenin by enamel epithelium of Hertwig's root sheath. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 1996; **81**: 703–709.
74. VANDENBOS T, BRONCKERS ALJJ, GOLDBERG HA, BEERTSEN W. Blood circulation as source for osteopontin in acellular extrinsic fiber cementum and other mineralizing tissues. *J Dent Res* 1999; **78**: 1688–1695.